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

TESE DE DOUTORADO

Biomarcadores periféricos, toxicidade sistêmica e regulação transcricional no transtorno bipolar: identificação de vias moleculares associadas com a sua fisiopatologia e potenciais alvos terapêuticos

BIANCA PFAFFENSELLER

Porto Alegre

2016

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

Biomarcadores periféricos, toxicidade sistêmica e regulação transcricional no transtorno bipolar: identificação de vias moleculares associadas com a sua fisiopatologia e potenciais alvos terapêuticos

BIANCA PFAFFENSELLER

Orientador: Prof. Dr. Fábio Klamt

Coorientador: Prof. Dr. Flávio Kapczinski

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

Porto Alegre

2016

CIP - Catalogação na Publicação

Pfaffenseller, Bianca
Biomarcadores periféricos, toxicidade sistêmica e regulação transcricional no transtorno bipolar: identificação de vias moleculares associadas com a sua fisiopatologia e potenciais alvos terapêuticos / Bianca Pfaffenseller. -- 2016.
248 f.

Orientador: Fábio Klamt.
Coorientador: Flávio Kapczinski.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2016.

1. transtorno bipolar. 2. EGR3. 3. regulação transcricional. 4. biomarcadores. 5. toxicidade. I. Klamt, Fábio, orient. II. Kapczinski, Flávio, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

“Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrepende.”

(Leonardo da Vinci)

AGRADECIMENTOS

Ao meu orientador, Professor Fábio Klamt, pela orientação, ensinamentos e dedicação a minha formação acadêmica e especialmente pela sua amizade e empolgação com a pesquisa.

Ao meu coorientador, Professor Flávio Kapczinski, pela orientação e importante referência na área da pesquisa e pela oportunidade de aprendizado e crescimento profissional.

Aos colegas e amigos do Laboratório de Psiquiatria Molecular, particularmente ao pessoal da bancada, com quem é muito bom compartilhar experiências e momentos divertidos no laboratório e fora dele. Destaco os amigos que estão no exterior, Gabriel, Gabi e Laura, que contribuíram muito na minha trajetória acadêmica.

Em especial, agradeço à Bi Aguiar, minha xará, grande amiga, braço direito e parceria de sempre há mais de dez anos, com quem sempre pude contar. Um obrigado também muito especial à Gi, minha amiga-irmã, pelas trocas de ideias, apoio e consideráveis ajudas que foram fundamentais para a conclusão deste trabalho. Também agradeço à Bruna Maria, Emily e Thaís pelas conversas e parceria. E, mais recentemente, aos queridos amigos Ellen e Rafa Colombo. Pessoal, valeu pela amizade, companheirismo e pelo apoio de sempre.

Aos demais colegas e professores do Laboratório de Psiquiatria Molecular, pela amizade e troca de experiência e conhecimentos, principalmente ao Pedro Magalhães pela importante contribuição neste trabalho.

Aos amigos e colegas do Departamento de Bioquímica, principalmente à Pati, Dai, Ivi, Fer, que me auxiliaram no aprendizado e discussão de técnicas. E um obrigado essencial ao Marco Antônio pela sua disponibilidade, tranquilidade e por compartilhar seu conhecimento na área da bioinformática. Da mesma forma, agradeço ao Mauro Castro pela fundamental participação neste trabalho com seu admirável conhecimento e dedicação.

As minhas grandes amigas da Biomed: Bianca, Roberta e Bárbara pela amizade e parceria desde a faculdade.

À CAPES e ao CNPq pelas bolsas de estudo e financiamento de projetos. Ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica pela excelência em educação e ensino. À Cleia pela simpatia, auxílio e esclarecimentos constantes.

Um agradecimento mais que especial ao Adriano pela companhia, amor e carinho. Muito obrigada por me apoiar e compreender as minhas ausências e preocupações.

A minha família, pela paciência, confiança, dedicação e compreensão em todos os momentos desta trajetória, principalmente naqueles em que estive mais ausente. Destaco meu pai Eliseu, que sempre incentivou o estudo e a busca pelo conhecimento. Agradeço por tudo!

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 artigos científicos e um capítulo com resultados, as respectivas metodologias e discussão;

Parte III: Discussão, Conclusões e Perspectivas.

Além disso, a seção de Anexos compreende: a) artigos científicos citados na tese, publicados em coautoria durante o período do doutorado; b) um capítulo de livro escrito e publicado durante a realização do doutorado; c) um artigo científico submetido para publicação, realizado em coautoria durante o doutorado (o qual complementa um dos capítulos da tese); e d) uma lista com informação complementar ao capítulo 3 .

Os trabalhos que compõem esta tese foram desenvolvidos entre os anos de 2012 e 2016 em dois laboratórios: Laboratório de Biologia Celular (Laboratório 24) do Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul, sob orientação do Prof. Dr. Fábio Klamt, e no Laboratório de Psiquiatria Molecular, localizado no Centro de Pesquisas Experimentais do Hospital de Clínicas de Porto Alegre, sob orientação do Prof. Dr. Flávio Kapczinski. Este estudo foi apoiado pelos fundos brasileiros CNPq/MS/SCTIE/DECIT - Pesquisas sobre doenças neurodegenerativas (466989 / 2014-8) e MCT/CNPq INCT-TM (573671/2008-7).

PARTE I

Introdução e objetivos

RESUMO

Evidências sugerem que o transtorno bipolar esteja associado a uma toxicidade sistêmica, representada por alterações periféricas em marcadores de inflamação, estresse oxidativo e neurotrofinas, a qual parece estar associada aos episódios de humor e à progressão da doença levando a prejuízos sistêmicos e na neuroplasticidade. Os trabalhos apresentados nesta tese tiveram como objetivo revisar estas alterações e explorar possíveis mecanismos responsáveis por estes achados, com enfoque em uma desregulação transcricional no transtorno bipolar. No primeiro capítulo, revisamos os biomarcadores periféricos associados aos episódios de humor, a relação destes com a toxicidade sistêmica e os possíveis mecanismos subjacentes a esta toxicidade. Seguimos ilustrando, no capítulo 2, um exemplo de alterações estruturais cerebrais em um paciente bipolar com experiência de múltiplos episódios, como um possível exemplo da neuroprogressão no transtorno bipolar. Em seguida, buscamos por vias de regulação transcricional disfuncionais no córtex pré-frontal de pacientes que poderiam estar associadas a essas alterações e neuroplasticidade prejudicada. A partir de abordagem inovadora de bioinformática, no capítulo 3, identificamos algumas unidades regulatórias (regulons) associadas com as duas assinaturas gênicas do transtorno bipolar avaliadas, obtidas a partir de bancos de dados de microarranjo de pré-frontal *postmortem*. Em uma análise mais rigorosa, identificamos apenas o regulon do gene *early growth response 3* (*EGR3*) enriquecido nas duas assinaturas da doença em duas redes transcricionais do pré-frontal, estando reprimido no fenótipo bipolar. Nossos resultados sugerem o regulon do *EGR3* como um alvo importante no transtorno bipolar. Considerando seu papel fundamental na resposta ao estresse e na translação de estímulos ambientas em mudanças na expressão gênica neuronal, propomos que uma disfunção em vias biológicas envolvendo *EGR3* poderia levar a uma resposta prejudicada ao estresse e influenciar no risco para o transtorno bipolar. No quarto capítulo, então, caracterizamos o perfil de expressão gênica do modelo de células SH-SY5Y diferenciadas e avaliamos o comportamento do regulon do *EGR3* de acordo com o protocolo de diferenciação. Além disso, identificamos moléculas com potencial de modular os regulons enriquecidos no transtorno bipolar visando testar o efeito destas drogas no referido modelo celular. Nossos resultados reforçam o fenótipo neuronal deste modelo *in vitro* e demonstram que o regulon do *EGR3* está enriquecido nas células diferenciadas, sugerindo que ele é importante nesse processo e que este modelo experimental é adequado para estudar este regulon e as moléculas selecionadas pela análise de mapa de conectividade. Nós ainda avaliamos nas células SH-SY5Y o efeito do soro de pacientes bipolares, para investigar o papel da toxicidade sistêmica em células neuronais. O soro de pacientes, especialmente em estágio tardio da doença, causou toxicidade às células, reduzindo a densidade de neuritos e a viabilidade celular. Esses achados representam uma forma de desafio celular relacionado à toxicidade sistêmica do transtorno bipolar, propondo as células SH-SY5Y diferenciadas como um modelo *in vitro* para estudo desta doença. Em suma, os resultados desta tese sugerem que a toxicidade sistêmica relacionada aos episódios recorrentes de humor pode influenciar nas alterações anatômicas cerebrais associadas com a progressão do transtorno bipolar, e a disfunção no regulon do *EGR3* poderia estar envolvida com aspectos desta neuroprogressão considerando o papel de *EGR3* na resposta ao estresse e na neuroplasticidade. Estas hipóteses, que também sugerem alvos interessantes para o desenvolvimento de novos tratamentos, devem ser apropriadamente validadas em modelos experimentais, como o modelo de células SH-SY5Y diferenciadas estudado neste trabalho.

ABSTRACT

Evidence suggests that bipolar disorder is associated with a systemic toxicity, represented by peripheral changes in markers of inflammation, oxidative stress and neurotrophins, which appears to be associated with mood episodes and illness progression leading to systemic damage and impaired neuroplasticity. The work presented in this thesis aimed to review these changes and explore possible mechanisms responsible for these findings, focusing on transcriptional regulation in bipolar disorder. In the first chapter, we review the peripheral biomarkers associated with mood episodes, their relationship with systemic toxicity and the possible mechanisms underlying this toxicity. We have shown, in Chapter 2, an example of structural brain changes in a bipolar patient with multiple episodes experience, as a possible example of 'neuroprogression' in bipolar disorder. Then we investigated dysfunctional transcriptional regulatory pathways in the prefrontal cortex of patients that could be associated with these changes and impaired neuroplasticity. Using innovative bioinformatics approaches, in Chapter 3, we identified some regulatory units (regulons) associated with the two gene signatures of bipolar disorder evaluated, obtained from microarray data sets from prefrontal postmortem studies. With a more rigorous analysis, we only identified the regulon of early growth response 3 gene (*EGR3*) enriched in the two bipolar signatures in the two transcriptional prefrontal networks evaluated, being *EGR3* repressed in bipolar phenotype. Our results suggest the *EGR3* regulon as an important target in bipolar disorder. Considering its key role in response to stress and translation of environmental stimuli into long-term changes in neuronal gene expression, we propose that a dysfunction in biological pathways involving *EGR3* could lead to an impaired response to stress and influence on the risk for bipolar disorder. Then, in Chapter 4, we characterized the gene expression profile of differentiated SH-SY5Y cells and evaluated the *EGR3* regulon according to the differentiation protocol. Furthermore, we have identified molecules with potential to modulate the regulons enriched in bipolar disorder, using connectivity map analysis, in order to test the effect of these drugs on this cellular model in future studies. Our results consolidated the neuronal phenotype of this *in vitro* model and demonstrated that the *EGR3* regulon is enriched in differentiated cells, suggesting that it is important in this process and that this experimental model is suitable for studying this regulon and molecules selected by connectivity map analysis. Moreover, in Chapter 5, we evaluated the effect of serum of bipolar patients on differentiated SH-SY5Y cells to investigate the role of systemic toxicity in neuronal cells. The serum of patients, especially at late stages of illness, caused toxicity to cells, reducing neurite density and cell viability. These findings represent a strategy of challenging cells related to the systemic toxicity of bipolar disorder, proposing differentiated SH-SY5Y cells as an *in vitro* model to study this disorder. Therefore, the results of this thesis suggest that systemic toxicity related to recurrent mood episodes may influence on the brain anatomical changes associated with the bipolar disorder progression, and dysfunction in *EGR3* regulon could be involved with aspects of 'neuroprogression' considering the role of *EGR3* in response to stress and neuroplasticity. These hypotheses, which also suggest interesting targets for the development of new treatments, should be further properly validated in experimental models such as the differentiated SH-SY5Y cells model studied in this work.

