UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

TESE DE DOUTORADO

O papel da resiliência celular, estresse e epigenética na patofisiologia, progressão e resposta ao tratamento nos transtornos de humor

GABRIEL RODRIGO FRIES

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Ao Lipe, meu mano querido.

If I have seen further it is by standing on the shoulders of giants
[Se vi mais longe, foi por estar sobre ombros de gigantes]
(Isaac Newton)

Das Leben ist kein Ponyhof.
(Ditado popular alemão)

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SUMÁRIO

APRESENTAÇÃO

A presente tese de doutorado está organizada em três partes, conforme a seguir:

Parte I: resumo, *abstract*, Introdução e Objetivos;

Parte II: resultados apresentados na forma de quatro artigos científicos;

Parte III: Discussão, Conclusões e Referências bibliográficas.

Além disso, a seção de Anexos compreende: a) lista de figuras; b) lista de tabelas, c) um capítulo de livro escrito e aceito para publicação durante a realização do doutorado; d) Termos de Consentimento Livre e Esclarecido para pacientes e controles; d) um editorial publicado durante o período do doutorado (o qual complementa um dos tópicos discutidos no corpo do texto principal); e e) um artigo científico publicado em co-autoria durante o período do doutorado.

Os trabalhos que compõem esta tese foram desenvolvidos entre os anos de 2011 e 2014 em duas localidades: no Laboratório de Psiquiatria Molecular, localizado no Centro de Pesquisas Experimentais do Hospital de Clínicas de Porto Alegre, sob orientação da Profª Drª Márcia Kauer Sant'Anna e do Prof. Dr. Flávio Kapczinki, e no Instituto Max Planck de Psiquiatria, em Munique, Alemanha, sob orientação do Dr. Theo Rein (doutorado sanduíche).

PARTE I

Introdução e objetivos

RESUMO

Evidências sugerem o envolvimento de mecanismos de resiliência celular, estresse e de alterações epigenéticas na patofisiologia dos transtornos de humor, como o transtorno bipolar (TB) e o transtorno depressivo maior. Os estudos apresentados nesta tese tiveram como objetivo explorar esses mecanismos de forma translacional, partindo da avaliação da morte e sobrevivência celular até a correlação dos achados com a resposta ao tratamento. O primeiro capítulo se propôs a revisar dados da literatura com relação à progressão do TB, os quais apontam para uma série de alterações biológicas envolvendo resiliência celular em pacientes com diferentes estágios do transtorno. O segundo capítulo teve como objetivo avaliar parâmetros de morte celular em pacientes com TB, onde um aumento na frequência de células em apoptose inicial foi detectado em comparação a controles. Em seguida, considerando os efeitos potenciais dos glicocorticóides na indução de apoptose, o terceiro capítulo avaliou a atividade do eixo hipotálamo-pituitária-adrenal (HPA) em pacientes com TB, seus irmãos e em controles saudáveis, além de mecanismos moleculares associados ao eixo. Nossos resultados sugerem uma disfunção do eixo HPA em pacientes associada a uma hiporresponsividade do receptor de glicocorticóide, maiores níveis da proteína ligante de FK506 de 51 kDa (FKBP51) e um aumento da metilação do gene *FKBP5*. Nós ainda observamos que os pacientes com maior número de episódios apresentaram uma disfunção do eixo mais pronunciada do que aqueles em estágio inicial. No quarto e último capítulo nós realizamos um estudo para verificar os mecanismos moleculares pelos quais o estresse pode induzir alterações epigenéticas envolvendo a metilação do DNA, onde descrevemos o efeito oposto das cochaperonas FKBP51 e FKBP52 sobre a fosforilação da enzima DNA metiltransferase 1 (DNMT1). Além disso, a redução da fosforilação da DNMT1 correlacionou-se com uma melhor resposta clínica ao tratamento em pacientes com transtorno depressivo maior. Em suma, os resultados desta tese sugerem que a resiliência celular, a resposta ao estresse e a metilação do DNA desempenham importantes papéis na patofisiologia, progressão e resposta ao tratamento de pacientes com transtornos de humor. Novos tratamentos com alvo em mecanismos epigenéticos, na modulação do receptor de glicocorticóide e no aumento da resiliência celular devem ser priorizados em estudos futuros.

ABSTRACT

Evidence suggests the involvement of cellular resilience mechanisms, stress, and of epigenetic alterations in the patophysiology of mood disorders, such as bipolar disorder (BD) and major depressive disorder. The studies presented in this thesis aimed at exploring these mechanisms in a translational fashion, going from the assessment of cellular death and survival to the correlation of findings with the response to treatment. The first chapter aimed at reviewing data from the literature regarding the progression of BD, which point to a series of biological alterations involving cellular resilience in patients at different stages of the disorder. The second chapter aimed at assessing cell death parameters in patients with BD, in which a higher frequency of cells in early apoptosis was detected when compared to controls. In addition, considering the potential effects of glucocorticoids in the induction of apoptosis, the third chapter assessed the activity of the hypothalamus-pituitary-adrenal (HPA) axis in patients with BD, their siblings, and in healthy controls, in addition to the molecular mechanisms associated with the axis. Our results suggest a dysfunction of the HPA axis in patients associated with a hyporesponsiveness of the glucocorticoid receptor, increased FK506-binding protein of 51 kDa (FKBP51) levels, and increased methylation levels at the *FKBP5* gene. We also noted that patients with a greater number of previous episodes presented a more pronounced dysfunction of the axis when compared to early-stage patients. In the fourth and last chapter we performed a study to verify the molecular mechanisms by which stress can induce epigenetic alterations involving DNA methylation, where we describe opposing effects of the cochaperones FKBP51 and FKBP52 on DNA methyltransferase 1 (DNMT1) phosphorylation. In addition, the reduction of DNMT1 phophorylation was correlated with a better clinical response to treatment in patients with major depressive disorder. In summary, the results of this thesis suggest that cellular resilience, response to stress and DNA methylation play key roles in the pathophysiology, progression and response to treatment in patients with mood disorders. Novel treatments targeting epigenetic mechanisms, the modulation of the glucocorticoid receptor, and enhancing cellular resilience should be prioritized in future studies.

Lista de abreviaturas

AVP	arginina vasopressina
ACTH	hormônio adrenocorticotrófico (do inglês *adrenocorticotropic hormone*)
CDK5	cinase dependente de ciclina 5 (do inglês *cyclin-dependent kinase 5*)
CRH	hormônio liberador de corticotrofina (do inglês *corticotropin-releasing hormone*)
CTQ	Questionário sobre Traumas na Infância (do inglês *Childhood Trauma Questionnaire*)
DAMP	padrão molecular associado ao dano (do inglês *damage-associated molecular pattern*)
DNMT	DNA metiltransferase
FAST	Escala Breve de Funcionamento (do inglês *Functioning Assessment Short Test*)
FKBP51	proteína ligadora do FK506 de 51 kDa (do inglês *FK506-binding protein of 51 kDa*)
FKBP52	proteína ligadora do FK506 de 52 kDa (do inglês *FK506-binding protein of 52 kDa*)
GR	receptor de glicocorticóide (do inglês *glucocorticoid receptor*)
GRE	elemento de resposta a glicocorticóides (do inglês *glucocorticoid responsive element*)
HDRS	*Hamilton Depression Rating Scale*
HPA	hipotálamo-pituitária-adrenal

HSP90	proteína de choque térmico de 90 kDa (do inglês *heat shock protein of 90kDa*)
MR	receptor de mineralocorticóide (do inglês *mineralocorticoid receptor*)
NAA	N-acetil-aspartato
PBMC	célula mononuclear de sangue periférico (do inglês *peripheral blood mononuclear cell*)
PVN	núcleo paraventricular do hipotálamo (do inglês *paraventricular nucleus of hypothalamus*)
RE	retículo endoplasmático
TB	transtorno bipolar
TNF-α	fator de necrose tumoral alfa (do inglês *tumor necrosis factor alpha*)
TRP	repetição de tetratricopeptídeos (do inglês *tetratricopeptide repeat*)
TSD	teste de supressão com dexametasona
YMRS	*Young Mania Rating Scale*

1. INTRODUÇÃO

1.1 Transtorno bipolar

O transtorno bipolar (TB) é um transtorno psiquiátrico crônico, multifatorial e potencialmente grave cuja prevalência na população mundial está estimada em cerca de 2 % (Geddes e Mikliwitz, 2013). Segundo a Organização Mundial da Saúde, o TB é a sexta principal causa de incapacitação entre todas as condições médicas gerais (World Health Organization, 2011), sendo caracterizado pela ocorrência de sintomas maníacos e/ou hipomaníacos e depressivos. Na prática, os pacientes com TB raramente experienciam apenas um único episódio, sendo que a taxa de relapso para novos episódios pode chegar a até 70 % em cinco anos (Gitlin *et al*., 1995). De acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais, 4ª edição (DSM-IV-TR), o TB pode ser dividido em quarto diferentes subtipos: TB tipo I, TB tipo II, ciclotimia e TB sem outra especificação (Tabela 1).

Tabela 1. Subtipos diagnósticos do Transtorno Bipolar (TB)

Transtorno	Definição
TB tipo I	Episódio maníaco ou misto com ou sem psicose e/ou depressão maior
TB tipo II	Episódio hipomaníaco com depressão maior; sem história de episódios maníacos ou mistos
Ciclotimia	Sintomas hipomaníacos e depressivos que não fecham critérios para TB tipo II; ausência de episódios de depressão maior
TB sem outra especificação	Não fecha critérios para depressão maior, TB tipo I, TB tipo II ou ciclotimia (p. ex., menos de uma semana com sintomas maníacos sem psicose ou hospitalização)

Fonte: Price e Marzani-Nissen. Bipolar Disorders: a review. *Am Fam Physician*. 2012; 85(5):483-93.

Os episódios de mania caracterizam-se pela presença de humor persistentemente elevado, expansivo ou irritável, com duração de pelo menos uma semana (ou qualquer

duração se uma hospitalização for necessária), durante a qual três (ou mais) dos seguintes sintomas precisam estar presentes: autoestima inflada ou sentimentos de grandiosidade, diminuição da necessidade de dormir (p.ex., se sente descansado após apenas três horas de sono), taquilalia, fuga de ideias, experiência subjetiva de que os pensamentos estão acelerados, distratibilidade, agitação psicomotora, e envolvimento excessivo em atividades prazerosas com alto potencial para consequências danosas (Price & Marzani-Nissen, 2012).

Os episódios de depressão, por outro lado, caracterizam-se pela presença de cinco (ou mais) dos seguintes sintomas durante um período de pelo menos duas semanas: humor deprimido na maior parte do dia, diminuição do interesse ou prazer em todas (ou quase todas) as atividades do dia, perda ou ganho significativo de peso (ou alterações significativas no apetite), insônia ou hipersonia quase todos os dias, agitação ou retardo psicomotor, fadiga ou perda de energia, sentimentos de invalidez ou culpa excessiva ou inapropriada, habilidade diminuída de pensar ou de se concentrar, além de pensamentos recorrentes de morte, ideação suicida recorrente ou tentativa de suicídio (Price & Marzani-Nissen, 2012).

Na maioria dos casos, os pacientes com TB apresentam sintomas residuais de depressão por até um terço de suas vidas (Judd *et al*., 2002), sendo a depressão a principal causa de incapacitação entre os pacientes. Além disso, eles comumente apresentam sintomas psicóticos, prejuízos no funcionamento, comprometimento da qualidade de vida e estigma (Geddes & Miklowitz, 2013). O tratamento do TB comumente se divide em dois tipos: 1) estabilização dos episódios agudos, no qual o objetivo é trazer um paciente em mania ou depressão aguda à remissão dos sintomas (eutimia); e 2) tratamento de manutenção, onde o objetivo é prevenir o relapso, reduzir os sintomas subsindrômicos, e aumentar o funcionamento social e ocupacional dos pacientes. O tratamento de ambas as fases pode ser complexo, visto que os mesmos tratamentos que tratam a depressão podem causar mania,

hipomania ou ciclagem rápida, da mesma forma que os tratamentos que reduzem os sintomas maníacos podem causar episódios 'rebotes' de depressão (Geddes & Miklowitz, 2013).

1.1.1 Progressão do TB

Evidências clínicas sugerem que alguns pacientes com TB apresentam um curso progressivo. Esta progressão (decorrente, por exemplo, da recorrência de episódios agudos) está comumente associada a vários desfechos clínicos desfavoráveis, incluindo uma redução nos intervalos inter-episódicos, redução na resposta ao tratamento (especialmente ao lítio e à terapia cognitivo-comportamental), maiores taxas de comorbidades, prejuízos no funcionamento, aumento do risco de suicídio e hospitalização, além de um pior desfecho no tratamento com psicoeducação familiar (Berk *et al*., 2011; Hawton *et al*., 2005; Matza *et al*., 2005; Reinares *et al*., 2010; Rosa *et al*., 2012; Swann *et al.*, 1999). Além disso, os prejuízos cognitivos tendem a piorar com episódios cumulativos, afetando funções executivas e outros testes cognitivos, incluindo a memória verbal, resposta inibitória, atenção sustentada, velocidade psicomotora e abstração (Kessing *et al*., 2004; Torres *et al*., 2007).

Vários modelos de estadiamento clínico tem sido propostos para auxiliar no manejo e no tratamento dos pacientes em diferentes estágios da doença (revisados em Fries *et al*., 2012, que compõe o capítulo 1 desta tese). Diferenças à parte, todos os modelos descrevem o transtorno como um *continuum* progredindo de uma forma latente ou assintomática (estágio 0) até uma forma crônica e resistente ao tratamento (estágio 4). Simplificadamente, os pacientes com TB podem também ser divididos em pacientes em estágios iniciais do transtorno (incluindo, por exemplo, aqueles que apresentaram poucos episódios agudos e uma curta duração da doença) e pacientes em estágios avançados ou tardios (pacientes crônicos). Uma série de estudos tem identificado diferenças neuroanatômicas e biológicas entre esses dois grupos de pacientes, reforçando a teoria de que a progressão do TB altera não apenas

aspectos clínicos do transtorno (como o funcionamento, cognição, sintomatologia subssindrômica e resposta ao tratamento), como também seus aspectos patofisiológicos (Fries *et al*., 2012; Pfaffenseller *et al.*, 2014).

1.2 Transtorno depressivo maior

O transtorno depressivo maior, também conhecido como depressão maior, é um dos transtornos psiquiátricos mais prevalentes na população mundial (Kessler *et al*., 2007). Os atuais critérios diagnósticos incluem um humor depressivo ou irritável durante a maior parte do dia, quase todos os dias, por pelo menos duas semanas, além de sintomas adicionais que podem consistir em anedonia, preocupação excessiva, sentimentos de culpa, ideação suicida, alterações psicomotoras, alterações no sono, peso, apetite e em parâmetros cognitivos (American Psychiatric Association, 2000).

Cerca de 50% dos pacientes com depressão maior experienciam uma resolução inadequada dos seus sintomas com os tratamentos farmacológicos típicos (Gaynes *et al*., 2011). Como consequência, aqueles com respostas parciais ou inexistentes ao tratamento sofrem significativas quedas na qualidade de vida e funcionamento (Mauskopf *et al*., 2009). Além disso, embora os tratamentos atualmente disponíveis para a depressão maior sejam seguros, existe uma grande variabilidade na resposta ao tratamento entre diferentes indivíduos. Cerca de 60% dos pacientes não apresentam melhoras significativas após o tratamento com um único antidepressivo, e 20% desses pacientes não responde à nenhuma intervenção disponível (Labermaier *et al*., 2013). Atualmente não existem medidas clinicamente úteis que podem predizer com relativa certeza – *a priori* ou no início do tratamento – se um paciente em particular responderá a um determinado antidepressivo. Entre os fatores que já foram mostrados como moduladores da resposta ao tratamento estão a gravidade, maior duração e frequência dos episódios, comorbidade com transtorno de

ansiedade, e uma idade de início tardio da doença. No entanto, devido à sua baixa sensibilidade e especificidade, preditores bioquímicos ou genéticos de utilidade clínica suficiente ainda não foram identificados (Labermaier *et al*., 2013).

1.3 O impacto do estresse nos transtornos de humor

A progressão dos transtornos de humor, caracterizada pela recorrência de episódios agudos, pode ser comparada a modelos de sensibilização ao estresse e a modelos de *kindling* eletrofisiológico, como revisado por Post (2007). Esse fenômeno de aceleração dos episódios foi originalmente descrito por Kraepelin em 1899, sugerindo que episódios iniciais são frequentemente iniciados por estressores psicossociais, enquanto novas recorrências podem se tornar autônomas e independentes de gatilhos ambientais (revisado em Post, 2007).

Especificamente, a progressão do TB tem sido relacionada a um aumento de 'carga alostática', a qual pode ajudar a explicar a carga médica cumulativa associada com os episódios recorrentes de humor (Kapczinski *et al*., 2008). Baseando-se nesta teoria, acredita-se que pacientes com TB são cronicamente expostos a eventos estressores e precisam ativar mecanismos para lidar com os mesmos. A ativação crônica de mecanismos alostáticos (por exemplo, a ativação do eixo hipotálamo-pituitária-adrenal (HPA) e posterior redução dos níveis de cortisol de volta aos seus níveis basais) pode, por si só, levar à redução nos mecanismos de resiliência[1]. Esse processo pode, em última instância, estabelecer um ciclo vicioso de progressão, no qual os pacientes se tornam mais vulneráveis ao estresse e a gatilhos para novos episódios à medida que a doença progride (Figura 1).

[1] O termo "resiliência" será empregado nesta tese em dois diferentes contextos: 1) resiliência ao estresse (também conhecida como "resiliência psicológica"), a qual se refere à habilidade de um indivíduo de lidar e se adaptar a um evento estressor e adversidade (Rutten *et al*., 2013); e 2) resiliência celular (discutida em maiores detalhes na seção 1.5), a qual se refere à capacidade de uma célula em lidar (e, assim, sobreviver) com eventos estressores.

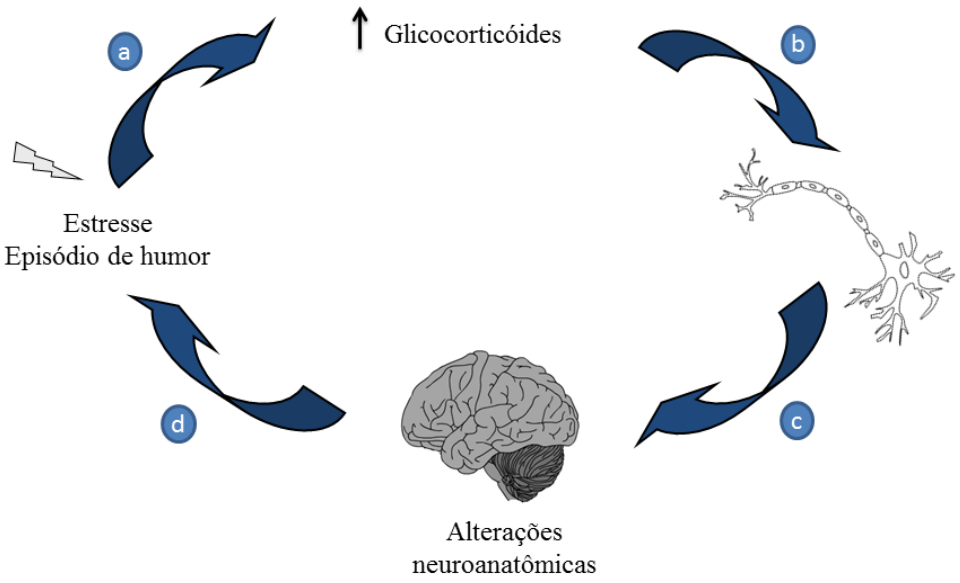

Figura 1. Modelo teórico proposto para a progressão do Transtorno Bipolar (TB). A) Eventos estressores podem funcionar como gatilhos para episódios agudos de humor (principalmente no início do transtorno), ativando o eixo do estresse e induzindo a liberação de altos níveis de glicocorticóides na circulação. B) Altos níveis de cortisol podem, a longo prazo, induzir disfunções celulares, podendo culminar com morte celular (apoptose) ou reorganização de dendritos, no caso de neurônios. C) Essa reorganização pode levar, em última instância, à alterações neuroanatômicas significativas, como o aumento no volume da amígdala e a diminuição no volume do hipocampo e do córtex pré-frontal (como descrito para pacientes com TB em estágios tardios). D) Essas alterações, por consequência, culminam com uma diminuição na capacidade de lidar com estressores (menor resiliência) e, portanto, maior vulnerabilidade à ocorrência de novos episódios agudos de humor. Modificado de Kapczinski *et al*., 2008.

1.3.1 O eixo HPA e a resposta ao estresse

O principal componente endócrino da resposta ao estresse envolve a ativação do eixo HPA, o qual constitui-se em uma cascata neuroendócrina que culmina na síntese e secreção de glicocorticódides (primariamente o cortisol em humanos e corticosterona em ratos, camundongos e outras espécies). A ação fisiológica primária dos glicocorticóides é induzir a redistribuição de energia e aumentar a disponibilidade de combustível para diferentes tecidos, promovendo a capacidade de sobrevivência frente à percepção da ameaça (estressor). Por outro lado, a grande ação catabólica dos glicocorticóides requer um controle fino e muito bem regulado, visto que esta pode ter efeitos deletérios quando cronicamente ativada. A regulação da secreção de glicocorticóides, portanto, está sujeita a um controle por retroalimentação do eixo HPA, como detalhado em seguida (Figura 2) (Juruena *et al.*, 2004).

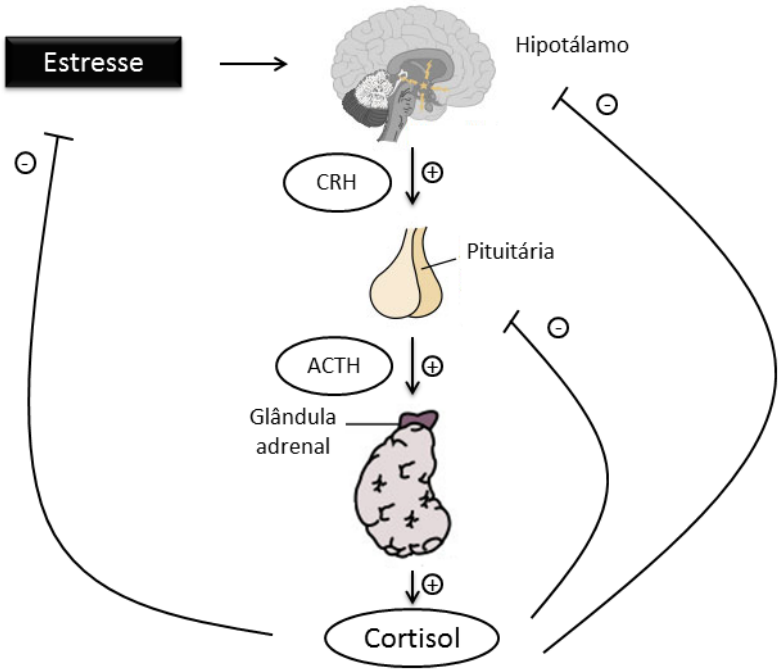

Figura 2. Eixo hipotálamo-pituitária-adrenal. A percepção do estresse estimula o hipotálamo a secretar o hormônio liberador de corticotrofina (CRH), que estimula as células da pituitária anterior a liberar o hormônio adrenocorticotrófico (ACTH). Este age no córtex da adrenal para aumentar a liberação de cortisol para a corrente sanguínea. O cortisol é, então, responsável por uma alça de retroalimentação negativa sobre o hipocampo, hipotálamo e pituitária, consequentemente reduzindo seus próprios níveis. Modificado de <embryology.med.unsw.edu.au/embryology/index.php>.

A ativação do eixo HPA é controlada por uma pequena fração de neurônios parvocelulares localizados no núcleo paraventricular (PVN) do hipotálamo. Após a estimulação pelo estresse ou por ritmos circadianos, esses neurônios liberam fatores na circulação porta-hipofisária, como o hormônio liberador de corticotrofina (CRH) e a arginina vasopressina (AVP). Esses fatores, então, atingem a pituitária anterior (ou hipófise) e causam a liberação do hormônio adrenocorticotrófico (ou corticotrofina, ACTH), que é liberado para a circulação sistêmica e causa a síntese e secreção de glicocorticóides pelo córtex da glândula adrenal. Uma vez liberados, os glicocorticóides são capazes de se ligar ao receptor de mineralocorticóide (MR, do inglês *mineralocorticoid receptor*) de alta afinidade ou ao receptor de glicocorticóide (GR, do inglês *glucocorticoid receptor*) de baixa afinidade, que funcionam como fatores de transcrição para regular a expressão gênica. Devido à sua alta afinidade, os MR estão extensivamente ligados em situações de baixos níveis de

glicocorticóides. Por outro lado, o GR apresenta-se ligado apenas em situações de altos níveis de glicocorticóides circulantes, como durante o estresse. Em função disso, a maioria dos mecanismos de retroalimentação negativa do eixo HPA induzidos pelos glicocorticóides é mediada pelo GR.

1.3.2 Alterações do eixo HPA no TB

Diferentes estudos relataram anormalidades nos níveis de cortisol em pacientes com depressão maior e TB (Havermans *et al*., 2011; Cervantes *et al*., 2001; Gibbons & McHugh, 1962; Gibbons, 1964; McClure, 1966; Platman *et al*., 1971; Sachar *et al*., 1972). Especificamente, uma grande proporção de pacientes suprime de forma ineficiente a liberação de cortisol em resposta ao teste de supressão com dexametasona (TSD). Ainda, eles apresentam níveis séricos de cortisol aumentados (Sher, 2006), independente da fase da doença (Watson *et al*., 2004). Essas observações indicam um prejuízo na alça de retroalimentação negativa do eixo HPA, o qual persiste mesmo após a remissão da depressão aguda. Pacientes apresentando anormalidades no eixo HPA são mais susceptíveis a relapsos depressivos durante a remissão, tanto na depressão unipolar (Zobel *et al*., 2001) quanto na depressão bipolar (Vieta *et al*., 1997).

Além da já conhecida disfunção no eixo HPA na depressão, evidências sugerem que tal disfunção também ocorre durante a mania. Estudos prévios usando o TSD sugerem uma alteração na função dos glicocorticóides em episódios maníacos com características mistas (Evans & Nemeroff, 1983; Krishnan *et al*., 1983; Godwin, 1984; Swann *et al*., 1992). Schmider e colaboradores (1995) realizaram o teste de dexametasona/CRH em pacientes com mania aguda e relataram uma resposta aumentada ao teste quando comparados com controles saudáveis, mesmo após a remissão dos sintomas. Da mesma forma, Vieta e colaboradores (1999) foram capazes de predizer relapsos de mania baseando-se no teste de estimulação com CRH em pacientes com TB em remissão. Estudos com perfis de secreção de cortisol também

relataram alterações no eixo HPA durante a mania. Linkowski e colaboradores (1994) encontraram níveis plasmáticos de cortisol significativamente elevados durante a noite em pacientes maníacos em comparação com controles saudáveis. Mais recentemente, Cervantes e colaboradores (2001) mostraram que pacientes com TB em diferentes fases – depressão, mania/hipomania e eutimia – apresentavam níveis elevados de cortisol quando comparados com controles, não havendo diferenças entre os pacientes.

Esta deficiência no eixo HPA em regular os níveis de cortisol circulantes resulta em um mecanismo que leva a um aumento exagerado dos níveis de cortisol durante o estresse e diminui a habilidade do eixo em retornar estes a níveis basais (Tatro *et al*., 2009). O cortisol aumentado, consequentemente, pode ter importantes consequências a longo prazo em pacientes com TB, visto que glicocorticóides apresentam importantes papéis no processo pelo qual mediadores alostáticos interagem com sistemas de neurotransmissores e peptídeos cerebrais (McEwen, 2004). Além de alterações na neuroplasticidade, a disfunção do eixo HPA pode alterar vários aspectos do ritmo circadiano, incluindo a regulação do sono. De fato, a maioria destes parâmetros já foi associada à patofisiologia do TB e da depressão maior (Murray & Harvey, 2010).

1.3.3 A regulação do GR e a proteína FKBP51

Conforme discutido nas seções anteriores, o GR é o principal ligante responsável pela alça de retroalimentação negativa do eixo HPA. Este receptor é um membro da superfamília de fatores de transcrição que medeiam a atividade fisiológica de seus ligantes em múltiplos sistemas, incluindo o respiratório e o sistema nervoso central. O GR não-ligado (inativo) está associado a um complexo oligomérico contendo proteínas regulatórias no citoplasma, incluindo a proteína de choque térmico de 90 kDa (HSP90, do inglês *heat shock protein of 90 kDa*), proteína ligante de FK506 de 51 kDa (FKBP51, do inglês *FK506-binding protein of 51*

kDa), e a pequena fosfoproteína p23 (Jääskeläinen *et al*., 2011). GRs são compostos por vários elementos estruturais conservados, incluindo um domínio de ligação C-terminal (que apresenta os resíduos necessários para a dimerização e transativação gênica hormônio-dependente), uma região contendo um sinal de localização nuclear, um domínio central de dedo de zinco (para a ligação ao DNA), e uma região variável importante na região N-terminal (Figura 3).

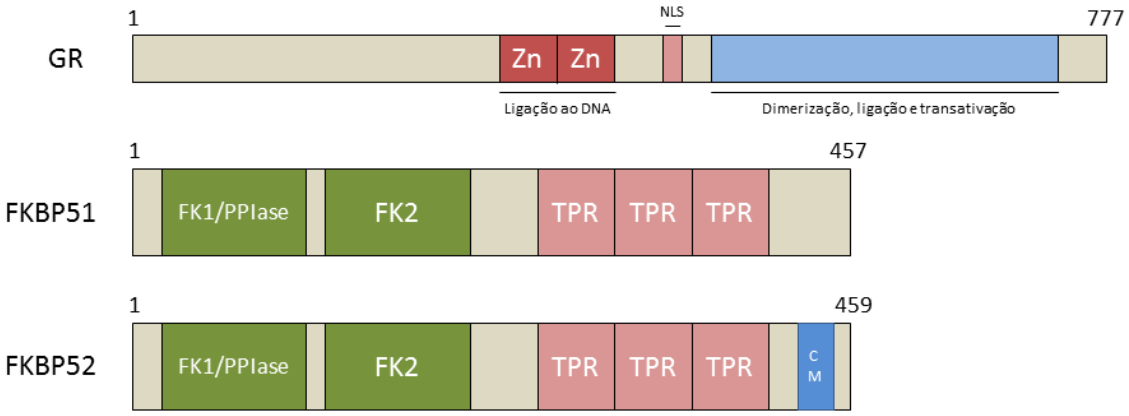

Figura 3. Estruturas do receptor de glicocorticóide (GR) e das proteínas ligantes de FK506 de 51 kDa (FKBP51) e 52 kDa (FKBP52). O GR apresenta um domínio C-terminal necessário para a dimerização, ligação e transativação do receptor, um sinal de localização nuclear (NLS, do inglês *nuclear localization signal*), um domínio de dedo de zinco para ligação ao DNA, e uma região N-terminal variável. As proteínas FKBP51 e FKBP52 apresentam ambas uma região C-terminal contendo domínios de repetição de tetratricopeptídeos (TPR) necessários para sua interação com a proteína de choque térmico de 90 kDa (HSP90), assim como dois domínios de ligação ao FK506 (FK1 e FK2), dos quais o FK1 apresenta atividade de peptidil-prolilisomerase (PPIase). A proteína FKBP52 ainda apresenta um domínio de ligação a cálcio-calmodulina (CM), o qual não está presente na FKBP51.

A interação entre o GR e a HSP90 é necessária para manter o seu domínio C-terminal em uma conformação favorável para a ligação de glicocorticóides. A ligação hormonal libera o GR de suas interações com o complexo inibitório e induz uma mudança conformacional na proteína, resultando na exposição do seu sinal de localização nuclear. Após a ativação, o GR transloca-se para o núcleo e se liga ao DNA em uma forma dimérica, ativando a transcrição de genes específicos (Jääskeläinen *et al*., 2011).

Dentre as cochaperonas do GR, a proteína FKBP51 pertence às imunofilinas, uma família de proteínas que se ligam a drogas imunosupressoras, como o macrolídeo FK506, a rapamicina e a ciclosporina A (Jääskeläinen *et al*., 2011). As maiores imunofilinas FKBP51 e FKBP52 (nomeadas de acordo com suas massas moleculares) são ubiquamente expressas e apresentam estruturas bem conservadas (Figura 3). Seus domínios C-terminais de repetição de tetratricopeptídeos (TPR)[2] são responsáveis por suas interações com as chaperonas HSP90 e a proteína de choque térmico de 70 kDa (HSP70, do inglês *heat shock protein of 70 kDa*), assim como com outras proteínas (Figura 3). As regiões N-terminais do FKBP51 e FKBP52 contêm dois domínios de ligação ao FK506 (FK), dos quais o mais N-terminal possui atividade de peptidil-prolilisomerase. Ele pode catalisar a conversão cis-trans de ligações Xaa-Pro, apresentando um importante papel no dobramento de proteínas (revisado em Jääskeläinen *et al*., 2011). O FKBP51 pode ser encontrado tanto no citoplasma quanto no núcleo [e, como recentemente descrito, também na mitocôndria (Gallo *et al*., 2011)], podendo, portanto, modular vários passos da sinalização esteroidal. De uma forma geral, o FKBP51 atenua as atividades do GR (Figura 4), do receptor de progesterona e do MR.

[2] A repetição de tetratricopeptídeos (TPR) é um motivo protéico de interação proteína-proteína encontrado em múltiplas cópias em diferentes proteínas. Dados estruturais sugerem que este motivo contém duas alfa-hélices antiparalelas que formam uma estrutura helicoidal com um canal anfipático propício para acomodar a região complementar da proteína alvo. A maioria das proteínas contendo TPRs estão associadas a complexos multiprotéicos, e há evidências indicando que os motivos TPR são importantes para o funcionamento de chaperonas, ciclo celular, transcrição, além do transporte de proteínas (Blatch GL & Lässl, 1999).

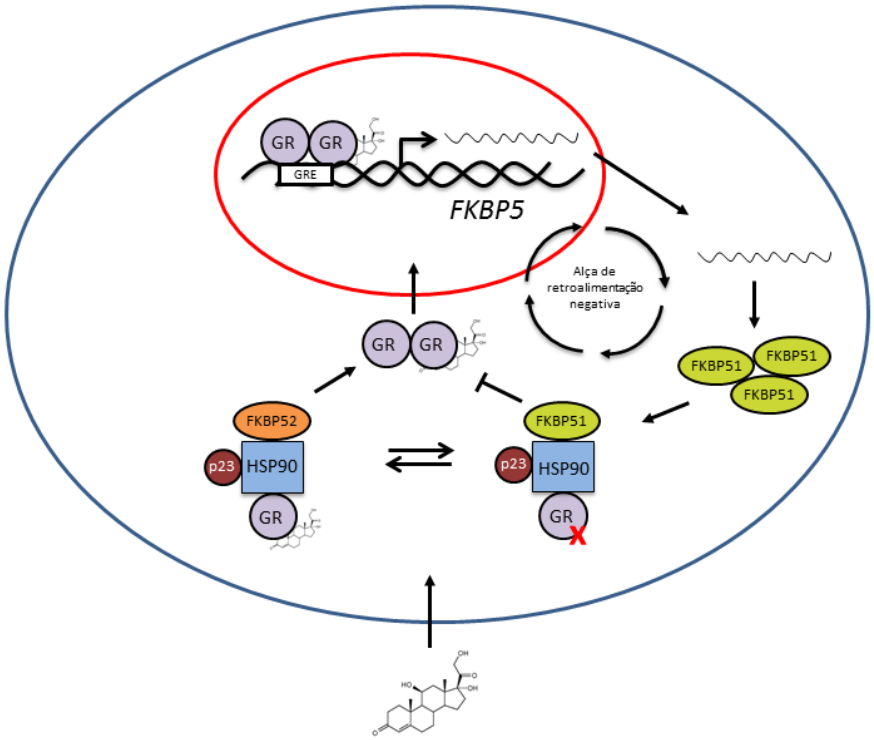

Figura 4. **Modelo esquemático para a regulação do receptor de glicocorticóide (GR) pelas proteínas de ligação ao FK506 de 51 kDa (FKBP51) e de 52 kDa (FKBP52).** O GR não-ligado forma um complexo com chaperonas (dímero de proteínas de choque térmico de 90 kDa (HSP90), p23, e FKBP51 ou FKBP52). A ligação com glicocorticóides transfere o equilíbrio do complexo para a ligação com o FKBP52, o que permite a dimerização do GR e a sua translocação nuclear. Em seguida, o GR ativa a transcrição de *FKBP5*, que pode então se ligar ao complexo citoplasmático e inibir a ação do glicocorticóide (uma alça de retroalimentação negativa intracelular). Modificado de Jääskeläinen *et al.*, 2011.

Devido à sua ação sobre o GR e a resposta ao estresse, vários estudos tem se focado nos efeitos de FKBPs sobre parâmetros neurais. Seu principal ligante, o macrolídeo FK506, apresenta ações neuroregenerativas e neuroprotetoras e é capaz de estimular o crescimento de neuritos *in vitro* (Quintá *et al*., 2010). Interessantemente, esses efeitos são intimamente modulados por FKBPs, de forma que o crescimento de neuritos é favorecido pela superexpressão de FKBP52 ou pelo silenciamento de FKBP51, e é prejudicado pelo silenciamento de FKBP52 ou pela superexpressão de FKBP51, indicando que um balanço entre essas duas proteínas tem um papel importante nos mecanismos iniciais de diferenciação neuronal (Quintá *et al*., 2010). Isto é particularmente interessante para a área de transtornos psiquiátricos, visto que a neurogênese adulta pode atenuar o efeito do estresse (Snyder *et al*.,

2011) e, dessa forma, ajudar um indivíduo a lidar com eventuais estressores. Em suma, uma expressão aumentada de FKBP51 em pacientes com TB poderia não só levar à resistência aos glicocorticóides e disfunção do eixo HPA, como também prejudicar a neurogênese e consequentemente reduzir mecanismos de resiliência ao estresse.

A expressão gênica de *FKBP5* parece ser finamente regulada por mecanismos epigenéticos. De fato, em cérebro de camundongos, glicocorticóides aumentaram a expressão gênica de *Fkbp5* em algumas regiões, e a exposição crônica a glicocorticóides levou à desmetilação de algumas regiões intrônicas do gene *Fkbp5*, modulação esta que persistiu mesmo após um período sem exposição ao hormônio (Scharf *et al*., 2011; Lee *et al*., 2010). Interessantemente, uma das regiões desmetiladas é homóloga à região do *FKBP5* humano que circunda a principal região intrônica regulada por glicocorticóides (Paakinaho *et al*., 2010), o que sugere uma interação entre a metilação do DNA e a ação do GR na regulação desse gene. A persistência da metilação do DNA (i.e. o estado epigenético alterado) no gene de camundongos sugere que o *FKBP5* humano possa se tornar sensível a níveis cronicamente elevados de glicocorticóides ou estresse (Jääskeläinen *et al*., 2011). Este cenário combina perfeitamente com a teoria da carga alostática no TB anteriormente mencionada. Cabe ressaltar que a metilação do *Fkbp5* já foi considerada uma medida da "carga de glicocorticóides" (Lee *et al*., 2010), sendo possivelmente este um novo mediador dos mecanismos de carga alostática.

1.4 Vulnerabilidade e resiliência: familiares

Diferentes estudos tem evidenciado a importância clínica da agregação familiar do TB. Helenius e colaboradores (2013), por exemplo, investigaram a frequência com que o TB ocorre em famílias afetadas em comparação a famílias controles, e mostrou que os fatores familiares correspondem à cerca de 20% da variação no desfecho da doença. Ainda, um

estudo dinamarquês mostrou que indivíduos com um familiar de primeiro grau com TB tem um risco quase 14 vezes maior de desenvolver TB do que aqueles que não apresentam história familiar (Mortensen *et al*., 2003). No entanto, ainda há taxas de incidência discordantes na literatura, visto que alguns estudos com filhos de pais com TB mostram uma taxa de incidência de 14-50% para transtornos do espectro bipolar nos EUA (Chang *et al*., 2000; Duffy *et al*., 1998), enquanto um estudo holandês encontrou uma incidência de 2,8% (Wals *et al*., 2004). Evidências ainda sugerem que, mesmo antes de desenvolverem qualquer diagnóstico, filhos de pais com TB mostram maiores taxas de sintomas psiquiátricos quando comparados com filhos de pais controles (Hirshfeld-Becker *et al*., 2006).

Indivíduos com alto risco genético para o TB (como gêmeos, irmãos, filhos e familiares de primeiro e segundo graus) apresentam também uma série de alterações biológicas, como traços neuroanatômicos e mudanças em marcadores bioquímicos (Frangou, 2012; Fusar-Poli *et al*., 2012). Um estudo de desafio emocional, por exemplo, reforçou a ocorrência de diferenças em grupos de irmãos de pacientes (diminuição no córtex frontal medial em pacientes e um aumento da mesma região em irmãos), sugerindo a presença de respostas do tipo compensatória nesses grupos de alto risco (Krüger *et al*., 2006). Além disso, achados de alterações cognitivas em familiares de primeiro grau em comparação a controles, principalmente em função executiva e em memória verbal (Arts *et al*., 2008), parecem estar de acordo com os correlatos neurológicos já descritos. Neste sentido, filhos de pacientes com TB apresentaram alterações no funcionamento do eixo HPA, exibindo uma secreção aumentada de cortisol ao longo do dia e uma maior vulnerabilidade para o desenvolvimento de transtornos afetivos (Ellenbogen *et al*., 2010). Um melhor entendimento do risco familiar para o TB através da identificação de alterações em familiares poderá auxiliar na identificação de endofenótipos do TB e na avaliação de como o risco familiar é clinicamente manifestado na sintomatologia e na qualidade de vida dos pacientes (Antypa & Serretti, 2014).

1.5 Resiliência celular[3]

Não só a resiliência ao estresse em termos fisiológicos (envolvendo a ativação do eixo HPA, por exemplo) parece estar prejudicada em pacientes (e, como sugerido por estudos preliminares, em indivíduos de alto risco), como também a resiliência celular. Especificamente, a resiliência celular pode ser definida como a habilidade de uma célula em lidar com estímulos danosos e sobreviver a estes que, em outras circunstâncias, levariam à sua morte (Hunsberger *et al.*, 2009). Mecanismos de resiliência são constantemente ativados por células em resposta a estímulos internos [como disfunção mitocondrial e estresse do retículo endoplasmático (RE)] e externos (como a ativação de certos receptores de membrana e excitotoxicidade) (Fulda *et al.*, 2010). Além disso, alguns tipos celulares podem modular o seu comportamento e conexões com outras células em resposta a estímulos, um processo conhecido como plasticidade celular. Especificamente, a chamada "neuroplasticidade" é responsável pela habilidade que neurônios apresentam de recuperar conexões axônicas e dendríticas sob circunstâncias específicas, assim como de formar novas conexões em resposta a estímulos ambientais, como o estresse. Mecanismos de neuroplasticidade são responsáveis por manter redes neurais e funções cognitivas, incluindo a memória e funcionamento. Como resultado, prejuízos em neuroplasticidade podem induzir uma diminuição de estruturas cerebrais devido à redução na conexão entre células ou na complexidade da rede de células cerebrais (Manji *et al.*, 2000).

A resposta celular a uma devida condição sempre vai depender do tipo e do nível do estímulo, e é a capacidade adaptativa da célula que, em última instância, determinará o seu destino. Diferentes respostas podem ser ativadas para lidar com o estresse, como a resposta das proteínas de choque térmico, a resposta a proteínas mal dobradas, a resposta ao dano ao

[3] Uma revisão detalhada sobre resiliência celular e suas implicações clínicas no TB pode ser encontrada no capítulo de livro intitulado "*Loss of cellular resilience and neurodegeneration and their impact on the treatment of bipolar disorder*", de Fries GR & Kapczinski F, anexo 3 desta tese.

DNA, e a resposta ao estresse oxidativo. Se o estresse permanecer não resolvido, a célula eventualmente ativa vias de morte celular (Fulda *et al*., 2010). Neste caso, esta pode ocorrer de diferentes maneiras: apoptose, necrose, ou morte celular autofágica (Fulda *et al*., 2010), as quais se diferenciam, sobretudo, em aspectos morfológicos e nas suas vias de ativação.

Uma série de estudos tem mostrado prejuízos em resiliência celular e marcadores de morte celular em pacientes com TB. Pacientes apresentaram níveis diminuídos de N-acetil-aspartato (NAA, marcador de viabilidade e integridade neuronal) no hipocampo e no córtex pré-frontal (Sassi *et al*., 2005), o que já mostrou ser revertido com o tratamento com lítio (Bertolino *et al*., 1999; Winsberg *et al*., 2000). Além disso, alterações em fatores apoptóticos já foram observadas em pacientes com TB, incluindo um aumento na atividade apoptótica do soro (Politi *et al*., 2008), dano ao DNA em sangue periférico (Andreazza *et al*., 2007), disfunção mitocondrial (Shao *et al*., 2008) e expressão diferenciada de moléculas envolvidas em sobrevivência celular (Herberth *et al*., 2011). Como consequência, células do neuroepitélio olfatório de pacientes com TB já foram descritas como sendo altamente vulneráveis à morte celular (McCurdy *et al*., 2006). Da mesma forma, células mononucleares de sangue periférico (PBMCs, do inglês *peripheral blood mononuclear cells*) de pacientes apresentaram uma menor viabilidade celular em resposta à indução de estresse do RE (Pfaffenseller *et al.*, 2014)[4].

1.6 Interação entre gene e ambiente: epigenética[5]

Uma série de evidências tem sugerido que a modulação da expressão gênica e da interação entre genes e ambiente podem ter papéis-chave na fisiopatologia dos transtornos de humor (Klengel & Binder, 2013; Petronis, 2003; Pregelj, 2011). Isso indica, de forma

[4] Artigo pertencente ao anexo 6 desta tese (Pfaffenseller B, Wollenhaupt-Aguiar B, Fries GR, Colpo GD, Burque RK, Bristot G, Ferrari P, Ceresér KM, Rosa AR, Klamt F, Kapczinski F. *Impaired endoplasmic reticulum stress response in bipolar disorder: cellular evidence of illness progression.* Int J Neuropsychopharmacol. 2014. No prelo).

[5] O conteúdo desta seção é baseado no capítulo "*Neuroprogression as the biological underpinning of staging in bipolar disorder*", de autoria de Fries GR, Magalhães PVS e Berk M, em consideração para publicação no livro "*Neuroprogression and Staging in Bipolar Disorder*", da Oxford University Press.

indireta, a importância da modulação da estrutura da cromatina por mecanismos epigenéticos no TB e no transtorno depressivo maior, visto que esta é a principal forma pela qual fatores ambientais podem modular a atividade gênica. Estudos tem mostrado também que certos eventos no início da vida, como traumas e abuso infantil, podem induzir alterações em marcadores epigenéticos de forma permanente em genes específicos, o que pode interagir com alterações genéticas (como polimorfismos) e, em última instância, levar a um fenótipo patológico (Szyf, 2013). O mecanismo epigenético mais estudado é a metilação do DNA, que pode inibir a transcrição gênica por induzir a formação de heterocromatina ao redor do promotor de um gene. A metilação do DNA é o marcador epigenético mais estável, e vários estudos pré-clínicos já mostraram que a metilação induzida por eventos precoce no desenvolvimento pode permanecer até a vida adulta em animais (Champagne, 2013).

Baseando-se na teoria da carga alostática, parece razoável supor que estressores cumulativos (por exemplo, episódios de humor recorrentes) podem agir como estímulos ambientais que induzem alterações específicas em marcadores epigenéticos, consequentemente interferindo com a habilidade de um paciente de responder e lidar com um novo estressor. Esses marcadores poderiam ser, então, responsáveis por diferenças na resiliência entre pacientes, possivelmente esclarecendo mecanismos pelos quais alguns deles acabam desenvolvendo graves prejuízos funcionais após alguns episódios, enquanto outros são capazes de superar os efeitos do estresse e lidar adequadamente com o mesmo.

De fato, alterações na metilação de vários genes já foram descritas em pacientes com TB e transtorno depressivo maior. A relevância da epigenética na patofisiologia destes transtornos também é reforçada pelos já conhecidos mecanismos de ação dos estabilizadores de humor e antidepressivos, os quais são capazes de modular várias enzimas e vias associadas com o remodelamento da cromatina. Por exemplo, o valproato de sódio pode inibir a enzima histona desacetilase, consequentemente induzindo a formação de eucromatina ao redor de

promotores específicos (Machado-Vieira *et al*., 2011; Monti *et al*., 2009). Além disso, esta droga já mostrou ser capaz de induzir a desmetilação do DNA em extratos nucleares de cérebro de camundongos adultos (Dong *et al*., 2010), sugerindo um novo mecanismo de alteração epigenética. Antidepressivos também já mostraram ser capazes de reverter alterações em histonas induzidas por um paradigma de estresse crônico em ratos (Tsankova *et al*., 2006), o que mostra a habilidade dessas drogas psicotrópicas em reverter alterações induzidas pelo ambiente.

1.6.1 Metilação do DNA

A metilação do DNA consiste na adição de um grupamento metil ao carbono 5 de citosinas localizadas em dinucleotídeos CpG (citosinas seguidas de guaninas no sentido 5'→ 3' em uma mesma fita de DNA). De forma geral, mamíferos apresentam um metiloma global consistindo principalmente de CpGs metilados (ou seja, com uma grande proporção de 5-metilcitosinas, 5mC). Estes altos níveis de metilação genômica são pontuados por pequenas regiões de DNA não metilado, muitas das quais correspondem a regiões de alto conteúdo de CpGs conhecidas como "ilhas CpG" (Reddington *et al*., 2013). Embora a maioria das ilhas CpG seja encontrada na sua forma não metilada na maioria dos tecidos, uma parte delas encontra-se metilada de maneira tecido-específica (Reddington *et al*., 2013).

Como via de regra, quando uma região próxima ao local de início da transcrição em um promotor contém uma grande quantidade de dinucleotídeos CpG, a presença de altos níveis de 5mC está geralmente associada à inatividade gênica. Neste caso, a função da metilação do DNA em promotores gênicos é atribuída principalmente a dois mecanismos: a) o efeito negativo que a metilação apresenta sobre a interação entre o DNA e certos fatores de transcrição; e b) sua capacidade de atrair proteínas ligadoras ao DNA metilado (Weber *et al*., 2007). Esta função canônica da metilação do DNA (repressão gênica) é essencial, por

exemplo, para o *imprinting* de genes específicos e para o processo de inativação do cromossomo X (Reddington *et al.*, 2013).

Além de promotores, a metilação do DNA pode ocorrer também em outras regiões localizadas dentro de genes, assim como em elementos distais regulatórios e em regiões do DNA cujas funções ainda não são conhecidas (Reddington *et al*., 2013). Embora o papel exato da metilação em regiões no "corpo" do gene (como em íntrons e exons) ainda não seja conhecido, duas funções potenciais já foram propostas: a) regular o processamento co-transcricional do RNA, como o *splicing* alternativo (Shukla *et al*., 2011); e b) prevenir o início da transcrição gênica a partir de regiões no corpo do gene (Brown *et al*., 2012). Coletivamente, essas observações aumentam as perspectivas para o quanto a metilação do DNA contribui para a regulação gênica e para a identidade celular. Em outras palavras, elas demonstram que a metilação do DNA pode ter funções regulatórias importantes que vão além do seu papel repressor em promotores gênicos, e, portanto, pode contribuir para a regulação do genoma de uma maneira multifatorial (Reddington *et al*., 2013).

1.7 Justificativa

O entendimento das bases biológicas dos transtornos de humor, embora em constante avanço científico, segue sendo insuficiente para o desenvolvimento de tratamentos altamente eficazes e específicos para pacientes. Isso se deve provavelmente a uma série de fatores, incluindo o fato de que pacientes com o mesmo diagnóstico apresentam grande heterogeneidade e, portanto, não respondem de maneira similar aos esquemas de tratamento. Além disso, diferentes estudos sugerem que estes transtornos são, além de multifatoriais (cuja gênese é determinada pela interação entre fatores genéticos e ambientais, até hoje ainda não esclarecidos), multisistêmicos, de forma que não só o sistema nervoso central está comprometido, mas também outros sistemas fisiológicos, incluindo o endócrino, imune,

cardiovascular, entre outros. Como consequência, pacientes com TB e transtorno depressivo maior apresentam uma série de alterações em marcadores periféricos consistentes com um perfil pró-inflamatório, aumento de estresse oxidativo, diminuição de fatores tróficos e, consequentemente, uma toxicidade sistêmica significativa (Kapczinski *et al*., 2010). Essa toxicidade pode, por si só, induzir alterações nos mesmos sistemas que inicialmente colaboraram para o seu desenvolvimento (por amplificar alterações inflamatórias e diminuir a resiliência celular, por exemplo), o que pode, em última instância, prejudicar ainda mais o tratamento dos pacientes e piorar significativamente o seu prognóstico.

Neste sentido, um melhor entendimento dos mecanismos responsáveis por tais alterações sistêmicas (como, por exemplo, os altos níveis de cortisol, o prejuízo na resiliência celular, e as alterações epigenéticas encontradas nesses pacientes) poderá servir como base para a identificação de populações mais homogêneas de pacientes e, portanto, para o desenvolvimento de tratamentos mais eficazes.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo geral desta tese foi avaliar parâmetros relacionados à resiliência celular e ao estresse, assim como seus mecanismos epigenéticos associados, em pacientes com transtornos de humor.

2.2 Objetivos específicos

- Revisar e discutir os mecanismos biológicos associados à progressão do TB a partir de dados da literatura;

- Avaliar parâmetros de resiliência e morte celular em células periféricas de pacientes com TB;

- Caracterizar a atividade do eixo do estresse em pacientes com TB, assim como os mecanismos moleculares associados envolvendo o GR e o FKBP51;

- Analisar mecanismos moleculares pelos quais as proteínas associadas ao estresse FKBP51 e FKBP52 podem induzir alterações epigenéticas em nível de metilação do DNA;

- Avaliar mecanismos epigenéticos associados à resposta clínica ao tratamento em pacientes com transtorno depressivo maior.

PARTE II

Artigos científicos

3. ARTIGOS CIENTÍFICOS

3.1 CAPÍTULO 1

Staging and Neuroprogression in Bipolar Disorder

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BIPOLAR DISORDERS (MA FRYE, SECTION EDITOR)

Staging and Neuroprogression in Bipolar Disorder

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Abstract The apparently progressive nature of a considerable proportion of cases of bipolar disorder (BD) has been acknowledged in recently proposed clinical staging models. This has been part of an attempt to facilitate and refine diagnosis, treatment selection, and establish a prognosis. The study of the progressive nature of some cases of BD has given raise to the hypothesis of neuroprogression, which postulates that different stages of BD are associated with distinct neurobiological underpinnings. Given that BD may be intimately associated with chronic stress response and coping mechanisms over the course of illness, we propose that cellular resilience mechanisms may play a key role in the neuroprogression in BD. In the present study, we review neuroanatomical evidence of the progression that occurs in many cases of BD, as well as cellular resilience mechanisms and peripheral biomarkers associated with distinct stages of this disorder. In summary, cellular resilience mechanisms seem to be less efficient at later stages of BD, especially mitochondrial and endoplasmic reticulum-related responses to stress. These insights may help in developing staging models of BD, with a special emphasis on the search for biomarkers associated with illness progression.

Keywords Bipolar disorder · BD · Staging · Clinical staging model · Neuroprogression · Cellular resilience · Neuroplasticity · Biomarkers · Allostatic load · Treatment · Remission · Psychiatry

Introduction

A growing body of evidence has suggested that bipolar disorder (BD) may present a progressive course [1•, 2••, 3]. As reviewed elsewhere [4•], the duration of interepisode intervals seems to be reduced with the recurrence of acute episodes [5, 6], and progression of BD may also be associated with several unfavorable clinical outcomes: lower responsiveness to treatment, especially with lithium and cognitive behavioral therapy [7–9], worse treatment outcome of family psychoeducation

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[10], higher rates of comorbidity [11], functional impairment [12], increased cognitive dysfunction [1•, 13, 14], and an augmented risk of suicide [15] and hospitalization [16].

Different hypotheses have been proposed to explain the mechanisms underlying the recurrence of mood episodes and the progressive nature of a significant percentage of cases of BD. Post (1992) [17] suggested that multiple episodes may lead to permanent alterations in neuronal activity, possibly resulting in a greater liability to relapse and a poorer response to medication [18]. In this same vein, BD progression has been compared to stress sensitization models and to electrophysiological kindling of seizures, used in providing a possible explanation for the increased vulnerability to episode occurrence and the transition from triggered to spontaneous episodes at later stages of illness [1•]. Moreover, an allostatic load theory has been proposed to account for the cumulative damage associated with BD [3, 19]. According to that theory, the chronicity involved in activating allostatic mechanisms to restore parameters after stressful events leads to a physiological wear-and-tear, which has been described as 'allostatic load' [20]. These effects are normally observed during aging and exposure to chronic stress, and seem to play a role in adaptive functions. In contrast, the progressive neural and physical dysfunction resulting from multiple mood episodes in BD could be seen as affecting allostatic responses, leading to an allostatic overload in a non-adaptive way. More recently, the allostatic load paradigm has been incorporated into a new concept of 'neuroprogression' [2••], focused on identifying the pathways associated with oxidative stress, inflammation, and neurotrophin expression, which may provide explanations for the progressive nature of BD. Neuroprogression will be used in this review as the pathological rewiring of the brain that takes place when a clinical and cognitive deterioration occurs in the context of the progression of psychiatric disorders.

Several neurobiological studies have linked chronic social stress with distinct biological features possibly underlying BD neuroprogression and its consequences. Therefore, this review aims to discuss neuroanatomical and neurobiological and genetic findings associated with BD staging and progression, emphasizing the potential role of dysfunctions in cellular resilience mechanisms as one of the pathways to neuroprogression. The clinical implications of these studies are discussed in light of the concept of clinical staging in BD.

Neuroanatomical Changes in BD

Different stages of BD have been associated with specific brain abnormalities related to cognitive and emotional functions. Due to the heterogeneity of groups and methodologies used in the studies available, it remains unclear whether changes predate the illness or are related to the consequences of illness progression [21].

Reductions of the gray [22–24] and white matter [23–25] in the prefrontal cortex have been described in first manic episode patients, becoming more pronounced after multiple episodes. Consistent results have been also obtained in the anterior cingulate cortex, pointing to a reduction of its volume [26] and also of gray matter volume [27] in BD patients, regardless of their stages. In particular, the subgenual prefrontal cortex has attracted special interest, because of its mood-regulating role, integrating cognitive and emotional information [28]. A study involving patients with a family history of BD has reported reductions in the volume, blood flow and glucose metabolism of this region [28]. A reduced number of glial cells at the same area has also been reported in mood disorders' patients [25]. This latter finding is especially interesting because the subgenual prefrontal cortex has connections with the amygdala, hypothalamus and midbrain periaqueductal structures that are related to emotional behavior and stress responses [25]. As a result, changes in the prefrontal cortex may partly explain the impairment of executive functions [13] and the emotional instability observed in patients with BD.

The limbic system is another neuroanatomical area of interest in BD due to its relationship with emotional responses. Some studies have suggested that the amygdala tends to increase in volume with the progression of illness [29], although results obtained at initial stages have shown reduced amygdala volumes [30]. Interestingly, the hippocampus appears to increase in volume in early stage BD, but progressively decreases with illness duration and number of episodes experienced by patients, ultimately becoming smaller than the hippocampus of healthy controls [31, 32]. Some studies have also shown that V2 and V3 regions of the cerebellar vermis are reduced in BD patients who have experienced multiple episodes when compared to first-episode patients [33].

The basal ganglia in general and the striatum in particular have also been shown to present altered shape [34] and volume [29, 35] in BD patients. These structural changes seem to take place at the onset of BD and to remain at later stages [35]. The corpus callosum also seems to be affected from illness onset. Studies involving first-episode bipolar patients [36], bipolar adolescents [37], and bipolar adults [38] have reported abnormalities in the white matter of the corpus callosum, possibly indicating altered myelination and ultimately leading to problems in interhemispheric communication in BD patients [38].

Finally, other studies have shown that total brain gray matter is reduced in BD patients [39, 40], and that the ventricles increase in size with illness duration [41]. As a

consequence, total brain volume is smaller in multiple-episode patients compared to first-episode patients and control subjects [40].

In sum, it seems that neuroanatomical alterations already present at the onset of BD are aggravated by the ocurrence of further episodes. However, other abnormalities seem to appear only with illness progression and may be characteristic of later stages of BD. Further studies are required to clarify these issues. Nonetheless, the findings above, along with additional evidence coming from neuroimaging and post-mortem brain studies that associate BD with structural anatomical changes in the size, number, and density of neurons and glia in specific brain areas [21, 42], suggest that BD neuroprogression may be associated with impairments of cellular resilience and neuroplasticity mechanisms.

Cellular Resilience

Considering cellular resilience as the ability of cells to adapt to different insults or stress episodes, an impaired resilience at the cellular level may be one possible explanation for the increased vulnerability of BD patients when exposed to stressful environmental conditions.

Evidence suggests that abnormalities in several intracellular signaling pathways may affect neuroplasticity and cellular resilience in BD. This would include neurotransmitters, glutamatergic and glucocorticoid signaling, neurotrophic cascades, anti-apoptotic factors, cell survival pathways, and calcium signaling, among others [43–45]. For instance, elevated and calcium levels have been found in peripheral blood cells of BD patients [46], and an increased vulnerability to cell death in cells of the olfactory neuroepithelium has also been observed [47]. Neuroimaging studies reporting decreased levels of N-acetylaspartate in the living brain [48] also support the hypothesis that BD patients present impaired neuronal viability and function, possibly causing alterations in cell number and density and changing gray matter volumes. The mechanisms leading to this reduced resilience are most likely involve specific cell signaling pathways and organelles typically responsible for maintaining cell homeostasis, such as the ER [49, 50] and the mitochondrion [51••]. *In vitro* and animal model studies have reported that chronic stress and chronic exposure to glucocorticoids can induce mitochondrial dysfunction, causing reductions in oxygen consumption, mitochondrial membrane potential and calcium holding capacity, ultimately leading to apoptosis [52, 53]. Of note, BD patients present mitochondrial dysfunction and an impaired hypothalamus-pituitary-adrenal (HPA) axis [54], as evidenced by decreased levels of high-energy phosphates and mitochondrial respiration, alterations in mitochondrial morphology, and downregulation of proteins involved in mitochondrial metabolism [51••]. These findings may be related to an impaired regulation of Ca^{2+} cascades [46], as apoptosis in BD is manifested by an increased expression of apoptotic genes [55]. No study has assessed mitochondrial functions in early- vs. late-stage BD patients, but the evidence of impaired mitochondrial functioning strongly supports a key role of this organelle in synaptic functioning, thus contributing to the atrophic changes underlying BD neuroprogression.

It remains unclear whether such alterations in cellular resilience pathways occur as a result of developmental abnormalities, illness progression toxicity of mood episodes, or due to treatment (or the lack of it). Few studies have examined resilience at the cell level in BD to determine whether illness duration and number of episodes may affect this process. One of such studies reported that the levels of synaptic subcellular markers of neuroplasticity were not only reduced in the anterior cingulate cortex of BD patients, but also negatively correlated with illness duration [56], suggesting progressive alterations in synaptic plasticity in BD. Another recent study showed that lymphocytes from early-stage-patients responded better to *in vitro*-induced ER stress (with induction of glucose-regulated protein of 78 kDa (GRP78) and phosphorylated eukaryotic initiation factor 2 (eIF2α-P), both essential in activating ER stress response signaling) when compared to patients at late stages of BD [57]. These findings suggests that protective cell mechanisms may become less efficient at more advanced stages of BD.

The loss of cell plasticity in BD is thought to be the result of a deficiency in trophic support or survival factors along with an impaired regulation of intracellular signaling cascades [58]. In fact, brain-derived neurotrophic factor (BDNF) levels have been shown to be reduced in postmortem brain from BD patients when compared to controls [59], and alterations in peripheral neurotrophic factors have been reported in BD during acute episodes, e.g., BDNF [60], neurotrophin-3 and 4/5 (NT-3 and NT-4/5) [61, 62], and glial-derived neurotrophic factor (GDNF) [63]. Furthermore, increased peripheral oxidative stress and higher levels of inflammatory markers have also been observed in BD patients [64]. As discussed below, these alterations in biochemical markers may be related to different stages of the disorder [65, 66].

Neuronal atrophy and reduced cellular resilience make certain neurons more vulnerable to insults and may be related to stress and chronic activation of the HPA axis [67] and/or a decreased expression of BDNF in some brain regions [67]. BDNF, as well as other neurotrophic factors, are necessary for neuronal survival and function, and are involved in neurogenesis and brain maturation during neurodevelopment. In adults, BDNF activates important intracellular pathways implicated in synaptic

plasticity and dendritic growth, especially in the cortex and hippocampus [68]. Therefore, reduced BDNF levels in late-stage BD may indicate decreased neuronal viability [66] resulting from illness progression.

Additional evidence comes from studies showing the effects of mood stabilizers on signaling pathways involved in the regulation of cell plasticity [69], such as mitogen-activated protein kinases, cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein, BDNF, and B-cell lymphoma 2 (Bcl-2) protein. Those studies have suggested long-term benefits associated with mood stabilizers as a result of their neurotrophic effects. In this same vein, the increased vulnerability to stress associated with disease progression may be explained by the progressive loss of neuronal resilience. These cell cascades are therefore considered important targets in the treatment of BD. Likewise, impairments in signal transduction pathways suggest that effective treatments will need to provide both trophic and neurochemical support, mainly in patients refractory to conventional medications. Because cell changes may progress over the course of BD, avoiding impairments by enhancing resilience mechanisms could probably delay or prevent illness progression. Further studies should focus on identifying resilience and susceptibility factors, and also on elucidating how such factors could contribute to treatment and to improve both staging of BD and interventions aimed at earlier stages of the disorder. In this sense, if we consider that these impairments provoke changes to different peripherally detectable molecules, the use of biomarkers could be a useful tool in optimizing clinical staging.

The use of biomarkers as a potential tool for staging BD

An ideal biomarker assay for BD staging should be sensitive, specific, cost-effective, fast, easily detected, and robust against inter-operator and inter-institutional variability [70]. It should also be clinically more relevant than the information already available at the time of diagnosis [70]. In addition, it is reasonable to consider that biomarkers for BD staging should be used after the stabilization of an acute episode (i.e., during euthymia), in order to avoid bias from episode-induced alterations. To the best of our knowledge, none of the biomarkers studied so far has adequately fulfilled these characteristics.

Therefore, we decided to review the main peripheral biomarkers in euthymic BD patients, including neurotrophins, inflammatory markers, oxidative stress, and telomere length. Given the lack of studies designed to evaluate these biomarkers in relation to the clinical staging of BD findings were divided into in early-stage alterations (stages I or II) and late-stage alterations (stages III or IV). The main findings are reported in Table 1.

Neurotrophins

Alterations in neurotrophic factors are well documented in BD [59], especially in association with acute mood symptomatology. During euthymia, decreased BDNF levels have been reported at late stages of BD, but not at early stages [66]. Recently, some studies have reported increased plasma levels of BDNF in patients with long-term BD [71, 72]. However, meta-analytic studies seem to agree that BDNF levels are reduced during mood episodes but not during euthymia, suggesting a decrease of this protein with age and length of illness [60, 73]. Another possible target is neurotrophin 4/5, which was found to be increased in euthymic BD patients at late stages [62]. In summary, along with the evidence of reduced neuroplasticity resulting from illness progression, the measure of peripheral neurotrophic factors may be useful to determine the stage of BD.

Inflammatory Markers

We found only two studies assessing inflammatory markers at early stages of BD, which reported increased serum levels of tumor necrosis factor alpha (TNF-alpha) [66, 74], as well as of interleukin-6 (IL-6) and interleukin-10 (IL-10) [66], in patients when compared to controls. Moreover, several studies report a pro-inflammatory imbalance at late stages of BD. The main inflammatory markers which seem to be increased are IL-6 [66, 75], TNF-alpha [66, 76, 77], high sensitive C-reactive protein (hs-CRP) [75, 76], IL-10 [78], and IL-1β [79]. Recently, increased plasma levels of CCL11, CCL24, and CXCL10, and decreased plasma levels of CXCL8 have been reported in late BD when compared to healthy controls [80]. In a peripheral profiling analysis for BD, approximately 60 differentially expressed molecules involved predominantly in cell death/survival pathways were identified. In peripheral blood mononuclear cells, this was manifested in cytoskeletal and stress response-associated proteins, whereas most serum analyses were associated with inflammatory response [81]. Therefore, the imbalance toward a pro-inflammatory state seems to be prominent at late stages of BD. More studies are warranted to further assess inflammatory markers in early stages of BD.