Lista de abreviaturas

AR	ácido retinóico
ARACNE	algoritmo para a reconstrução de redes celulares (do inglês *algorithm for the reconstruction of accurate cellular networks*)
ARC	proteína associada ao citoesqueleto regulada por atividade (do inglês *activity regulated cytoskeletal associated gene*)
BDNF	fator neurotrófico derivado do encéfalo (do inglês *brain-derived neurotrophic factor*)
CaN	calcineurina
CAT	catalase
CCL	ligante de quimiocina motivo CC (do inglês *chemokine (C-C motif) ligand*)
CMAP	mapa de conectividade (do inglês *connectivity map*)
COBL	*cordon-bleu WH2 repeat protein*
CRHBP	proteína ligadora do hormônio liberador de corticotrofina (do inglês *corticotropin releasing hormone binding protein*)
CXCL	ligante de quimiocina motivo CXC (do inglês *chemokine (C-X-C motif) ligand*)
DAT	transportador de dopamina (do inglês *dopamine active transporter*)
DGKB	diacilglicerol cinase beta (do inglês *diacylglycerol kinase beta*)
EGR	resposta de crescimento precoce (do inglês *early growth response*)
EPAC	proteína trocadora diretamente ativada por cAMP (do inglês *exchange protein directly activated by cAMP*)
GABRA	receptor de ácido gama-aminobutírico A (do inglês *gamma amminobutyric acid A receptor*)
GDNF	fator neurotrófico derivado da glia (do inglês *glial-derived neurotrophic factor*)
GEO	*Gene Expression Omnibus*
GO	ontologia gênica (do inglês *gene ontology*)
GSEA	método de enriquecimento gênico (do inglês *Gene Set Enrichment Analysis*)

GWAS estudos de associação em genoma completo (do inglês *genome-wide association studies*)

HPA hipotálamo-pituitária-adrenal

HTR receptor de hidroxitriptamina (serotonina) (do inglês *hydroxytryptamine receptor*)

IEG genes de expressão imediata (do inglês *immediate early genes*)

IL interleucina

ILF fator de ligação/potenciador de interleucina (do inglês *interleukin enhancer-binding factor*)

iPS células tronco de pluripotência induzida (do inglês *induced pluripotent stem cells*)

LTD depressão de longa duração (do inglês *long-term depression*)

MADD domínio de morte ativado por MAP cinase (do ingês *MAP-kinase activating death domain*)

MAPK proteína cinase ativada por mitógeno (do inglês *mitogen-activated protein kinase*)

MR regulador mestre (do inglês *master regulator*)

MRA análise de regulador mestre (do inglês *master regulator analysis*)

NETO proteína similar a neuropilina e tolloid (do inglês *neuropilin and tolloid-like*)

NeuN proteína de núcleo de neurônio (do inglês *neuronal nuclei*)

NGF fator de crescimento neural (do inglês *nerve growth factor*)

NMDA N-metil-D-aspartato

NRG neuregulina

NSE enolase específica de neurônio (do inglês *neuron specific enolase*)

OMG glicoproteína da mielina de oligodendrócitos (do inglês *oligodendrocyte myelin glycoprotein*)

PCA análise de componentes principais (do inglês *principal component analysis*)

PCR reação em cadeia da polimerase (do inglês *polymerase chain reaction*)

PKC proteína cinase C (do inglês *protein kinase C*)

RAPGEF fator trocador de nucleotídeos de guanina (do inglês *rap guanine nucleotide exchange factor*)

RE retículo endoplasmático

RGS proteína reguladora da sinalização por proteína G (do inglês *regulator of G-protein signaling*)

SFB soro fetal bovino

SLC família de proteínas carreadoras de soluto (do inglês *solute carrier family*)

SNC sistema nervoso central

SNP polimorfismo de nucleotídeo único (do inglês *single nucleotide polymorphism*)

SOD superoxide dismutase

TF fator de transcrição (do inglês *transcription factor)*

TH tirosina hidroxilase

TNF fator de necrose tumoral (do inglês *tumor necrosis factor)*

TSC22D família do domínio TSC22 (do inglês *TSC22 domain family*)

VEGF fator de crescimento endotelial vascular (do inglês *vascular endothelial growth factor*)

YBX proteína de ligação a Y-box (do inglês *Y-box binding protein*)

1. INTRODUÇÃO

1.1 Transtorno bipolar

O transtorno bipolar é uma doença psiquiátrica crônica e potencialmente grave, que afeta aproximadamente 2% da população mundial (Geddes e Mikliwitz, 2013) e está associada à alta morbidade clínica e elevados índices de suicídio e desemprego (Belmaker, 2004; Kupfer, 2005). Os sintomas envolvem alterações no humor, comportamento, cognição e funcionalidade, em intensidades variáveis, podendo levar à incapacitação do paciente. Segundo a Organização Mundial da Saúde, o transtorno bipolar é a sexta principal causa de incapacitação entre todas as condições médicas gerais (World Health Organization, 2011).

O curso clínico do transtorno bipolar é crônico, caracterizado por episódios agudos (mania ou depressão), quando há uma exacerbação dos sintomas, e por períodos subsindrômicos e de remissão dos sintomas (denominados eutimia). O diagnóstico é realizado a partir da ocorrência de pelo menos um episódio maníaco ou hipomaníaco durante a vida, sendo que a presença de episódio maníaco confere o diagnóstico de transtorno bipolar tipo I, enquanto a presença de episódio hipomaníaco confere o diagnóstico de transtorno bipolar tipo II, que são os dois principais subtipos diagnósticos do transtorno bipolar (Belmaker, 2004; Price e Marzani-Nissen, 2012).

Os episódios de mania caracterizam-se por humor elevado ou euforia (expansivo ou irritável) com duração de no mínimo uma semana, associado a três (ou mais) dos seguintes sintomas: hiperatividade, redução da necessidade de sono, distratibilidade, taquilalia, aceleração do pensamento e fuga de ideias, agressividade, impulsividade e grandiosidade, com comportamento excessivo a ponto de prejudicar o julgamento dos

pacientes e colocá-los em risco e aqueles a sua volta. Já os episódios de depressão bipolar caracterizam-se pela presença, durante um período de pelo menos duas semanas, de cinco (ou mais) dos seguintes sintomas: humor deprimido, perda de interesse, alterações no apetite e no sono, retardo psicomotor, diminuição da velocidade de pensamento e fala, baixa autoestima e ideação suicida (Belmaker, 2004; Price e Marzani-Nissen, 2012). A gravidade destes sintomas, durante episódios de mania ou depressão, pode variar consideravelmente entre os pacientes. Na eutimia, período entre episódios, há remissão dos sintomas e o paciente apresenta humor estável. Além disso, os pacientes frequentemente apresentam sintomas psicóticos, prejuízos no funcionamento, na cognição e na qualidade de vida (Geddes & Miklowitz, 2013).

O tratamento do transtorno bipolar baseia-se no manejo dos episódios agudos (para levar um paciente em mania ou depressão à remissão dos sintomas - eutimia) e no tratamento crônico de manutenção para prevenir a ocorrência de novos episódios, reduzir os sintomas subsindrômicos e aumentar a funcionalidade dos pacientes. Os fármacos disponíveis incluem o lítio (estabilizador de humor mais comumente utilizado), a carbamazepina, o ácido valpróico, e também lamotrigina, topiramato, gabapentina e antipsicóticos atípicos (Yatham *et al*., 2005). Além dos efeitos adversos e baixa eficácia em alguns pacientes, o tratamento pode ser complexo, já que fármacos que tratam a depressão podem causar mania/hipomania e vice-versa, tratamentos que reduzem os sintomas maníacos podem causar episódios de depressão (Geddes & Miklowitz, 2013). Assim, muitos estudos têm focado em novas terapias, buscando moléculas que atuem em vias associadas com os episódios de humor (conforme será discutido no capítulo 1), alternativas inovadoras como o uso de células-tronco mesenquimais no tratamento de transtornos psiquiátricos[*] e abordagens visando identificar indicações terapêuticas adicionais para fármacos já aprovados e utilizados na

clínica, por exemplo, através de uma ferramenta de bioinformática chamada de Mapa de Conectividade (como será discutido no capítulo 4 desta tese).

1.2 Biomarcadores periféricos no transtorno bipolar

Estudos bioquímicos a partir de amostras periféricas de pacientes têm sido importantes no esclarecimento da fisiopatologia do transtorno bipolar. Nesse contexto, um conjunto crescente de evidências sugere alterações em moléculas associadas com neuroplasticidade, estresse oxidativo e inflamação, especialmente durante os episódios de humor (Pfaffenseller *et al*., 2013), como será discutido detalhadamente no capítulo 1 desta tese.

Entre estas moléculas, destaca-se o papel das *neurotrofinas*, as quais compreendem uma classe de proteínas altamente abundantes no sistema nervoso com funções fundamentais na sobrevivência, crescimento e plasticidade neuronal (Huang and Reichardt, 2001). Inicialmente, um estudo mostrou níveis séricos diminuídos do fator neurotrófico derivado do encéfalo (BDNF) em pacientes em episódios agudos de mania e depressão comparados a indivíduos saudáveis (controles) ou pacientes em eutimia (Cunha *et al*., 2006). Outros resultados nessa área foram posteriormente avaliados por estudos de meta-análise (Fernandes *et al*., 2011; 2014), sugerindo um importante papel do BDNF no transtorno bipolar e na remissão dos sintomas. Além disso, estudos também indicam alterações em outras neurotrofinas e fatores tróficos nos pacientes bipolares, incluindo a neurotrofina-3, a neurotrofina-4/5, o fator neurotrófico derivado da glia (GDNF) e os fatores de crescimento neural (NGF) e endotelial vascular (VEGF) como revisado recentemente (Scola e Andreazza, 2015), reforçando a hipótese

* Para mais informações sobre este assunto, leia o artigo 'Mesenchymal stem cells for the treatment of neurodegenerative and psychiatric disorders'. Colpo GD, Ascoli BM, Wollenhaupt-Aguiar B, Pfaffenseller B, Silva EG, Cirne-Lima EO, Quevedo J, Kapczinski F, Rosa AR. An Acad Bras Cienc. 2015; 87:1435-1449.

de que prejuízos na neuroplasticidade estejam envolvidos na fisiopatologia do transtorno bipolar.

Além das neurotrofinas, outros marcadores biológicos têm sido analisados no transtorno bipolar. No caso do *estresse oxidativo*, já foram descritas alterações nas principais enzimas antioxidantes, como aumento da atividade da superóxido dismutase (SOD, E.C. 1.15.1.1) em pacientes em episódio de mania ou depressão, e diminuição da catalase (CAT, E.C. 1.11.1.6) em pacientes eutímicos medicados e aumento desta em pacientes maníacos não medicados (Steckert *et al*., 2010). Entre vários marcadores de estresse oxidativo já avaliados no transtorno bipolar, uma meta-análise recente mostrou que os níveis de peroxidação lipídica, óxido nítrico e dano ao DNA/RNA estão significativamente elevados em pacientes quando comparados a controles (Brown *et al*., 2014).