Oxidative Stress Markers

Many lines of evidence link BD with a fundamental abnormality in oxidative energy metabolism [82]. In early stages, increased lactate levels in the cerebrospinal fluid of patients possibly indicate increased extra-mitochondrial, anaerobic glucose metabolism, which is consistent with

Table 1 Peripheral biomarkers in early and late-stage euthymic BD patients

	Early	Late
Neurotrophins	-	⇓BDNF [66] ⇑NT-4/5 [62]
Inflammatory markers	⇑IL-6 [66] ⇑IL-10 [66] ⇑TNF-alpha [66, 74]	⇑IL-6 [66, 75] ⇑TNF-alpha [66, 76] ⇑IL-10 [78] ⇑hs-CRP [75, 76] ⇑IL-1β [79] ⇑CCL11, CCL24, CXCL10 [80] ⇓CXCL8 [80]
Oxidative stress	⇑3-Nitrotyrosine [65] ⇑PCC [85]	⇑Glutathione reductase [65] ⇑Glutathione S-transferase [65] ⇑3-Nitrotyrosine [65] ⇑TBARS [86] ⇑NO [86] ⇑Lactate [83] ⇑Total oxidants status [88]
Telomere length	-	Shorter telomeres [89, 90]

the impaired mitochondrial metabolism observed in some patients with schizophrenia and BD [83]. Impaired mitochondrial metabolism could lead to excess free radicals, causing an imbalance between oxidants and antioxidant mechanisms [84]. Two studies point to oxidative alterations in proteins in early BD, such as increased 3-nitrotyrosine [65] and protein carbonyl content [85]; some of these alterations seem to be maintained at late stages [65]. Nonetheless, the main findings have been reported for late stages of BD. Increased levels of thiobarbituric acid-reactive substances (TBARS) [86, 87], glutathione reductase, glutathione S-transferase [65], nitric oxide [86], and total oxidant status [88] point toward an increase in oxidative stress along with the progression of BD.

Telomere Length

Telomere length has been reported to be significantly shorter in patients with mood disorders, corresponding to as much as 10 years of accelerated aging when compared to controls [89]. Moreover, the load of short telomeres was found to be increased in patients with BD type II compared to healthy controls, possibly representing 13 years of accelerated aging. In this study, the authors found that the load of short telomeres and mean telomere length were associated with lifetime number of depressive episodes, but not with illness duration. Depressive episode-related stress may accelerate telomere shortening and aging. Longitudinal studies are needed to fully clarify the role of telomere shortening and its relationship with clinical variables in BD [90].

Clinical Staging Models

Different clinical staging models have been proposed for BD [91–93] (Table 2). Their common feature is placing the illness in a continuum progressing from a latent or asymptomatic form (stage 0 or latent) to a chronic, unremitting presentation (stage IV or unremitting). That is to say researchers agree that there is a great clinical need of selecting treatment interventions that are able to match patients' illnesses in terms of natural course, severity and underlying biology. This is the basis of staging models [93]. Effective staging methods should be able to predict what treatments should be used according to illness characteristics, and this would benefit the patient in terms of efficacy and tolerability. Nonetheless, at this point staging models proposed for BD specifically differ regarding emphasis on mood symptomatology and patterns of recurrence, functional disability and cognitive decline.

Simply using the total number of previous episodes, Berk and colleagues have been able to demonstrate the potential of clinical staging [4•]. When people with BD have had over ten previous episodes, for instance, they tend to have a more treatment-resistant illness, and their risk of relapse is much higher when compared to people with fewer than ten episodes [4•]. Furthermore, an analysis using data from STEP-BD shows that people with more than ten episodes tend to have worse outcomes across the board, having worse longitudinal functioning and quality-of-life measures in addition to traditional symptom outcomes [94]. In this same dataset, staging also predicted the likelihood of having a comorbid clinical condition, which is also in accordance with the notion of neuroprogression and staging [95]. While an

Table 2 Proposed clinical staging models for BD

Berk et al., 2007 [91]		Kapczinski et al., 2009 [92]		Reinares et al., 2012 [96]	
Stage	Description	Stage	Description	Stage	Description
0	at-risk, asymptomatic period, where a range of risk factors may be operating	Latent	mood and anxiety symptoms and increased risk for developing threshold BD; no cognitive impairment but polymorphisms that confer susceptibility		
1a 1b	mild or non-specific symptoms range of prodromal patterns	1	well-established periods of euthymia and absence of overt psychiatric morbidity between episodes, without cognitive impairment. High serum levels of tumor necrosis factor alpha (TNF-alpha) and 3-nitrotyrosine (3-NT) as biomarkers	Good outcome	low subsyndromal depressive symptoms, increased inhibitory control and estimated verbal intelligence
2	first threshold episode of illness, which can be of either polarity, but more commonly depressive	2	rapid cycling or current axis I or II comorbidities, transient impairment and high serum levels of TNF-alpha and 3-NT and low brain-derived neurotrophic factor (BDNF) as biomarkers		
3a 3b 3c	first relapse, subthreshold threshold illness subsequent pattern of remission and recurrences	3	clinically relevant pattern of cognitive and functioning deterioration as well as altered biomarkers (morphometric changes in brain may be persistent, high serum levels of TNF-alpha and 3-NT and low BDNF levels)	Poor outcome	residual depressive symptoms, increased episode density, low inhibitory control and estimated verbal intelligence
4	unremitting or treatment refractory course	4	cognitive and functioning impairment, unable to live autonomously and altered brain-scans and biomarkers (ventricular enlargement and/or white matter hyperintensities, high levels of TNF-alpha and 3-NT and low BDNF levels, increased levels of glutathione reductase and transferase)		

estimate of a quantity of episodes is possibly too simplistic to realistically reflect individual treatment needs, this line of research demonstrates how people with recent illness can differ from people with chronic illness in a number of features related to course and outcome. Taking into account interepisode functioning may be one viable alternative to create more realistic models [92, 96]. Table 2 demonstrates some features of one such model. What is hypothesized is that having a measure of disability and cognitive decline, for instance, would be a more direct measure of underlying neuroprogression that would be able to more accurately predict treatment needs [92]. This has been tested in a sample of people that underwent a course of psychoeducation. As predicted, being on a late stage predicted a worse outcome to this simple intervention [10], as would be predicted by the notion of staging [93].

Certainly, there is a large cross-over between current models. That is, possibly most people characterized to be in a late stage by chronicity would only be placed in a late stage using functioning measures. Nevertheless, the clinical implications of using these staging models need to be clarified, so models can be refined. Ideally, the utility of staging BD – as well as the utility of employing a specific model – should be demonstrated in randomized controlled trials. That would be the test of whether staging truly has heuristic potential for improving the treatment of BD.

Conclusions

Some cases of patients with BD seem to progress with the course of illness. As an attempt to explain the progression reported in BD without the kind of degeneration reported in patients with neurodegenerative diseases, we propose the hypothesis of neuroprogression. This progression has been acknowledged by different clinical staging systems, which all categorize the disorder in prodromal, early, and late stages of BD. In this vein, neuroprogression may help explain clinical, functional and cognitive alterations that occur with the course of illness. However, it is crucial to extend staging beyond clinical features to include biological correlates. In this light, a stage-specific treatment regimen might work not only to promote

regression to an earlier stage but also to prevent progression to more advanced stages, ultimately allowing the patient to obtain sustained full remission [97].

Based on available data, it is reasonable to assume that neuroprogression may occur along with a loss of cellular resilience. As discussed earlier, we consider that impairment of cellular resilience may play a key role in the pathological rewiring of specific brain areas, possibly accounting for the impaired resilience to stress observed in these patients. Chronic stress and increased allostatic load associated with neuroprogression may be implicated in cellular resilience impairments most likely by interfering with mitochondrial functions and trophic cell signaling pathways. In order to prevent these alterations, the identification of staging biomarkers becomes a priority. Although only longitudinal studies can confirm most of these alterations and their association with different stages of BD, the present findings strongly support the inclusion of biological underpinnings of BD neuroprogression in an effective and useful clinical staging model. Moreover, these data point toward new possible targets in the research for novel drugs potentially effective in treating later stages of BD, such as mitochondrial enhancers. Within this scenario, we believe that the modulation of mechanisms such as mitochondrial resilience and ER unfolded-protein response may allow for patients to effectively re-set stress-activated mechanisms, ultimately decreasing the allostatic load and possibly achieving sustained full remission.

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3.2 CAPÍTULO 2

Early apoptosis in peripheral blood mononuclear cells from patients with bipolar disorder

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Preliminary communication

Early apoptosis in peripheral blood mononuclear cells from patients with bipolar disorder

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ABSTRACT

Background: The pathophysiology of bipolar disorder (BD) includes several systemic alterations, such as inflammatory markers, oxidative stress, and DNA damage. Most of these parameters may be related to dysfunctions in cellular resilience mechanisms reported in patients, such as endoplasmic reticulum stress and mitochondrial damage. As a consequence, these impairments can ultimately lead to cell death. Therefore, the aim of this study was to assess cell death and viability in peripheral blood mononuclear cells (PBMCs) from patients with BD and controls.
Methods: Ten euthymic patients with BD type I and seven age- and sex-matched healthy controls were recruited and had peripheral blood collected by venipuncture in heparine tubes. PBMCs were isolated from total blood, followed by measurement of cell viability by trypan blue exclusion, and apoptosis and necrosis by anexin V/propidium iodide (PI) staining.
Results: Cell viability did not significantly differ between groups, as well as the percentage of cells in necrosis or in late apoptosis/necrosis. However, the percentage of cells in early apoptosis was higher in patients when compared with controls ($p=0.002$).
Limitations: This is a preliminary study with relatively small sample size.
Conclusions: The systemic toxicity along with dysfunctional cell resilience mechanisms reported in patients with BD may be inducing apoptosis in PBMCs. A deeper look into the clinical relevance of such findings is warranted.

1. Introduction

Bipolar disorder (BD) is a severe and chronic psychiatric disorder associated with increased morbidity and mortality due to general medical conditions, including obesity, metabolic syndrome, and cardiovascular diseases, among others (Kupfer, 2005; Roshanaei-Moghaddam and Katon, 2009). The pathophysiology of BD includes several systemic alterations, such as increased inflammatory markers, reduced neurotrophic factors, oxidative stress, and DNA damage (O'Brien et al., 2006; Andreazza et al., 2007; Andreazza et al., 2008; Fernandes et al., 2011), which characterize a so-called systemic toxicity (Kapczinski et al., 2010, 2011). Most of these systemic alterations may be related to dysfunctions in cellular resilience mechanisms reported in patients, such as endoplasmic reticulum stress and mitochondrial damage (Hayashi et al., 2009; Clay et al., 2011).

Cellular resilience is defined as the ability of a given cell to handle and adapt to a certain stimulus, mostly by activating protective and adaptive mechanisms. Therefore, impaired cellular resilience mechanisms would make cells more vulnerable to stressful situations, ultimately leading to cell death in toxic and stressful environments. Based on the stimulus, different types of cell death can take place, namely apoptosis, necrosis, autophagy, or associated with mitosis (Kroemer et al., 2009). These can be experimentally identified by their morphology, enzymological criteria, functional aspects, or immunological features (Kroemer et al., 2009). Necrosis, for instance, is characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular

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contents (Kroemer et al., 2009). Contrary to the previous concept of necrosis as a merely accidental cell death mechanism, it is currently considered to occur in a regulated manner, as well (Vandenabeele et al., 2010). Apoptosis, on the other hand, is typically characterized by rounding-up of the cell, reduction of cellular size (pyknosis), chromatin condensation, little or no ultrastructural modifications of cytoplasmic organelles, nuclear fragmentation, plasma membrane blebbing, and engulfment by resident phagocytes *in vivo* (Kroemer et al., 2009). Evidence has suggested that apoptotic factors are altered in BD, including increased DNA damage in peripheral blood of patients (Andreazza et al., 2007), increased apoptotic serum activity (Politi et al., 2008), altered expression of molecules involved in cell death/survival pathways in peripheral blood mononuclear cells (PBMCs) from patients (Herberth et al., 2011), as well as mitochondrial dysfunction (Shao et al., 2008). Altogether, these studies implicate the involvement of cell death in BD.

To further characterize the involvement of cellular resilience and death in BD, we aimed to assess cell viability, necrosis and apoptosis in PBMCs from patients with BD and controls. Moreover, we sought to correlate clinical features from patients with such cellular parameters, aiming at identifying the relevance of peripheral cell death in BD pathophysiology.

2. Methods

2.1. Patients and controls

The present study was approved by the Ethical and Research Committee of Hospital de Clínicas de Porto Alegre, Brazil, protocol number 12-0102. Ten euthymic patients with BD type I were recruited at the Bipolar Disorders Program (PROTAHBI), an outpatient program of Hospital de Clínicas de Porto Alegre, Brazil. Seven age- and sex-matched healthy controls without history of psychiatric illness and history of psychiatric or neurologic disorders in first-degree relatives were enrolled at the Blood Bank from the same hospital. Written informed consent was obtained from all participants after receiving a complete description of the study. All participants were at least 18 years old. Patients with BD were diagnosed according to DSM-IV Axis I (SCID-I) criteria. Euthymia was confirmed by the Hamilton Depression Rating Scale (HDRS) and Young Mania Rating Scale (YMRS). Exclusion criteria for both patients and controls included history of autoimmune diseases or chronic infection/inflammatory disorders, as well as any severe systemic disease or use of immunosuppressive therapy.

2.2. Analysis of cell death

Ten milliliters of peripheral blood were collected from all participants by venipuncture in heparine tubes. PBMCs were isolated from total blood with Ficoll-Hypaque (GE Healthcare) density gradient centrifugation, followed by cell counting and measurement of cell viability by trypan blue exclusion. Afterwards, one hundred cells were pelleted and submitted to the analysis of apoptosis and necrosis by annexin V/propidium iodide (PI) staining, according to manufacturer's instructions (BD Biosciences, USA). Analysis of stained cells was performed on a BD FACScalibur flow cytometer (BD Biosciences), by assessing the fluorescence median intensity of samples on a FL1 × FL2 plot. The percentage of cells in early apoptosis (annexin V+/PI−), necrosis (annexin V−/PI+), and late apoptosis/necrosis (annexin V+/PI+) was collected.

2.3. Statistical analyses

Data were fitted into a normal standard distribution and analyses were therefore performed by independent samples *t*-tests. Percentage of cell on necrosis did not fit a normal standard distribution and was analyzed by Mann–Whitney test. Sex difference between patients and controls was assessed by chi-square test, whereas age and scale scores were analyzed by independent samples *t*-test. Correlations were analyzed by Pearson's correlation test. *P* values lower than 0.05 were considered to indicate statistical significance.

3. Results

Patients and controls did not differ regarding sex and age ($p > 0.05$ for both comparisons, Table 1). Even though patients were euthymic, HDRS and YMRS scores were higher in patients when compared to controls (Table 1). Cell viability assessed by trypan blue exclusion did not significantly differ between groups (controls – 96.7% ± 2.09; patients – 93.8% ± 5.08, $t(14) = 1.552$, $p = 0.143$), as well as the percentage of cells in necrosis (controls – 0.6% ± 0.7; patients – 0.68% ± 0.47, $U = 33$, $Z = -0.195$, $p = 0.775$) or in late apoptosis/necrosis (controls – 6.99% ± 5.49; patients – 10.7% ± 5.92, $t(15)$`=-1.309, $p = 0.21$). However, the percentage of cells in early apoptosis was significantly higher in patients when compared with controls (controls – 20.11% ± 5.23; patients = 31.56% ± 7.05; $t(15) = -3.64$, $p = 0.002$; Fig. 1). Since typical antipsychotics and benzodiazepines have been shown to induce apoptosis, two patients that were on these medications were removed from the analysis. Even so, the difference between patients and controls remained significant ($t(13) = -3.806$, $p = 0.002$). No correlations were found between the cellular parameters and YMRS and HDRS scores, number of manic and depressive episodes, number of hospitalizations or number of suicide attempts ($p > 0.05$ for all analyses).

Table 1
Clinical and demographic characteristics of patients and controls.

Characteristic	Patients ($n = 10$)	Controls ($n = 7$)	*P*
Age (years)[a]	49.7 (6.1)	51.7 (5.1)	0.488
Gender (male/female)	3/7	2/5	0.949
HDRS[a]	3.8 (1.9)	0.57 (1.1)	0.002
YMRS[a]	0.57 (1.1)	0	0.044
Medications			
Mood stabilizers	70%	n/a	
Antidepressants	20%	n/a	
Atypical antipsychotics	60%	n/a	
Typical antipsychotics	10%	n/a	
Benzodiazepines	20%	n/a	

[a] Mean (SD).

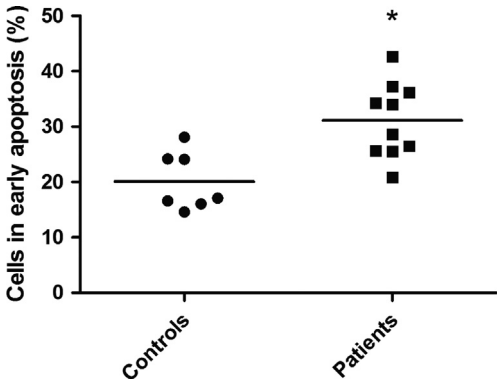

Fig. 1. Early apoptosis in BD patients and controls. *$P = 0.002$, independent *t*-test.

4. Discussion

On this preliminary study, we showed that euthymic patients with BD present an increased percentage of early apoptotic PBMCs when compared to controls, which was not seen in overall cell viability, necrosis or late apoptosis. Based on our results, one can hypothesize that the systemic toxicity along with dysfunctional cell resilience mechanisms reported in patients with BD (Kapczinski et al., 2010) may be inducing apoptosis in PBMCs. In fact, incubation of PBMCs from healthy subjects with serum from patients with BD has been shown to induce an increase in the number of apoptotic cells and a decrease in viable cells when compared to the effect observed for serum from control subjects on the same cells (Herberth et al., 2011).

In this sense, it is likely that peripheral molecules are acting as extracellular stress signals that are sensed and propagated by specific transmembrane receptors in PBMCs, ultimately activating extrinsic apoptosis (Galluzzi et al., 2012). One of these so-called 'lethal ligands' is tumor necrosis factor-alpha (TNF-α), which has been shown to be increased in serum from patients with BD (Brietzke and Kapczinski, 2008; Kauer-Sant'Anna et al., 2009) and is able to bind to death receptors on the cell membrane and induce activation of caspases (Galluzzi et al., 2012). Upon binding of these molecules, one of the mechanisms activated early in the apoptosis pathway is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which was the parameter measured in this study.

Previous studies have already suggested the association between BD and peripheral apoptosis (Gigante et al., 2011). Lymphocytes from patients with BD have been shown to present decreased expression of the anti-apoptotic factor HSP70 along with reduced BAX levels in the cytosolic fraction, suggesting that it had been translocated to the mitochondria to induce apoptosis (Bei et al., 2009). Moreover, increased apoptotic serum activity has been reported in patients (Politi et al., 2008). Of note, even though all of the patients on this study were on medication, most of these were mood stabilizers or atypical antipsychotics (Table 1), which have consistently been shown to induce the expression of antiapoptotic proteins *in vitro* (Nandra and Agius, 2012; Song et al., 2012). In addition, the difference between groups remained unchanged when excluding the patients on neuroleptics and benzodiazepines from the analysis. Moreover, even though early apoptosis is increased in BD, overall cell viability did not differ between groups. The trypan blue exclusion assay does not discriminate between apoptotic and necrotic cells, since both types of dying cells are able to take up the dye. Therefore, since the percentage of necrotic cells is not different between groups, it seems that the extent of apoptosis in PBMCs may not have been sufficiently high to reduce overall cell viability.

Our results show no correlation between cell death and YMRS and HDRS scores, which may be due to the fact that all patients were euthymic at the time of enrollment. Further studies should explore the percentage of dead cells in patients during acute episodes, considering the toxic systemic milieu patients face when acutely ill (Kapczinski et al., 2010) and the reported cell-protective effects of mood stabilizers (Bachmann et al., 2005). Once dead, these cells may end up releasing immunostimulatory molecules, such as damage-associated molecular patterns, and therefore induce alterations in inflammatory markers (Krysko et al., 2011). These peripheral alterations may be then responsible for detrimental effects on peripheral cells, ultimately inducing apoptosis and completing a vicious cycle of peripheral toxicity and reduced cellular resilience.

4.1. Limitations

The number of participants in both groups was considerably small. However, this did not limit the statistical power of the analyses due to the homogeneity of the population (Table 1). Moreover, the patients enrolled for the study were all on medication, which could itself interfere with apoptotic mechanisms and thus represent a potential bias in our results. Nonetheless, as previously mentioned, most of these were mood stabilizers, which have been shown to present antiapoptotic properties.

5. Conclusions

To our knowledge, this is the first study showing basal increased early apoptosis in cells from patients with BD. Such a finding points to the potential use of cell-protective agents in the treatment of BD, as well as the identification of novel targets for the unraveling of its pathophysiology. A deeper look into the clinical relevance of such findings is warranted.

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Conflict of interest

GRF, MPV, CG, BTMQS, ALSTR, BE, JS, BP declare no conflict of interest. FK has received grant/research support from Astra-Zeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD and Stanley Medical Research Institute; has been a member of the board of speakers for Astra-Zeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier. MK has received research grants from CNPq–INCT–TM, CNPq Universal, CAPES, SMRI, NARSAD, Astra–Zeneca, Eli Lilly and FIPE–HCPA.

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3.3 CAPÍTULO 3

The role of FKBP51 in the HPA axis dysfunction in patients with bipolar disorder and their siblings

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Abstract

Background: Impaired stress resilience and a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis are suggested to play key roles in the pathophysiology of bipolar disorder (BD), even though the mechanisms leading to this dysfunction have never been completely understood. To that end, this study aimed to examine the HPA axis activity and its underlying molecular mechanisms in patients with BD and their first-degree relatives.

Methods: Twenty four euthymic patients with BD, 18 siblings and 26 healthy controls were recruited for this study. All subjects performed a low-dose dexamethasone suppression test followed by analyses associated with the HPA axis and the glucocorticoid receptor (GR).

Results: Our results show that BD patients, particularly those at a late stage of illness, presented increased salivary post-dexamethasone cortisol levels when compared to controls (p=0.015). Accordingly, these patients presented reduced *ex vivo* GR responsiveness (p=0.008) and increased basal protein levels of FK506-binding protein of 51 kDa (FKBP51, p=0.012), a cochaperone known to desensitize GR, in peripheral blood mononuclear cells (PBMCs). Moreover, patients presented increased intronic methylation at the *FKBP5* gene, which may be partially responsible for the reduced GR-induced *FKBP5* expression seen in patients.

Conclusions: Our data suggest that the epigenetic modulation of the *FKBP5* gene along with increased FKBP51 levels are responsible for the GR hyporesponsiveness seen in patients, ultimately leading to a hyperactive HPA axis and an impaired resilience to stress.

Introduction

Evidence suggests that patients with BD present a reduced resilience to stress and an increased vulnerability to novel mood episodes as the illness progresses (1). In fact, stress resilience and coping mechanisms are mainly mediated by the HPA axis, which appears to be dysfunctional in patients with BD (2). Patients have been shown to have a hyperactive HPA axis, in some cases presenting high levels of cortisol (3) and a nonsuppression of its levels in the dexamethasone suppression test or in the dexamethasone/cortisol releasing hormone (CRH) test (4). In addition, high-risk subjects, such as first-degree relatives, have also been shown to present elevated baseline cortisol levels (5, 6) and abnormal responses to the dexamentasone/CRH test (7, 8). In this sense, HPA axis abnormalities are suggested to be a trait conferring vulnerability to mood disorders (9).

One of the main players on the modulation of the HPA axis is the GR, which upon hormone binding translocates from the cytosol to the nucleus and acts as a transcription factor. The function of GR depends on a large molecular complex entailing several chaperones and cochaperones, including the FKBP51 (10). *In vitro* overexpression of human FKBP51 reduces hormone binding affinity and nuclear translocation of GR (11), and high levels of FKBP51 lead to GR insensitivity accompanied by increased blood cortisol levels in New World Monkeys (12). Interestingly, glucocorticoids can induce the expression of FKBP51 as part of an intracellular ultra-short negative feedback loop for GR activity (13).

Given the reported familial and genetic component of BD pathophysiology (14), it is likely that most of these stress-related features reflect a particular genetic background. On this vein, several studies suggest that the genetic contribution to BD operates mostly through gene vs. environment interactions (15, 16); mechanistically, environmental impact reprograms gene activity by changing epigenetic modifications independent of the genotype, thus increasing

the risk for the disease in susceptible subjects or interfering with the course of one's illness. Among such epigenetic modifications, alterations in DNA methylation have been consistently reported in BD patients (17, 18). Of note, chronic exposure to glucocorticoids has been shown to induce alterations in DNA methylation at the murine *Fkbp5* gene and at the human *FKBP5* gene in patients with post-traumatic stress disorder (19, 20). Therefore, *FKBP5* methylation might be one of the mechanisms by which stress plays its role in BD pathophysiology.

In summary, stress resilience and coping mechanisms are suggested to play key roles in the development of the disease, as well as in the progressive course of illness in BD patients. However, the mechanisms causing this HPA axis dysfunction in patients are still poorly understood, as well as the role it plays in the risk for the disease in susceptible subjects. Therefore, this study aimed to examine the HPA axis and its underlying molecular mechanisms in patients with BD, first-degree relatives and in healthy controls, possibly identifying clinical and epigenetic mechanisms associated with the development and progression of BD.

Materials and methods

Subjects

The present study was approved by the Ethical and Research Committee of the Hospital de Clínicas de Porto Alegre (HCPA), Brazil, approval number 12-0102. Written informed consent was obtained from all participants after receiving a complete description of the study. All participants were at least 18 years old. Twenty four euthymic patients diagnosed with BD type I according to DSM-IV Axis I criteria were recruited at an outpatient program of the HCPA. Euthymia was confirmed by the Hamilton Depression Rating Scale (HDRS) and Young Mania Rating Scale (YMRS) (scores lower than 7 for each scale). To assess if the mechanisms assessed are also involved in BD progression, we divided patients into early (stages I and II) and late stages (stages III and IV) of illness, according to a previously published staging model of BD (21). For this purpose, a series of clinical parameters were taken into account in a semistructured interview, including data on course of illness, functioning, and comorbidities, as previously successfully employed (22, 23).

Moreover, 18 siblings from patients were included in the study, as well as 26 age- and sex-matched nonrelated healthy controls without any history of psychiatric illness or neurologic disorders. Exclusion criteria for patients, siblings, and controls included history of autoimmune diseases or chronic infection/inflammatory disorders, as well as any severe systemic disease or use of immunosuppressive therapy. All subjects were clinically interviewed by a trained psychiatrist and evaluated with the Functioning Assessment Short Test (FAST) and Childhood Trauma Questionnaire (CTQ) for the assessment of functioning and early childhood adverse events, respectively. Twenty milliliters of peripheral venous blood were collected from each subject for further analyses. All blood collections were performed at the same period of the day with all participants (late afternoon).

Dexamethasone suppression test

All subjects performed a low-dose dexamethasone suppression test with 1.0 mg dexamethasone for the assessment of the HPA axis activity. All subjects were informed that no food or drinks were supposed to be taken at least 30 min prior to each saliva collection. The first saliva sample was collected at 8 am on the first day (T1). On the same day, a saliva sample was collected at 11 pm (T2), followed by the oral administration of dexamethasone. On the next day, the third saliva sample was collected around 8 am, immediately after waking up (T3), and the last one at 4 pm (T4). Once collected, all samples were kept at 4 °C until brought to the laboratory, where they were centrifuged, aliquoted, and finally stored at -20 °C until further analysis.

Cortisol and adrenocorticotropic hormone (ACTH) levels

Salivary cortisol levels were measured with the Cortisol Enzyme Immunoassay kit (Arbor Assay, Ann Arbor, MI, USA), according to the manufacturer's instructions. A standard curve ranging from 3,200 to 0 pg/ml of cortisol was used for calculating the values for each sample. EDTA plasma samples were used for the measurement of ACTH levels with an ELISA kit (Calbiotech, Spring Valley, CA, USA), according to the manufacturer's instructions. A standard curve ranging from 0 to 517 pg/ml of ACTH was used for determining its levels in the samples.

Ex vivo GR responsiveness

The measurement of dexamethasone-induced *FKBP5* mRNA expression in PBMCs was used for estimating GR responsiveness, as previously described (13, 24, 25). PBMCs were isolated from total heparinized blood by Ficoll-Hypaque (GE Healthcare, Buckinghamshire, UK) density gradient centrifugation. Following counting, cells were

resuspended to a concentration of 0.5 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% charcoal-treated fetal bovine serum, 10 μg/ml gentamycin, and 0.25 μg/ml amphotericin B. PBMCs were than plated onto a 24-well plate in 0.5 ml aliquots (0.25 x 10^6 cells/well). Following overnight stabilization in culture, cells were treated for 24 hours with 10^{-9}, 10^{-8} or 10^{-7} M dexamethasone, as well as with vehicle. Total RNA was then isolated with the illustraRNAspin Mini RNA Isolation kit (GE Healthcare) and reverse transcribed using the Omniscript Reverse Transcriptase Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The newly synthesized cDNA was then amplified in a real time PCR performed on the LightCycler system (Roche Applied Science, Penzberg, Germany) using the QuantiFast SYBR Green PCR Kit (Qiagen). Primers used were 5'-ccattgctttattggcctct-3' (forward) and 5'-ggatatacgccaacatgttcaa-3' (reverse) for *FKBP5*, and 5'-agatgagtatgcctgccgtg-3' (forward) and 5'-tgcggcatcttcaaacctcc-3' (reverse) for the housekeeping gene (beta-2 microglobulin).

Western blot analysis

For protein analysis, isolated PBMCs were lysed in 50 μL of lysis buffer (60 mM Tris-HCl (pH 6.8), 2% SDS and 10% saccharose, supplemented with 1:100 protease (Sigma, St. Louis, MO, USA) and phosphatase (Roche) inhibitors, followed by sonication and determination of protein concentration using a BCA kit (Thermo Fisher Scientific, Rockford, IL, USA). For immunoblot detection, 15 μg of cell lysates' total protein were separated by SDS-PAGE under denaturing conditions, followed by transfer onto nitrocellulose membranes. After blocking with 5% nonfat milk in Tris-buffered saline-Tween buffer, membranes were incubated overnight with a monoclonal antibody against FKBP51 (1:1000, Bethyl, Montgomery, TX, USA), followed by a horseradish peroxidase-conjugated antibody against rabbit IgG (Cell Signaling, Danvers, MA, USA). Signals were visualized using ECL detection

reagent (Millipore, Billerica, MA, USA) and monitored in the ChemiDoc MP Imaging System (BioRad, Hercules, CA, USA). Band intensities were normalized by the intensity of a reference sample (pooled PBMCs) that was loaded onto each gel to ensure accurate comparison of samples loaded on different gels.

FKBP5 DNA methylation

FKBP5 methylation was analyzed by bisulfite pyrosequencing, as previously described (19, 26). Genomic DNA from total blood was isolated using the illustra blood genomicPrep Mini Spin Kit (GE Healthcare), followed by spectrometric quantification (NanoDrop, Thermo Fisher, Rockford, IL, USA). Approximately 300 ng of DNA were converted with bisulfite using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), and bisulfite-converted DNA samples were used as templates in PCR reactions for the amplification of intron 2, intron 7, and the putative promoter region of the *FKBP5* gene (19). All sites analyzed were located close to glucocorticoid response elements (GREs).

Statistical analyses

Statistical analyses were performed using PASW Statistics Version 18.0. Descriptive statistics were used to report demographic and clinical characteristics of the sample. Normality of data distribution was assessed with Shapiro-Wilk test and histogram visualization. Categorical variables were compared by Chi-Square or Fischer's exact test. One-way ANOVA followed by post-hoc test of Tukey was performed for the comparison of parametric variables between patients, siblings and controls, and independent *t*-tests were performed for analyzing differences on clinical and demographical data between early- and late-stage patients. Analyses of covariance were conducted adjusting for the potential confounding variables age, sex, BMI, and years of education. Kruskal-Wallis test followed by

Mann-Whitney tests were used to compare non-parametric variables between independent samples. Bonferroni correction was applied for controlling multiple comparisons. Correlations between continuous variables were assessed by Pearson's or Spearman's correlation tests. Significance was set at $p<0.05$, except when otherwise specified.

Results

Sample

Characteristics of patients, siblings, and healthy controls are shown in Table 1. Groups did not differ in regards to age, gender, BMI, smoking, years of education, or CTQ scores. Even though all participants were euthymic at enrollment, patients and siblings showed increased scores of mania and depression scales when compared to controls (YMRS – $F(2.65)=4.9$, $p=0.01$; HDRS – $F(2.65)=17.9$, $p<0.001$). Moreover, patients presented higher FAST scores when compared with siblings and controls ($F(2)=22.24$, $p<0.001$), as well as a higher prevalence of hypothyroidism ($\chi^2=15.6$, $p<0.001$).

Cortisol and HPA axis activity

Results from the dexamethasone suppression test are shown in Table 2. All groups presented significantly lower post-dexamethasone salivary cortisol levels when compared to basal levels (T1 *vs.* T3; $p<0.05$ for all groups). Moreover, the cortisol suppression ratio did not differ between groups. Basal salivary cortisol levels (T1) did not differ between groups, as well as bedtime cortisol levels (T2). However, BD patients presented increased post-dexamethasone cortisol levels (T3) when compared with controls ($F(2)=4.21$, $p=0.015$, Figure 1a). The findings remained similar when age, sex, BMI and education were included as covariates. A negative correlation was also found between post-dexamethasone cortisol levels and the total number of episodes in patients ($r=-0.329$, $p=0.017$). No differences were found for the last time point (T4). The mean decline of cortisol levels throughout the day (diurnal slope) also did not differ between groups, nor did plasma ACTH levels.

GR responsiveness

Considering the role of GR on the cortisol-induced negative feedback of the HPA axis, we hypothesized that the increased post-dexamethasone cortisol levels in patients could be related to a less responsive GR. Accordingly, patients induced significantly less *FKBP5* mRNA levels in response to the lower concentration of dexamethasone when compared to the control group (U=22, Z=-2.66, p=0.008, Figure 1b). No differences were found between groups for the two other dexamethasone concentrations. Siblings also showed no differences from controls or patients. No significant correlations were found between GR responsiveness and salivary cortisol levels at any of the assessed time points, nor with the number of episodes, length of illness, or mood symptoms.

Baseline *FKBP5* mRNA levels

Vehicle-treated cells from the previous experiment were also collected for the analysis of baseline *FKBP5* mRNA levels. After log-transformation of data, no differences were found for any of the comparisons (p>0.05; Figure 1c). No significant correlations were found between baseline *FKBP5* mRNA levels and clinical parameters.

FKBP51 protein levels

The reduced induction of *FKBP5* mRNA expression in patients might be due to a reduced ability of GR to translocate into the nucleus and act as a transcription factor on *FKBP5* gene (i.e., a GR hyporesponsiveness). Considering that FKBP51 itself is a negative regulator of GR, we assessed basal FKBP51 protein levels in isolated PBMCs from all subjects. Our results showed that BD patients presented increased protein levels of FKBP51 when compared to siblings (U=62, Z=-2.505, p=0.012), and a tendency was also found for the comparison with controls (U=113, Z=-1.959, p=0.05, Figure 1d). Interestingly, a significant

correlation was found between FKBP51 and post-dexamethasone cortisol levels (Spearman's ρ=0.313, p=0.049). No correlations were found between FKBP51 protein and basal mRNA levels, nor with GR responsiveness or other clinical parameters.

FKBP5 methylation

Based on previous evidence of epigenetic mechanisms modulating the FKBP51 ultrashort feedback loop, we assessed the DNA methylation of the *FKBP5* gene on three different loci (intron 7, intron 2 and promoter) in all subjects. Differences were found at two CpG sites in intron 7 and one CpG in intron 2 of the *FKBP5* gene, but only the differences on CpG 6 of intron 7 and CpG 2 of intron 2 remained significant after post-hoc tests (Table 3). Patients showed increased percentage of methylation of intron 7 compared with siblings (CpG 6, $F(2,57)=5.58$; p=0.007, Figure 1e), and a tendency was also found for the comparison with controls (p=0.054). Patients also showed increased methylation of intron 2 at CpG 2 when compared with controls ($F(2,53)=3.7$, p=0.04, Figure 1f). No further differences regarding individual CpG sites were found in our sample. Interestingly, significant negative correlations were found between the number of previous manic episodes and the methylation status of intron 7 at two CpG sites (CpG 1 – r=-0.444, p=0.034; CpG5 – r=-0.426, p=0.043). As previously reported (19), analyses were also performed after summarizing the percentage of the 16 CpG sites into seven bins, according to their spatial proximity to the consensus GRE sites. No differences were found between groups for the percentage of methylation in these bins. Moreover, results remained the same after controlling for potential confounders. No correlations were found between *FKBP5* methylation and CTQ or FAST scores.

Analyses according to staging

To assess if these mechanisms are also involved in BD progression, we divided patients into early and late stages of illness, and further analyzed our data comparing the two staging groups. Late-stage patients presented higher number of total episodes ($t(14.7)=-4.27$, $p=0.001$), both manic/mixed ($t(13.6)=-3.57$, $p=0.003$) and depressive episodes ($t(15.8)=-2.91$, $p=0.01$), number of hospitalizations ($t(13.3)=-2.73$, $p=0.017$), and FAST scores ($t(21)=-3.32$, $p=0.003$) when compared with early-stage patients, but did not differ in regards to the length of illness, age at illness onset, nor in other sociodemographic and clinical variables (Table 4).

Our results show that late-stage patients presented increased post-dexamethasone cortisol levels when compared with controls ($F(3)=2.96$, $p=0.033$, Figure 2a), which was not the case for early-stage patients. Accordingly, late-stage patients presented reduced GR responsiveness at 10^{-9}M dexamethasone when compared with controls ($U=6$, $Z=-2.603$, $p=0.009$, Figure 2b), whereas no difference was found between early-stage patients and controls ($U=16$, $Z=-1.854$, $p=0.064$), nor between early- and late-stage patients ($U=16$, $Z=-0.714$, $p=0.475$). Both groups did not differ regarding basal *FKBP5* mRNA or FKBP51 protein levels (Figures 2c and 2d). However, early-stage patients showed increased methylation at intron 7 when compared with siblings (CpG 6, $F(3,56)=4.33$; $p=0.008$, Figure 2e) and controls ($p=0.046$).

Discussion

For the first time alterations in FKBP51 levels and epigenetics were identified in BD patients. The results were also extended to first-degree relatives, considering the relevance of epigenetic mechanisms in the modulation of one's susceptibility to mood disorders. Taken together, our results provide evidence for a FKBP51-mediated and epigenetically-induced modulation of the stress axis in BD.

Increased post-dexamethasone cortisol levels reflect a diminished ability of dexamethasone to reduce cortisol levels overnight, possibly accounting for a dysfunctional HPA axis in BD patients. This seems to be the case specifically for late-stage patients, and the positive correlation between post-dexamethasone cortisol levels and the total number of episodes suggests that such a dysfunction is related to the progression of BD. High post-dexamethasone cortisol has been previously associated with persistent deficits in executive performance in patients with mood disorders (27), as well as with the severity of depression (28). As reported for patients with major depressive disorder (MDD) (29), this can be one of the mediators of the increased recurrence of episodes, considering that the number of previous mood episodes predicts the risk of recurrence in patients with BD (30). On this sense, dexamethasone suppression test results and the HPA axis activity might present a prognostic value in BD.

Given that patients, siblings and controls did not show differences in ACTH levels, we hypothesized that the HPA axis dysfunction seen in patients might be mostly due to the role of GR in the cortisol-induced negative feedback. We have taken the *ex vivo FKBP5* mRNA induction by dexamethasone as a measure of GR responsiveness, and our results show that PBMCs from patients induce significantly less *FKBP5* mRNA expression after dexamethasone stimulation when compared with controls, which is specifically the case for

late-stage patients. Among possible mechanisms leading to this GR resistance, we found that patients with BD presented increased basal protein levels of FKBP51. In fact, we found a positive correlation between basal FKBP51 protein expression and post-dexamethasone cortisol levels. Increased FKBP51 protein levels have also been associated with an increased recurrence of depressive episodes in patients with MDD (29), which also fits the current evidence of increased recurrence of episodes along with BD progression. Interestingly, no such differences were found in mRNA levels, which suggests that the increased protein levels in patients are independent of the mRNA expression levels in our sample. Such a discrepancy between mRNA and protein has already been reported in a sample of patients with MDD (29), which may rely on an enhanced translation or protein stability in patients.

Taking the methylation status of *FKBP5* into account, BD patients appear to present increased methylation levels when compared to controls and siblings, mainly in distal intronic GREs (intron 2 and 7). Considering that distal intronic elements significantly contribute to the transcriptional regulation of *FKBP5* by glucocorticoids (31), increased methylation in these regions might reduce the inducibility of *FKBP5* expression after dexamethasone stimulation. Of note, no correlation between DNA methylation and basal *FKBP5* mRNA and protein levels were found, which suggests that methylation at these regions may be important for GR-mediated *FKBP5* expression, but not for basal expression (for which transcription factors other than GR might be relevant). Interestingly, illness' progression seems to correlate with reduced intronic methylation, based on negative correlations found between the number of episodes and the methylation status of *FKBP5*, and the fact that methylation was only increased in early-stage patients. This might be a result of chronic stress and hypercortisolemia, based on the fact that GR activation can induce alterations in *Fkbp5* methylation, and chronic exposure to dexamethasone can lead to DNA demethylation in intronic CpGs in the *FKBP5* gene (19).

Our results also suggest that some of the parameters analyzed regarding HPA axis activity and its modulation might be altered in BD siblings. Post-dexamethasone cortisol results indicate that siblings present an HPA axis activity somewhere between controls and BD patients, which was also the case for GR responsiveness. Moreover, they presented significantly less FKBP51 protein levels than patients, even though no differences were found on *FKBP5* basal mRNA levels or on gene methylation.

Some limitations of our study need to be mentioned. The relatively small sample size reduced the statistical power for secondary analyses; thus, it would be interesting to see what results will look like in larger samples, in particular for the comparisons close to significance in our study. Moreover, even though groups did not differ regarding medication use, it is possible that different medications may have an impact on the parameters analyzed due to error type I. On the other hand, the strengths of this study include the assessment of multiple cortisol parameters, which enabled us to discuss the HPA axis activity from different points of view. Moreover, the inclusion of both patients and siblings in the analysis allows the discussion of the role of environmental effects on the disorder. Improvements to the present study include a larger sample, the recruitment of acutely ill patients, the analysis of *FKBP5* polymorphisms, and a longitudinal approach of the current analyses.

In summary, our findings call the attention to the role of the HPA axis in the pathophysiology of BD from a novel perspective: dysfunctional negative feedback of the HPA axis and impaired GR responsiveness due to increased FKBP51 levels and increased *FKBP5* intronic methylation. These results add much to previous evidence of altered levels of cortisol in mood disorders, which may be crucial to understanding BD pathophysiology, development and progression. A clearer understanding of the environmental and physiological stimuli leading to these alterations is essential for strategies aiming at

preventing the development of BD by high risk subjects and reversing the deleterious effects associated with its progression.

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Figure legends

Figure 1. *HPA axis activity and FKBP51 alterations in BD.* A) Post-dexamethasone salivary cortisol levels; one-way ANOVA followed by post-hoc test of Tukey. B) *Ex vivo* GR responsiveness assay. Isolated PBMCs were incubated with increasing concentrations of dexamethasone, and the increase in *FKBP5* mRNA levels was assessed after 24 hours; Kruskall-Wallis followed by Mann-Whitney test and Bonferroni correction. C) Basal *FKBP5* mRNA levels; one-way ANOVA followed by post-hoc test of Tukey. D) FKBP51 protein levels; Kruskall-Wallis followed by Mann-Whitney test and Bonferroni correction. E-F) Methylation status of the *FKBP5* gene at two specific CpG dinucleotides; one-way ANOVA followed by post-hoc test of Tukey. **p*=0.008 (patients *vs.* controls). N = 20 – 26 controls; 11-16 siblings; 19 – 24 patients.

Figure 2. *HPA axis activity and FKBP51 alterations in BD patients according to staging.* A) Post-dexamethasone salivary cortisol levels; one-way ANOVA followed by post-hoc test of Tukey. B) Ex vivo GR responsiveness assay; Kruskall-Wallis followed by Mann-Whitney test and Bonferroni correction. C) Basal FKBP5 mRNA levels; Kruskall-Wallis followed by Mann-Whitney test and Bonferroni correction. D) FKBP51 protein levels; Kruskall-Wallis followed by Mann-Whitney test and Bonferroni correction. E-F) Methylation status of the *FKBP5* gene at two specific CpG dinucleotides; one-way ANOVA followed by post-hoc test of Tukey. N = 20 – 26 controls; 11-16 siblings; 8-10 early BD; 11 – 14 late BD.

Table 1. Characteristics of controls, siblings and BD patients

Characteristic	Controls (n = 26)	Siblings (n = 18)	Patients (n = 24)	*P*
Age (years)[a,d]	46.9 (7.08)	51.1 (13.2)	46.9 (7.08)	0.453
Gender (male/female)[c]	8 / 18	6 / 12	7 / 17	0.959
HDRS[a,d]	0.65 (1.3)	3.56 (3.11)	4.17 (2.14)	< **0.001**
YMRS[a,d]	0.08 (0.27)	1.06 (1.3)	0.96 (1.6)	**0.01**
BMI[a,d]	28.1 (4.7)	27.3 (5.8)	30.3 (6.12)	0.183
Smoking[c]	33.3%	7.7%	25%	0.094
Years of education[a,d]	11.54 (3.95)	10.33 (4.34)	11.13 (3.6)	0.611
FAST score[a,d]	5.92 (6.37)	11.93 (12.3)	23.7 (9.92)	< **0.001**
CTQ score[a,d]	33.04 (9.7)	35.12 (7.9)	39.04 (11.0)	0.131
Age at illness onset[a]			25.67 (10.7)	
Length of euthymia (months)[b]			11 (35.3)	
Length of illness (years)[a]			21.04 (10.8)	
Numer of manic/mixed episodes[a]			5.3 (5.8)	
Number of depressive episodes[a]			8.5 (8.4)	
Numer of total episodes[a]			13.8 (11.5)	
Number of hospitalizations[a]			5.38 (8.7)	
Comorbidities				
Hypothyroidism[c]	0%	11.1%	41.6%	< **0.001**
Hyperthension[c]	0%	16.6%	25%	0.03
Diabetes mellitus[c]	0%	0%	16.6%	0.02
Dyslipidemia[c]	3.8%	33.3%	20.8%	0.036
Obesity[c]	0%	0%	4.17%	0.394
Others[c]	7.7%	27.7%	33.3%	0.073
Medications				
Mood stabilizers			66.6%	
Antidepressants			4.16%	
Atypical antipsychotics			33.3%	
Typical antipsychotics			12.5%	
Benzodiazepines			8.33%	

[a]Mean (SD); [b]Median (IQR); [c]Chi-squared test; [d]One-way ANOVA.

Table 2. Cortisol and HPA axis parameters

Characteristic	Controls (n = 26)	Siblings (n = 18)	Patients (n = 24)	*P*
Cortisol (pg/μl)				
T1, basal levels[a]	2.84 (2.16)	3.13 (2.24)	3.1 (1.62)	0.881
T2, bedtime levels[a]	1.16 (0.66)	0.98 (0.73)	1.89 (1.94)	0.102
T3, postdexamethasone levels[a]	0.23 (0.11)	0.32 (0.22)	0.41 (0.21)	**0.02**
T4[a]	0.26 (0.08)	0.38 (0.08)	0.37 (0.2)	0.087
Cortisol suppression ratio[a,b]	13.47 (11.13)	11.55 (8.86)	9.64 (6.96)	0.462
Diurnal slope, mean decline[a,c]	0.15 (0.13)	0.22 (0.13)	0.13 (0.08)	0.163
Plasma ACTH levels (pg/ml)[a]	18.99 (11.57)	15.8 (7.25)	20.32 (12.2)	0.469

[a]Mean (SD); [b]Suppression ratio = cortisol T1 / cortisol T3; [c]Diurnal slope = (cortisol T1 – cortisol T2) / (time T2 – awakening time). T1 = 8 am at day 1; T2 = 11 pm at day 2 (pre-dexamethasone); T3 = 8 am at day 2; T4 = 4 pm at day 2.

Table 3. *FKBP5* methylation in patients, siblings, and controls (%)

	Controls (n = 26)	Siblings (n = 18)	Patients (n = 24)	*P*
Intron 7				
P1_CpG1[a,c]	69.6 (3.6)	70.7 (5.7)	68.3 (3.9)	0.239
P1_CpG2[a,c]	90.6 (3.4)	93.2 (3.8)	91.3 (4.1)	0.12
P1_CpG3[b,d]	100 (3.4)	99.9 (2.7)	97.6 (4.6)	**0.041**
P1_CpG4[b,d]	79.6 (6.2)	78.2 (3.7)	79.9 (4.6)	0.249
P1_CpG5[a,c]	47.8 (4.2)	48.5 (2.3)	49.0 (2.9)	0.482
P1_CpG6[a,c]	56.9 (4.3)	55.5 (3.7)	60.1 (5.1)	**0.006**
Intron 2				
P4_CpG1[a,c]	66.6 (2.4)	66.7 (3.05)	66.9 (1.9)	0.893
P4_CpG2[a,c]	59.8 (2.1)	59.9 (2.05)	61.6 (2.6)	**0.031**
P4_CpG3[a,c]	48.6 (1.9)	50.4 (2.8)	50.4 (3.0)	0.058
P4_CpG4[a,c]	61.2 (1.9)	61.4 (1.8)	60.6 (1.3)	0.322
Promoter				
GRE3_CpG1[b,d]	94.8 (6.2)	94.7 (4.4)	94.4 (5.5)	0.235
GRE3_CpG2[a,c]	47.1 (1.5)	46.9 (1.6)	46.8 (1.9)	0.801
GRE3_CpG3[a,c]	100	100	100	1.000
GRE3_CpG4[a,c]	92.5 (3.5)	90.9 (2.8)	92.3 (3.5)	0.315
GRE3_CpG5[a,c]	84.2 (1.6)	84.3 (2.2)	85.0 (2.1)	0.427
GRE3_CpG6[a,c]	62.2 (1.9)	62.4 (1.7)	63.5 (1.7)	0.059

[a]Mean (SD); [b]Median(IQR); [c]One-way ANOVA test; [d]Kruskal-Wallis test.

Table 4. Characteristics of early and late BD patients

Characteristic	Early BD (n = 10)	Late BD (n = 14)	*P*
Age (years)[a,c]	44.4 (7.38)	48.79 (6.51)	0.138
Gender (male/female)[d]	3 / 7	4 / 10	0.993
HDRS[a,c]	3.4 (2.22)	4.71 (1.97)	0.141
YMRS[a,c]	1.2 (1.93)	0.79 (1.13)	0.544
BMI[a,c]	27.8 (4.95)	32.2 (6.3)	0.081
Smoking[d]	10%	35.7%	0.151
Years of education[a,c]	12.4 (3.83)	10.21 (3.37)	0.154
Age of illness onset[a,c]	23.22 (10.87)	26.07 (10.27)	0.532
Length of euthymia (months) [b,f]	10 (62.5)	12 (33.3)	0.815
Length of illness (years)[a,c]	19.7 (12.59)	22 (9.78)	0.619
Number of manic/mixed episodes[a,c]	1.7 (0.82)	7.93 (6.45)	**0.003**
Number of depressive episodes[a,c]	3.9 (2.72)	11.79 (9.59)	**0.01**
Number of total episodes[a,c]	5.6 (2.63)	19.71 (11.95)	**0.001**
Number of hospitalizations[a,c]	0.9 (0.99)	8.57 (10.4)	**0.017**
FAST score[a,c]	17.2 (8.16)	28.69 (8.27)	**0.003**
CTQ score[a,c]	40.8 (11.9)	37.7 (10.6)	0.516
Comorbidities			
Hypothyroidism[e]	30%	50%	0.421
Hyperthension[e]	10%	35.7%	0.341
Diabetes mellitus[e]	20%	14.3%	1.000
Dyslipidemia[e]	30%	21.4%	0.615
Obesity[e]	0%	7.14%	1.000
Others[e]	30%	28.6%	0.673
Medications			
Mood stabilizers[e]	90%	50%	0.079
Antidepressants[e]	10%	0%	0.417
Atypical antipsychotics[e]	30%	35.7%	1.000
Typical antipsychotics[e]	0%	21.4%	0.239
Benzodiazepines[e]	10%	7.14%	1.000

[a]Mean (SD); [b]Median (IQR); [c]Student *t*-test; [d]Chi-squared test; [e]Fischer's exact test; [f]Mann-Whitney test.

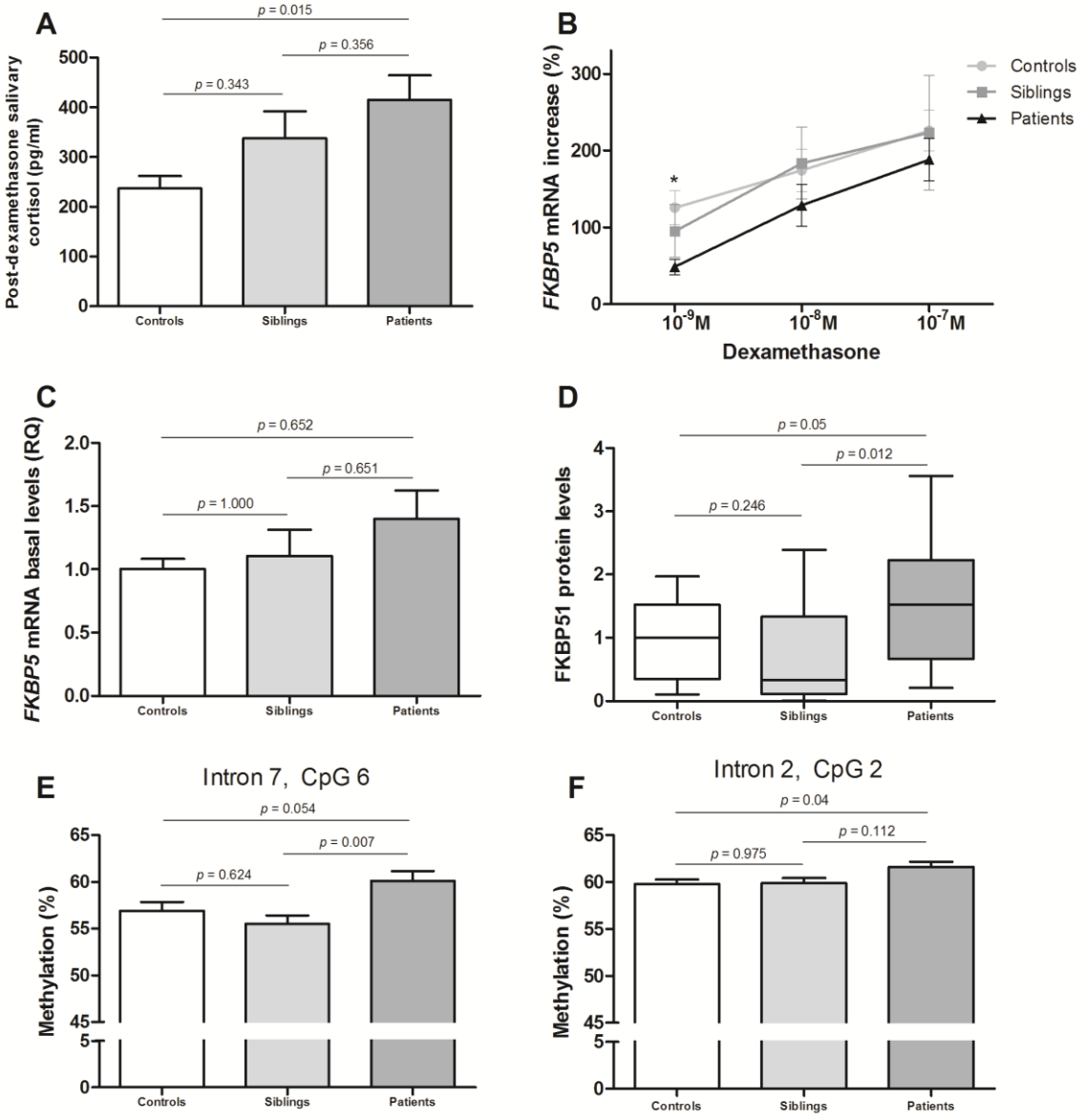

Figure 1. *HPA axis activity and FKBP51 alterations in BD.*

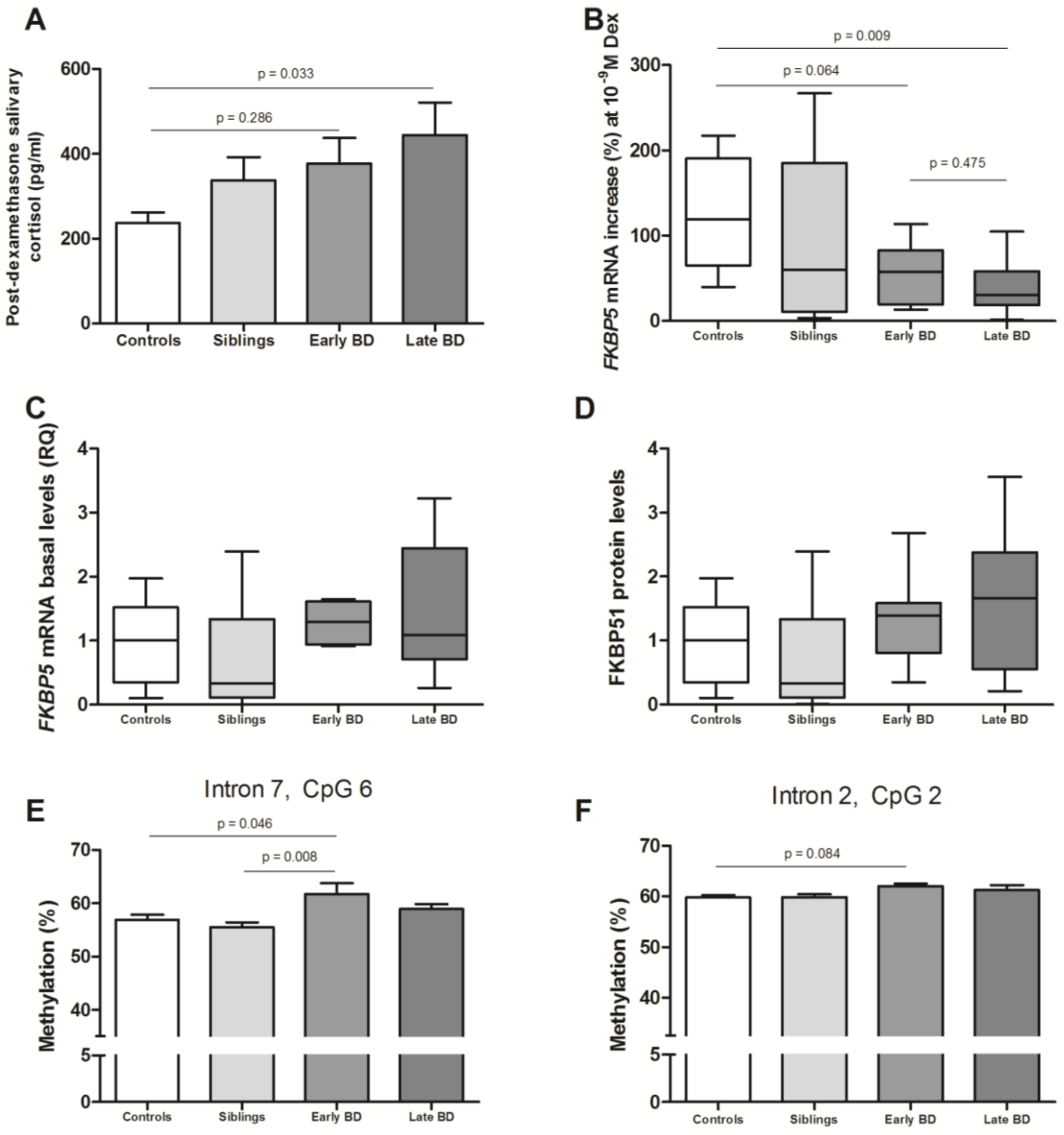

Figure 2. *HPA axis activity and FKBP51 alterations in BD patients according to staging.*

3.4 CAPÍTULO 4

The interplay between FKBP51 and FKBP52 modulates DNMT1 phosphorylation and mediates antidepressant response in patients with major depressive disorder

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Title: The interplay between FKBP51 and FKBP52 modulates DNMT1 phosphorylation and mediates antidepressant response in patients with major depressive disorder

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Abstract

Evidence suggests that epigenetic regulation plays a critical role in the pathophysiology of psychiatric disorders, such as major depressive disorder (MDD). Recent studies have shown that chronic stress can lead to considerable alterations in epigenetic markers, including DNA methylation, and that some antidepressants can inhibit the activity of DNA methyltransferase 1 (DNMT1). On this sense, the cross-talk between stress-related proteins and DNMT1 might account for the effects of stress on epigenetic mechanisms, orchestrating signaling pathways that may ultimately shape one's illness and predict treatment response. To that end, this study aimed to assess the mechanisms by which the stress-related chaperones FK506-binding protein of 51 kDa (FKBP51) and of 52 kDa (FKBP52) modulate DNMT1 phosphorylation, as well as the implications of such mechanisms in predicting the clinical antidepressant (AD) response in patients with MDD. Our results show that both FKBPs compete with each other for the binding to cyclin-dependent kinase 5 (CDK5) and, thereby, present opposing effects on DNMT1^{S154} phosphorylation (known to increase the activity of DNMT1). Moreover, our results suggest that paroxetine shifts the equilibrium towards a stronger physical interaction between FKBP51 and CDK5, ultimately reducing pDNMT1^{S154} levels. Accordingly, we found a correlation between clinical response to treatment in MDD patients and a reduction in pDNMT1^{S154} levels in peripheral blood mononuclear cells (PBMCs). In addition, the *ex vivo* effects of paroxetine on PBMCs from patients were also able to predict their clinical response to treatment. In summary, these results emphasize the effects of stress-modulated chaperones on epigenetic programming in the context of a major psychiatric disorder.

Introduction

Gene vs. environment interactions have been suggested to play key roles in the pathophysiology of several psychiatric disorders, including major depressive disorder (MDD) (*1*). The mechanisms mediating such interactions are still unclear, but candidates include epigenetic changes by DNA methylation or histone modifications, which are responsive to both environmental stimuli and to genetic variations. These alterations do not only seem to be crucial in shaping the phenotype of such complex disorders, but may also play a role in one's response to certain medications (*2*). In this sense, the methylation status of the brain-derived neurotrophic factor (*BDNF*) and of the interleukin-11 genes has been shown to predict the clinical response to antidepressants in patients with MDD (*3*, *4*).

DNA methylation is a process regulated by DNA methyltransferases (DNMTs), whose family comprises three subtypes in mammalian cells with different specificities and functions (DNMT1, DNMT3a and DNMT3b) (*5*). While some antidepressants (ADs) can inhibit DNMT1 activity (the major DNMT to execute the maintenance DNA methylation during the S-phase of DNA replication) by reducing the levels of the histone methyltransferase G9a (*6*), evidences also suggest that the activity of this enzyme can be modulated by post-translational modifications, such as phosphorylation (*7-10*). Among several reported alterations, the phosphorylation of DNMT1 at Serine 154 by cyclin-dependent kinase 5 (CDK5) seems to be crucial for its activity and stability (*11*), and thus might play a role in the AD-induced effects on DNMT1.

The most consistently reported environmental stimuli associated with MDD include trauma and stress (*1*). Stress response is regulated by a complex of chaperones and cochaperones that modulate the function of the glucocorticoid receptor (GR) and ultimately regulate the expression of stress-responsive genes. Among them, FK506-binding protein of 51

kDa (FKBP51) and 52 kDa (FKBP52) are major players on this process, and have been shown to present opposing effects on GR function (*12*). Of note, human genetic studies have linked the *FKBP5* gene to MDD pathophysiology and AD response (*13*). Therefore, the cross-talk between these proteins and DNMT1 might account for the effects of stress on epigenetic mechanisms, orchestrating signaling pathways that may ultimately predict AD response. To that end, this study aimed to assess the mechanisms by which the stress-related cochaperones FKBP51 and FKBP52 modulate DNMT1 phosphorylation at Serine 154, as well as the implications of these mechanisms in predicting the clinical AD response in patients with MDD.

Results

FKBP51 and FKBP52 differentially interact with DNMT1 and modulate its phosphorylation

To check if FKBP51 and FKBP52 can interfere with the DNMT1 phosphorylation by CDK5, we initially tested for physical interactions between these proteins in HEK293 cells. Our results obtained from coimmunoprecipitation studies show that both FKBP51 and FKBP52 are able to bind to CDK5 (Figure 1A and 1B), although only FKBP52 binds to DNMT1 (Figure 1B). Subsequently, we tested the dynamics of these interactions by co-expressing both FKBPs in HEK cells and found that they compete with each other for the binding to CDK5 (Figure 1C and 1D). Considering the reported effects of CDK5 on DNMT1 (*11*), we proceeded on analyzing DNMT1 phosphorylation at Serine 154 (pDNMT1^{S154}) after the overexpression of FKBP51/52. As shown in Figure 1E, pDNMT1^{S154} was significantly reduced after the overexpression of FKBP51 and was increased after FKBP52 overexpression. To check for the relevance of FKBPs on DNMT1 phosphorylation, we assessed pDNMT1^{S154} levels in *Fkbp*51$^{-/-}$ and *Fkbp*52$^{-/-}$ knockout mouse embryonic fibroblasts (MEF), and found that pDNMT1^{S154} levels were significantly reduced in *Fkbp*52$^{-/-}$ MEF cells when compared with both wild-type and *Fkbp*51$^{-/-}$ cells (Figure 1F).

ADs modulate DNMT1 phosphorylation and activity by means of FKBP51

Based on previous evidence of an inhibitory effect of ADs on DNMT1 (*6*), we sought to assess if this inhibition is also mediated by the effects of FKBP51/52 and CDK5 on DNMT1 phosphorylation. Initially, we tested if paroxetine (an AD shown to inhibit DNMT1) would interfere with the interaction between FKBPs and CDK5. Our results show that paroxetine strengthens the interaction between FKBP51 and CDK5 and reduces the

interaction between FKBP52 and CDK5 (Figure 2A). On this sense, we next checked for the effects of paroxetine on pDNMT1 levels and found that, as hypothesized, it induced a reduction of pDNMT1^{S154} levels in wild type MEF cells, but not in *Fkbp51*$^{-/-}$ ones (Figure 2B). Altogether, it seems that ADs shift the equilibrium between FKBP51 and FKBP52 towards a stronger interaction between FKBP51 and CDK5, ultimately reducing the phosphorylation of DNMT1 at Serine 154 by CDK5. To confirm these results, we checked for similar results in the hippocampus of wild-type and *Fkbp51*$^{-/-}$ mice chronically treated with paroxetine. Our results show that chronic treatment with paroxetine induced a reduction of pDNMT1^{S154} levels only in the presence of FKBP51 (although not statistically significant) (Figure 2C).

pDNMT1^{S154} *levels correlate with FKBP51 levels in PBMCs from healthy individuals*

To check if the effects of ADs on the crosstalk between FKBP51/52 and DNMT1 can also be seen *in vivo* in humans, we checked for the levels of these parameters in peripheral blood mononuclear cells (PBMCs) from healthy individuals. Initially, we found that FKBP51 and pDNMT1^{S154} levels were negatively correlated in PBMCs ($r = -0.652$, $p = 0.00135$, Figure 3A). In adittion, we checked for the effects of ADs on the same cells by treating them *ex vivo* with paroxetin and amitryptilin for 72 hours and assessing the alterations on pDNMT1^{S154} after treatment. As seen in Figure 3B and 3C, higher basal FKBP51 levels were significantly associated with a paroxetine-induced reduction in pDNMT1^{S154} levels ($r = -0.495$, $p = 0.0265$), and a tendency was also found after treatment with amitryptiline ($r = -0.414$, $p = 0.0693$), further corroborating our previous results that suggest the relevance of FKBP51 for the effects of ADs on DNMT1 phosphorylation.

pDNMT1^{S154} levels predict clinical treatment response in patients with MDD

We next sought to check if these FKBP51-dependent changes in DNMT1 phosphorylation by ADs are of relevance for their clinical effects in patients with MDD. To that end, we checked for these parameters on PBMCs from patients at baseline (when they were symptomatic, shortly after admission to the hospital) and after six weeks of treatment, and correlated our laboratory findings with their clinical AD response (percentage of increase in the Hamilton Depression Rating Scale (HDRS) scores throughout the treatment scheme). Our sample was comprised of 17 males and 23 females, aged 48 ± 14.7 years, with a mean HDRS score of 24.23 ± 5.5 at admission and 10.45 ± 8.9 after 6 weeks. As expected, we found a significant negative correlation between clinical AD response and the reduction on pDNMT1^{S154} levels ($r = -0.434$, $p = 0.006$, Figure 4A). We also treated PBMCs from patients with paroxetine and checked for its ability to induce changes in pDNMT1^{S154} *ex vivo*. As shown in Figure 4B, we found a correlation between the cellular pDNMT1^{S154} changes induced by paroxetine and the clinical AD response ($r = -0.389$, $p = 0.016$). Moreover, we also found a positive correlation between the cellular changes on pDNMT1^{S154} levels induced by paroxetin and the changes on pDNMT1^{S154} induced by the treatment of patients *in vivo* ($r = 0.396$, $p = 0.014$, Figure 4c). In summary, these results suggest that a reduction of DNMT1 phosphorylation at Serine 154 throughout treatment is required for a proper clinical response to ADs. Moreover, these effects could also be seen after an *ex vivo* treatment of PBMCs, further suggesting a possible role of such mechanisms in predicting AD response in MDD.