Já em relação às rotas de *inflamação*, os achados mostram o aumento nos níveis periféricos de citocinas pró-inflamatórias no transtorno bipolar. Os dados mais consistentes sugerem um aumento nos níveis de interleucina (IL)-6 e do fator de necrose tumoral alfa (TNF-α) durante a mania e a depressão (Brietzke *et al*., 2009; Modabbernia *et al*., 2013). Entre pacientes eutímicos, já se verificou que o TNF-α e a IL-6 encontravam-se elevados de forma mais pronunciada nos estágios mais avançados de progressão do transtorno (Kauer-Sant'Anna *et al*., 2009). Já a IL-10, uma citocina anti-inflamatória, foi encontrada aumentada somente no estágio precoce. Mais recentemente, observou-se um aumento dos níveis plasmáticos das quimiocinas CCL11, CCL24 e CXCL10, e uma diminuição de CXCL8 em pacientes crônicos comparados a controles (Barbosa *et al*., 2012). A origem destas alterações imunológicas ainda é desconhecida, entretanto a principal relação entre a alta presença de comorbidades clínicas (doenças

cardiovasculares, síndrome metabólica e diabetes) e o transtorno bipolar parece ser a inflamação sistêmica crônica (McIntyre *et al*., 2010; Weiner *et al*., 2011). Devido ao número crescente de evidências que sugerem uma inflamação crônica leve na periferia e no cérebro de pacientes (Hamdani *et al*., 2012), alguns autores tem se referido ao transtorno bipolar como uma doença inflamatória multissistêmica (Leboyer *et al*., 2012; Stertz *et al*., 2013).

1.2.1 Toxicidade sistêmica no transtorno bipolar

As evidências biológicas acima discutidas sugerem alterações em marcadores periféricos no transtorno bipolar, especialmente durante os episódios de humor. No entanto, estes parâmetros têm sido usualmente avaliados de maneira individual, contrariando a ideia de que biomarcadores isolados são provavelmente insuficientes para compreender distúrbios complexos. Para avaliar estes biomarcadores simultaneamente, nosso grupo conduziu uma avaliação *en bloc* de um conjunto de alvos relacionados ao *estresse oxidativo*, *neurotrofinas* e *inflamação*, previamente descritos como alterados no transtorno bipolar. Os resultados demonstraram correlações significativas entre a maioria dos biomarcadores, os quais foram então utilizados para extrair um índice de toxicidade sistêmica. Pacientes em episódios maníacos e depressivos apresentaram toxicidade sistêmica aumentada quando comparados a pacientes eutímicos e controles, mas inferior à toxicidade apresentada por pacientes com sepse (grupo controle "positivo" para doença periférica extrema) (Kapczinski *et al*., 2010; 2011).

Ao associar os episódios agudos com a toxicidade sistêmica, este estudo corroborou com a ideia de que o transtorno bipolar possa ser visto como uma doença multissistêmica, na qual a fisiopatologia periférica é um componente importante (Soreca *et al*., 2009). Acredita-se que esta toxicidade observada sistemicamente possa causar alterações celulares. De fato, o tratamento de células periféricas sanguíneas de indivíduos saudáveis com o soro de pacientes bipolares, cuja expressão sérica apresentava alterações na expressão de proteínas pro-/anti-inflamatórias, levou a uma diminuição na viabilidade celular (Herberth *et al*., 2011).

Entretanto, estes dados não explicam como estas mudanças periféricas se correlacionam com alterações cerebrais. Sabe-se que a plasticidade neural é sensível ao dano sistêmico (McEwen e Gianaros, 2011). A fisiopatologia central e periférica poderia estar conectada em estados pro-oxidativos e apoptóticos (Gigante *et al*., 2011), possivelmente através de mudanças na permeabilidade da barreira hematoencefálica. Considerando o papel fundamental da barreira hematoencefálica na proteção do Sistema Nervoso Central (SNC) contra compostos neurotóxicos, tem aumentado o interesse em avaliar a função desta barreira nas doenças psiquiátricas. Para o transtorno bipolar, foi proposto um modelo de perda da integridade da barreira hematoencefálica associada com aumento da permeabilidade a substâncias pro-inflamatórias do sangue periférico, o que poderia levar à ativação microglial e causar danos neurais (Patel e Frey, 2015). Embora não tenha nenhum estudo avaliando diretamente a integridade da barreira no transtorno bipolar, um trabalho recente mostrou níveis aumentados de MMP9, enzima envolvida na degradação da matriz extracelular e que parece aumentar a permeabilidade da barreira em estados pro-inflamatórios na depressão bipolar (Rybakowski *et al*., 2013). Nesse sentido, a descoberta recente de estruturas similares aos vasos linfáticos nas meninges (Louveau *et al*., 2015) relacionando o SNC e o sistema imune periférico

possivelmente irá mudar a forma como entendemos a interação neuro-imune nas doenças mentais e a conexão entre a fisiopatologia central e periférica destes transtornos.

Os possíveis mecanismos subjacentes à toxicidade sistêmica associada aos episódios de humor no transtorno bipolar serão discutidos no capítulo 1 desta tese.

1.3 Progressão do transtorno bipolar[1]

A progressão do transtorno bipolar está associada com uma redução na resposta ao tratamento (Swann *et al*., 1999; Ketter *et al*., 2006), maiores taxas de comorbidades clínicas (Matza *et al*., 2005), prejuízos em funcionalidade e cognição (Torres *et al*., 2007; Rosa *et al*., 2012) e um risco aumentado de suicídio (Hawton *et al*., 2005). Além disso, existem diferenças bioquímicas significativas entre pacientes em estágios iniciais e tardios da doença, incluindo alterações neuroanatômicas (Strakowski *et al*., 2002; Pfaffenseller *et al*., 2012), marcadores inflamatórios e neurotróficos (Kauer-Sant'Anna *et al*., 2009; Panizzutti *et al*., 2015), e em parâmetros de estresse oxidativo (Andreazza *et al*., 2009). Com base nestas observações, um modelo de estadiamento tem sido proposto para o transtorno bipolar (Kapczinski *et al*., 2009a; 2009b; Vieta *et al*., 2011), considerando variáveis clínicas, comorbidades, cognição/funcionalidade e biomarcadores no período de remissão.

Desta forma, sugere-se que o transtorno bipolar leve ao acometimento gradual da saúde geral do paciente, através de um processo progressivo no tecido central ('neuroprogressão') e periférico com o curso da doença, o qual é episódio-dependente (Kapczinski *et al*., 2008; Berk *et al*., 2011; Post *et al*., 2012). Uma das teorias propostas para explicar a recorrência dos episódios de humor e o consequente dano progressivo/cumulativo (Kapczinski *et al*., 2008; Vieta *et al*., 2012) é a chamada 'teoria

[1] Uma revisão detalhada sobre a progressão do transtorno bipolar e estadiamento encontra-se no artigo pertencente ao Anexo 1 (Fries GR, Pfaffenseller B, Stertz L, Paz AV, Dargél AA, Kunz M, Kapczinski F. Staging and neuroprogression in bipolar disorder. Curr Psychiatry Rep. 2012 Dec;14(6):667-75).

da carga alostática', que propõe que a ativação crônica de mecanismos para restaurar a homeostase após condições estressoras gere um desgaste no organismo (McEwen e Wingfield, 2003). Esses mecanismos adaptativos são vitais, mas também podem promover efeitos mal adaptados na plasticidade cerebral, assim como nos sistemas metabólico, imune e cardiovascular (McEwen e Gianaros, 2011).

Conforme mencionado anteriormente, episódios agudos no transtorno bipolar têm sido associados com toxicidade sistêmica e sugere-se que esses efeitos possam ser cumulativos, sendo sutis após os primeiros episódios, porém mais pronunciados após múltiplos episódios (Magalhães *et al*., 2011; Grande *et al*., 2012). Portanto, as disfunções sistêmicas e neurais progressivas resultantes de múltiplos episódios poderiam ser consideradas como estados cumulativos de carga alostática no transtorno bipolar (Kapczinski *et al*., 2008), deixando o paciente mais suscetível a novos estressores e, assim, também a novos episódios, completando um ciclo de progressão da doença.

1.4 Resiliência e plasticidade celular[2]

Embora as alterações neuroanatômicas observadas em pacientes bipolares, como a redução da massa cinzenta no córtex pré-frontal e o alargamento dos ventrículos laterais, tornam-se pronunciadas com a progressão do transtorno (Strakowski *et al*., 2002; Pfaffenseller *et al*., 2012), ainda não está esclarecido se refletem a perda de neurônios em si e/ou uma diminuição na conectividade entre estas células. Nesse contexto, é possível sugerir que a fisiopatologia do transtorno bipolar envolva prejuízos na resiliência celular (*i.e*., habilidade das células de se adaptarem e sobreviverem frente a diferentes estímulos de dano), incluindo prejuízos na plasticidade celular, quando se

[2] O conteúdo desta seção é baseado no capítulo pertencente ao Anexo 2: "Fisiopatologia do Transtorno Bipolar", de autoria de Fries GR, Pfaffenseller B e Kapczinski F, publicado no livro "Transtorno bipolar: teoria e clínica". 2ed. Artmed. 2015.

refere aos tipos celulares que podem modular a comunicação com outras células de acordo com estes estímulos, como os neurônios.

Entre os achados indicando alterações que poderiam afetar a resiliência celular no transtorno bipolar, já se demonstrou que pacientes apresentam níveis diminuídos de N-acetil-aspartato no córtex pré-frontal dorsolateral (Sassi *et al*., 2005). Isso pode indicar um prejuízo na plasticidade neuronal destes pacientes, já que esta molécula é abundante em neurônios maduros e representa integridade neuronal. Também relacionado à integridade celular, níveis aumentados de fatores apoptóticos em tecidos periféricos e centrais no transtorno bipolar tem sido relatados, assim como uma diminuição de fatores anti-apoptóticos (Gigante *et al*., 2011). Além disso, um estudo recente do nosso grupo reportou um aumento de apoptose em células periféricas de pacientes bipolares (Fries *et al*., 2014) (Anexo 3 desta tese).

Ainda mais relevante, tem se demonstrado que as células de pacientes bipolares, quando desafiadas, são mais suscetíveis frente a diferentes insultos, como estresse oxidativo (Roedding *et al*., 2012) e estresse do retículo endoplasmático (Pfaffenseller *et al*., 2014). Em conjunto, os achados sugerem que as células destes pacientes apresentam uma menor resiliência, possivelmente associada com maior morte celular. De fato, um estudo mostrou que células do neuro-epitélio olfatório de pacientes apresentam maior vulnerabilidade à morte celular em comparação a indivíduos saudáveis (McCurdy *et al*., 2006). O prejuízo na resiliência celular pode ser um dos fatores responsáveis pelas alterações em estruturas cerebrais nos pacientes bipolares (pela redução no número ou na complexidade das conexões neurais) e provavelmente relacionado às alterações bioquímicas e clínicas.

1.5 Genética do transtorno bipolar

O componente genético parece ter um papel muito importante na fisiopatologia do transtorno bipolar. É bastante comum os pacientes apresentarem histórico familiar de transtornos de humor ou psicóticos. Para exemplificar, um estudo mostrou que os fatores familiares correspondem à cerca de 20% da variação no desfecho da doença, comparando-se a frequência de transtorno bipolar em famílias já afetadas em relação a famílias não acometidas pelo transtorno (Helenius *et al*., 2013). Indivíduos com um familiar de primeiro grau com transtorno bipolar tem um risco aproximadamente 14 vezes maior de desenvolver a doença do que aqueles que não possuem história na família (Mortensen *et al*., 2003). Além de possuir um risco aumentado para o desenvolvimento de transtorno bipolar, parentes de indivíduos bipolares apresentam maior risco para vários outros transtornos psiquiátricos relacionados, como depressão unipolar e esquizofrenia (Tsuang & Faraone, 1990), o que indica que estas doenças compartilham alguma susceptibilidade genética. Também nesse sentido, o histórico familiar de transtorno bipolar tem sido sugerido como um preditor clínico para o possível desenvolvimento deste transtorno em indivíduos que apresentam inicialmente alguns episódios de depressão (Craddock & Sklar, 2013).