Discussion

Previous reports on the association between stress-related illness and epigenetic alterations support the notion that molecular mechanisms shape the pathophysiology of these disorders and the response to treatment in patients. The results reported in this study shed light into the mechanisms by which stress-modulated proteins can interfere with the epigenetic machinery, suggesting possible targets for reversing and/or preventing the effects of stress.

Briefly, our results suggest that a differential interaction between the chaperones FKBP51 and FKBP52 with DNMT1 regulates the effect of CDK5 over DNMT1 (Figure 5). Of note, several studies have shown that the levels of FKBP51 are differentially modulated in psychiatric disorders, mainly based on specific genotypes of the *FKBP5* gene (*13-16*). Coincidently or not, most of these disorders have been shown to present alterations in DNA methylation at specific genes (*17*). Our study suggests that such alterations are molecularly linked, possibly based on the effects of CDK5 on DNMT1. In addition, we found that both FKBP51 and FKBP52 compete with each other for their effect on DNMT1 via scaffolding of CDK5, suggesting that an equilibrium between their levels are of key relevance for this mechanism. Accordingly, the occurrence of opposing effects for both immunophilins has already been shown in the regulation of corticosteroid receptors (*12, 18*), neurite outgrowth (*19*) and in the regulation of microtubules (*20, 21*). Even though they share 70% similarity and adopt similar conformations (*22*), FKBP51 and FKBP52 present differences on the residues of the proline-rich loop located at their FKBP12-like domain 1 (FK1) (*23*), which affect their interactions with large peptide substrates and might therefore account for their differences regarding DNMT1 interaction.

The fact that CDK5 requires FKBP52 for its phosphorylating effect on DNMT1 suggests that this kinase might undergo structural changes upon binding to this immunophilin. It might also be the case that, once bound to FKBP52, CDK5 may have a better access to DNMT1 (since the latter can also bind to FKBP52). On the contrary, by binding to CDK5 and not to DNMT1, FKBP51 might reduce the availability of DNMT1 to CDK5, ultimately leading to a reduction of DNMT1 phosphorylation.

Our results also show that ADs strength the interaction between FKBP51 and CDK5, thereby reducing DNMT1 phosphorylation at Serine 154. This mechanism, in combination with the reduction of G9a levels previously shown to occur after AD treatment *in vitro* (*6*), supports the inhibitory role played by ADs on DNMT1 activity and function. Accordingly, inhibitors of DNA methylation have been shown to induce antidepressant-like behavior in rats (*24*). Moreover, our data also suggest that FKBP51 is required for the effects of AD, given that no such effect was seen in cells depleted of this gene. This is in line with previous observations that the *FKBP5* genotype associated with higher expression of FKBP51 is associated with a faster AD response in patients (*13*).

On this sense, studies on MDD have reported the relevance of differential DNA methylation patterns on the response to ADs (*3, 4, 25*). The results found in PBMCs from patients suggest that a significant reduction on the levels of DNMT1 phosphorylation at Serine 154 are required for a clinical AD response; i.e., patients that already presented low levels of $pDNMT1^{S154}$ at the beginning of treatment and that did not show a significant reduction of these levels throughout treatment did not show high clinical AD responses. In other words, it seems that ADs require high levels of $pDNMT1^{S154}$ prior to the treatment so that they can reduce them and ultimately induce symptom resolution. Interestingly, this has also been shown for the methylation levels of the interleukin-11 gene in MDD patients (*3*),

where only patients with high methylation levels at onset of treatment showed high clinical responses to medications.

Limitations of this study include the lack of data on DNMT1 activity and on DNA methylation on the same experiments in which $pDNMT^{S154}$ was assessed. Nonetheless, the activating effect of this phosphorylation had been consistently shown in a previous study (*11*). In addition, there should be noted that phosphorylation at Serine 154 is not the only relevant post-translational modification reported for DNMT1. Phosphorylation at Serine 515 has been also shown to activate the enzyme (*8*), whereas phosphorylation at Serine 127 has been shown to reduce its activity (*26*), suggesting that multiple alterations need to be simultaneously orchestrated for the determination of DNMT1's activity. Although consistent with our results, the specific modulation of CDK5 should also be explored in more details in further studies.

In summary, our results emphasize the effects of stress-related chaperones on epigenetic programming in the context of MDD. In addition, the *ex vivo* modulation of $pDNTM1^{S154}$ levels by ADs in PBMCs might predict the clinical response to these medications in patients. The identification of genes epigenetically modulated by ADs may help clarify the mechanisms underlying symptom resolution and the effects of stress on MDD.

Materials and Methods

Cell lines

Human embryonic kidney cells (HEK-293, ATCC CRL-1573) and MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS and 100 units/ml penicillin and streptomycin, respectively. Preparation of *Fkbp51*$^{-/-}$ and *Fkbp52*$^{-/-}$ MEFs has been previously described (*27*).

Preparation of rat primary astrocytes

Enriched astroglial cultures were prepared from postnatal day 1 rat pups (Sprague-Dawley, Charles River, Sulzfeld, Germany) and handled as described elsewhere (*28*).

Transfection of astrocytes and HEK cells

Detached HEK cells or cortical astrocytes (2 x 10^6) were resuspended in 100 µl transfection buffer (50 mM HEPES pH 7.3, 90 mM NaCl, 5 mM KCl, 0.15 mM $CaCl_2$) (*29*). A maximal amount of 10 µg plasmid DNA expressing FKBP51-FLAG or FKBP52-FLAG (*12*) was added to the cell suspension, and electroporation was carried out using the Amaxa Nucleofactor system. Cells were re-plated at a density of 10^5 x cm^{-2} and further processed for Western blot analysis.

Co-immunoprecipitation (CoIP)

CoIPs of FLAG-tagged FKBP51/52 and CDK5 were performed in HEK293 cells. Briefly, 5×10^6 cells were electroporated with 5 µg of the respective expression plasmids using a GenePulser (Bio-Rad, USA) at 350 V/700 µF in 400 µl of electroporation buffer (50 mM K_2HPO_4/KH_2PO_4, 20 mM CH_3CO_2K, pH 7.35, 25 mM $MgSO_4$). After three days of

cultivation in DMEM/10% FCS, cells were lysed in CoIP-buffer [20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Igepal complemented with protease inhibitor cocktail (Sigma)] for 20 min at 4 ºC with constant mixing. The lysate was cleared by centrifugation, the protein concentration was determined, and 1.2 mg of the lysate was incubated with 2.5 µg FLAG or Cdk5 antibody overnight at 4 °C with constant mixing. Subsequently, 20 µl of BSA-blocked Protein G Dynabeads (Invitrogen, 100-03D) were added to the lysate–antibody mix followed by 3 h incubation at 4 °C. Beads were washed 3 times with PBS, and bound proteins were eluted with 100 µl of 1× FLAG-peptide solution (Sigma, 100–200 µg ml-1, F3290) in CoIP buffer for 30 min at 4 °C. 5–15 µg of the cell lysates or 2.5 µl of the immunoprecipitates were separated by SDS-PAGE.

Western blot analysis

Western blot analysis was conducted as previously described (*6*). Briefly, protein extracts were obtained by lysing cells in 62.5 mM Tris, 2% SDS and 10% sucrose, supplemented with protease (Sigma, P2714) and phosphatase (Roche, 04906837001) inhibitor cocktail, followed by sonication of samples and heating at 95 ºC for 5 min. Proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Blots were placed in Tris-buffered saline supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature, followed by an incubation with the primary antibody (diluted in TBS/0.05% Tween) overnight at 4 ºC. The following primary antibodies were used: anti-FLAG (1:7000, Rockland, 600-401-383), anti-FKBP51 (1:1000, Bethyl, A301-430A), anti-FKBP52 (1:2000, Bethyl, A301-427A), anti-DNMT1 (1:1000, Imgenex, IMG-261A), anti pDNMT1^{S154} (1:1000, Abnova, PAB4924), and anti-Hsc70 (heat-shock cognate 70; 1:2000, Santa Cruz Biotechnologies, sc-7298). Subsequently, blots were washed and probed with the respective horseradish peroxidase-conjugated secondary antibody for 1 h at

room temperature. ECL detection reagent (Millipore) was applied to visualize the immunoreactive bands at ChemiDoc MP (Millipore).

Chronic paroxetine treatment

Paroxetine (GlaxoSmithKline, Munich, Germany) was administered via drinking water to the micefor 28 days. Briefly, the paroxetine solution was diluted in tap water to a final concentration of 0.16 mg/ml. With average water consumption of 5ml/mouse/day, the daily dose of paroxetine was ~20 mg/kg body weight. Fluid intake was monitored daily and the variation of fluid intake was found to be <10% over the course of experiment.

Subjects and preparation of human PBMCs

Twenty one healthy male volunteers and forty depressed patients from the Munich Antidepressant Response Signature (MARS) study (*30*) were included in this study. Written informed consent was obtained from all participants after the nature and possible consequences of the procedure were explained. Peripheral blood was collected via venipuncture, diluted with PBS and carefully loaded on Biocoll solution (BioChrom AG, L6113) and centrifuged at 800 x g for 20 min. PBMCs were enriched by selecting the interphase of the Biocoll gradient, followed by washing two times with ice-cold PBS. Cells were then re-suspended in RPMI and plated at $4 \times 10^5 \times cm^{-2}$. After recovery for 6 h, cells were treated with either 365 nM paroxetine, 888 nM imipramine or vehicle. This concentration has been chosen to match therapeutic concentrations in the serum according to the consensus guidelines for therapeutic drug monitoring in psychiatry (*31*).

Statistical analysis

Statistical analyses were performed with SigmaPlot 11.0. Student's *t*-tests were applied to compare two groups, whereas one- or two-way ANOVA were performed for comparisons between three or more groups, followed by Tukey's or Duncan's post-hoc test, as appropriate. Correlations between variables were analyzed using the Pearson correlation coefficient. P values lower than 0.05 were considered statistically significant.

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Figure legends

Figure 1. *FKBP51 and FKBP52 differentially interact with DNMT1 and modulate its phosphorylation.* A) Western blots of the interaction analyses between CDK5 and FKBPs after CDK5 immunoprecipitation (in triplicate) in HEK cells. B) FLAG-tagged FKBPs were overexpressed in HEK cells and then immunoprecipitated with an anti-FLAG antibody for the analysis of their interactions. C) Representative Western blots of the competition assay between FKBP51 and FKBP52, which are quantified in D. Bars represent the mean ± SEM of three independent experiments performed in triplicate. *Student *t*-test. E) Representative Western blots of the effects of increasing levels of FKBP51 (0, 2.5, 5, 7.5 or 10 μg plasmid) and FKBP52 (0, 5 or 10 μg plasmid) on pDNMT1^{S154} levels in rat astrocytes. F) Representative Western blots and quantification of pDNMT1^{S154} levels in *Fkbp51*$^{-/-}$ and *Fkbp52*$^{-/-}$ MEF cells. * $P < 0.05$ (one-way ANOVA followed by post-hoc test of Tukey). IP = immunoprecipitation; C = control immunoprecipitation.

Figure 2. *Paroxetine modulates DNMT1 phosphorylation by means of FKBP51*. A) Paroxetine treatment induced a stronger interaction between FKBP51 and CDK5, and reduced the interaction between FKBP52 and CDK5 in HEK cells. *Student *t*-test. Moreover, it reduced pDNMT1^{S154} levels in wild type MEF cells (B) and in the hippocampus of chronically treated wild-type mice (C), but not in the absence of FKBP51. *$P < 0.05$ (two-way ANOVA followed by post-hoc test of Tukey).

Figure 3. *Expression of FKBP51 in human peripheral blood mononuclear cells (PBMCs) correlates with pDNMT1^{S154} levels in healthy volunteers.* A) FKBP51 protein levels negatively correlated with pDNMT1^{S154} basal levels in PBMCs. B) PBMCs were treated *ex*

vivo with paroxetine for 72 hours and the percentage of change of pDNMT1^{S154} levels was significantly correlated with FKBP51 levels. C) A tendency was also found for the same parameter after treatment of PBMCs with amitriptyline. Correlations were analyzed using Pearson correlation coefficient.

Figure 4. *pDNMT1^{S154} levels predict clinical treatment response in patients with MDD.* A) The changes on pDNMT1^{S154} levels on PBMCs throughout a 6-weeks treatment scheme with ADs significantly correlated with the clinical response to treatment in patients with MDD. B) PBMCs from patients were treated *ex vivo* with paroxetine and the change on pDNMT1^{S154} levels was significantly correlated with the clinical AD response, as well as with the changes in pDNMT1^{S154} levels induced *in vivo* by the treatment of patients. Correlations were analyzed using Pearson correlation coefficient.

Figure 5. *Schematic model for the effects of FKBP51 and FKBP52 on DNMT1 phosphorylation at Serine 154.* FKBP52, by interacting with both CDK5 and DNMT1, leads to the phosphorylation of DNMT1 by CDK5 and, therefore, to its increased activity. FKBP51, on the other hand, competes with FKBP52 for binding with CDK5 and, by not binding to DNMT1, reduces the phosphorylating effect of CDK5 over DNMT1, ultimately reducing its activity. Antidepressants shift the equilibrium towards a stronger interaction between FKBP51 and CDK5, ultimately reducing DNMT1^{S154} phosphorylation. Black-filled circles represent methylated CpGs, whereas non-filled ones represent nonmethylated CpGs at the DNA.

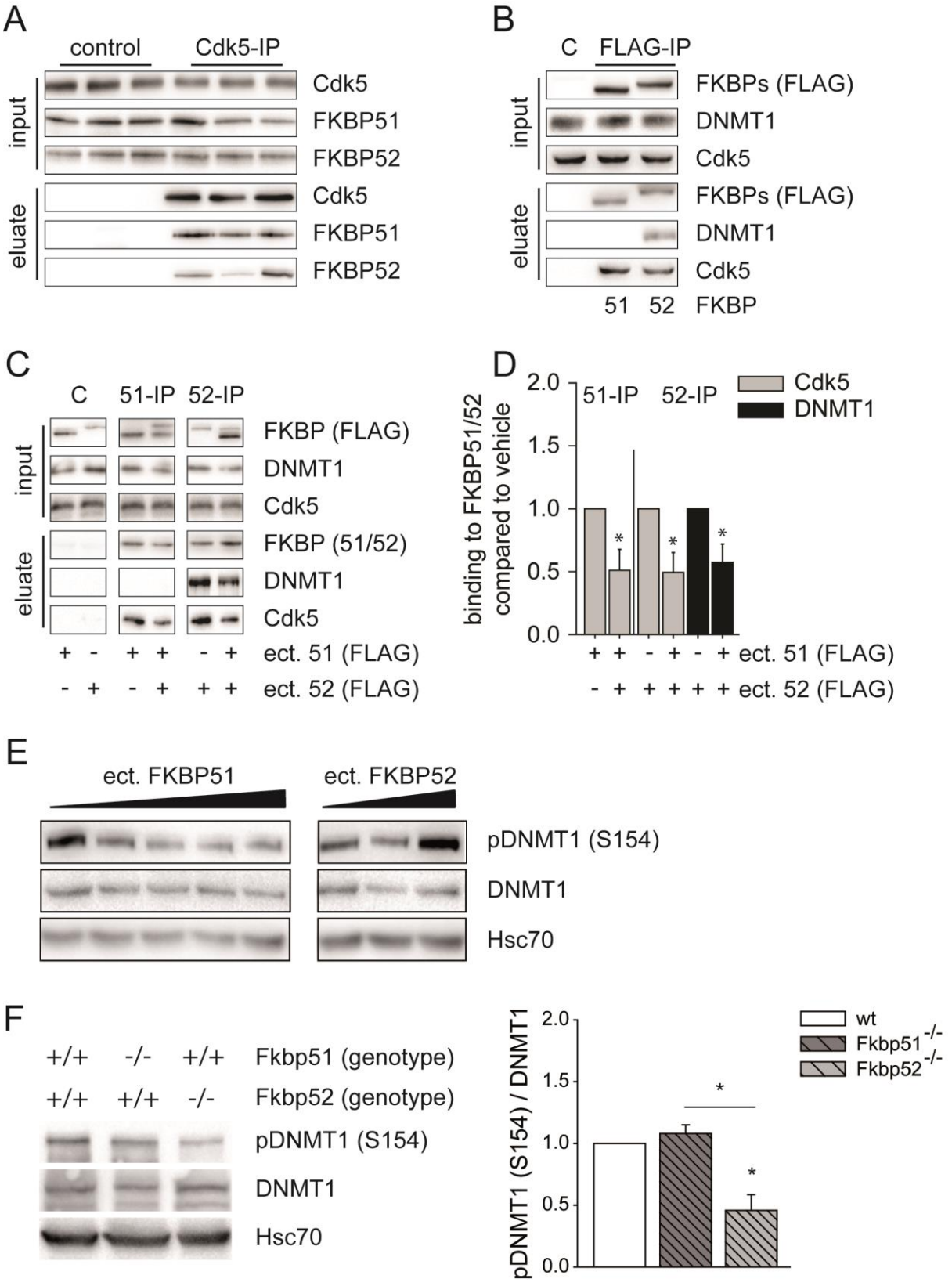
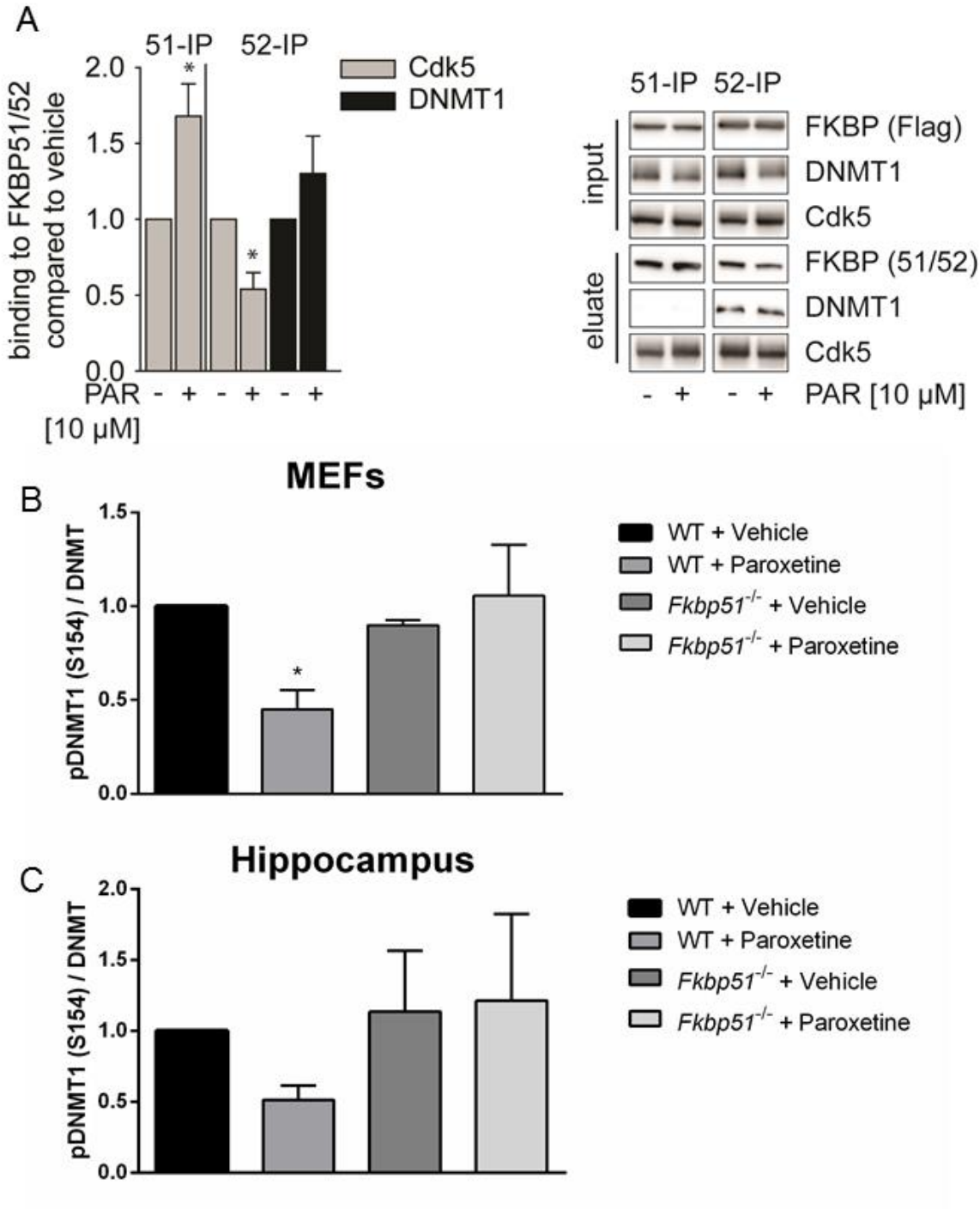


Figure 1. *FKBP51 and FKBP52 differentially interact with DNMT1 and modulate its phosphorylation.*



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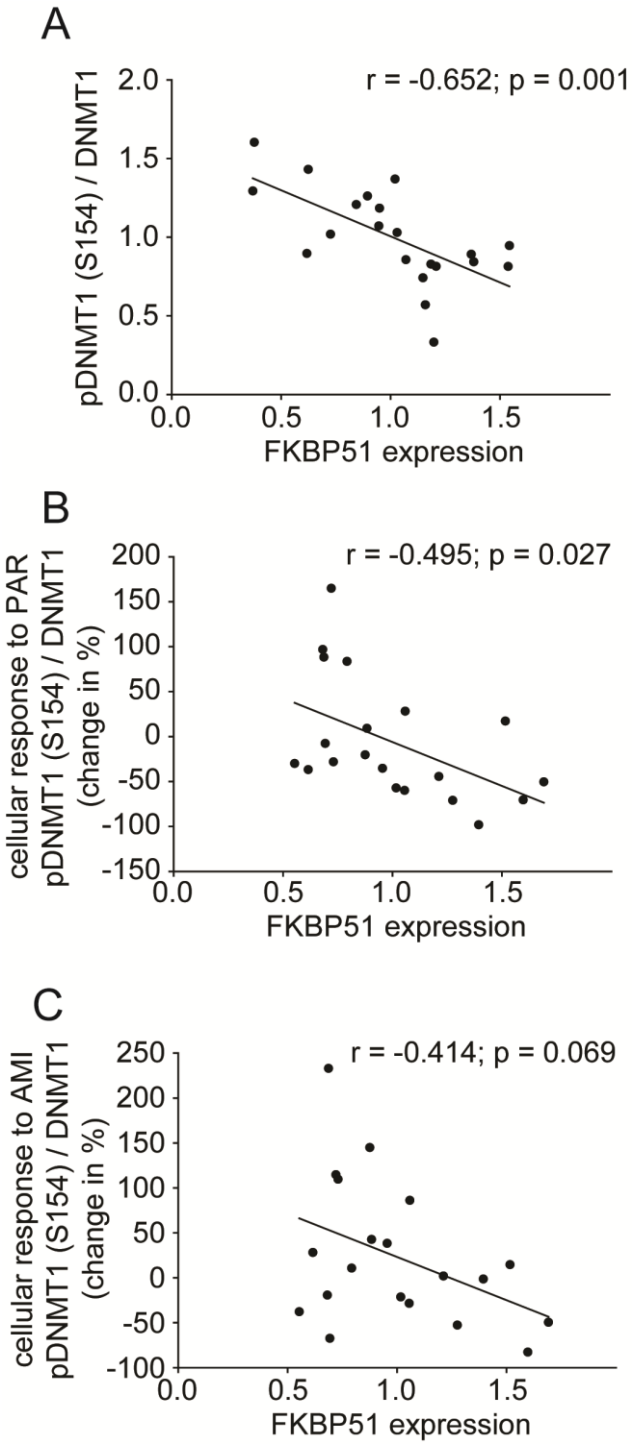

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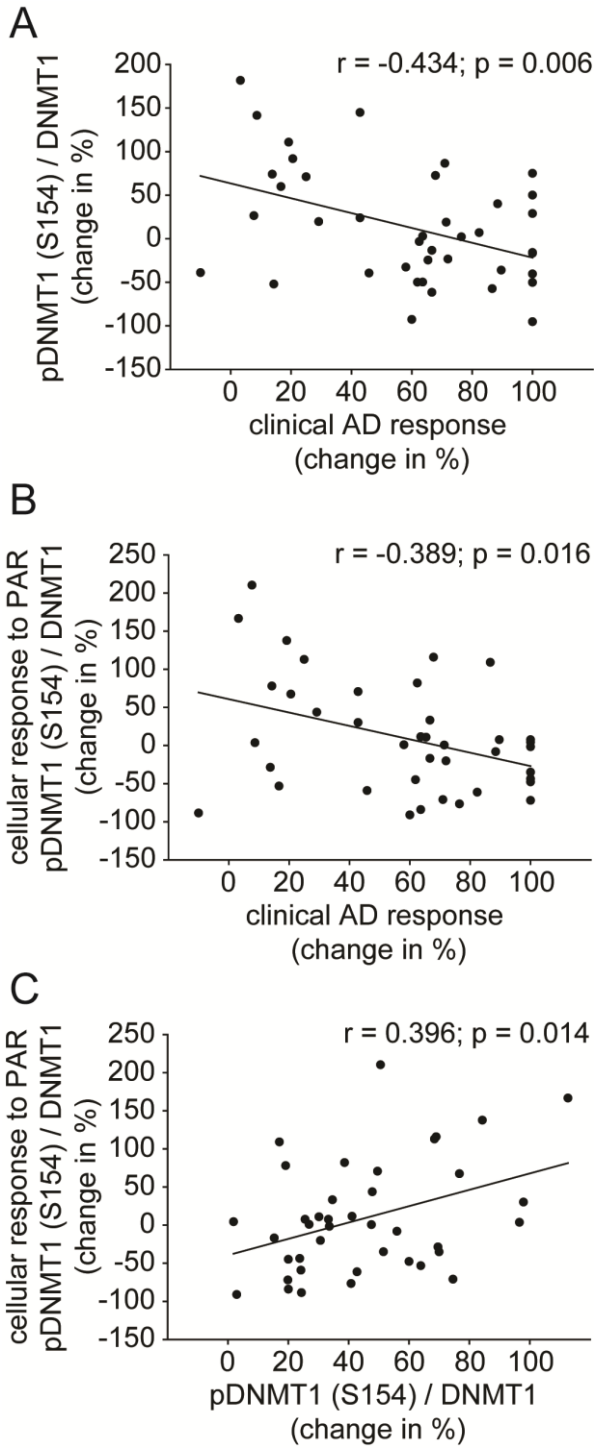

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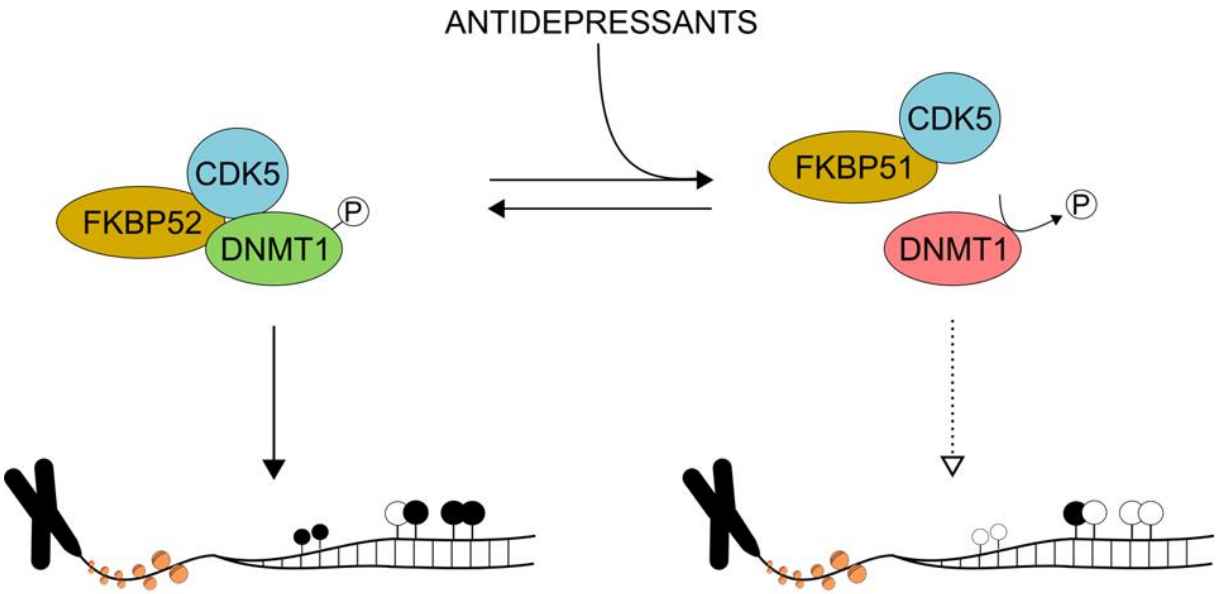

Figure 5. *Schematic model for the effects of FKBP51 and FKBP52 on DNMT1 phosphorylation at Serine 154.*

PARTE III

Discussão e conclusões

4. DISCUSSÃO

O estresse e suas consequências desempenham papéis-chave na fisiopatologia dos transtornos de humor. Como sugerido pelos resultados desta tese, a forma como os pacientes respondem a estímulos estressores, tanto em nível fisiológico quanto celular, pode definir o curso da doença, modular parâmetros periféricos e, ainda, induzir modificações epigenéticas em genes específicos.

Como discutido anteriormente, os transtornos de humor, particularmente o TB, apresentam um fenótipo extremamente complexo e heterogêneo marcado por altos níveis de toxicidade sistêmica. Neste contexto, nós avaliamos um dos principais desfechos desta toxicidade medindo parâmetros de resiliência e morte celular em PBMCs de pacientes com TB. Os pacientes apresentaram um aumento de cerca de 50% na contagem de células em apoptose inicial em comparação a controles saudáveis pareados. Nossos resultados são corroborados por evidências na literatura que mostram uma diminuição nos níveis de Bcl-2 (proteína anti-apopotótica) e um aumento nos níveis do promotor de morte associado à Bcl-2 (BAD, do inglês *Bcl-2-associated death promoter*), da proteína X associada à Bcl-2 (BAX, do inglês *Bcl-2-associated X protein*), de caspase-9 e de caspase-3 na área 9 de Brodmann em pacientes com TB (Kim *et al*., 2010; Gigante *et al*., 2011). Ainda, Benes e colaboradores (2006) mostraram um aumento na expressão de 19 genes associados à apoptose concomitante a uma redução na expressão de genes antiapoptóticos no hipocampo de pacientes. Especificamente em células periféricas, há evidências indiretas de apoptose baseadas no aumento de dano ao DNA (Andreazza *et al*., 2007) e disfunção mitocondrial (Shao *et al*., 2008).

Bioquimicamente, o processo de morte celular por apoptose inclui os seguintes mecanismos: a) ativação de caspases; b) condensamento da cromatina; c) ativação de

endonucleases, causando clivagem do DNA internucleossomal e levando à extensiva fragmentação do DNA; d) aparecimento de uma morfologia celular distinta com preservação de organelas; e) diminuição do tamanho celular (*shrinkage*); e f) fragmentação nuclear e formação de corpos apoptóticos (Wlodkowic *et al*., 2011). Além disso, outras alterações já foram identificadas como sendo marcadores de estágios específicos de apoptose, como a dissipação do potencial de membrana mitocondrial e alterações na membrana plasmática, incluindo mudanças na composição lipídica e na permeabilidade da membrana a pequenas sondas catiônicas (van Engeland *et al*., 1998). A assimetria da bicamada lipídica se altera logo nos estágios iniciais da apoptose, quando a fosfatidilserina, que constitui menos de 10% do total de fosfolipídios de membrana, é exposta na camada externa da membrana (van Engeland *et al*., 1998). Esta exposição na superfície celular sinaliza para macrófagos, que são então atraídos para a célula e iniciam o processo de fagocitose das células e corpos apoptóticos em questão (Wlodkowic *et al*., 2011).

Embora a exposição da fosfatidilserina na superfície celular seja um marcador de apoptose comumente utilizado, ele não permite a identificação das causas ou origens da morte celular. De forma geral, a apoptose detectada nos pacientes pode ser consequência da insuficiência de fatores de crescimento, aumento de estresse oxidativo, aumento nos níveis de cálcio intracelular, entre outros mecanismos. No caso específico da via extrínseca da apoptose, por exemplo, receptores de membrana específicos (como, por exemplo, os receptores conhecidos pelo nome sugestivo de ‘receptores de morte’) são ativados por ligantes extracelulares, como o fator de necrose tumoral-alfa (TNF-α, do inglês *tumor necrosis factor - alpha*) (Wlodkowic *et al*., 2011). Considerando os altos níveis séricos de TNF-α descritos em pacientes com TB (Munkholm *et al*., 2013), é possível que este seja um dos responsáveis pela apoptose aumentada encontrada nas PBMCs. Essa teoria é reforçada por um estudo mostrando que o soro de pacientes com TB induz uma maior porcentagem de apoptose *in*

vitro em PBMCs de controles saudáveis em comparação ao soro de controles (Herberth *et al*., 2011), sugerindo um potencial tóxico (pró-apoptótico) inerente ao sangue periférico no TB.

Além disso, evidências indicam que alguns tipos celulares de pacientes com TB apresentam prejuízos na regulação de cascatas do Ca^{2+} (Soeiro-de-Souza *et al*., 2012), o qual é capaz de orquestrar vias apoptóticas de forma significativa (Mattson & Chan, 2003). Especificamente, disfunções no canal de cálcio, na função do RE e na captação de cálcio pela mitocôndria já foram descritas em pacientes com TB (Kato *et al*., 2003; Manji *et al*., 2003). Além disso, níveis basais elevados de Ca^{2+} já foram encontrados em plaquetas e linfócitos de pacientes com TB (Dubovsky *et al*., 1989; Emamghoreishi *et al*., 1997).

Da mesma maneira, como discutido anteriormente, a disfunção mitocondrial pode desempenhar um importante papel na apoptose inicial observada nos pacientes. Em resumo, a via mitocondrial para ativação apoptótica envolve a liberação inicial de citocromo c do espaço intermembrana mitocondrial e indução de uma cascata citoplasmática que culmina com a ativação de caspases. A relevância desta via é sugerida por diferentes estudos mostrando a presença de disfunção mitocondrial em células de pacientes com TB (Quiroz *et al*., 2008).

Neste sentido, um dos potenciais mecanismos descritos para a indução de disfunção mitocondrial é a exposição crônica a altos níveis de glicocorticóides, como o cortisol (Du *et al*., 2009). Glicocorticóides elevados mostraram ser capazes de induzir prejuízos na oxidação mitocondrial, no potencial de membrana mitocondrial, e na capacidade de armazenamento de cálcio (De *et al*., 2009). De fato, um dos principais indutores de apoptose em linfócitos *in vitro* é a dexametasona, um análogo sintético do cortisol (Smith *et al*., 2013). Uma vez liberado pelo córtex da adrenal em resposta a eventos estressores (através da ativação do eixo HPA), o cortisol mobiliza glicose e lipídios para a circulação sanguínea e pode, assim, induzir um estresse metabólico significativo (visto que uma mobilização energética de longo prazo pode promover disglicemia e resistência à insulina) (Picard *et al*., in press). Para reduzir esses

efeitos, os níveis de cortisol são finamente regulados por uma alça de retroalimentação negativa no eixo HPA que faz com que estes retornem ao nível basal inicial após o pico induzido pelo evento estressor. De fato, os efeitos não neurológicos do estresse predizem a ocorrência de doenças cardiovasculares, assim como a diabetes mellitus, obesidade e síndrome metabólica (Sinha & Jastreboff, 2013). Neste contexto, considerando os achados prévios de altos níveis de cortisol em pacientes com TB e seus familiares e as potenciais consequências descritas para a hipercortisolemia, nós realizamos um estudo para melhor explorar a disfunção do eixo do estresse nesses indivíduos e identificar possíveis mecanismos que poderiam explicar essa disfunção.

Nossos resultados, apresentados no capítulo 3 desta tese, indicam que a disfunção do eixo HPA em pacientes está relacionada a uma hiporresponsividade do GR, o qual deveria estar ativo para uma eficiente alça de retroalimentação negativa do eixo. Nossos dados ainda indicam que esta hiporresponsividade em pacientes está associada a dois mecanismos: altos níveis protéicos de FKBP51 (uma cochaperona do GR que atua negativamente sobre o GR) e um aumento na metilação do gene *FKBP5* em regiões intrônicas distais. Em outras palavras, uma vez no citoplasma, o cortisol encontra um GR citoplasmático inibido pelos altos níveis de FKBP51 e, portanto, não pode induzir adequadamente uma translocação do receptor ao núcleo. Os GRs que translocam-se para o núcleo, por outro lado, encontram altos níveis de metilação nos elementos de resposta a glicocorticóides (GRE, do inglês *glucocorticoid response element*) no gene *FKBP5*, o que impede sua ligação ao gene e diminui a sua atuação como fator de transcrição (Klengel *et al*., 2013). Coletivamente, esses dois mecanismos colaboram para uma função reduzida do GR e, portanto, permitem que o cortisol permaneça em altos níveis por longos períodos após o evento estressor (consistente com o aumento de cortisol salivar pós-dexametasona em pacientes).

A disfunção do eixo HPA já foi anteriormente considerada um possível endofenótipo no TB, visto que esta já foi observada em pacientes assintomáticos (como no nosso estudo) e em familiares de primeiro grau de pacientes (sugerindo uma herdabilidade e segregação entre famílias). Nossos achados indicam que irmãos de pacientes com TB não apresentam prejuízos significativos na atividade do eixo HPA ou na responsividade do GR, mas apresentam resultados intermediários entre pacientes e controles para estes parâmetros. Isso reforça achados prévios, e, mais do que isso, os sugere como sendo características importantes na vulnerabilidade ao TB.

Ainda, considerando os aspectos relativos à progressão do TB, nós estendemos nossas análises principais e dividimos os pacientes em dois estágios, baseados num modelo de estadiamento clínico anteriormente proposto (Kapczinski *et al*., 2009): estágio inicial e estágio tardio. Nossas análises mostraram que apenas os pacientes em estágio tardio diferiram significativamente de controles nos níveis de cortisol salivar pós-dexamentasona e na responsividade ao GR, sugerindo que os mecanismos de disfunção do eixo HPA devido a uma hiporresponsividade do GR devam se dar em paralelo à progressão do TB. No entanto, não houve diferenças entre esses grupos quanto aos níveis de FKBP51 (mRNA e proteína), o que talvez seja consequência do baixo tamanho amostral por grupo que obtivemos ao realizar o estadiamento. Além disso, ao contrário da nossa hipótese *a priori*, a metilação do gene *FKBP5* se mostrou aumentada em um dinucleotídeo CpG apenas nos pacientes em estágio inicial. Isso vai de encontro à hipótese proposta para o TB, onde propusemos que o aumento na metilação diminui a ação do GR sobre o *FKBP5* e, consequentemente, a responsividade do GR. Como encontramos uma correlação negativa entre o número de episódios maníacos e a metilação do intron 7, podemos supor que a progressão do TB é acompanhada de um mecanismo que reduz esta metilação, possivelmente como uma tentativa de manter a responsividade do GR. Como este ainda se mantém hiporresponsivo em pacientes no estágio

tardio, é possível que outros mecanismos se tornem relevantes e se sobressaiam ao efeito compensatório na metilação (por exemplo, alteração de outras proteínas reguladoras do GR ou uma diminuição dos níveis do próprio receptor). Futuros estudos são necessários para esclarecer esses mecanismos.

Independentemente se a metilação do *FKBP5*, de fato, atua na disfunção do eixo HPA observada nos estágios tardios da doença, nossos resultados sugerem que a hiporresponsividade do GR está associada à progressão do TB. Este fato pode ter outras consequências importantes, considerando que os glicocorticóides, quando em situações normais de liberação e ativação de seus receptores, são moléculas com ação anti-inflamatória (Dejager *et al*., 2014). Uma vez estando os pacientes resistentes aos glicocorticóides, a inflamação apresentada pelos mesmos pode ser, então, amplificada. De fato, vários estudos já mostraram que pacientes com TB apresentam altos níveis de citocinas inflamatórias (Munkholm *et al*., 2013). Além disso, as próprias citocinas inflamatórias podem interferir no efeito dos glicocorticóides (Dejager *et al*., 2013), o que forma uma alça de retroalimentação positiva entre inflamação e resistência ao GR no TB (e, possivelmente, na sua progressão). A origem dessa alça ainda não está clara, mas possivelmente deva se iniciar com o aumento da inflamação, visto que pacientes em estágios iniciais do transtorno já apresentam níveis aumentados de citocinas em comparação a controles, permanecendo aumentados em estágios tardios (Kauer-Sant'Anna *et al*., 2009). Esta inflamação, por sua vez, pode ser resultado de uma série de fatores, inclusive de uma resposta à liberação de moléculas conhecidas como padrões moleculares associados ao dano (DAMPs, do inglês *damage-associated molecular patterns*) em consequência à morte celular (Figura 5). Essa hipótese é corroborada por estudos mostrando um aumento nos níveis de diferentes DAMPs em pacientes com TB (Stertz, 2014).

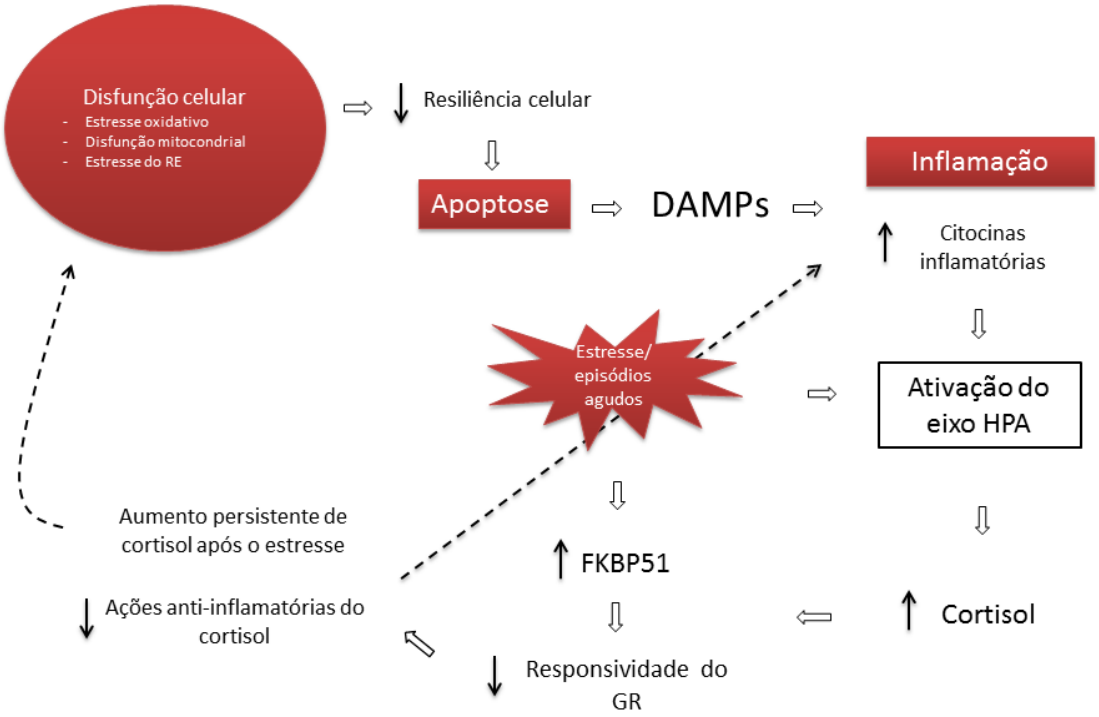

Figura 5. **Modelo hipotético para a relação entre a resistência a glicocorticóides e inflamação em pacientes com Transtorno Bipolar.** Prejuízos em certos processos celulares podem ser responsáveis pela diminuída resiliência celular apresentada por pacientes, os quais apresentam uma maior quantidade de células em apoptose (capítulo 2). Como consequência da morte celular, moléculas conhecidas como padrões moleculares associados ao dano (DAMPs) são liberadas na circulação sanguínea, as quais induzem uma resposta inflamatória. A inflamação estabelecida prejudica o efeito de glicocorticóides e, assim, colabora no estabelecimento de uma disfunção do eixo hipotálamo-pituitária-adrenal (HPA) e na ação de glicocorticóides (capítulo 3). Em paralelo, a baixa ação anti-inflamatória dos mesmos amplifica a inflamação nos pacientes, o que, em última instância, pode aumentar os efeitos danosos em nível celular e, dessa forma, estabelecer um ciclo vicioso. DAMPs – padrões moleculares associados ao dano; FKBP51 – proteína de ligação ao FK506 de 51 kDa; GR – receptor de glicocorticóide; HPA – hipotálamo-pituitária-adrenal.

Além destes mecanismos, outras vias já foram descritas como estando alteradas ao longo do curso da doença em pacientes com TB, conforme discutido no capítulo 1 desta tese. Mais do que isso, essas alterações podem vir a se tornar marcadores de estadiamento e prognóstico, possivelmente também de resposta ao tratamento. Em resumo, os achados da lituratura com relação à progressão do TB reforçam a resiliência celular e seus mecanismos associados como alvos-chave no desenvolvimento de tratamentos que sejam eficazes também em pacientes crônicos. Neste sentido, nós propomos o uso de moléculas que aumentem a resiliência celular, como os chamados ‘potencializadores mitocondriais’, para a reversão dos efeitos associados à progressão do TB (anexo 5).

Ainda neste contexto, estudos avaliando a interação entre aspectos genéticos e eventos ambientais estão sendo cada vez mais determinantes no entendimento da patofisiologia dos transtornos psiquiátricos. Já é consenso que alterações genéticas em genes individuais só exercem um efeito modesto, sendo os fenótipos determinados por complexas interações entre múltiplos genes e fatores ambientais (Zannas & Binder, 2014). Isso reforça a relevância da metilação do DNA (a qual é responsiva tanto ao ambiente quanto à variação genética) nestes transtornos. De fato, várias alterações na metilação do DNA já foram descritas em pacientes com transtornos de humor, como sumarizadas na Tabela 2. Aumentando ainda mais a complexidade de tais achados, alguns estudos tem mostrado também que a metilação gênica é capaz de interagir com alterações na sequência primária do DNA para determinar a atividade de um gene (Klengel *et al*., 2013).

A complexidade de tais alterações sugere a existência de mecanismos muito finamente regulados para a metilação do DNA. Esta reação é catalisada por enzimas conhecidas como DNA metiltransferases (DNMTs), as quais são divididas em três subtipos em células de mamíferos: DNMT1, DNMT3a e DNMT3b. A DNMT1 é a principal DNMT a executar a metilação de manutenção do DNA durante a replicação na fase S do ciclo celular, enquanto as DNMT3a e 3b são principalmente envolvidas na metilação *de novo* (Zimmermann *et al*., 2012). Assim como as alterações na metilação do DNA descritas na tabela anterior, alterações nos níveis das DNMTs também já foram descritas em pacientes com transtornos de humor (Tabela 3). No entanto, como sugerido por estudos mais recentes, a principal regulação dessas enzimas se dá na sua atividade (através de modificações pós-traducionais, por exemplo) (Estève *et al*., 2011; Lavoie & St-Pierre, 2011; Sun *et al*., 2007).

Tabela 2. Alterações na metilação do DNA em transtornos do humor

Transtorno	Gene analisado	Célula/tecido	Método	Achados	Referência
Transtorno depressivo maior	*ACE* (enzima conversora de angiotensina)	Leucócitos e cérebro pós-mortem	Sequenciamento de bissulfito	Aumento na metilação do gene *ACE* em pacientes. Correlação inversa entre marcadores inflamatórios e metilação.	Zill *et al*., 2012
	BDNF (fator neurotrófico derivado do cérebro)	Leucócitos	Pirosequenciamento de bissulfito	Altos níveis de metilação do gene *BDNF* foram associados à história prévia de tentativa de suicídio e com ideação suicida Além disso, a metilação foi capaz de predizer uma pior melhora na ideação suicida durante o tratamento, independentemente da alteração nos sintomas depressivos.	Kang *et al*., 2013a
	BDNF	Sangue periférico	Pirosequenciamento de bissulfito	A metilação do gene *BDNF* foi associada com piora dos sintomas depressivos durante um ano de seguimento (correlação positiva entre metilação e escore de depressão).	Kim *et al*., 2013
	BDNF	Sangue periférico	Análise de metilação por MassARRAY	Ausência de correlação entre metilação e escores de depressão. O perfil de metilação do exon I distinguiu pacientes e controles (marcador de diagnóstico).	Fuchikami *et al*., 2011
	BDNF	Área de Wernicke pós-mortem	Pirosequenciamento de bissulfito	Aumento da metilação do gene *BDNF* em suicidas, alguns dos quais tinham o diagnóstico de transtorno depressivo maior.	Keller *et al*., 2010
	Subunidade α1 do $GABA_A$	Córtex frontopolar pós-mortem	Sequenciamento de bissulfito	Aumento na metilação do gene que codifica para a subunidade α1 do receptor $GABA_A$ em suicidas com histórico de transtorno depressivo maior.	Poulter *et al*., 2008
	IL11 (interleucina 11)	Sangue periférico	Análise de metilação por MassARRAY	A metilação do gene *IL11* foi capaz de predizer a resposta a antidepressivos (baixos níveis de metilação foram associados a uma melhor resposta).	Powell *et al*., 2013

	SLC6A4 (transportador de serotonina)	Leucócitos	Pirosequenciamento de bissulfito	Alta metilação foi associada a adversidades na infância e se correlacionou com história familiar de depressão, maior percepção do estresse e apresentações mais graves de psicopatologia.	Kang *et al*., 2013b
	MAOA (monoamina oxidase A) e *NR3C1* (receptor de glicocorticóide)	Saliva	Análise de metilação por MassARRAY	Hipometilação do gene *MAOA* em mulheres deprimidas. Morte parental no início da vida e privação familiar foram associados com uma hipermetilação no gene *NR3C1*.	Melas *et al*., 2013
	Transportador de serotonina (*SERT)*	Linhagens de células linfoblastóides	Análise de metilação por MassARRAY	Mulheres apresentaram altos níveis de metilação do gene *SERT* em comparação aos homens. Tendência para a associação entre a metilação e os sintomas de depressão.	Philibert *et al*., 2008
	Vários genes	Sangue periférico	Microarranjo de metilação	Para alguns processos (p.ex., desenvolvimento cerebral e metabolismo do triptofano) os pacientes apresentaram altos níveis de metilação (e baixos níveis de expressão desses genes). De forma contrária, para outros processos (p.ex., lipoproteínas, atividade de hydrolase) os pacientes apresentam menores níveis de metilação (e alta expressão gênica). Houve uma correlação inversa entre metilação e os níveis séricos de IL-6, e entre a metilação do gene *IL-6* e os níveis séricos de proteína C-reativa.	Uddin *et al*., 2011
	Vários genes. Após validação: *LASS2*, *CPSF3*, *ZNF263* e *PRIMA1*	Córtex frontal pós-mortem	Arranjo de larga escala para metilação relativa (CHARM) e validação por pirosequenciamento de bissulfito	438 regiões diferencialmente metiladas entre pacientes e controles. Após validação, regiões próximas dos genes *LASS2*, *CPSF3*, *ZNF263*, and *PRIMA1* permaneceram diferentemente alteradas entre os grupos.	Sabunciyan *et al*., 2012
	Trk B-T1(tirosina cinase B)	Córtex frontal	Imunoprecipitação de DNA metilado (meDIP) seguido de microarranjo	Aumento da metilação do gene *Trk B-T1* em suicidas (alguns dos quais com transtorno depressivo maior).	Ernst *et al*., 2009

Transtorno bipolar (TB)	*5HTT* (transportador de serotonina)	Cérebro pós-mortem	PCR quantitativo específico para metilação (MSP)	Tendência para hipermetilação da região promotora do gene *5HTT* em pacientes com TB livres de antipsicóticos.	Abdolmaleky *et al*., 2014
	5HTR1A (transportador de serotonina tipo-1A)	Leucócitos	Desnaturação de alta resolução (HRM)	Hipermetilação do gene *5HTR1A* no TB.	Carrard *et al*., 2011
	BDNF	Células mononucleares de sangue periférico	MSP	Aumento da metilação do gene *BDNF* em pacientes com TB tipo II (mas não tipo I), em comparação com controles. Correlação negativa entre metilação e expressão gênica em pacientes com TB tipo II.	D'Addario *et al*., 2012
	Metilação global	Células linfoblastóides transformadas	ELISA	Metilação reduzida em pacientes com TB e seus familiares em comparação com controles, permanecendo desta forma após o tratamento com lítio em pacientes, mas não em familiares.	Huzayyin *et al*., 2013
	Metilação global, *COX-2*, *BDNF*, proteína tipo debrina	Cérebro pós-mortem	MSP e ELISA (para metilação global)	Hipermetilação global, hipometilação do gene *COX-2*, hipermetilação do gene *BDNF*, hipermetilação do promotor do gene que codifica a proteína tipo debrina.	Rao *et al*., 2012
	HCG9 (antígeno leucocitário humano grupo 9)	Cérebro pós-mortem e células de sangue periférico	Pirosequenciamento de bissulfito	Baixa metilação do gene *HCG9* em pacientes comparados a controles.	Kaminsky *et al*., 2012
	HTR2A (receptor de serotonina tipo 2)	Lobo frontal pós-mortem	MSP	Hipermetilação do promotor do gene *HTR2A* ao redor da região polimórfica -1438A/G, e hipometilação da região T102C em pacientes em comparação a controles.	Abdolmaleky *et al*., 2011
	HTR2A (receptor de serotonina tipo 2A)	Saliva	MSP e sequenciamento de bissulfito	Hipometilação do gene *HTR2A* na região polimórfica T102C em pacientes.	Ghadirivasfi *et al*., 2011
	MB-COMT (catechol-O-metiltransferase ligada à membrana)	Saliva	MSP	Hipometilação do promotor do gene *MB-COMT* em pacientes comparados a controles.	Nohesara *et al*., 2011

	MB-COMT	Lobo frontal pós-mortem	MSP e sequenciamento de bissulfito	Hipometilação do promotor do gene *MB-COMT* em pacientes comparados a controles.	Abdolmaleky *et al*., 2006
	RELN (REELIN)	Encéfalo pós-mortem	Análise por enzimas de restrição e PCR	Ausência de correlação entre os níveis de metilação do gene *RELN* e idade em pacientes, ao contrário do encontrado em controles saudáveis (sugerindo a possibilidade de que uma aberração epigenética do status normal de metilação do gene *RELN* pode conferir uma maior susceptibilidade a transtornos psiquiátricos).	Tamura *et al*., 2007
	Vários genes, especialmente *ST6GALNAC1* (membro da família de moléculas sialiltransferase)	Sangue periférico e cérebro pós-mortem	Ensaio de metilação Infinium	Hipometilação da região promotora do gene *ST6GALNAC1* em gêmeos afetados com psicose. Esta hipometilação também foi encontrada em cérebros pós-mortem de pacientes.	Dempster *et al*., 2011
	SLC6A4 (transportador de serotonina)	Linhagens de célula linfoblastóide e cérebros pós-mortem	Sequenciamento de bissulfito	Hipermetilação do gene *SLC6A4* no TB (em gêmeos com TB e na comparação entre casos e controles).	Sugawara *et al*., 2011
	Regiões *upstream* da espermina sintase (*SMS*) e peptidilprolilisomerase tipo E (*PPIEL*) (CN265253)	Células linfoblastóides transformadas	Análise de diferença representacional sensível à metilação (MS-RDA)	Metilação aberrante em regiões *upstream* dos genes que codificam a espermina sintase (*SMS*) e a peptidilprolilisomerase tipo E (*PPIEL*) em pacientes.	Kuratomi *et al*., 2008
	Cromossomo X	Mucosa bucal e leucócitos periféricos	Análises por enzimas de restrição	Gêmeos discordantes para TB mostraram ser os mais discordantes para a metilação dos alelos maternos e paternos do cromossomo X, especialmente em comparação a gêmeos concordantes para o mesmo transtorno.	Rosa *et al*., 2008
	Metilação global	Leucócitos	Método de extensão de citosinas.	Sem diferenças na metilação global entre pacientes e controles.	Bromberg *et al*., 2009

Tabela 3. Alterações na expressão de DNMTs descritas em transtornos de humor

Transtorno	Célula/tecido	Alteração	Referência
Transtorno depressivo maior	Córtex frontopolar	Diminuição e aumento na expressão de DNMT1 e DNMT3b, respectivamente, em pacientes com depressão vítimas de suicídio.	Poulter *et al*., 2008
	Núcleo paraventricular	Aumento na expressão de DNMT em pacientes com depressão vítimas de suicídio.	Poulter *et al*., 2008
	Células mononuclares de sangue periférico	Redução e aumento nos níveis de mRNA da DNMT1 DNMT3b, respectivamente, em pacientes com depressão aguda (mas não em remissão).	Higuchi *et al*., 2011
	Amígdala	Redução na expressão de DNMT1 e DNMT3B em pacientes com depressão vítimas de suicídio.	Poulter *et al*., 2008
Transtorno bipolar	Córtex pré-frontal	Aumento de neurônios positivos para mRNA de DNMT1 em pacientes psicóticos (incluindo pacientes com TB e esquizofrenia) comparados a indivíduos não psicóticos.	Guidotti *et al*., 2013
	Células mononucleares de sangue periférico	Redução na expressão do mRNA da DNMT1 na depressão bipolar, mas não na eutimia.	Higuchi *et al*., 2011
	Área 9 de Brodmann e neurônios GABAérgicos	Aumento de S-adenosil metionina e expressão de mRNA de DNMT1 em pacientes com TB e esquizofrenia.	Guidotti *et al*., 2007
	Área 9 de Brodmann e neurônios GABAérgicos	Aumento dos níveis de mRNA e protéicos da DNMT1 em pacientes com TB com psicose.	Veldic *et al*., 2005

Neste contexto, o quarto e último capítulo desta tese se propôs a avaliar mecanismos pelos quais o estresse (modulado pelo GR e suas proteínas regulatórias, incluindo o FKBP51) pode influenciar na atividade da DNMT1 (podendo, em última instância, influenciar o padrão

de metilação de genes específicos com relevância clínica). Este estudo se baseou em achados prévios da literatura sugerindo um efeito inibidor de alguns antidepressivos sobre a atividade da DNMT1 (Zimmermann *et al*., 2012), além dos efeitos do tipo antidepressivo apresentados por inibidores da metilação do DNA em estudos pré-clínicos (Sales *et al*., 2011). Em resumo, nossos resultados sugerem um mecanismo de competição entre as proteínas FKBP51 e FKBP52 para a ligação à cinase dependente de ciclina 5 (CDK5, do inglês *cyclin-dependent kinase* 5) e, consequentemente, efeitos opostos de ambas na fosforilação (e possivelmente atividade) da DNMT1. Mais ainda, os dados apresentados sugerem que o FKBP51 é fundamental para o efeito dos antidepressivos *in vitro* e *in vivo*.

Conforme avaliado em células periféricas de pacientes com transtorno depressivo maior, esses mecanismos parecem ser relevantes e determinantes para a resposta clínica dos mesmos ao tratamento. Ainda, nós pudemos predizer a resposta clínica dos pacientes em um ensaio *ex vivo* com PBMCs baseando-se nos mesmos parâmetros (modulação da fosforilação da DNMT1 na Serina 154 em resposta ao tratamento com antidepressivos). Entre diversas implicações clínicas, esses resultados sugerem o uso de tal ensaio (e avaliação da pDNMT1^{S154}) como um marcador laboratorial de resposta ao tratamento com antidepressivos.

De forma geral, nós pudemos obervar papéis diferentes para o FKBP51 nos dois transtornos de humor estudados nesta tese. No caso do TB, altos níveis de FKBP51 foram 'responsabilizados' pela disfunção do eixo HPA observada em pacientes. Por outro lado, no caso do transtorno depressivo maior, altos níveis de FKBP51 parecem ser necessários para o efeito de antidepressivos e uma melhor resposta clínica em pacientes. Embora pareça discordante, uma situação semelhante já foi observada em pacientes com depressão maior, onde o genótipo do gene *FKBP5* que leva a maiores níveis de FKBP51 foi associado a uma maior recorrência de episódios agudos e, da mesma forma, a uma melhor resposta clínica ao tratamento (Binder *et al*., 2004). Isso reforça a complexidade dos mecanismos associados a

esta proteína e a importância de futuros estudos que dêem seguimento aos resultados apresentados nesta tese.

5. CONCLUSÕES

Com base nos resultados apresentados nesta tese, podemos concluir que:

- A progressão do TB parece envolver, além da disfunção do HPA, uma série de outros mecanismos biológicos envolvendo resiliência celuar, fatores neurotróficos, inflamação, estresse oxidativo e encurtamento de telômero;
- Pacientes com TB apresentam uma maior porcentagem de PBMCs em apoptose inicial em comparação a controles, possivelmente decorrente da toxicidade sistêmica e de prejuízos em mecanismos de resiliência celular, como a disfunção mitocondrial e o estresse do RE;
- Pacientes com TB apresentam uma disfunção no eixo HPA associada a uma menor responsividade do GR em células mononucleares de sangue periférico. Esta hiporresponsividade do GR pode ser resultado dos altos níveis protéicos de FKBP51 e do aumento de metilação intrônica no gene *FKBP5*;
- As proteínas FKBP51 e FKBP52 competem entre si para a ligação à CDK5 e, consequentemente, apresentam efeitos opostos sobre a fosforilação da DNMT1, sugerindo um mecanismo molecular para os efeitos do estresse sobre marcadores epigenéticos;
- Antidepressivos exercem seus efeitos sobre a fosforilação da DNMT1 de forma dependente da proteína FKBP51. Neste sentido, a redução nos níveis de DNMT1 fosforilada foi correlacionada com uma melhor resposta clínica ao tratamento em pacientes com transtorno depressivo maior.

Em conclusão, nós sugerimos que a resiliência celular, a resposta ao estresse e a metilação do DNA desempenham importantes papéis na patofisiologia, progressão e resposta ao tratamento de pacientes com transtornos de humor. Novos tratamentos com alvo na modulação do GR, no aumento da resiliência celular e em mecanismos epigenéticos devem ser priorizados em estudos posteriores.

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ANEXOS

ANEXO 1

Lista de figuras

apresentam uma maior quantidade de células em apoptose (capítulo 2). Como consequência da morte celular, moléculas conhecidas como padrões moleculares associados ao dano (DAMPs) são liberadas na circulação sanguínea, as quais induzem uma resposta inflamatória. A inflamação estabelecida prejudica o efeito de glicocorticóides e, assim, colabora no estabelecimento de uma disfunção do eixo hipotálamo-pituitária-adrenal (HPA) e na ação de glicocorticóides (capítulo 3). Em paralelo, a baixa ação anti-inflamatória dos mesmos amplifica a inflamação nos pacientes, o que, em última instância, pode aumentar os efeitos danosos em nível celular e, dessa forma, estabelecer um ciclo vicioso.....110

ANEXO 2

Lista de tabelas

ANEXO 3

Capítulo aceito para publicação no livro "*Bipolar Disorder: Millennium Update*", da Oxford University Press (OUP), editado por Aysegul Yildiz, Charles Nemeroff e Pedro Ruiz.

Título: *Loss of cellular resilience and neurodegeneration and their impact on the treatment of bipolar disorder*

Autores: Gabriel Rodrigo Fries, Flávio Kapczinski

Capítulo de livro: Loss of cellular resilience and neurodegeneration and their impact on the treatment of bipolar disorder

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Abstract

Impairments in cognition and functioning reported in patients with bipolar disorder (BD) have been associated with several neuroanatomical alterations in brain structures known to play key roles in coping and stress resilience mechanisms. In this scenario, a question has been raised as to whether such alterations are associated with neuroplasticity impairments or neurodegenerative-like mechanisms. Patients with BD present several biochemical signs of cell death (both in the central nervous system and in the periphery), but impairments in neuroplasticity mechanisms, including the role of neurotrophic factors, are usually the most striking findings. This chapter discusses different potential mechanisms of impaired cellular resilience in BD, emphasizing the relevance of these processes for illness progression and treatment.

Keywords: cellular resilience, neurodegeneration, neuroprogression, neuroplasticity, apoptosis, neurotrophic factors, endoplasmic reticulum stress

List of abbreviations: BAD, Bcl-2-associated death promoter; BAX, Bcl-2-associated X protein; BD, bipolar disorder; BDNF, brain-derived neurotrophic factor; ER, endoplasmic

reticulum; HPA, hypothalamus-pituitary-adrenal; NAA, N-acetyl-aspartate; PBMC, peripheral blood mononuclear cell; UPR, unfolded protein response.

1. Introduction

Bipolar disorder (BD) is often associated with pronounced cognitive impairments involving executive functions and verbal memory (Martinez-Aran et al., 2000; Robinson et al., 2006). Most notably, these impairments seem to be progressive in nature, increasing with number of episodes and length of illness (Martinez-Aran et al., 2004). Several studies have shown that the cognitive decline observed in BD is associated with neuroanatomical alterations, including gray matter reduction in the dorsolateral prefrontal cortex (Ekman et al., 2010) and enlargement of the lateral ventricles. Notwithstanding, a question has been raised as to whether such alterations are associated with neuroplasticity impairments or neurodegenerative mechanisms (Jakobsson et al., 2013). Even though the exact mechanisms underlying these findings are unknown, cellular resilience and plasticity do seem to play a role in the establishment and progression of BD.

Cellular resilience can be defined as the ability of a given cell to cope with and survive in response to stimuli that would otherwise lead to its death. Resilience mechanisms are constantly activated by cells in response to internal stimuli, such as mitochondrial dysfunction and endoplasmic reticulum (ER) stress, as well as external stimuli, such as activation of certain membrane receptors and excitotoxicity, among others. Moreover, cells can modulate their behavior and connections with other cells in response to stimuli, a process known as cell plasticity. In particular, the so-called neuroplasticity is responsible for the neurons' ability to restore axonal and dendritic connections under specific circumstances, as well as to form new connections in response to environmental stimuli, e.g., stress. Neuroplasticity mechanisms are responsible for maintaining neuronal networks and cognitive functions, including memory

and functioning. As a result, impairments in neuroplasticity can lead to shrinkage of brain structures due to a reduction in the connection between cells or in the complexity of the brain cell network.

As opposed to neuroplasticity impairments, neurodegeneration is defined as neuronal loss of function with an underlying neuropathology, and mostly associated with a cell death outcome. Typical neurodegenerative diseases show markers of protein aggregation and neuronal death/toxicity, e.g., Lewy bodies in Parkinson's disease and beta-amyloid peptides in Alzheimer's disease. Of note, no evidence of Alzheimer-type neurodegeneration (assessed by levels of amyloid precursor protein metabolites in cerebrospinal fluid) was found in patients with BD (Jakobsson et al., 2013). Even so, neuropathological findings strongly support the occurrence of degenerative-like mechanisms in BD, as will be discussed over the next sections. This chapter aims to review different findings on neurodegeneration and neuroplasticity in BD, aiming at identifying the most probable mechanisms underlying BD progression.

2. Neuroimaging and neuropathological findings in BD

Neuroimaging studies undertaken to better understand BD pathophysiology and progression have suggested that neuroanatomical alterations occur in specific brain regions rather than in the whole brain. One of the neuroanatomical alterations most consistently reported in patients with BD is lateral ventricle enlargement (Kempton et al., 2008), which has also been positively correlated with the occurrence of multiple episodes (Strakowski et al., 2002; Brambilla et al., 2001a). In fact, several alterations seem to take place with BD progression. For instance, illness duration has been correlated with smaller left putamen and reduced left inferior prefrontal gray matter volume (Lopez-Larson et al., 2002), as well as with smaller total brain gray matter volume (Frey et al., 2008). Hippocampal volume has also

been reported to progressively decrease as illness progresses (Javadapour et al., 2010), accompanied by an apparent increase in amygdala volume (Bora et al., 2010). Changes in the shape and volume of the basal ganglia in general and of the striatum in particular have also been described in patients with BD, and seem to take place at onset but remain at later stages (Hwang et al., 2006; Bora et al., 2010; Strakowski et al., 1999). In addition, an inverse correlation has been found between age and total gray matter volume in individuals with BD, suggesting faster age-related cortical neuronal loss in BD (Brambilla et al., 2001b). Given that most of the observable cerebral changes in BD involve the gray matter, the occurrence of neurodegenerative processes in association with illness progression has been suggested (Frey et al., 2008). Conversely, no changes in global brain volume have been detected (Hoge et al., 1999; Strakowski et al., 2005).

Neuropathological studies have identified a reduction in glial cell number and density, as well as some alterations in the density and size of specific types of cortical neurons (Rajkowska, 2002). There is also evidence to suggest that both inhibitory and excitatory neurons in the prefrontal and limbic cortical regions are reduced in BD. Finally, postmortem studies have reported reductions in neuronal cell density, apparently more subtle than the corresponding glial alterations (Rajkowska, 2002). It remains to be known whether most of these changes actually represent loss of cells or simply a consequence of reduced branching and connections between them.

3. Biochemical evidence of cell death and loss of cell resilience in BD

Along with morphometric studies, several biochemical findings suggest the involvement of cell death and loss of resilience in the pathophysiology of BD. For instance, these patients have been shown to present bilateral decreased levels of N-acetyl-aspartate (NAA) in the hippocampus and in the dorsolateral prefrontal cortex (NAA is a molecule

found in mature neurons, considered a measure of neuronal as opposed to glial viability, function, and integrity). Patients also present decreased NAA levels in the dorsolateral prefrontal cortex, which may reflect an underdevelopment of dendritic arborizations and synaptic connections (Sassi et al., 2005). Lithium is able to increase NAA levels in the brain of patients with BD (Bertolino et al., 1999; Winsberg et al., 2000), which further confirms the relevance of this molecule for BD treatment.