Além dessas observações clínicas, pesquisas genéticas epidemiológicas com famílias e gêmeos têm demonstrado evidências reforçando a predisposição genética para o transtorno bipolar; a herdabilidade estimada é alta assim como a taxa de concordância entre gêmeos monozigóticos (Kieseppa *et al*., 2004; Craddock & Sklar, 2013). Isto indica a importância dos fatores genéticos sobre a susceptibilidade ao transtorno bipolar (Quadro 1). Entretanto, como a concordância entre gêmeos monozigóticos é bem menor que 100%, outros fatores não genéticos também contribuem para a fisiopatologia da doença e devem ser levados em consideração. Desta forma, assim como a maioria das

doenças, o transtorno bipolar se caracteriza por seguir um padrão de herança poligênico multifatorial, decorrente de fatores genéticos (como polimorfismos e mutações) e fatores não genéticos (como estilo de vida e exposição ao estresse, eventos traumáticos e a agentes infecciosos ou químicos).

Quadro 1. Risco genético para o transtorno bipolar.

- Indivíduo da população sem histórico familiar de transtornos psiquiátricos: 0,5 – 1,5%
- Parente de primeiro grau de um indivíduo bipolar: 5 – 10%
- Gêmeos monozigóticos: 40 – 70%

Fonte: Craddock & Jones (1999).

1.6 Metodologias para estudo da genética em transtornos psiquiátricos

Atualmente, nos estudos de genética molecular, tem-se utilizado basicamente dois tipos de metodologia:

- abordagem com genes candidatos: baseia-se na pesquisa de genes possivelmente implicados na fisiopatologia de transtornos psiquiátricos, um de cada vez. Por exemplo, genes relacionados à transmissão sináptica e neuroplasticidade. No geral, esta metodologia não tem gerado resultados robustos.

- abordagem com sequenciamento genômico global (*genome-wide association studies* - GWAS): baseia-se na análise completa do genoma a partir de marcadores conhecidos. Neste caso, a avaliação não está focada apenas em um ou poucos genes candidatos, mas sim no genoma todo que é comparado entre indivíduos com a doença em estudo e indivíduos saudáveis. A vantagem deste método é testar o efeito geral de vários genes envolvidos em um processo biológico ao invés de um polimorfismo de nucleotídeo único (SNP), variação na sequência de DNA que afeta somente uma base, por vez.

O GWAS permite a análise simultânea da variação genética ao longo de todos os cromossomos, através da genotipagem de milhares de SNP em milhares de indivíduos. Essa metodologia tem possibilitado um significativo progresso nos achados genéticos sobre a fisiopatologia de vários transtornos psiquiátricos, incluindo o transtorno bipolar. Entretanto, os resultados obtidos não tem sido satisfatórios para explicar o risco genético destas doenças, pois os tamanhos de efeito em SNP individuais são muito pequenos e não atingem significância no GWAS. Essas análises poligênicas são consistentes com centenas ou milhares de variantes de susceptibilidade para o transtorno bipolar, mas o risco de cada uma é muito pequeno para apresentar um efeito forte independentemente (Craddock *et al*., 2013). Nesse sentido, estudos de metanálise de dados provenientes de GWAS a partir de milhares de pacientes com transtorno bipolar e esquizofrenia, comparados com indivíduos saudáveis, têm demonstrado associações com efeito fraco (Manolio *et al*., 2009; McClellan & King, 2010).

Tipicamente, os SNP associados na análise GWAS explicam apenas uma pequena proporção da variação genética da população, o que tem conduzido à questão da "hereditariedade em falta" (Manolio *et al*., 2009). Sugere-se que a variação conferida pelos SNP pode explicar aproximadamente 20-30% da herdabilidade estimada em estudos de família e de gêmeos (Purcell *et al*., 2009; Lee *et al*., 2011). Além disso, associações anteriores com base em genes candidatos não têm sido replicadas nas análises em grande escala do GWAS (Gershon *et al*., 2011).

Apesar do progresso obtido até o momento na medicina com a revolução da biologia molecular, a questão da “falta de herdabilidade” em certas doenças, como o transtorno bipolar, tem sido o foco da pesquisa nessa área científica (Maher, 2008; Manolio *et al*., 2009). A associação entre genótipo e fenótipo nas doenças psiquiátricas

é altamente complexa como a própria fisiopatologia destas doenças. Para o transtorno bipolar, análises matemáticas já indicavam há anos que este transtorno não pode ser avaliado por um único gene ou um conjunto de raros genes de susceptibilidade (Craddock *et al*., 1995). Como esta doença envolve vários *loci* gênicos e mecanismos genéticos complexos interagindo com fatores não genéticos (ambientais), é necessário estudar vias moleculares inteiras, ao invés de genes individuais ou vários genes com baixo efeito estatístico. Isso tem aumentado o interesse por abordagens alternativas, que possibilitem um maior entendimento do envolvimento genético na fisiopatologia de doenças complexas.

1.6.1 Redes de regulação transcricional e análise de 'reguladores mestres'

Estudos de expressão gênica em doenças psiquiátricas são muito importantes na investigação de alterações relacionadas com a fisiopatologia e na busca por alvos com potencial terapêutico. Nestes estudos, o uso de tecnologia *high-throughput* (triagem de alta produtividade), como o microarranjo de RNA, possibilita a avaliação da expressão diferencial do DNA genômico na forma de RNA mensageiro, o que potencialmente pode fornecer assinaturas gênicas de doenças (Altar, Vawter & Ginsberg, 2009). É importante destacar que a análise de RNA pode ser mais informativa do que aquela oriunda da sequencia de genes em si, pois o RNA reflete um estado celular mais funcional influenciado não só por polimorfismos, mas também por uma modulação da transcrição gênica.

O campo da biologia de sistemas tem emergido como uma nova forma de pensar e realizar a pesquisa científica na era pós-genoma. Cada vez mais, cresce a necessidade de ir além da abordagem de um único gene ou proteína e focar na visão integral de todas as fases de pesquisa, incluindo a coleta de dados, processamento de informação e

análises, interpretação, geração de hipóteses e subsequente delineamento experimental para testar as hipóteses geradas. Além disso, há o atual desafio de como utilizar e analisar a quantidade substancial de dados produzidos por tecnologias *high-throughput* nessa era pós-genoma a fim de gerar resultados com significado biológico. Nesse contexto, destacam-se abordagens focadas em fatores de transcrição (*transcription factors* - TF) visto que são moléculas 'chave' no controle da expressão gênica e assim no funcionamento celular. Sob a regulação de TF, cada gene influencia a atividade celular através de mRNA que guiam a síntese proteica coordenando reações bioquímicas e processos moleculares. Esse mecanismo complexo de controle da expressão gênica por TF pode ser representado através de redes de regulação gênica (Figura 1), as quais podem ser utilizadas para descrever o comportamento fenotípico de um sistema em estudo (uma doença, por exemplo) (Lee & Tzou, 2009).

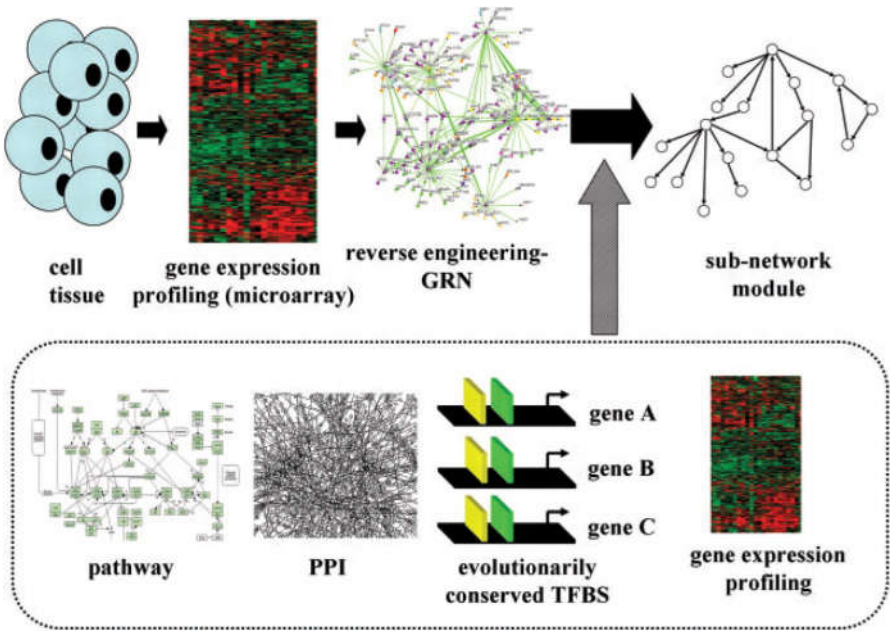


Figura 1. Inferindo redes de regulação gênica a partir de dados do perfil de expressão gênica. A partir de mRNA extraído de células/tecidos, realizam-se experimentos de microarranjo para obter os dados do perfil de expressão gênica das amostras. Estes dados são utilizados para inferir uma rede regulatória usando métodos computacionais. Nesse processo, diferentes tipos de bancos de dados (perfil de expressão gênica, proteica, vias celulares) podem ser consultados para filtrar a análise de acordo com o interesse do estudo. O objetivo é, então, obter uma sub-rede que serve de modelo de trabalho para gerar hipóteses e desenhar experimentos (Lee & Tzou, 2009).

Os fenótipos são avaliados pela atividade dinâmica destas redes de genes coregulados. Como a expressão gênica é regulada por TF, associações estatísticas entre os níveis de mRNA do gene, embora não reflita diretamente a concentração de proteínas ativadas, devem fornecer pistas para desvendar mecanismos regulatórios dos genes. Assim, estudos tem focado em utilizar os dados de técnicas de alto rendimento (como os microarranjos), que possibilitam mensurar simultaneamente os níveis de mRNA em um genoma inteiro, para construir modelos de redes regulatórias que permitam entender melhor as influências reguladoras que os genes exercem uns sobre os outros e em um determinado processo fisiológico ou patológico (Margolin *et al*., 2006a; 2006b).

Várias abordagens robustas para realizar a 'engenharia reversa' de redes a partir de dados de expressão gênica tem sido propostas (van Someren *et al*., 2002; Margolin *et al*., 2006b), a fim de gerar uma representação de alta fidelidade da rede gênica, em que os genes são representados como vértices e são conectados por arestas que representam as interações regulatórias diretas (Figura 2). Entre estas abordagens, encontra-se o algoritmo ARACNE (*Algorithm for the Reconstruction of Accurate Cellular Networks*), método computacional para identificação de interações transcricionais entre produtos gênicos usando dados de perfil de expressão gênica por microarranjo. Semelhante a outros algoritmos, ARACNE prevê associações funcionais em potencial entre genes, ou novas funções para os genes não caracterizados, através da identificação de dependências estatísticas entre os produtos gênicos (Margolin *et al*., 2006a).

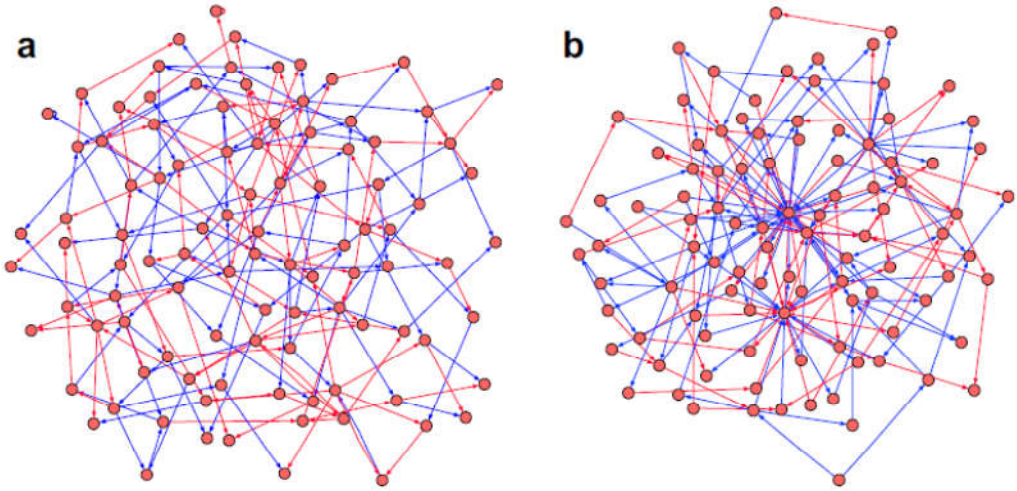


Figura 2. Representação de redes de regulação gênica. Arrestas azuis e vermelhas correspondem à interação gênica de ativação e inibição. (A) Cada gene é igualmente conectado a cada outro gene da rede. (B) Rede caracterizada por interações desiguais entre os genes, com genes apresentando muitas conexões. Adaptado de Margolin *et al*., 2006a.