A growing body of evidence also points to increased levels of apoptotic factors in BD, including increased apoptotic serum activity (Politi et al., 2008), DNA damage in peripheral blood (Andreazza et al., 2007), mitochondrial dysfunction (Shao et al., 2008), and altered expression of molecules involved in cell survival (Herberth et al., 2011). In fact, a recent study has reported an increased percentage of peripheral blood mononuclear cells (PBMCs) in early apoptosis in patients with BD when compared to controls (Fries et al., 2013).

With regard to the nervous system, a postmortem study has found signs of apoptosis in olygodendrocytes from the frontal lobe (BA 10) of brains of patients with BD, including decreased nuclear size, cell shrinkage, and condensation of nuclei and nuclear chromatin (Uranova et al., 2001). Other findings already reported include decreased levels of Bcl-2 in BA 9, decreased levels of brain-derived neurotrophic factor (BDNF) (which can act as an antiapoptotic factor), and increased levels ofBcl-2-associated death promoter (BAD), Bcl-2-associated X protein (BAX), caspase-9, and caspase-3, all known to induce apoptosis (Kim et al., 2010; Gigante et al., 2011). These results are corroborated by the findings of a microarray study in the hippocampus of patients with BD, in which an up-regulation of 19/44 genes related to apoptosis were identified, along with a down-regulation of antiapoptotic genes (Benes et al., 2006). As a consequence of these alterations, cells from patients with BD are considered to be less resilient to different insults. For instance, cells from the olfactory

neuroepithelium of patients with BD have been shown to present an increased vulnerability to cell death (McCurdy et al., 2006).

In sum, all of these studies support the involvement of cell death in BD and partly explain some of the neuroanatomical findings previously discussed. The mechanisms by which these alterations take place, however, are far from being clearly understood.

4. Potential mechanisms

Several mechanisms may be responsible for the alterations here described, but a few altered pathways have been consistently reported in patients with BD and seem to play key roles in the modulation of cell death/survival and resilience. In this section, we will discuss evidence of biological alterations often found in BD, which could ultimately be involved in these processes, including neurotrophic factors, excitotoxicity, glucocorticoids, mitochondrial dysfunction, and ER stress.

4.1 Neurotrophic factors

Neurotrophic factors comprise a family of proteins that are highly abundant in the nervous system and play key roles in the survival, growth, and plasticity of neuronal cells. BDNF is the most abundant neurotrophin in the adult mammalian brain, and it has been shown to be crucial for neuroplasticity and for the mechanisms of action of antidepressants, antipsychotics, and mood stabilizers (Grande et al., 2010). Because of the ability of this neurotrophin to cross the blood-brain barrier, peripheral BDNF levels have been consistently used as a biomarker of disease activity and progression, combined with several clinical variables in patients with BD. For instance, BDNF has been associated with cognitive deficits (Rybakowski et al., 2003), and peripheral BDNF levels have been found to be reduced during acute episodes and to return to normal levels after symptom remission (Fernandes et al., 2011;

Tramontina et al., 2009). Moreover, lower BDNF levels have been observed in patients at late stages of illness when compared to early-stage patients and healthy controls (Kauer-Sant'Anna et al., 2009).

Once disrupted, impaired BDNF signaling can lead to decreased dendritic spines and arborization, neuritic dystrophy and degeneration, neuronal atrophy, and reduced neurogenesis (Teixeira et al., 2010). Animal studies have consistently described that these alterations in BDNF can lead to cognitive impairments and depressive behavior, mostly reversible after re-establishment of normal BDNF functions (Teixeira et al., 2010). A scenario of reduced neurotrophic signaling, along with decreased levels of antiapoptotic factors and antioxidant molecules, may partially explain the reduced cell resilience found in BD.

Furthermore, the implication of BDNF in the pathophysiology of BD is strongly supported by studies showing that mood stabilizers are able to increase BDNF levels both *in vitro* and *in vivo*(Frey et al., 2006; Yoshimura et al., 2006; Yasuda et al., 2009). Interestingly, BDNF alterations in BD seem to be associated with epigenetic changes, as an increased methylation at BDNF gene promoter I has been found in PBMCs from patients with BD type II compared with controls. This mechanism may link genetic alterations in BDNF to external stimuli (D'Addario et al., 2012), underscoring the key role of the environment in BD pathophysiology.

4.2 Calcium, excitotoxicity, and ER stress

The neuroprotective properties of mood stabilizers and antidepressants have been consistently explained, among other mechanisms, by their ability to counteract glutamate-induced excitotoxicity *in vitro* (Chuang, 2004; Leng et al., 2013). This feature is of great relevance for BD due to the key roles apparently played by glutamate and calcium in the disorder. Increased serum glutamate levels have been reported in patients with BD during

mania and depression (Altamura et al., 1993; Hoekstra et al., 2006), and increased glutamate has also been found in the frontal cortex of these patients (Hashimoto et al., 2007). Moreover, individuals with BD have been shown to present increased glutamate and/or glutamine levels in the plasma and cerebrospinal fluid (Zarate Jr et al., 2003), which in turn may explain the increased intracellular calcium levels. Sufficiently high concentrations of calcium can induce excessive cytosolic calcium mobilization and cause overactivity of several calcium-dependent enzymes (Sapolsky, 2000). Finally, this could lead to a scenario of cytoskeletal degradation, protein misfolding, and oxygen reactive species generation, ultimately leading to cell death. Elevated calcium levels have been found in PBMCs from patients with BD (Perova et al., 2008), and disturbances of intracellular calcium homeostasis have been consistently reported in BD (Warsh et al., 2004).

Cells are strictly regulated by several mechanisms to prevent the consequences of increased intracellular calcium levels. Therefore, alterations in intracellular calcium levels, as commonly reported in patients with BD, may be induced by dysfunction of specific cellular organelles, e.g., ER and mitochondria, which typically play key roles in intracellular calcium sequestration, buffering, and storage (Warsh et al., 2004). For instance, the ER presents calcium ATPases that pump calcium from the cytosol into the ER lumen, whereas mitochondria may sequester the ion via an electrogenic transporter located on its inner membrane.

ER dysfunction in BD has been suggested by several studies assessing the so-called unfolded protein response (UPR), a mechanism typically activated by the ER to restore its function after the accumulation of unfolded proteins in the lumen. Typically, the ER responds to a transient dysfunction by up-regulating chaperones, which can handle the increasing amount of unfolded proteins, and decreasing overall protein synthesis until homeostasis is restored. Cells from patients with BD have been shown to present an impaired UPR,

characterizing ER stress (So et al., 2007), a scenario that can ultimately lead to the activation of apoptotic pathways. In fact, ER stress has been considered one of the mechanisms against which cells from patients with BD are considered to be the least resilient (Fries et al., 2012). Of note, this mechanism seems to be central for the action of mood stabilizers: chronic valproate and lithium treatment, for example, can increase the expression of ER chaperones *in vivo* (Chen et al., 2000; Shao et al., 2006), and thus help counteract ER stress.

4.3 Stress, glucocorticoids, and mitochondrial dysfunction

Several studies have suggested that chronic stress plays a key role in the pathophysiology of BD. Most of the effects of stress are mediated by cortisol, which is released and controlled by the hypothalamus-pituitary-adrenal (HPA) hormonal axis. Typically, high circulating levels of cortisol are able to bind to glucocorticoid receptors in the hypothalamus and repress cortisol release by the adrenal cortex. However, this negative feedback loop can be impaired in some patients, culminating with chronic increased cortisol levels and/or an enhanced cortisol response to stress. Patients with BD have been shown to present impairments in the HPA axis, as seen by the inability of a high number of patients to suppress cortisol release in response to dexamethasone (Daban et al., 2005). Glucocorticoids can also induce regression of dendritic processes in neurons and inhibit neurogenesis (Sapolsky, 2000). Of note, adult neurogenesis has been linked to stress buffering and antidepressant-like behavior (Snyder et al., 2011). Finally, glucocorticoids can increase glutamate concentrations in hippocampal synapses, potentially contributing to excitotoxic mechanisms, as discussed previously.

In addition, increased cortisol levels may have important long-term consequences at the cellular level. For instance, *in vitro* and animal studies have shown that chronic stress and chronic exposure to high levels of glucocorticoids can lead to mitochondrial dysfunctions,

including reductions in oxygen consumption, mitochondrial membrane potential, and calcium holding capacity (Du et al., 2009). Once dysfunctional, mitochondria can induce the opening of the mitochondrial permeability transition pore, thus releasing cytochrome c from the intermembrane space and inducing apoptotic cascades. Several studies have reported mitochondrial dysfunctions in BD, including impaired energy metabolism, alterations in respiratory chain complex enzymes, and down-regulation of mitochondria-related genes, among others (Quiroz et al., 2008). Cerebrospinal fluid lactate concentrations have also been shown to be significantly higher in patients with BD than in controls, suggesting increased extra-mitochondrial and anaerobic glucose metabolism (indicators of impaired mitochondrial metabolism) (Regenold et al., 2009). In this scenario, the use of 'mitochondrial enhancers' as an add-on treatment in the management of BD would be desirable, aiming at preventing mitochondrial dysfunction and thus increasing cellular resilience (Fries & Kapczinski, 2011).

5. Clinical implications and treatment impact

Most of the mechanisms here described seem to worsen with illness progression. In other words, neuroprogression in BD seems to be closely associated with a reduction in resilience mechanisms, making patients more vulnerable to new mood episodes and less responsive to medications (Fries et al., 2012; Kapczinski et al., 2008). The type and direction of the association between resilience at the cellular level and stress and coping mechanisms in patients with BD remains to be elucidated; however, considering that neuroprogression is implicated in several of the aforementioned biological mechanisms, one can infer that both scenarios could interfere with each other.

With regard to coping mechanisms, patients with BD are significantly less likely to use adaptive coping strategies and more likely to use maladaptive ones when compared to healthy controls (Fletcher et al., 2013). These coping strategies have been hypothesized to be

influenced by the neurotoxicity associated with illness progression and allostatic load (Kapczinski et al., 2008), which corroborates our hypothesis of impaired coping mechanisms being caused by reduced cell resilience. A summary of this process is illustrated in Figure 1. Briefly, external stimuli can induce cell signaling pathways that need to be dealt with. Depending on the resilience of a given cell, the activated mechanisms can result in cell survival and ultimately lead to a favorable clinical status of coping and cognition. Conversely, in cases of impaired resilience, the same stimuli can lead to cell death and ultimately to clinically observed impairments.

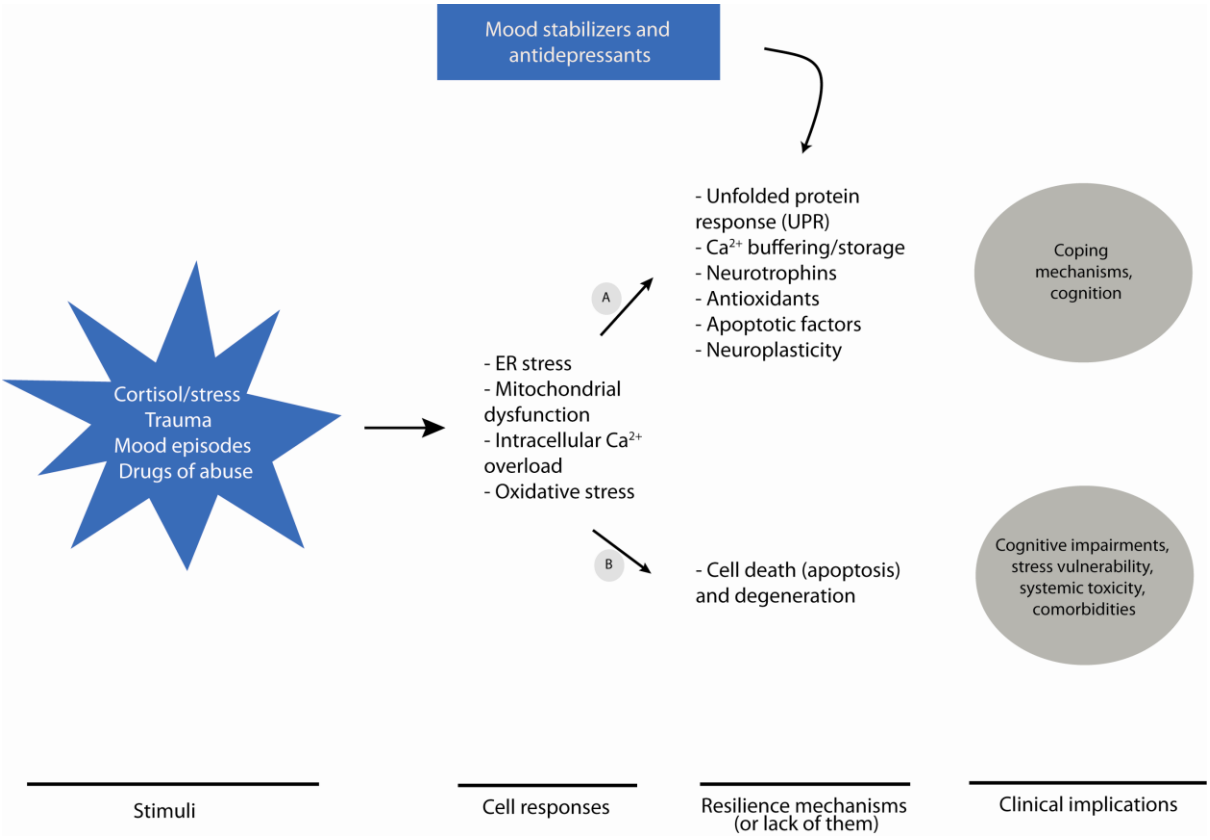

Figure 1. Cellular resilience and plasticity in BD. Different stimuli interfere with cellular responses and functions, e.g., in neurons and peripheral blood mononuclear cells (PBMCs). Such insults may lead to several cellular responses, such as endoplasmic reticulum (ER) stress, mitochondrial dysfunction, intracellular calcium overload, and oxidative stress. Depending on the cells' ability to respond and cope with these responses, they can be resilient

(A) or ultimately die (B). Resilience mechanisms include activation of the unfolded protein response (UPR) in the ER, buffering and storage of excessive intracellular calcium, release of neurotrophins, as well as the induction and/or activation of antioxidant molecules and antiapoptotic factors. In neurons, these mechanisms can lead to enhanced neuroplasticity and possibly the formation of new dendrites. Of note, most of these responses have been shown to be induced by mood stabilizers and antidepressants. As a consequence, cellular resilience and plasticity mechanisms may end up improving cognitive tasks and coping mechanisms. Conversely, impaired resilience mechanisms can lead to enhanced vulnerability to stress and impaired cognitive functions.

In addition, deficits in neuroplasticity, as suggested by reduced levels of neurotrophic factors, may be directly associated with several of the cognitive impairments reported in patients with BD. These deficits are seen in all mood states (including euthymia), and may include attention, verbal memory, and executive function impairments (Martínez-Arán et al., 2004; Vieta et al., 2013). Some aspects of cognition, e.g., executive measures, have also been found to be associated with illness duration (Torrent et al., 2012). Among the neurobiological underpinnings of cognition, BDNF has been considered a key mediator of long-term potentiation and other neuroplasticity mechanisms. Therefore, it is possible that the reduction in BDNF levels that takes place with illness progression (Kauer-Sant'Anna et al., 2009) may mediate some of the cognitive impairments reported in patients. This is a crucial aspect when considering the mechanisms of action of mood stabilizers and antidepressants.

It is currently a consensus that mood stabilizers improve cell viability, resilience, and plasticity, both *in vitro* and *in vivo* (Bachmann et al., 2005). For instance, lithium has the ability to increase the levels of Bcl-2 (an antiapoptotic protein), heat shock protein 70, and BDNF, thus protecting cells against stimuli that would otherwise activate apoptosis. Lithium

can also inhibit the enzyme glycogen-synthase kinase 3β, involved in several cellular processes, e.g., inflammation and apoptosis. In this same vein, mood stabilizers have been shown to increase the levels of neurotrophic factors (part of their neuroprotective properties). Finally, by suppressing microglial activation and neuroinflammation, these drugs end up reducing neuronal toxicity and ultimately attenuating neurodegeneration (Yu et al., 2012). In summary, the mechanisms of action of mood stabilizers seem to counteract most of the loss of neuroplasticity and neurodegenerative-like mechanisms described in patients with BD, which once again underscores the relevance of such alterations for the pathophysiology and treatment of BD.

The fact that illness progression has been associated with reduced responsiveness to treatment (Swann et al., 1999) raises an important question regarding neuroplasticity and neurodegeneration mechanisms in BD. One can hypothesize that epigenetic programming may occur with illness progression, possibly by means of DNA methylation at specific genes whose expression would normally be induced by mood stabilizers. As a consequence, these drugs would find a repressed chromatin, and would fail to induce important neuroprotective genes. As previously mentioned, methylation has been found in BD not only at the *BDNF* promoter but also at other genes of relevance. Nonetheless, further studies are required to assess the relationship between BD neuroprogression and epigenetic alterations.

6. Conclusions

In summary, there is evidence to suggest that both neuroplasticity impairments and neurodegenerative-like mechanisms take place in BD and are strongly associated with illness progression. However, BD does not seem to fit into the category of neurodegenerative disorders, as is the case with Alzheimer's and Huntington's disease, because the neurodegenerative features observed seem to be more subtle than the neuroplasticity

impairments and their consequences. Moreover, BD presents a different pattern of neuronal and glial cell pathology when compared to typical neurodegenerative disorders (Jakobsson et al., 2013).

Some hypotheses have recently been proposed to explain the role of apoptosis and cell death in BD. An interesting approach is the assumption that apoptosis may be engaged locally in synapses rather than in the cell body, in a process described as 'synaptic apoptosis' (Mattson & Duan, 1999). This would provide a pathophysiological explanation for the apoptotic markers found in postmortem studies in BD, as well as for the synaptic remodeling observed in some brain structures and the reduced plasticity reported in patients. Of note, lithium has been shown to increase hippocampal dendritic arborization *in vivo* (Watase et al., 2007), possibly due to its antiapoptotic and neurotrophic properties.

A better understanding of the mechanisms associated with BD neuroprogression, whether involving neurodegeneration or neuroplasticity impairments, may shed light on the mechanisms by which patients become more vulnerable to stress and new mood episodes, thereby establishing a vicious cycle of dysfunction. By enhancing resilience at the cellular level, alterations in neuronal networks and much of the cognitive and functional impairment observed could probably be prevented. Moreover, in peripheral cells, an increased resilience would contribute to reducing inflammation and the systemic toxicity reported in patients, ultimately reducing systemic comorbidities and improving the patients' quality of life.

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ANEXO 4

Termos de consentimento livre e esclarecido para pacientes e controles, conforme aprovado pelo Grupo de Pesquisa e Pós-Graduação (GPPG) do Hospital de Clínicas de Porto Alegre, sob o número 12-0102.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PACIENTES

AVALIAÇÃO DO EIXO HIPOTÁLAMO-PITUITÁRIA-ADRENAL (HPA), RECEPTOR DE GLICOCORTICÓIDE E FUNÇÃO MITOCONDRIAL EM PACIENTES COM TRANSTORNO DE HUMOR BIPOLAR

Nome do Paciente: _____Data de Nascimento:_____
Pesquisador Responsável: Prof. Dra. Márcia Kauer Sant'Anna
Pesquisador Executor: MSc. Gabriel Rodrigo Fries

Antes de participar deste estudo, gostaríamos que você tomasse conhecimento do que ele envolve. Damos abaixo alguns esclarecimentos sobre dúvidas que você possa ter. Em caso de qualquer outra dúvida quanto ao estudo e o que ele envolve ou sobre os seus direitos, você poderá contatar a Dra. Márcia Kauer Sant'Anna, pelo telefone: (51) 3359-8845, ou o pesquisador Gabriel Fries, pelo telefone: (51) 3359-8021.

Qual o objetivo desta pesquisa?
Esta pesquisa tem como objetivo ampliar os conhecimentos acerca das causas e das características do Transtorno de Humor Bipolar (THB). Existe uma possível relação entre alterações no sangue, estresse e THB, porém mais estudos devem ser feitos para confirmar esta associação. Neste estudo, vamos também avaliar se as alterações encontradas no sangue estão relacionadas com características da doença e com algumas características genéticas. Para participar desse estudo, você deverá ter o diagnóstico de THB tipo I, ter mais de 18 anos, e se for mulher, não estar grávida ou amamentando.

O que acontecerá neste estudo?
Inicialmente, um psiquiatra da equipe fará perguntas sobre o seu histórico médico, e fará um questionário para avaliar seus sintomas psiquiátricos. Se você tiver as características para participar deste estudo, você responderá a algumas questões através de questionários, fará uma coleta de sangue de 20 ml, e receberá um kit contendo dois (2) tubos para coleta de saliva e um comprimido (dexametasona 1,5mg), o qual deverá ser ingerido conforme instruções contidas no kit.

Quais os benefícios em participar deste estudo?
Participando deste estudo, você terá uma avaliação psiquiátrica completa, a qual poderá identificar possíveis problemas antes não conhecidos, sendo assim tratada de maneira mais específica e adequada. Além disso, este estudo ajudará a desenvolver um maior conhecimento sobre o THB, principalmente sobre as causas dessa doença e seu funcionamento, ajudando no desenvolvimento de um melhor plano de atendimento aos demais pacientes. Você não terá despesas para participar deste estudo. Caso seja solicitado que você venha ao hospital fora do horário de suas consultas habituais, os pesquisadores irão reembolsar o valor das passagens.

Quais são os direitos dos participantes?
Qualquer dado coletado de seus registros médicos será tratado confidencialmente. Os resultados desse estudo poderão ser publicados em jornal científico, e em nenhum momento você será identificado por nome. Sua participação no estudo é voluntária, tendo a liberdade de desistir do estudo a qualquer momento, sem fornecer um motivo, assim como pedir maiores informações sobre o estudo e o procedimento a ser feito. Isto de maneira alguma irá influenciar na qualidade do atendimento a qual você tem direito.

Quais são os riscos que envolvem este estudo?
Este estudo possui riscos mínimos que são típicos da coleta de sangue, como mal-estar passageiro e/ou manchas roxas no local também são passageiras. A coleta de sangue será feita com material esterilizado e descartável por profissionais da área de saúde com competência técnica para tal. O teste de supressão com dexametasona não apresenta efeitos adversos significativos.

Quais são as responsabilidades dos participantes?
Os participantes desse estudo comprometem-se a responder, sem omitir informações, as escalas aplicadas pelos médicos pesquisadores e as escalas auto-aplicáveis.

Declaro que:

1. Recebi uma explicação completa do objetivo do estudo, dos procedimentos envolvidos e o que se espera de minha pessoa. O médico me explicou os possíveis problemas que podem surgir em conseqüência da minha participação neste estudo.
2. Estou ciente de que tenho total liberdade de desistir do estudo a qualquer momento e que essa desistência não irá, de forma alguma, afetar meu tratamento ou atendimento médico futuro na instituição.
3. Estou ciente de que a informação nos meus registros médicos é essencial para a avaliação dos resultados desse estudo. Concordo em liberar esta informação sob o entendimento de que ela será tratada confidencialmente, ou seja, não serei referido por nome em qualquer relatório relacionado a esse estudo. Da minha parte, não devo restringir, de forma alguma, o uso dos resultados que possam surgir deste estudo.
4. Concordo total e voluntariamente em fazer parte desse estudo.
5. Tenho mais de 18 anos.

_____	_____
Assinatura do Paciente ou responsável legal	Assinatura do Pesquisador
Nome:	Nome:
Data:	Data:

Pesquisador responsável: Márcia Kauer Sant'Anna
Endereço: Laboratório de Psiquiatria Molecular, Hospital de Clínicas de Porto Alegre / Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos, 2350 – CPE – 1º. andar – Porto Alegre – RS – CEP 90035-903. Telefone: (51) 3359-8845

Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre
Endereço: Rua Ramiro Barcelos, 2350 – Porto Alegre – RS – CEP 90035-903
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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA CONTROLES

AVALIAÇÃO DO EIXO HIPOTÁLAMO-PITUITÁRIA-ADRENAL (HPA), RECEPTOR DE GLICOCORTICÓIDE E FUNÇÃO MITOCONDRIAL EM PACIENTES COM TRANSTORNO DE HUMOR BIPOLAR

Nome: _____ Data de Nascimento: _____
Pesquisador Responsável: Prof. Dra. Márcia Kauer Sant'Anna
Pesquisador Executor: MSc. Gabriel Rodrigo Fries

Você está sendo convidado a participar desta pesquisa por não possuir diagnóstico de Transtorno de Humor Bipolar (THB). Em caso de qualquer dúvida quanto ao estudo e o que ele envolve ou sobre os seus direitos, você poderá contatar a Dra. Márcia Kauer Sant'Anna, pelo telefone: (51) 3359-8845, ou o pesquisador Gabriel Fries, pelo telefone (51) 3359-8021.

Esta pesquisa tem como objetivo ampliar os conhecimentos acerca das causas e das características do THB. Existe uma possibilidade de associação de alterações em células do sangue e no eixo hormonal do estresse com o Transtorno de Humor Bipolar, mas mais estudos devem ser feitos para constatar tal afirmação. Além disso, vamos correlacionar alterações encontradas no sangue com marcadores genéticos. Nesse estudo, a participação dos voluntários será através do preenchimento de algumas questões, uma coleta de sangue e a realização do teste de supressão com dexametasona. O sangue coletado será armazenado e as alterações serão avaliadas em conjunto com alguns dados coletados dos pacientes.

Este estudo aumentará o conhecimento sobre o THB, principalmente sobre as causas dessa doença e seu funcionamento, auxiliando no desenvolvimento de um melhor plano de atendimento aos pacientes. Você não terá despesas para participar deste estudo. Caso seja solicitado que você venha ao hospital, os pesquisadores irão reembolsar o valor das passagens. Os riscos deste estudo são mínimos e inerentes ao procedimento de coleta de sangue, como mal-estar passageiro e/ou manchas roxas no local. A coleta de sangue será feita com material esterilizado e descartável por profissionais da área de saúde com competência técnica para tal. O teste de supressão com a dexametasona não apresenta efeitos adversos significativos. Seu nome e seus dados serão mantidos em sigilo pelos pesquisadores, sendo esses dados utilizados somente para pesquisa.

Eu, _____, fui informado(a) dos objetivos especificados acima e da justificativa desta pesquisa de forma clara e detalhada. Recebi informações específicas sobre cada procedimento no qual estarei envolvido, dos desconfortos ou riscos previstos, como também dos benefícios esperados. Todas as minhas dúvidas foram respondidas com clareza e sei que poderei solicitar novos esclarecimentos a qualquer momento. Além disso, sei que terei liberdade de retirar meu consentimento de participação na pesquisa, a qualquer momento, sem que isto me traga prejuízos. Confirmo ter mais de 18 anos.

_____	_____
Assinatura do voluntário	Assinatura do pesquisador
Nome:	Nome:
Data:	Data:

Pesquisador responsável: Márcia Kauer Sant'Anna
Endereço: Laboratório de Psiquiatria Molecular, Hospital de Clínicas de Porto Alegre / Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos, 2350 – CPE – 1º. andar – Porto Alegre – RS – CEP 90035-903. Telefone: (51) 3359-8845

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ANEXO 5

Editorial publicado na *Revista Brasileira de Psiquiatria* sugerindo o papel da N-acetilcisteína como potencializador mitocondrial no TB.

Rev Bras Psiquiatr. 2011;33:321-322

Revista Brasileira de Psiquiatria
RBPPsychiatry
Official Journal of the Brazilian Psychiatric Association
Volume 33 • Número 4 • Dezembro/2011

EDITORIAL

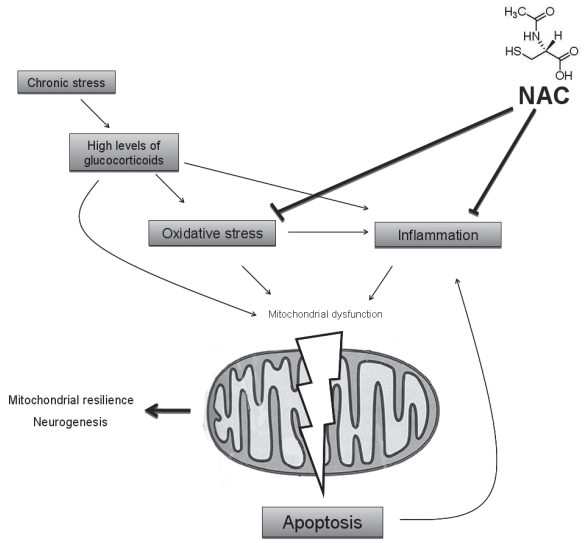
N-acetylcysteine as a mitochondrial enhancer: a new class of psychoactive drugs?

N-acetilcisteína como potencializador mitocondrial: uma nova classe de drogas psicoativas?

The limited available agents effective on the treatment of depressive episodes in bipolar disorder warrants further research on the field. Of note, several drugs have been studied in the light of their mood stabilizing efficacy, including well known supplements commonly used as add-on therapy for a number of conditions. For the past years, attention has been given to N-acetylcysteine (NAC), a chemical with antioxidant properties primarily used as a mucolytic agent and in the treatment of paracetamol overdose. A study by Magalhães et al.[1] published in this issue of RBP shows that add-on NAC therapy improved depressive symptoms and functional outcomes in bipolar disorder patients, which increases the interest in studying the mechanisms by which this drug may present neuroprotective effects.

Among its known mechanisms of action, NAC-induced brain glutathione (GSH) replenishment is the most studied. Glutathione is the primary endogenous antioxidant of the cell, and has the ability to scavenge oxygen and nitrogen species, therefore maintaining the oxidative balance. In addition to restoring GSH levels, NAC modulates inflammation by exerting anti-inflammatory actions, and presents direct effects on glutamatergic and dopaminergic neurotransmission. The inflammatory-modulating effects of NAC may be important for its mood stabilizing efficacy, mainly due to the recently described relevance of systemic inflammation in bipolar disorder pathophysiology (Kapczinski et al.[2]). More specifically, its usefulness on depressive episodes may be linked to innovative mechanisms of action, which we speculate to be taking part throughout its treatment. Among them, modulation of cellular signaling pathways by NAC may ultimately increase mitochondrial resilience, as supported below.

Chronic stress has been thought to play a key role in the pathophysiology of bipolar disorder, although the exact reasons for this association have not yet been fully clarified. The effects of stress are mediated mainly through glucocorticoids, which exert several body changes commonly known as 'stress response'. Dysregulation of glucocorticoids is associated with cognitive impairments and depressive disorder, supporting the notion that stress response may be impaired in affective and mood disorders. In this same vein, chronic stress has been shown to induce mitochondria dysfunction, whereas recent studies have described a biphasic effect of glucocorticoids on mitochondrial function, ultimately leading to the control of apoptosis on neuronal populations. Apoptosis may link chronic stress-induced neuronal death and inflammation, mainly through the release of damage-associated molecular patterns. All together, these features may be responsible for the systemic toxicity found in



NAC may counterbalance chronic stress-induced oxidative stress and inflammation, both pathways known to lead to mitochondrial dysfunction and apoptosis. Thus, NAC-induced neuroprotective mechanisms may increase mitochondrial resilience and neurogenesis.

Figure 1 Putative role of NAC as a mitochondrial enhancer.

patients (Kapczinski et al.[3]) and underlie the burden associated with the progression of mood disorders.

More recently, adult hippocampal neurogenesis has been found to buffer stress responses and depressive behavior (Snyder et al.[4]). In addition, inflammation downregulates neurogenesis, mainly through modulation of mitochondrial viability (Volobouéva et al.[5]). Thus, neuroprotective properties of NAC may be related to its neurogenesis-inducing ability, which is likely related to mitochondria-protective mechanisms. Of note, mitochondrial dysfunction has been described as one of the pathways underlying neuroprogression in bipolar disorder (Berk et al.[6]). Further studies are warranted in light of NAC effects on treating depressive episodes in bipolar disorder. As far as it seems, NAC may be the first pharmacological intervention that increases mitochondrial resilience and prevents allostatic load in psychiatry (Kapczinski et al.[7]).

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Disclosures

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* Modest

** Significant

*** Significant: Amounts given to the author's institution or to a colleague for research in which the author has participation, not directly to the author.

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ANEXO 6

Artigo científico publicado na revista *International Journal of Neuropsychopharmacology* sugerindo a redução na resiliência celular em resposta ao estresse do RE associada à progressão do TB.

International Journal of Neuropsychopharmacology, Page 1 of 11. © CINP 2014
doi:10.1017/S1461145714000443

ARTICLE

Impaired endoplasmic reticulum stress response in bipolar disorder: cellular evidence of illness progression

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Abstract

Bipolar disorder (BD) is a severe chronic psychiatric disorder that has been associated with cellular dysfunctions related to mitochondria, neurotrophin levels, and oxidative stress. Evidence has shown that endoplasmic reticulum (ER) stress may be a common pathway of the cellular changes described in BD. In the present study we assessed unfolded protein response (UPR) and the effects of this cellular process on lymphocytes from patients with BD. We also evaluated whether the stage of chronicity of BD was associated with changes in UPR parameters. Cultured lymphocytes from 30 patients with BD and 32 age- and sex-matched controls were treated with tunicamycin, an ER stressor, for 12 or 24 h to measure levels of UPR-related proteins (GRP78, eIF2α-P, and CHOP) using flow cytometry, and for 48 h to analyse ER stress-induced cell death. In healthy controls but not in patients we found an increase in levels of GRP78, eIF2α-P, and CHOP after ER stress induction. In addition, tunicamycin-induced cell death was significantly higher in patients compared to controls. More importantly, early-stage patients did not differ from controls while the late-stage patients showed an impaired ER stress response. Thus, dysfunction in ER-related stress response may be associated with decreased cellular resilience in BD and illness progression.

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Key words: Bipolar disorder, endoplasmic reticulum stress, illness progression, neuroprogression, unfolded protein response.

Introduction

Bipolar disorder (BD) is a severe chronic illness characterized by recurrent episodes of mania and depression. It affects around 2.4% of the population worldwide (Merikangas et al., 2011) and is associated with high clinical morbidity (Kupfer, 2005). The long-term outcome of BD is of concern, given that even subsyndromic symptoms are frequently associated with persistent cognitive and functional impairment (Martinez-Aran et al., 2007; Rosa et al., 2012). There is evidence that some patients may suffer changes in neuronal cell resilience and connectivity (Rajkowska, 2002). Such brain rewiring may be related to the chronicity of illness and has been called neuroprogression (Berk, 2009). Biological factors including anatomical brain structures (Strakowski et al., 2002; Lyoo et al., 2006), plasma levels of inflammatory and neurotrophic markers (Kauer-Sant'Anna et al., 2009), as well as mitochondrial and oxidative stress parameters (Andreazza et al., 2009), may also significantly change from early to late stages of the disorder. Such changes have been construed as potential pathways of neuroprogression (Berk et al., 2011).