Neste contexto de redes gênicas, tem sido demonstrado que um pequeno número de TF atua como 'reguladores mestre' (*master regulators* – MR) coordenando o comportamento celular e exercendo grandes influências sobre um determinado fenótipo. MR podem ser identificados através da construção de redes regulatórias centradas em TF (Basso *et al*., 2005), usando algoritmos como o ARACNE comentado anteriormente, onde cada TF na rede é conectado a um conjunto de genes que ele regula diretamente (constituindo uma unidade regulatória ou regulon). A associação de uma assinatura gênica relevante aos regulons pode indicar os TF que atuem como MR do fenótipo avaliado (*master regulator analysis*, MRA). Para esta análise, utiliza-se o método de enriquecimento gênico (*Gene Set Enrichment Analysis* - GSEA) que avalia dados de microarranjo por conjuntos de genes definidos com base no conhecimento biológico prévio (vias bioquímicas ou interação gênica relatada em experimentos anteriores) (Subramanian *et al*., 2005). O objetivo dessa análise é determinar se integrantes de um conjunto determinado de genes (*gene set S*) tendem a aparecer em direção ao topo ou à base de uma lista (*list L*) na qual os genes estão ordenados de acordo com a sua

expressão diferencial entre duas classes analisadas, sendo que cada extremo da lista representa um fenótipo (caso x controle, por exemplo). Neste caso, o conjunto de genes é correlacionado com o fenótipo de uma das classes, então se diz que estes genes estão enriquecidos neste fenótipo (Figura 3).

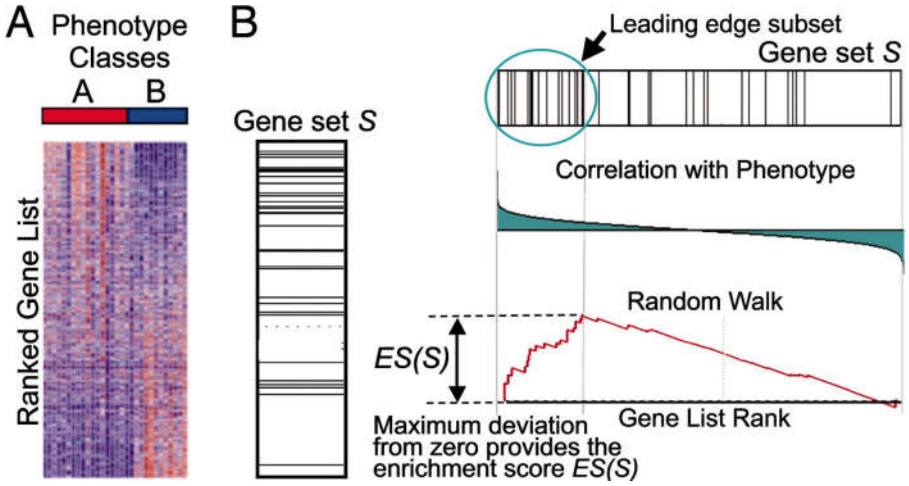


Figura 3. Visão geral do método de GSEA. (A) Um conjunto de dados de expressão ordenados pela correlação com o fenótipo (A ou B), o correspondente 'heat map' e a localização de genes de um conjunto S dentro da lista ordenada. (B) Grafo resultante da análise, mostrando a correlação com o fenótipo, incluindo a localização da pontuação máxima de enriquecimento (ES) (Subramanian *et al*., 2005).

Assim, por exemplo, o GSEA pode ser usado para avaliar se um dado regulon está enriquecido para genes que estão diferencialmente expressos entre duas classes de microarranjos, testando a associação entre tal regulon e diferentes fenótipos (por exemplo, assinaturas gênicas de doenças). A grande vantagem deste método, comparado a análises de genes únicos, é permitir que a interpretação de dados em larga escala seja mais compreensível e informativa, através da identificação de vias e processos biológicos inteiros e específicos.

Recentemente, tem aumentado o interesse no desenvolvimento de métodos computacionais para determinar grupos de genes, e vias biológicas inteiras, coordenados por um pequeno número de TF, permitindo identificar MR importantes com assinaturas

gênicas tecido-específicas. Estas abordagens têm obtido resultados interessantes em doenças como diabetes e câncer (Carro *et al*., 2010; Piao *et al*., 2012; Castro *et al*., 2016), identificando regulons que estão comprometidos nestas condições. Assim como nestas áreas médicas tradicionais, a psiquiatria tem focado no estudo de vias biológicas, seguindo a tendência de utilização de abordagens por bioinformática que fornecem hipóteses apropriadas para aperfeiçoar o estudo destas redes gênicas e, posteriormente, seguir com a validação experimental com maior embasamento científico.

1.7 Modelos celulares para estudo do transtorno bipolar

Modelos *in vitro* são amplamente utilizados para avaliar os mecanismos moleculares/bioquímicos envolvidos em processos patológicos e o potencial neuroprotetor/neurotóxico de diferentes compostos (Bal-Price *et al*., 2008). Há um grande interesse na utilização de modelos celulares para estudar doenças psiquiátricas. No caso do transtorno bipolar, se tem utilizado modelos celulares não-neurais (linhagens de células linfoblastóides, cultura primária de células do sangue e de fibroblastos) e modelos neurais (células do neuroepitélio olfatório e mais recentemente, células neurais diferenciadas a partir de células tronco de pluripotência induzida - iPS) (Viswanath *et al*., 2015).

Todos estes modelos apresentam limitações. No caso dos modelos não-neurais, embora muitos estudos demonstrem o envolvimento sistêmico no transtorno bipolar, ainda não se conhece as diferenças de expressão gênica e resposta celular entre tecidos periféricos e centrais. Em relação aos modelos neurais, células do neuroepitélio são fáceis de cultivar e não apresentam alterações genéticas, entretanto sua coleta é

invasiva. Já as iPS podem ser obtidas a partir de células fáceis de serem coletadas, mas são alteradas geneticamente e difíceis de manter em cultura (Viswanath *et al*., 2015).

Neste contexto, é crescente o interesse na utilização de linhagens celulares humanas que possam ser diferenciadas em células neurais, para aquisição, triagem e avaliação de dados que auxiliem na compreensão dos processos patológicos que envolvem o SNC (Radio e Mundy, 2008). A linhagem do neuroblastoma humano SH-SY5Y destaca-se por apresentar várias vantagens, como a sua origem humana, a facilidade para crescimento e manutenção e a presença de características neuronais que podem ser obtidas a partir de um processo de diferenciação *in vitro*. Nosso grupo padronizou a diferenciação do neuroblastoma humano SH-SY5Y em neurônios dopaminérgicos e colinérgicos e vem utilizando este modelo em neurociências (Lopes *et al*., 2010; 2012; Schönhofen *et al*., 2014).

A diferenciação dopaminérgica da linhagem SH-SY5Y é induzida por ácido retinóico, o qual promove *in vitro* a diferenciação e inibe a divisão celular, regulando a transição das células precursoras para células diferenciadas. Este processo resulta no surgimento de neuritos, modificação de sua morfologia epitelial para uma forma estrelada e expressão de marcadores neuronais, como a tirosina hidroxilase (TH), enolase específica de neurônio (NSE), proteína de núcleo de neurônio (NeuN) e o transportador de dopamina (DAT) (Lopes *et al*., 2010). Assim, a linhagem do neuroblastoma humano SH-SY5Y diferenciada com ácido retinóico se tornou uma ferramenta importante no estudo de doenças relacionadas ao SNC, incluindo o transtorno bipolar, complementando outros modelos experimentais na busca pela compreensão da fisiopatologia destas doenças e dos mecanismos de ação tanto de drogas clássicas como de novas moléculas para guiar estratégias terapêuticas.

1.8 Justificativa

Apesar dos avanços científicos obtidos na área da psiquiatria nos últimos anos, ainda é necessário esclarecer vários aspectos das bases biológicas do transtorno bipolar a fim de desenvolver tratamentos mais eficazes e personalizados que possam contribuir para uma melhor qualidade de vida dos pacientes. Desvendar a fisiopatologia do transtorno bipolar segue sendo um desafio uma vez que esta doença é altamente complexa, de caráter multifatorial (determinada pela interação entre fatores genéticos e ambientais) e sistêmico (envolvendo não só o sistema nervoso central, mas também outros sistemas fisiológicos periféricos).

Os pacientes apresentam várias alterações em biomarcadores periféricos (aumento de marcadores pró-inflamatórios, estresse oxidativo, diminuição de fatores tróficos), o que tem associado o transtorno a uma toxicidade sistêmica significativa (Kapczinski *et al*., 2010). Essa toxicidade pode estar relacionada a alterações nestes sistemas fisiológicos periféricos em si e possivelmente influenciar também em mudanças no sistema nervoso central, contribuindo para uma diminuição na resiliência celular periférica e central e, em última análise, a uma maior vulnerabilidade dos pacientes ao estresse e piora clínica no seu prognóstico. Neste contexto, a melhor compreensão dos mecanismos responsáveis por tais alterações sistêmicas e centrais (por exemplo, através do estudo de vias de regulação transcricional) poderá auxiliar no desenvolvimento de novas terapias que sejam mais eficazes no tratamento do transtorno bipolar.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo geral desta tese foi propor biomarcadores periféricos associados à toxicidade sistêmica no transtorno bipolar e estudar alterações na regulação transcricional nesta doença, a fim de identificar vias moleculares relevantes para a sua fisiopatologia e potenciais alvos terapêuticos.

2.2 Objetivos específicos

- Revisar e discutir os biomarcadores periféricos associados com a toxicidade sistêmica dos episódios de humor, os possíveis mecanismos responsáveis por esta toxicidade e novas terapias, a partir de dados da literatura científica (Capítulo 1);

- Ilustrar alterações anatômicas cerebrais associadas com a progressão do transtorno bipolar (Capítulo 2);

- Identificar vias de regulação transcricional disfuncionais no córtex pré-frontal de pacientes bipolares e propor alvos de pesquisa e com potencial terapêutico, através de abordagens de bioinformática baseadas em redes de unidades regulatórias (Capítulo 3);

- Buscar, através de análise de mapas de conectividade, fármacos com perfil de modular as assinaturas moleculares do transtorno bipolar (Capítulo 4);

- Avaliar o perfil de expressão gênica do modelo de células SH-SY5Y diferenciadas para caracterização neuronal e analisar se este modelo *in vitro* é adequado para estudar os alvos identificados no estudo de regulação transcricional (Capítulo 4);

- Investigar se o soro de pacientes bipolares, pelas suas alterações bioquímicas descritas, induz toxicidade nas células SH-SY5Y diferenciadas em células com fenótipo neuronal (Capítulo 5).

PARTE II

Artigos científicos

3. ARTIGOS CIENTÍFICOS

3.1 CAPÍTULO 1

Neurotrophins, inflammation and oxidative stress as illness activity biomarkers in bipolar disorder

Bianca Pfaffenseller, Gabriel R Fries, Bianca Wollenhaupt-Aguiar, Gabriela D Colpo, Laura Stertz, Bruna Panizzutti, Pedro VS Magalhães, Flávio Kapczinski

Artigo publicado na revista *Expert Review of Neurotherapeutics* em 2014.

EXPERT REVIEWS

Neurotrophins, inflammation and oxidative stress as illness activity biomarkers in bipolar disorder

Expert Rev. Neurother. 13(7), 827–842 (2013)

Bianca Pfaffenseller[1,2], Gabriel R Fries[1,2], Bianca Wollenhaupt-Aguiar[1,2], Gabriela D Colpo[1,3], Laura Stertz[1,2], Bruna Panizzutti[1,3], Pedro VS Magalhães[1,2] and Flávio Kapczinski*[1,2]

[1]*Laboratório de Psiquiatria Molecular, Instituto Nacional de Ciência e Tecnologia – Translacional em Medicina (INCT), Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[2]*Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[3]*Programa de Pós-Graduação em Ciências Médicas: Psiquiatria, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
**Author for correspondence:*
Tel.: +55 512 101 8845
Fax: +55 512 101 8846
flavio.kapczinski@gmail.com

Recent studies highlight the presence of systemic toxicity as an integral dimension of bipolar disorder pathophysiology, possibly linking this mood disorder with other medical conditions and comorbidities. This review summarizes recent findings on possible peripheral biomarkers of illness activity, with a focus on neurotrophins, inflammation and oxidative stress. The possible mechanisms underlying the systemic toxicity associated with acute episodes in bipolar disorder are also discussed. Finally, the authors outline novel therapies that emerge from this new research and the assessment of multiple biomarkers as a potential approach to improving management strategies in bipolar disorder.

Keywords: bipolar disorder • endoplasmic reticulum stress • glial dysfunction • illness activity • inflammation • mitochondrial dysfunction • neurotrophins • novel therapies • oxidative stress • peripheral biomarkers • systemic toxicity

Bipolar disorder (BD) is a severe chronic illness in which recurrent episodes of mania and depression alternate with periods of clinical remission (euthymia). BD has been commonly associated with significant disability, morbidity, and premature mortality [1,2]. The recurrence of acute episodes and illness progression often translate into worse long-term outcomes, for example, higher rates of clinical comorbidities, functional and cognitive impairments and lower responsiveness to treatment [3–6]. Moreover, patients with BD are at higher risk for developing a wide range of medical conditions, including cardiovascular and cerebrovascular disease, neurological disorders and metabolic syndrome [7].

One of the hypotheses that has been proposed to explain the mechanisms underlying the heavy medical burden and cumulative damage related to BD is the allostatic load theory [8–10]. According to this theory, the chronic activation of mechanisms to restore homeostasis after stressful conditions leads to wear-and-tear in the body and brain that has been called allostatic load [8,11]. These events are vital adaptive functions, but they may also promote maladaptive effects on brain plasticity, as well as on metabolic, immune, and cardiovascular pathophysiology, whenever mediators are excessive in number or remain active [12]. Recently, the allostatic load paradigm has been incorporated into a new concept of neuroprogression in BD, described as a pathological brain rewiring process-taking place when clinical and cognitive deterioration is observed as a result of disease progression [13]. In this sense, there is a growing interest in understanding the systemic pathophysiological mechanisms that contribute to dysfunction resulting from multiple mood episodes in BD, and especially in identifying the pathways associated with allostatic mediators involved in neuroprotection, oxidative stress and inflammation.

Within this scope, several studies have been performed to detect peripheral biomarkers that could work as indicators of cellular impairment and toxicity in patients with BD [14,15]. Different biomarkers could be associated with illness activity (indicating whether the illness is active or in remission), illness neuroprogression, or both. Of note, systemic markers have already been implicated in BD as mediators of allostasis [8,13,16]. These studies may be important in improving our understanding of illness activity

 10.1586/14737175.2013.811981 ISSN 1473-7175

and progression, and also in providing insights for new approaches to treatment and biomarkers.

The aim of the present communication is to review current evidence available on possible biomarkers of illness activity in BD. Special attention will be given to neurotrophins, inflammation and oxidative stress, as well as to the possible mechanisms whereby these metabolic routes are activated in acute mood episodes. Finally, the authors will outline emerging therapeutic opportunities in the field of BD. For recent reviews on the role of systemic pathophysiology and neuroprogression in BD, please see the reports of Grande *et al.* [9] and Fries *et al.* [17].

Peripheral biomarkers in bipolar disorder

There is considerable interest in incorporating biomarkers into psychiatry [18], using them as biological indicators to more accurately assess psychiatric conditions. A biological marker or biomarker is a feature that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (National Institutes of Health Definition Working Group, 2001) [19]. Biomarkers may be genes, proteins or other molecules, as well as morphological characteristics identified on the basis of physiological or biological mechanisms.

In addition to improving diagnosis, biomarkers could assist in predicting illness prognosis and the potential risk of developing a disorder, with valuable applications in monitoring illness status and responses to a therapeutic intervention or management strategy [20]. Moreover, biomarkers could help discover new therapeutic targets and are likely to contribute to uncover illness mechanisms in complex psychiatric disorders [21]. Particularly in BD, biomarkers may become useful tools in detecting illness activity associated with different mood states (a state marker) or in identifying specific features of the long-term course of illness (a trait marker) [22].

Only validated biomarkers can be used in the clinical setting. In other words, in order to be used clinically, a biomarker has to prove it is accurate, has high sensitivity and specificity for the expected outcome, is highly reproducible in standardized, cost effective, fast assays, is minimally invasive and acceptable to the patient, and also that it provides a clinically relevant result, with easily interpretable information [20]. Among the strategies adopted for biomarker discovery and application in BD, a great interest in peripheral biomarkers is observed: certain proteins found in peripheral blood may be transported through the blood–brain barrier and enter the CNS [23]. For instance, proteins of the neuregulin (NRG) family, that could enhance myelination of neurites, and brain-derived neurotrophic factor (BDNF) could enter the spinal cord and brain by a saturable receptor-mediated mechanism [24,25]. A recent study has found a correlation between cerebrospinal fluid and plasma BDNF levels in drug-naive, first-episode psychotic subjects [26].

This approach, using peripheral biomarkers, has several advantages, including easy collection, low cost, wide availability and feasibility for large-scale studies. Several peripheral markers have been studied as mediators of allostasis in BD [8,13]. Studies have focused primarily on biological pathways related to neuroplasticity in BD, including the role of neurotrophins, inflammation, oxidative stress and underlying processes.

Neurotrophins

Neurotrophic factors are small-secreted proteins that act in a range of biological functions related to interaction with different receptors, their local distribution and transport in the CNS [27]. Nerve growth factor was the first neurotrophin to be identified, by Levi-Montalcini in 1966. After that, several studies have discovered other neurotrophins, such as BDNF, glial cell-line derived neurotrophic factor (GDNF), neurotrophin 3 and neurotrophin 4/5 (NT-4/5), all playing major roles in synaptic plasticity, dendritic arborization, and neuronal connectivity. In addition, all have been shown to be altered in BD, as will be discussed below [8,28].

BDNF is the most abundant and widely distributed neurotrophin in the CNS and also the most studied one; current studies show that an altered expression of BDNF contributes to several disorders, including BD. A correlation between serum BDNF levels and other markers of CNS injury has also been suggested [29,30]. Moreover, a growing body of evidence points towards a relationship between peripheral BDNF levels and illness activity in BD.

Serum BDNF has been found to be reduced in BD during manic and depressive episodes when compared with euthymic patients and healthy controls, even in drug-free patients [31–34]. In unmedicated manic children and adolescents, a decrease in both mRNA levels of lymphocyte-derived BDNF and protein levels in platelets has been found in relation to healthy controls [35]. Despite some discrepancies between studies [15,36–39], meta-analyses have been performed to measure the effect size of differences in BDNF levels between patients in different mood states and controls [40,41]. The latest of these meta-analyses demonstrated that peripheral BDNF levels decrease during manic and depressive states and those patients who have experienced more episodes present lower BDNF levels.

In this context, the discrepancies found in the peripheral levels of BDNF could be associated with the difference on the methodology used in these studies. Further, this could be related to a study that verified that patients at different stages of the illness differ in the BDNF levels, showing changes in the late stages but not in early stages of illness compared with controls [42]. In addition, another study observed that BDNF levels were inversely related with age and length of illness [43]. Taken together, these findings suggest that the toxicity and cognitive impairment observed in patients with BD would be related to the number of episodes; each new episode would lead to further damage and therefore lower levels of BDNF [44].

Regarding treatment, mood stabilizers have been shown to increase BDNF levels [40]. Patient recovery from a manic episode after treatment with lithium has been associated with an increase in serum BDNF levels [45]. In the same vein, Rybakowski and Suwalska found that excellent lithium responders showed higher plasma BDNF levels compared with non-responders, and similar

levels compared with controls [46]. Other data have shown a significant increase in BDNF levels after lithium monotherapy for the management of manic episodes, suggesting a direct role of the regulatory effects of lithium on BDNF levels in mania [47]. A very recent open-label longitudinal trial in previously medication-free patients measured serum BDNF sequentially for 16 weeks. Relevantly, BDNF levels tended to increase with treatment, but only in patients acutely depressed at baseline. Those in manic or mixed episodes, in turn, showed a decrease in BDNF levels in the first weeks of treatment. These results suggest that manic and mixed episodes may be particularly toxic compared with depression, perhaps requiring a longer treatment time for BDNF to return to its baseline levels [9]. All these studies describing an important role of BDNF in the pathophysiology of BD have led to further research into novel targets of this neurotrophin, in addition to the already known therapeutic action of mood stabilizers.

Changes in other neurotrophic factors have also been reported in patients with BD, such as increased serum neurotrophin 3 levels during manic and depressive episodes compared with euthymic patients and healthy controls [48,49] and increased serum NT-4/5 levels in patients versus healthy controls, regardless of symptomatic state [50]. A recent work found increased GDNF plasma levels in euthymic patients compared with manic patients and healthy controls [51], although another study observed increased levels of GDNF in manic and depressive patients but not in euthymic patients when compared with control group [52]. Moreover, a previous study had found decreased serum levels of GDNF in patients during mania and depression, and increased levels after remission [53]. In line with this, a study has shown decreased levels of GDNF in remitted patients [54]. Taken together, these findings reinforce the implication of GDNF in the BD pathophysiology, but with a still unclear role. Additional evidence is needed to assess whether peripheral levels of GDNF are correlated with CNS levels of the neurotrophin [55].

Inflammation

Over the past few years, the number of publications focusing on immunological abnormalities involved in the pathophysiology of BD has grown substantially. Immune disturbances have been related to the severity and recurrence of mood episodes [42], illness progression [56,57], high rates of comorbidities [56] and drug effects [58,59].

Overall, mood episodes have been characterized as pro-inflammatory states [22], based on findings that show increased peripheral levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, during depressive episodes, and of IL-2, IL-4, IL-6 and TNF-α in mania when compared with euthymic patients and healthy subjects [60–62]. A recent review and meta-analysis has found that, during mania, patients show increased levels of TNF-α, soluble TNF receptor type 1 (sTNF-R1) and soluble IL-2 receptor (sIL-2R) when compared with healthy subjects, and high levels of sTNF-R1 and TNF-α when compared with euthymic patients [63]. Another recent meta-analysis has found that manic patients show increased TNF-α and sIL-2R levels and a trend toward higher sTNF-R1 concentrations when compared with euthymic patients, in addition to increased IL-1 receptor antagonist (IL-1RA) and a trend toward higher IL-6 levels when compared with healthy controls. Increased IL-10 levels were also found in patients in depression versus controls, however not reaching significance between acute phases. Finally, some cytokines, such as IL-1RA, have shown altered levels during euthymia compared with controls [64].