Studies have shown that dysfunction of several intracellular signaling pathways may affect neuroplasticity and cellular resilience in BD, including mechanisms involving neurotransmitters, glucocorticoids, neurotrophic and anti-apoptotic factors, cell survival pathways, and calcium signaling, among others (Schloesser et al., 2008; Hunsberger et al., 2009). For instance, increased levels

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of calcium have been found in platelets, lymphocytes, and lymphoblastoid cells from patients with BD (Dubovsky et al., 1992; Perova et al., 2008), and an increased susceptibility to cell death in olfactory neuroepithelium has also been reported (McCurdy et al., 2006). Considering that peripheral markers related to oxidative stress, inflammation, and neurotrophins are altered in patients with BD (Kapczinski et al., 2011), studies with peripheral samples may yield promising findings in the assessment of possible pathways involving cell resilience in BD. Cell resilience is the ability of cells to adapt to an insult or stress; as a result, the impaired cell resilience observed in BD, both in neurons (Rajkowska, 2002) and peripheral cells (Naydenov et al., 2007) may be associated with an increased vulnerability of these patients to situations of cellular stress.

The mechanisms underlying cellular dysfunctions observed in BD have not been fully elucidated. For instance, it remains to be discovered whether they are due to a specific cellular defect or to changes in the function of organelles involved in the maintenance of cell resilience, such as mitochondria and the endoplasmic reticulum (ER). Evidence has pointed toward an association between ER and BD, including pharmacological experiments suggesting that the levels of ER-related chaperones are modulated by mood stabilizers used in the treatment of the disorder (Chen et al., 2000; Shao et al., 2006; Kakiuchi et al., 2009). Moreover, mefloquine, an antimalarial drug known to cause ER dysfunction, has been shown to induce mania in vulnerable individuals (Dow et al., 2005).

ER is responsible for the synthesis, folding, and post-translational modification of proteins (Rutkowski and Kaufman, 2004). When perturbations occur in the ER lumen (these may be alterations in redox state and in calcium homeostasis, or defects in post-translational modifications), the function of ER is compromised and unfolded proteins accumulate; this condition is known as ER stress (Lai et al., 2007; Walter and Ron, 2011). The cellular response to ER stress is called unfolded protein response (UPR), an adaptive process in which cells activate protective mechanisms to restore homeostasis in the ER lumen (Lai et al., 2007; Kimata and Kohno, 2011). UPR takes place via different ways, such as induction of ER chaperones, inhibition of protein synthesis, induction of a protein degradation pathway, and induction of apoptosis (Lai et al., 2007). In mammals, the first response to ER stress is transient global translation attenuation, mediated by the PERK signaling pathway (Kimata and Kohno, 2011). Under normal conditions, the ER transmembrane protein PERK is associated with the chaperone GRP78. When unfolded proteins accumulate during ER stress, GRP78 dissociates from PERK, allowing PERK to autophosphorylate and dimerize (Harding et al., 1999; Bertolotti et al., 2000). Activated PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2α), inhibiting global protein synthesis (Harding et al., 1999). However, some mRNAs are preferentially translated when eIF2 is limited. One of these mRNAs encodes the transcription factor ATF4, which regulates transcription factor C/EBP homologous protein (CHOP) (Walter and Ron, 2011), involved in ER stress-related apoptosis.

The UPR has important physiological functions, and prolonged ER stress leads to cell death. Some previous studies have suggested an involvement of UPR dysfunction in the pathophysiology of BD. For instance, genetic studies have observed an association of a polymorphism in the promoter region of XBPI (a transcription factor that induces the expression of ER chaperones) (Kakiuchi et al., 2003) and in GRP78 (Kakiuchi et al., 2005) with BD. This suggests that alterations in these pathways may be a risk factor for developing the disorder. In addition, a decreased response of XBP1 and CHOP was found in lymphoblastoid cells from patients exposed *in vitro* to two ER stress inducers (So et al., 2007). Other findings have confirmed these results, reporting a reduction in stress-induced splicing of XBP1 and GRP94 expression in patients with BD (Hayashi et al., 2009).

Taken together, these findings suggest that patients with BD may present a dysfunctional UPR, which may impair homeostasis in the ER whenever key cells are exposed to stress. In the present study we assessed UPR parameters in cultured lymphocytes from patients with BD compared to healthy controls. More specifically, we assessed time-dependent expression of GRP78, phosphorylated eIF2α (eIF2α-P), and CHOP, as well as ER stress-induced cell death. We also assessed whether the stage of chronicity of BD was associated with changes in ER-stress parameters.

Method

Subjects

Thirty patients with BD (20 euthymic, six depressive, and four hypomanic) were recruited at the outpatient Bipolar Disorders Program of Hospital de Clínicas de Porto Alegre (HCPA), Brazil. Thirty-two age- and sex-matched healthy controls, with no history of psychiatric illness and no history of psychiatric or neurologic disorders in first-degree relatives, were selected at the blood bank of the same hospital. Patients were classified into different stages (I–IV) considering that the illness is in a continuum progressing from a mild form to a chronic and unremitting presentation. This staging model, previously proposed by our group (Kapczinski et al., 2009), emphasizes mood symptoms and functional and cognitive decline, as well as patterns of recurrence and the gravity of these clinical characteristics. According to this model, patients with bipolar disorder are classified, during the interepisodic period, into: Stage I, patients who present well established periods of euthymia and absence of cognitive impairment and psychiatric morbidities between episodes; Stage II, patients who present rapid

cycling or current axis I or II comorbidities; Stage III, patients who present a clinically relevant pattern of cognitive and functioning impairment, as well as altered biomarkers; and Stage IV, patients who are unable to live autonomously because of cognitive and functioning decline and who show altered brain scans and biomarkers. In the present work, sample size could limit interpretation of analysis such as correlation with multiple clinical features according with each stage. Thus, patients were stratified into early (stage I or II) or late stage (stage III or IV) since it was possible to observe two homogeneous and similar clinical groups. This stratification was performed to evaluate potential differences in the capacity of patients at early *vs.* late stages to activate the UPR cascade in response to ER stress.

Patients were diagnosed according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Depressive and manic symptoms were evaluated using the Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) and the Young Mania Rating Scale (YMRS) (Young et al., 1978), respectively. The following exclusion criteria were considered for both patients and controls: history of autoimmune disease or chronic infectious/inflammatory disorder, as well as any severe systemic disease or use of immunosuppressive therapy.

The study protocol was approved by the research ethics committee of HCPA (project no. 10-0191). All subjects provided written informed consent before their inclusion in the study.

Sample collection, cell isolation, and cell culture

Between 10 and 20 ml of peripheral blood was collected by venipuncture into heparin tubes from both patients and controls. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (GE Healthcare, USA) density gradient centrifugation, according to the manufacturer's instructions. The resulting PBMC suspension, containing mostly lymphocytes (Boyum, 1976), was washed twice with phosphate buffered saline (PBS) and resuspended in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 20% heat-inactivated fetal bovine serum (Life Technologies, USA), gentamicin (100 μg/ml), and amphotericin B (0.25 μg/ml). Cells were cultured at a concentration of 500 000 cells/ml in different culture plates, according to the experiment, and maintained at 37 °C in a humidified 5% CO_2 atmosphere for up to 72 h. All culture conditions were determined based on preliminary tests.

Drug treatment

For the pharmacological induction of ER stress, lymphocytes were incubated with 2.4 μM tunicamycin (Sigma-Aldrich) for 12, 24, or 48 h. Tunicamycin was chosen because it prevents the glycosylation of newly synthesized proteins in the ER, causing the accumulation of unfolded proteins (Tordai et al., 1995). To determine optimal tunicamycin concentration for the induction of UPR proteins, cells isolated from healthy subjects (n=3) were incubated with three different doses of tunicamycin (1.2, 2.4, and 13.5 μM), for three different incubation times (4, 12, and 24 h), after which ER stress parameters were analysed. Viability tests were performed using tunicamycin-treated cells to define optimal concentration and time of treatment (data not shown).

Intracellular staining

For intracellular detection of GRP78, eIF2α-P, and CHOP levels, lymphocytes were seeded in 12-well culture plates at a density of 500 000 cells/well overnight and treated with tunicamycin (2.5 μM) for 12 or 24 h. Control cells remained untreated. Afterwards, 2×10^6 cells were collected for immunostaining. Cells were washed twice with PBS and blocked with 10% goat serum and 10% human serum in PBS for 20 min at 4 °C. Then, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, USA) for 20 min at 4 °C. Subsequently, cells were washed with Perm/Wash buffer (BD Biosciences), incubated with 10% goat serum in PBS for 20 min at 4 °C, followed by incubation with primary antibodies in a total volume of 50 μl in Perm/Wash at 4 °C for 2 h (250 000 cells were used for each staining). The primary antibodies used were rabbit anti-human GRP78 (1:100), rabbit anti-human eIF2α-P (1:100), and rabbit anti-human CHOP (1:200) (all from Sigma-Aldrich). Subsequently, cells were washed and stained with fluorescent secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit (Life Technologies) at 1:400 in a total volume of 50 μl in Perm/Wash for 30 min at 4 °C protected from light. Finally, stained cells were washed and analysed by flow cytometry.

Flow cytometric acquisition

Data acquisition and analysis was performed using a FACScalibur (BD Biosciences) flow cytometer. Data were analysed using the CellQuest Pro software (BD Biosciences). Lymphocytes were identified by FSC/SSC pattern, and 10 000 events were collected per gate from each staining. Baseline autofluorescence control and isotype-matched negative control fluorescent antibodies were used as reference to set fluorescence thresholds for positivity. The median fluorescence intensity (MFI) of positive cells was analysed, and data were expressed as x-fold increase over control (MFI of untreated cells for each staining).

Cell viability

Cell viability was evaluated by the quantification of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by

cellular dehydrogenases, as previously described (Mosmann, 1983), with minor modifications. Lymphocytes were seeded in 96-well plates at a density of 100 000 cells/well. After overnight incubation, cells were treated with or without tunicamycin (2.4 μM) for 48 h. Untreated cells were used as controls. Each treatment condition was tested in four replicate wells. After treatment, 20 μl of MTT (5 mg/ml in PBS; Sigma-Aldrich) was added to each well and further incubated for 4 h at 37 °C. Then, the medium was discarded, and dimethyl sulfoxide was added to solubilize the formazan crystals. Absorbance was determined at 560 and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices, USA). Data were expressed as % of control (untreated cells).

Statistical analysis

Statistical analysis was performed using the Statistical Product and Service Solutions, v.18.0 (SPSS Inc, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. Wilcoxon test and Friedman's ANOVA (followed by Wilcoxon test with Bonferroni correction, when significant) were used to compare non-parametric variables between related samples. Mann–Whitney and Kruskal–Wallis tests were used to compare non-parametric variables between independent samples. Parametric variables were compared with one-way ANOVA (with Bonferroni correction, when significant) and the independent *t* test. Gender differences between groups were evaluated using the χ^2 test or Fisher's exact test. Differences in medications used by euthymic patients were evaluated using Fisher's exact test. Correlations were analysed by Pearson's or Spearman's correlation tests according to data distribution. Quantitative variables were expressed as mean±S.D. or as median and interquartile range, according to data distribution. In all experiments, $p<0.05$ was considered statistically significant, except when Bonferroni correction was used ($p<0.01$ was considered in these cases).

Results

Clinical parameters and drug treatments

There were no statistical differences in age or gender between patients and controls. Other relevant sample characteristics are listed in Table 1. Clinical features between patients at early *vs.* late stages of BD are shown in Table 2. There was no statistical age difference between these two groups, but they presented different lengths of disease ($U=20$, $Z=-2.275$, $p=0.023$) and number of episodes ($U=2.5$, $Z=-3.611$, $p<0.0001$). We also tested the variable 'illness onset', comparing the age of the onset of the disorder between the late and the early groups, and we observed no significant differences between them ($F=6.552$, $t=0.597$, df=18, $p=0.558$). In an attempt to avoid the potential confounding effects of age in the analysis, we tested possible correlations between age and biochemical data. No correlations were found between the age and cellular parameters ($p>0.05$ for all analyses), except between age and length of disease ($r=0.523$, $p=0.003$).

Table 1. Characteristics of bipolar disorder and control groups

	Controls ($n=32$)	Patients ($n=30$)	p
Age	42.94±12.17	46.47±11.96	0.255[a]
Female	20	19	1.00[b]
Mood state			
Euthymia	n/a	20	–
Depression	n/a	6	–
Hypomania	n/a	4	–
Medications (%)			
Lithium	0	43.3%	–
Other mood stabilizers	0	56.7%	–
Antipsychotics	0	76.7%	–
Antidepressants	0	20%	–

[a] Independent-samples *t* test. Data expressed as mean±S.D.
[b] χ^2 test. Data expressed as % of patients on medications.
n/a=not applicable.

The dose–response curve of tunicamycin (1.2, 2.4, and 13.5 μM) was obtained for lymphocytes isolated from healthy subjects so as to establish the best conditions to evaluate the UPR cascade in response to ER stress (time of treatment, cellular toxicity, GRP78, eIF2α-P, and CHOP protein levels). Based on these results, we found that incubation of 2.4 μM of tunicamycin for 12 and 24 h was able to induce early and late UPR, respectively (manifested as increased GRP78 and eIF2α-P protein levels and CHOP expression, respectively), but did not significantly affect cell viability (data not shown). Cell viability was also determined after 48 h of treatment, to establish the capacity of cells to cope with ER stress.

UPR protein levels and ER stress-induced cell death

In control group, there were significant differences in GRP78 ($\chi^2=18.667$, df=2, $p<0.0001$), eIF2α-P ($\chi^2=21.733$, df=2, $p<0.0001$) and CHOP ($\chi^2=8.236$, df=2, $p=0.016$) levels between different incubation times of cells with tunicamycin. Post-test analysis showed that treatment of lymphocytes from the control group with tunicamycin for 12 and 24 h significantly increased GRP78 ($Z=-3.920$, $p<0.0001$; $Z=-3.408$, $p=0.001$, respectively, Fig. 1*a*) and eIF2α-P levels ($Z=-4.187$, $p<0.0001$; $Z=-3.516$, $p<0.0001$, respectively, Fig. 1*b*). CHOP protein levels increased only after 24 h of tunicamycin treatment ($Z=-2.880$, $p=0.004$, Fig. 1*c*). In contrast, no differences in UPR parameters were found in lymphocytes from patients between cell treatments (GRP78: $\chi^2=0.018$, df=2, $p=0.991$; eIF2α-P: $\chi^2=1.939$, df=2, $p=0.379$; CHOP: $\chi^2=2.529$, df=2, $p=0.282$; Fig. 1*a–c*). We also observed differences between patients and controls in the

Table 2. Clinical features of euthymic patients at early vs. late stages of bipolar disorder

Euthymic patients	Early (n=10)	Late (n=10)	p
Age	43.4±9.3	52.6±14.1	0.103[a]
Female	80%	40%	0.170[b]
Duration of illness	7.5 (4.5–13.75)	23.5 (14.25–32)	0.023[c]
Number of episodes	2 (1–3)	11 (5.75–22.75)	<0.0001[c]
Medications (%)			
Lithium	60%	30%	0.370[b]
Other mood stabilizers	50%	70%	0.650[b]
Antipsychotics	60%	90%	0.303[b]
Antidepressants	30%	10%	0.582[b]

[a] Student's t test. Data expressed as mean±S.D.

[b] Fisher's exact test. Data expressed as % of patients.

[c] Mann–Whitney test. Data expressed as median and interquartile range (25th and 75th percentiles).

Comparisons between euthymic patients at early (n=10) *vs.* late stage (n=10) and controls (n=32) were not significant for gender (p=0.200) or age (p=0.090).

tunicamycin-treated cells for 12 and 24 h in GRP78 (U=101.000, Z=−3.942, p<0.0001; U=21.000, Z=−4.817, p<0.0001, respectively) and eIF2αP levels (U=131.000, Z=−2.162, p=0.031; U=14.000, Z=−4.486, p<0.0001, respectively).

There were significant differences in cell death between untreated cells, controls and patients tunicamycin-treated cells (F=199.994, df=2, p<0.0001). Treatment with tunicamycin for 48 h induced cell death at significant levels in both controls and patients (p<0.0001 for both groups; Fig. 1*d*). However, cell death levels found in tunicamycin-treated lymphocytes isolated from patients were almost two-fold higher when compared to the control group (50.4 and 28.67%, respectively; p<0.0001).

Once differences in UPR between cells isolated from controls and patients with BD were confirmed, we decided to investigate the role of illness progression in this imbalance. When analysing UPR parameters in euthymic patients according to stage of chronicity of BD compared to controls, we found differences in GRP78 (χ^2=13.975, df=2, p=0.001, Fig. 2*a*) and eIF2α-P expression (χ^2=6.405, df=2, p=0.041, Fig. 2*b*) between controls and patients at late stages, but no differences between controls and patients at early stages in cells treated with tunicamycin for 12 h. After 24 h of treatment with tunicamycin, differences were observed between controls and patients both at early and late stages, for GRP78 (χ^2=21.635, df=2, p<0.0001 and p=0.003, respectively, Fig. 2*a*) and eIF2α-P levels (χ^2=18.640, df=2, p=0.004 and p=0.001, respectively; Fig. 2*b*). No changes in CHOP protein levels were observed between groups (Fig. 2*c*) in 12 h (χ^2=0.295, df=2, p=0.863) and 24 h (χ^2=2.625, df=2, p=0.269). Cell death levels in response to tunicamycin between euthymic patients at early *vs.* late stages were also evaluated, and differences were found between controls and patients at both early and late stages (χ^2=22.891, df=2, p=0.01 and p<0.0001, respectively; Fig. 2*d*).

Regarding the different mood states assessed (depressive *vs.* euthymic *vs.* hypomanic), no differences were found in the parameters evaluated among groups (data not shown).

Discussion

As far as we are aware, this is the first study to evaluate both cellular markers of UPR and cell death in lymphocytes from patients with BD and control cells subjected to pharmacologically induced ER stress. As expected, the control group experienced an early increase in GRP78 and eIF2α-P protein levels after treatment with tunicamycin, and this increase was maintained after 24 h of treatment. We also observed late induction of transcription factor CHOP in this group, indicating a coordinated modulation of UPR elements in response to ER stress in healthy individuals. In contrast, no induction of UPR-related proteins was found in the BD group, suggesting a dysfunctional response to ER stress among patients with BD.

In healthy individuals, the classic UPR cascade was activated (or at least the evaluated PERK pathway) in response to tunicamycin, as demonstrated by the increased levels of GRP78 and eIF2α-P and by the later increase in CHOP protein levels (Walter and Ron, 2011). Conversely, no induction of UPR-related proteins was observed in patients, indicating a possible dysfunction in the PERK pathway, as GRP78 and eIF2α-P levels remained unchanged. In addition, the lack of CHOP induction may reflect changes in the components of signaling pathways that control its transcription,

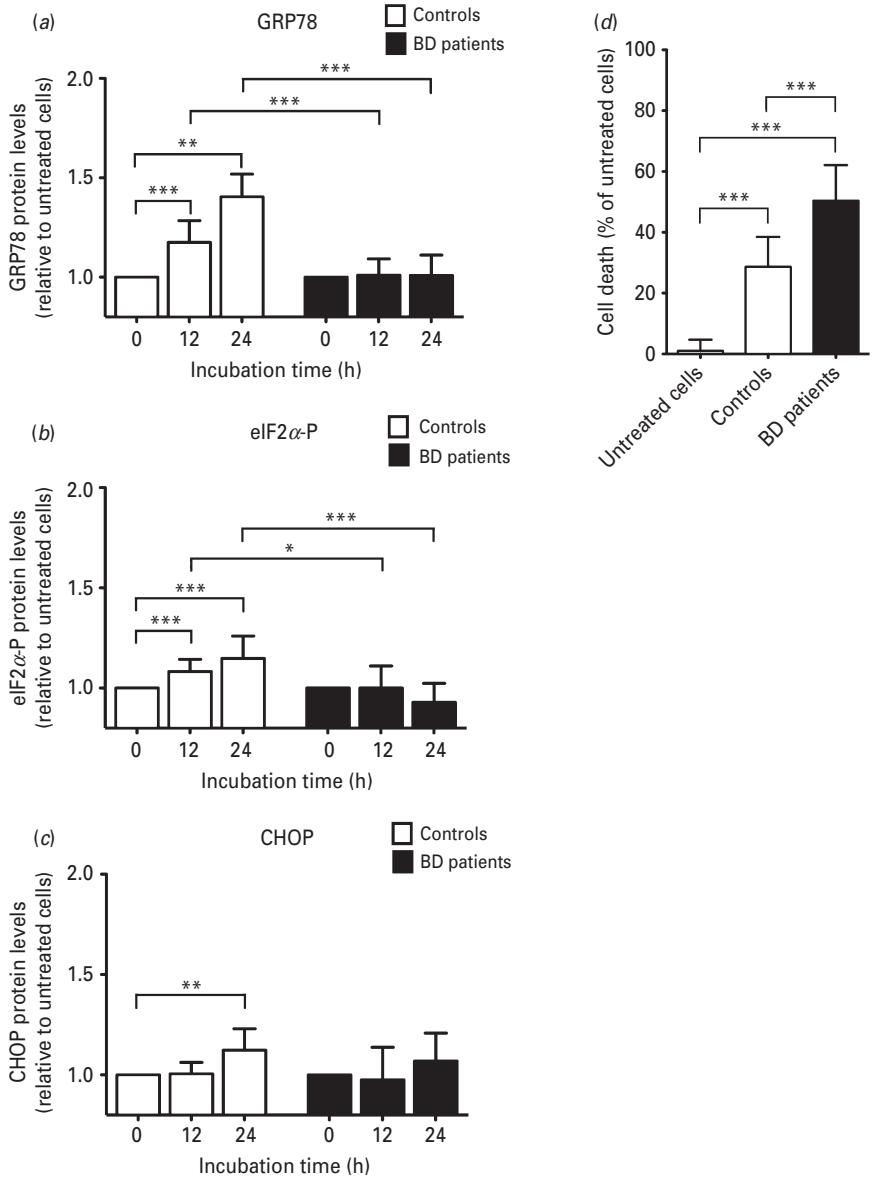

Fig. 1. Endoplasmic reticulum (ER) stress response in lymphocytes from patients with bipolar disorder (BD) and healthy control subjects. Levels of UPR-related proteins GRP78 (*a*), eIF2*α*-P (*b*), and CHOP (*c*) in lymphocytes from patients with BD and healthy subjects treated with 2.4 μM tunicamycin for 12 or 24 h. The median fluorescence intensity (MFI) of positive cells was analysed and data were calculated as x-fold increase over control (MFI of untreated cells for each staining). Data are expressed as median and interquartile range for a minimum of 15 subjects in each treatment. (*d*) ER stress-induced cell death in lymphocytes from patients and healthy subjects treated with 2.4 μM tunicamycin for 48 h. Data were calculated as % of control (untreated cells) and are expressed as mean ± S.D. for a minimum of 20 subjects in each treatment. *$p<0.05$ or 0.01; **$p<0.001$; ***$p<0.0001$.

predominantly regulated by the PERK/eIF2*α*/ATF4 cascade (Okada et al., 2002; Oyadomari and Mori, 2004). This impaired response to ER stress may also be related to several impairments in neural function reported in patients with BD, given that UPR components are also involved in neural development and plasticity, maturation and transport of several receptors, and calcium signaling (Verkhratsky, 2002; Vandenberghe et al., 2005; Weng et al., 2011).

The results of the present study differ from those of So et al. (2007) and Hayashi et al. (2009), who found no differences in GRP78 expression in lymphoblastoid cells between patients with BD and controls after treatment with tunicamycin and thapsigargin. Our findings are similar to those of Kakiuchi et al. (2003), who also observed a reduced GRP78 induction in response to ER stress in patients. In line with our study, the same study by So et al., also reported a decreased CHOP expression in response to ER stress in patients with BD when compared to controls (So et al., 2007). These differences across studies are likely due to differences in patient populations and cell culture conditions. Regarding cell culture, we

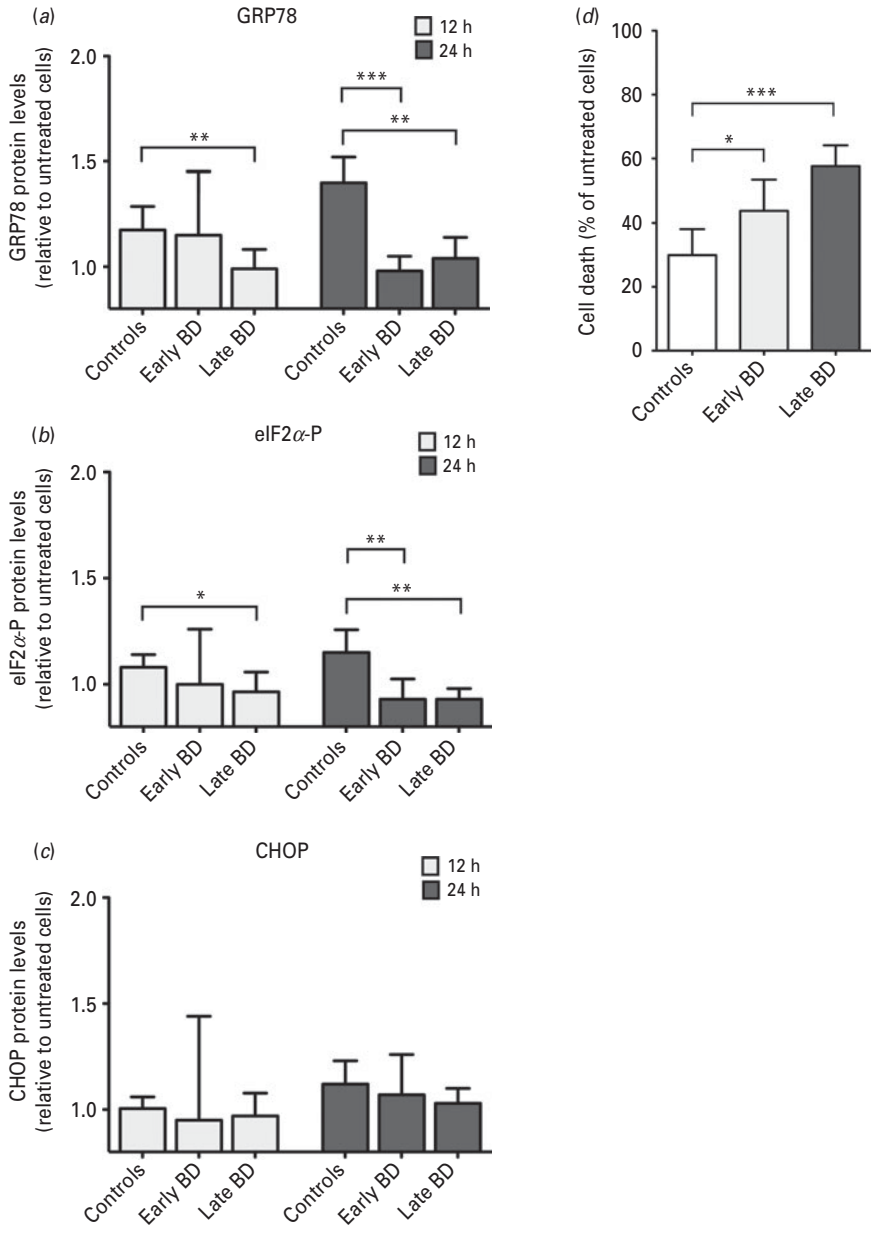

Fig. 2. Endoplasmic reticulum (ER) stress response in lymphocytes from euthymic patients according to stages of bipolar disorder (BD). Levels of UPR-related proteins GRP78 (*a*), eIF2α-P (*b*), and CHOP (*c*) in lymphocytes from patients at early and late stages of BD and healthy subjects. Data represent cells treated with 2.4 μM tunicamycin for 12 or 24 h and were calculated as x-fold increase over untreated cells. Data are expressed as median and interquartile range for a minimum of seven subjects in each treatment. (*d*) ER stress-induced cell death in lymphocytes from patients at early and late stages of BD and controls. Data represent cells treated with 2.4 μM tunicamycin for 48 h and were calculated as % of untreated cells. Data are expressed as median and interquartile range for a minimum of eight subjects in each treatment. *p<0.05 or 0.01; **p<0.001; ***p<0.0001.

evaluated UPR in primary cultures of lymphocytes, in contrast with other studies where lymphoblastoid cells derived from patients were used (the latter cells were obtained after *in vitro* transformation of lymphocytes with Epstein–Barr virus, and acquired cell line characteristics). Although lymphoblastoid cells are widely used in the investigation of neurological diseases (Sie et al., 2009), there is some concern about the possible occurrence of relevant genetic changes during the generation and maintenance of these cells. Even techniques designed to maintain lymphoblastoid cells, such as freezing, may impair protein folding, resulting in ER stress (Hayashi et al., 2009). Thus, our study, performed with primary lymphocyte cultures obtained from patients with BD and healthy subjects, adds to the existing body of knowledge and contributes toward a better understanding of the involvement of ER stress in BD.

Lymphocytes obtained from patients with BD showed higher cell death levels in response to tunicamycin treatment when compared to healthy controls, suggesting

that UPR dysfunction may reflect an increased cellular susceptibility. In fact, the lack of GRP78 induction in patients may be related to this finding, as this protein plays a major role in cell survival processes, including protein folding, calcium binding, anti-apoptotic responses, and regulation of UPR signaling pathways (Rao et al., 2002; Ni et al., 2011).

We found increased CHOP levels in the control group after 24 h of treatment with tunicamycin. Prolonged activation of CHOP can cause apoptosis in both physiological and pathophysiological settings (Tabas and Ron, 2011). In the first scenario, as probably was the case with cells from our healthy subjects, UPR-induced apoptosis could potentially offer a means of clearing out cells that remained with impaired RE functioning. In patients with BD, in turn, we did not observe induction of CHOP, although their lymphocytes were more susceptible to tunicamycin-induced cell death than controls. Even though CHOP expression does not increase in response to ER stress in eIF2α and PERK$^{-/-}$ mutants, these cells can still activate apoptotic pathways (Scheuner et al., 2001; Harding et al., 2003). Moreover, CHOP gene defects have been show to delay but not to prevent ER stress-induced apoptosis (Oyadomari et al., 2002). Therefore, induction of CHOP does not seem to be the only pathway involved in ER stress-associated apoptosis. Cells can activate apoptosis by other pathways mediated by protein c-Jun NH2-terminal kinase (JNK) through IRE1 activation and caspases (Schroder and Kaufman, 2005; Tabas and Ron, 2011), more specifically caspase-12. Considering that PERK is essential to induce CHOP transcription (Harding et al., 2000; Okada et al., 2002) and that this pathway appears to be impaired in patients with BD, it is possible that the marked cell death observed in this group, if apoptotic, may have been induced by other mechanisms rather than solely by CHOP. Of note, high apoptosis in BD has already been demonstrated both in post-mortem tissues (Kim et al., 2010) and in peripheral blood samples (Andreazza et al., 2007), including peripheral blood mononuclear cells (Fries et al., 2014).

We analysed UPR parameters in ER stress response according to different mood states in BD. Even though the number of patients in acute phases was rather small, the results showed a similar UPR pattern in acute and euthymic patients, indicating that ER stress response is independent of mood state. With regard to the stage of chronicity of BD, only patients at early stages responded to stress with induction of GRP78 and eIF2α-P under moderate ER stress (i.e. treatment with tunicamycin for 12 h). However, after a longer period of ER stress (treatment with tunicamycin for 24 h), no UPR modulation was found in patients. Cell death was also more pronounced in patients at late stages than in those at early stages or in healthy controls. These observations showed that ER response seems to depend on stress chronicity, and that different illness stages determine different cellular responses. Thus, although patients at early stages could possibly still respond to moderate ER stress, such response would not occur in patients at advanced stages. In fact, previous studies have shown that patients at early stages present much better clinical outcomes than those with multiple episodes (Tohen et al., 1990; Schuepbach et al., 2008) and that there are differences in biochemical parameters according to stage of chronicity of BD (Andreazza et al., 2009; Kauer-Sant'Anna et al., 2009). In order to better understand the contrast between early and late stage of BD, we tested the variable 'illness onset' comparing the age at the onset of the disorder between the late and the early groups, and we observed no significant differences between them. This suggests that our results are not related to an early or late onset of disorder, but appear to be more related, at least in this sample, to the illness progression. Although the age difference between the two groups is not statistical significant (approximately 10 yr), we found a positive correlation between age and length of disease, contributing to explaining the different lengths (approximately 16 yr) of disease between groups even with the age at illness onset being similar. This result points to the importance of an early diagnosis and treatment to avoid a possible faster progression and so provide a better prognosis.

An interesting point to the discussion of our results concerns the effects of psychotropic medications on the ER stress pathway, such as the mood stabilizer lithium (Shao et al., 2006). The results presented in this study were obtained from lymphocytes cultivated in the absence of any psychotropic medication and their treatment with ER stress inductor followed an overnight incubation in treatment-free medium to stabilize them (a wash-out period); however, we understand that the effects of the medications on cells while in the blood may significantly interfere with their response to an *in vitro* stimulation (or even by means of a potential residual effect). The inclusion of drug-free or drug-naïve patients is rather complicated, and was certainly not feasible in our study. Precisely because of this difficulty, the medication has always been a limitation in studies of this kind. Our sample size is unfortunately too small to break down our results according to medication, which seems to be the best way to overcome this potential limitation. The impact of different kinds of medication on biochemical parameters could not be examined because most patients receive polypharmacy. However, we have been careful to verify the percentage of patients taking lithium and to compare between the early and late stages, and we did not observe significant differences.

Taken together, our findings suggest that dysfunction in ER-related stress response may be associated with decreased cellular resilience in BD, and that protective cellular mechanisms may become less effective with the progression of the illness. Even though we assessed peripheral tissue, we believe this pathway might be of relevance also for the central nervous system. More studies

are needed to verify the possible relation between our findings and central mechanisms that could support the concept of neuroprogression suggested for BD (Kapczinski et al., 2008; Grande et al., 2012). In this vein, ER stress may be a novel and promising target for interventions aimed at reducing the biological impact of BD illness progression (Berk, 2009; Berk et al., 2011).

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Conflict of Interest

Professor Kapczinski has received grant/research support from Astra-Zeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD, and the Stanley Medical Research Institute; has been a member of the speakers' boards for Astra-Zeneca, Eli Lilly, Janssen, and Servier; and has served as a consultant for Servier. Professor Klamt has received a fellowship from MCT/CNPq. Dr Rosa receives a scholarship from CNPq through the program Ciência Sem Fronteiras, Bolsa Jovem Talento. Dr Ceresér has received grants from CNPq, FAPERGS, and CAPES. Dr Colpo, Mrs Ferrari, and Mrs Bristot are supported by scholarships from CNPq.

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Mr Burque declares no conflicts of interest.

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