An aberrant inflammatory gene expression signature has also been demonstrated in monocytes from patients with BD; some of them were related to the cytokines most commonly correlated with BD, namely, TNF and IL-6 [65]. In this study, mRNA expression of chemokine ligand 2 and MAPK-6 was found to be significantly greater in monocytes during manic and depressive episodes. In addition, IL-6, PTX3, and cell survival/apoptosis signaling genes *EMP1* and *BCL2A1* were overexpressed during the depressive phase compared with euthymic patients, suggesting a differentiated activation of the inflammatory response system. The study mentioned above showed that the inflammatory state in monocytes from patients is familial, which means that similar results were found in the offspring of these patients, but the study did not evaluate the possible interaction between the existence of an aberrant proinflammatory gene expression signature and environmental factors. In a follow-up study, Padmos *et al.* have shown that the pro-inflammatory activation of monocytes in monozygotic and dizygotic twins is most likely due to shared environmental factors [66]. In the same direction, this group showed that schizophrenic patients also present an inflammatory activation of monocytes. This signature is such like the one found in patients with BD, an upregulation of ATF3, DUSP2, EGR3 and MXD1, and differs of BD signature in PTPN7 and NAB2 [67].

However, this increased inflammatory signature at the transcriptomic level has not been demonstrated at the protein level in patients with BD. In addition, Herberth *et al.* identified altered expression of seven pro-inflammatory and five pro-/anti-inflammatory protein analytes in the serum of euthymic patients [68]. This serum was used to treat peripheral blood mononuclear cells and was observed to decrease cell viability, pointing to an increased inflammatory response and likely cell death in the immune system of patients with BD.

In accordance with the aforementioned abnormalities, adolescents with BD have also been shown to present some type of immune disturbance. Preliminary findings obtained in a sample of adolescents with BD have indicated an association between severity of manic symptoms and high-sensitivity C-reactive protein (hsCRP), as well as a negative association between serum IL-6 and BDNF protein levels [32]. In the same vein, Padmos *et al.* demonstrated that the offspring of patients with BD also had an altered expression of inflammation-related genes [65].

Changes mentioned above serve as a source of information on the biological bases of BD, however these changes have not been found every time when tested and the same cytokines are not always implicated. Therefore, the use of inflammatory markers as biomarkers for predicting prognosis is still limited, but these findings could point to targets for treatment and monitoring of these patients in order to improve their quality of life.

The origin of immunological imbalance in BD is still unknown. However, some studies have pointed to factors such as sleep and circadian rhythm alterations, stress, immune activation by retrovirus infection or autoimmune dysfunction [69], unhealthy lifestyle, long-term exposure to drugs and some specific mechanisms that should be the focus of further studies [70,71].

Patients with BD are known to be at a higher risk of developing medical comorbidities, including cardiovascular disease, metabolic syndrome and diabetes [32,72,73]. The main connection between these disorders seems to be the presence of chronic systemic inflammation, or high levels of the inflammatory markers mentioned above. In fact, it is precisely because of the growing evidence suggesting chronic mild inflammation in the periphery and brain of patients with BD [60,74,75] that BD has been referred to as a multisystemic inflammatory disease by some authors [56,76].

A major confounding factor present in almost all studies designed to investigate inflammation in BD is the exposure of patients to drugs. Some studies have proposed that lithium can restore the inflammatory imbalance observed in BD [77]. Guloksuz *et al.* found a correlation between lithium response and TNF-α levels, where patients with a poor response to lithium showed increased serum TNF-α levels [78]. Further studies are needed to elucidate the relationship between inflammatory markers, treatment and the development of medical comorbidities in BD.

Oxidative stress

A growing body of evidence has demonstrated that oxidative stress plays an important role in the pathophysiology of BD [79–81]. Oxidative stress is defined as an imbalance between oxidant and antioxidant agents, potentially leading to cellular damage. Decreased levels of antioxidants or an increased production of pro-oxidants will result in an oxidative stress state, ultimately causing damage to macromolecules such as lipids, proteins (receptors and enzymes), carbohydrates and DNA [82].

The CNS is particularly vulnerable to oxidative injury, due to high oxygen consumption and hence the generation of free radicals, and also because of the relatively low antioxidant capacity of this structure [83]. Increased neuronal oxidative levels may have deleterious effects on signal transduction, plasticity and cellular resilience [84]. The antioxidant system is the major line of defense against oxidative stress, and can be divided into the enzymatic system, comprising the key enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx), and the nonenzymatic system [85]. The most important nonenzymatic cellular antioxidant and redox-regulator is glutathione (GSH), the brain's dominant antioxidant [86].

Under physiological conditions, mitochondria are a major source of free radicals (oxidants), produced in electron transport chain complexes [87]. In BD, the prevalent hypothesis is that a greater burden of oxidative stress is generated as a result of a disturbed mitochondrial function [13]; this hypothesis has been supported by postmortem studies reporting alteration in mitochondrial complex I activity [88] and decreased levels of GSH [89] in the prefrontal cortex of patients. Mitochondrial dysfunction in BD will be reviewed in another section.

Clinical studies have demonstrated systemic alterations in diverse oxidative stress parameters and antioxidant enzymes in patients with BD. Some of these changes have been related to mood episodes. For instance, Andreazza *et al.* reported that SOD activity is increased during manic and depressive phases, but not in euthymia [90]. This finding was confirmed by Machado-Vieira *et al.* [33] who showed increased SOD activity in unmedicated manic patients, as well as by Kunz *et al.* [91] who also reported increased SOD activity in acute phases of BD, but not during euthymia. However, others studies have shown decreased SOD activity in acute phases of BD and in the euthymia [92–94]. Furthermore, Raffa *et al.* did not found differences in the SOD levels in patients when compared with healthy controls [95]. Catalase activity was also decreased in euthymic patients [90,94,95], but increased in medication-free patients during mania [33]. These results suggest that alteration in antioxidant enzymes can change due the treatment and the phases of the illness.

An increased frequency of DNA damage possibly caused by oxidative stress has been shown in patients with BD and was correlated with severity of depression and manic symptoms [96]. Conversely, a meta-analysis investigating markers of oxidative stress in BD showed that thiobarbituric acid reactive substance, a marker of lipid peroxidation and nitric oxide, a reactive nitrogen species, were significantly elevated in all phases of BD [79], suggesting a relevant role of these parameters as possible biomarkers of illness traits.

Parenthetically, evidence from preclinical, clinical and epidemiological studies suggests a benefit for adjunctive antioxidant compounds in BD [97]. *N*-acetylcysteine, for instance, proved safe in two randomized trials as an adjuvant to mood stabilizers [98–100]. Preliminary data also suggest clinical effects of antioxidant compounds in mania and depression, and a particularly strong effect in patients with comorbid medical conditions [100,101].

Mechanisms underlying cellular alterations & toxicity in bipolar disorder

Mechanisms leading to reduced resilience to stressful conditions associated with acute episodes in BD probably involve cell signaling pathways and organelles that are typically responsible for maintaining cellular homeostasis, for example, the mitochondrion and endoplasmic reticulum (ER), and could affect cells from both the periphery and the CNS, for example, neurons and glial cells. Basic research experiments have significantly contributed to the understanding of these mechanisms, partially explaining the toxicity related to cumulative mood episodes in BD. In this section, the authors attempt to summarize some of the mechanisms underlying toxicity in BD.

Mitochondrial dysfunction & the role of chronic stress

A growing body of evidence has suggested a key role of mitochondrial dysfunction in BD [102]. Impaired energy metabolism, alterations in respiratory chain complex enzymes, altered levels of

cytoplasmic calcium, and downregulation of mitochondria-related genes are some of the abnormalities reported [102]. In addition, several postmortem, imaging, and genetic studies have pointed to an association between mitochondrial dysfunction and BD [103]. Mean cerebrospinal fluid lactate concentrations are significantly higher in patients when compared with controls, which indicates increased extra-mitochondrial and anaerobic glucose metabolism and is consistent with impaired mitochondrial metabolism in BD [41]. More recently, a decrease in attachment of hexokinase 1 to the outer mitochondrial membrane in postmortem brain parietal cortex tissue of individuals with BD has been reported, associated with increased activity of an alternative anaerobic pathway of glucose metabolism [104]. In the same vein, alterations in mitochondrial shape and distribution could be one of the underlying causes of energy dysfunction in BD, as shown in the prefrontal cortex of postmortem brains and in peripheral cells from patients with BD [103]. The role of mitochondrial dysfunction in BD is further supported by studies reporting that known mood stabilizers and antidepressants can enhance mitochondrial function [102,105,106]. For instance, lithium has been shown to stimulate the activity of mitochondrial respiratory chain enzymes at clinically relevant concentrations [107].

To a greater extent, abnormalities may be associated with the consequences of chronic exposure to stress, which seems to play a role in the pathophysiology of BD [108]. The stress hormone axis, more commonly known as the hypothalamic–pituitary–adrenal axis, is clearly altered in mood disorders, as suggested by the high number of patients with BD that inefficiently suppress cortisol release on the dexamethasone suppression test [109]. This deficiency of the hypothalamic–pituitary–adrenal axis results in a feed-forward production of cortisol in response to stress and in a decreased ability to return to resting levels once stress exposure is ceased [110]. As a consequence, patients with BD in the three phases of the disorder present similarly increased levels of cortisol, higher than those observed in controls [111]. These increased cortisol levels may have important long-term consequences in patients. For instance, *in vitro* and animal model studies have shown that chronic stress and chronic exposure to glucocorticoids can induce mitochondrial dysfunction, causing reductions in oxygen consumption, mitochondrial membrane potential, and calcium holding capacity and ultimately leading to apoptosis [112,113]. Glucocorticoids may also aggravate inflammation and induce toxicity in the CNS, making neurons less capable of removing glutamate from the synapse and quenching free radicals [114]. In addition, neuronal toxicity and damage could be generated by an increase in synergists of inflammation, oxidative stress and mitochondrial dysfunction [115].

Altogether, the authors hypothesize that some of the impairments in mitochondrial functions in patients with BD are induced and further stimulated by chronic stress. As a consequence, dysfunctional mitochondria are likely to impair cellular resilience to environmental stimulus, ultimately inducing activation of caspases and apoptosis. Once dead, these cells may end up releasing immunostimulatory molecules and therefore induce alterations in inflammatory markers. These alterations may be then responsible for detrimental effects on peripheral cells, possibly inducing apoptosis and completing a vicious cycle of peripheral toxicity and reduced cellular resilience.

ER stress

The ER plays a central role in Ca^{2+} storage and signaling, and also in the synthesis, folding and quality control of secretory and membrane proteins [116]. Alterations in the ER luminal environment, such as changes in the redox state and in calcium homeostasis, nutrient deprivation, or defects in protein post-translational modifications, may affect the function of this organelle and subsequently result in accumulation of unfolded proteins. This condition is known as ER stress, and the cellular response to this condition is called unfolded protein response (UPR), an adaptive physiological process in which cells activate protective mechanisms to restore homeostasis in the ER. Prolonged ER stress (e.g., when UPR is not sufficient to restore the balance) leads to cell death [117,118].

Some studies have suggested an involvement of UPR dysfunction in the pathophysiology of BD. For instance, a decreased response of XBP1 (a transcription factor that induces the expression of ER chaperones) and CHOP (a transcription factor that induces ER stress-induced apoptosis) was found in lymphoblastoid cells from patients exposed to two ER stress inducers [119]. Other findings have confirmed these results, reporting a reduction in stress-induced splicing of XBP1 and in the expression of GRP94 (another ER chaperone) in patients with BD [120]. Moreover, pharmacological evidence suggests that mood stabilizer valproate modulates ER stress response [121–123]. In a recent study, lymphocytes from patients with BD, in contrast to healthy controls, failed to induce UPR-related proteins and presented higher cell death levels in response to *in vitro*-induced ER stress, suggesting that this dysfunctional response to ER stress may reflect an increased cellular susceptibility [124].

Taken together, these findings suggest that patients with BD show a dysfunctional ER stress response, inappropriate and insufficient to maintain homeostasis. This impaired response to ER stress may be related to several neural function impairments reported for these patients, given that UPR components are also involved in neural development and plasticity, maturation and transport of several receptors and calcium signaling [125–127].

The ER is closely linked with mitochondria, both morphologically and functionally; Ca^{2+} exchange is possibly the main way of communication between both organelles [128]. ER-derived Ca^{2+} signals modulate mitochondrial bioenergetics. As a result, alterations in ER–mitochondria interactions, such as changes in cellular Ca^{2+} levels, influence the regulation of cellular metabolism and could cause mitochondrial dysfunction, metabolic imbalance and ultimately lead to cell death [129]. Of note, changes in intracellular calcium levels are a consistent finding in BD [130].

Harmful crosstalk between both organelles has also been shown to be involved in oxidative damage [131]. Taking into consideration the prolonged ER stress and mitochondrial dysfunction observed in BD, the disruption of ER–mitochondria interactions may

potentially be responsible for metabolic alterations and peripheral toxicity associated with the disorder. ER stress may also be related to neurotrophic pathways [132,133] that may contribute to maintaining oxidative damage and systemic inflammation in BD, as these processes are intimately interrelated [134,135].

Glial alterations

In 1858, Rudolf Virchow described glial cells as a connective tissue that binds nervous elements together [136]. As we know today, the role of these cells goes far beyond: glial cells are functional components of the nervous system. Sometimes called neuroglia, some of their functions include maintaining homeostasis (astrocytes), forming myelin (oligodendrocytes), and providing support and protection for neurons in the brain (microglia). Glial cells are capable of responding to changes in the cellular and extracellular environment, and, possibly through a glial network, have communication skills that complement those of the neurons [136]. Given the fact that these cells play an important role in the CNS, it is natural to think that they will also play an important role in the establishment and development of neurological disorders. Indeed, several studies have demonstrated alterations in glial cells in psychiatric disorders, including a decreased glial density in the amygdala of patients with major depression [137] and upregulation of extracellular matrix proteins in astrocytes of the amygdala and entorhinal cortex of schizophrenic patients [138]. More directly in BD, the results in the last 5 years are scarce.

Histological observations as well as imaging studies support findings of myelin abnormalities and glial alterations in BD [125]. Oligodendrocytes express transferrin, an iron mobilization protein that acts as a trophic and survival factor for neurons and astrocytes, pointing to another important function of oligodendrocytes in addition to myelination [139]. A postmortem study has shown that transferrin is underexpressed in the internal capsule of patients with BD; in contrast, two astrocyte-associated genes (*GFAP* and *ALDH1L1*) showed higher mean levels in all brain regions [140]. These results could indicate an impaired functioning of oligodendrocytes and some degree of astrocytosis (increase in astrocyte markers). Another study has reported that astroglial and microglial markers (glial fibrillary acidic protein, inducible nitric oxide synthase, c-fos and CD11b) were significantly upregulated in the postmortem frontal cortex of patients with BD, in particular the IL-1 receptor (IL-1R) cascade involved in microglial activation [141]. Microglia are the brain resident macrophages, which become activated in response to tissue damage or brain infections [142]. Moreover, the fact that neuregulin (*NRG*), a gene involved in oligodendrocyte development and myelination of the CNS, is located at one of the genetic loci for BD [143] is another indicator that glial alterations deserve further attention. In fact, it is possible that glial dysfunction in BD could result in abnormal neuronal–glial interactions, as already reported for mania [144].

We speculate that the abnormalities described above could be interrelated, affecting cellular resilience and function both in the periphery and in the brain of patients with BD. In line with previous hypotheses [8], there is likely a set of complex, interacting processes occurring in BD that could lead to cell endangerment and be related to the toxicity found in patients during acute episodes [15]. In order to better understand the mechanisms underlying toxicity in BD, further studies addressing the association between these processes and mood states are required.

Novel therapies for bipolar disorder

In light of the pathways known to be implicated in illness activity, novel therapies can be designed and proposed for a better management of BD. Thinking of the more immediate future, interesting alternatives may involve adjuvant therapies that act on the pathways mentioned in this review [14,15]. Some of these agents, with antioxidant, anti-inflammatory and neuroprotective effects, will be described in more detail below.

N-acetylcysteine (NAC), a precursor of GSH, has been shown, in both basic and clinical studies, to attenuate oxidative stress, modulate inflammation and act on neurogenesis and glutamatergic and dopaminergic pathways [59,99]. Supplementation of conventional treatment for BD with substances that act on oxidative stress has been investigated in clinical trials. NAC treatment adjunctive to usual medication for BD in the maintenance phase significantly improved depressive symptoms, quality of life and functioning in a double-blind, randomized, placebo-controlled trial with large effect sizes [99]. A secondary exploratory analysis revealed that adjunctive NAC showed promising effectiveness for participants with a syndromal diagnosis of bipolar depression [100]. More recently, a double-blind, randomized, placebo-controlled trial investigating the maintenance effects of NAC failed to find significant differences in recurrence or symptomatic outcomes during the maintenance phase [98]. Further randomized trials assessing adjunctive NAC for BD are required to more reliably determine the effect size of this treatment approach.

In addition to influencing the redox state, the neuroprotective properties of NAC may be associated with its ability to induce neurogenesis, which is likely related to mitochondria-protective mechanisms [145]; also, the modulating effects of NAC on inflammation [146] may be fundamental for its efficacy as a mood-stabilizing agent, considering the already described relevance of systemic inflammation in BD pathophysiology [15]. Therefore, even though very few studies have investigated the use of anti-inflammatory agents as an adjunct therapy for BD, inflammatory pathways seem to be another group of potential new therapeutic targets for the development of more effective treatments for BD. Conventional mood stabilizers have been described to have effects on both pro- and anti-inflammatory cytokines [78,147]. Among anti-inflammatory drugs, cyclooxygenase-2 (COX-2) inhibitor celecoxib was studied in a double-blind, randomized, placebo-controlled study as an adjunct in the treatment of patients with BD during depressive or mixed episodes. Treatment with celecoxib was associated with a more rapid improvement of depressive symptoms after 1 week compared with placebo, but the difference was statistically significant only for subjects who completed the full 6-week trial. This

finding suggests a potential antidepressant effect of COX inhibitors [58]. In this context, studies have demonstrated that mood stabilizers approved for the treatment of BD decrease expression of markers of the rodent brain arachidonic metabolic cascade, and reduce excitotoxicity and neuroinflammation-induced upregulation of these markers [148]. Recent papers demonstrating neuroinflammation, excitotoxicity [141] and upregulated arachidonic acid metabolism [149] in the postmortem brain of patients with BD support the hypothesis of altered arachidonic acid cascade in BD.

Another compound currently under investigation is minocycline, a tetracycline antibiotic that crosses the blood–brain barrier and has shown antioxidant, anti-inflammatory and neuroprotective effects [150]. Given that these pathways overlap with the pathophysiological mechanisms observed in BD, the use of minocycline has been pointed out as a potential adjunctive treatment. More specifically, minocycline inhibits microglia-mediated release of proinflammatory cytokines IL-1b, TNF-α, IL-6 and p38, and promotes the release of anti-inflammatory cytokine IL-10 [151]. It is also an effective scavenger of reactive oxygen species and protects against glutamate-induced excitotoxicity [152]. Case reports of individuals with psychiatric disorders have shown benefits of minocycline treatment for the severity of symptoms. Currently, a clinical trial is testing the efficacy of minocycline and/or aspirin in the treatment of bipolar depression and evaluating the anti-inflammatory effects of these compounds [153].

Supplementation with ω-3 polyunsaturated fatty acids (ω-3 PUFAs) has also been considered a potential new treatment for BD, as these fats have shown neuroprotective and antioxidant capacity in animal models [154]. A recent review of clinical trials using nutraceuticals in combination with standard treatment for BD has shown that ω-3 PUFAs improved bipolar depression symptoms [155]. The BDNF signaling pathway is one of the possible mechanisms of action by which ω-3 PUFAs mediate mood regulation in patients with BD [156]. Further double-blind, placebo-controlled, randomized clinical trials with long follow-up periods and adequate power-effect sizes are needed before we can gain a better understanding of this relationship and of the therapeutic role of ω-3 PUFAs in BD.

Neurotrophic factors are emerging as promising therapeutic targets in BD. Lithium, the classical mood stabilizer, has been shown to be effective in restoring peripheral BDNF levels in patients with BD [40,47]. In this sense, studies that attempt to prevent, treat and reverse molecular impairments are interesting therapeutic avenues for novel and improved therapies in BD [157]. In particular, delivery of neurotrophic factors from biomaterial scaffolds seems to be a promising area of research for the treatment of any disorder affecting the CNS. This drug delivery system allows to control the site and time of release of therapeutic agents, ensuring that biologically active agents, for example, neurotrophic factors, will be transported to the desired location to help treat a disorder [158]. In a recent review, the advantages and challenges associated with different drug delivery systems were evaluated, and the possibility to combine drug delivery systems with gene therapies was raised, suggesting that the drug delivery device could be adjusted to provide a controlled release of neurotrophic factors [159].

Regarding the mechanisms of action of the mood stabilizers traditionally used for the treatment of BD (lithium and valproic acid), hypothesis involving pathways discussed in this review are often highlighted. A recent work has reviewed preclinical findings showing that these drugs, in addition to other roles, regulate the transcription and expression of factors involved in neuroprotective, neurotrophic and anti-inflammatory effects. Moreover, oxidative stress pathways and cell survival signaling cascades may further underlie beneficial actions of these already established treatments [160].

In summary, the identification of specific therapeutic targets commonly modulated by these drugs may reveal new avenues for the effective use of add-on therapies, with the primary aim of treating acute mood episodes and preventing their recurrence.

Peripheral biomarkers & illness activity in bipolar disorder

As discussed above, an increasing body of evidence points to changes in neuroplasticity, oxidative stress and inflammation pathways in BD, mainly during mood episodes. However, these peripheral biomarkers have usually been investigated individually, contrary to the proposal that single biomarkers are unlikely sufficient to identify complex disorders. Rather, research should be geared towards sets of biomarkers, reflecting different processes implicated in a given condition [18,20].

To evaluate these biomarkers simultaneously, Kapczinski *et al.* conducted an *en bloc* assessment of a set of targets related to oxidative stress, neurotrophins and inflammation, all previously described as individual biomarkers of mood episodes in BD. The results demonstrated significant correlations among most biomarkers, which were then used to extract a systemic toxicity index. Patients in manic and depressive episodes showed higher systemic toxicity than euthymic patients and healthy controls, but lower systemic toxicity was seen when compared with patients with sepsis ('positive' control group for extreme peripheral illness; Figures 1 & 2) [14,15].

The findings above associating acute episodes with significant systemic toxicity in BD corroborate the idea that BD can be seen as a multisystemic illness of which peripheral pathophysiology is a major component [1]. However, these data alone are unable to explain how peripheral changes correlate with brain changes. The brain coordinates all physiological processes and is therefore sensitive to systemic damage [12]. Of note, central and peripheral pathophysiology could be connected in pro-oxidant states [161], possibly via changes in blood–brain barrier permeability. Peripheral toxicity has been shown to significantly alter brain oxidative stress [162]. Indeed, as mentioned above, there may be a link between inflammation, oxidative stress and neuroplasticity pathways in BD. For instance, inflammation has been demonstrated to cause oxidative stress through activation of calcium-dependent proteins and direct inhibition of the mitochondrial electron transport chain [163]. Changes in oxidative status, in

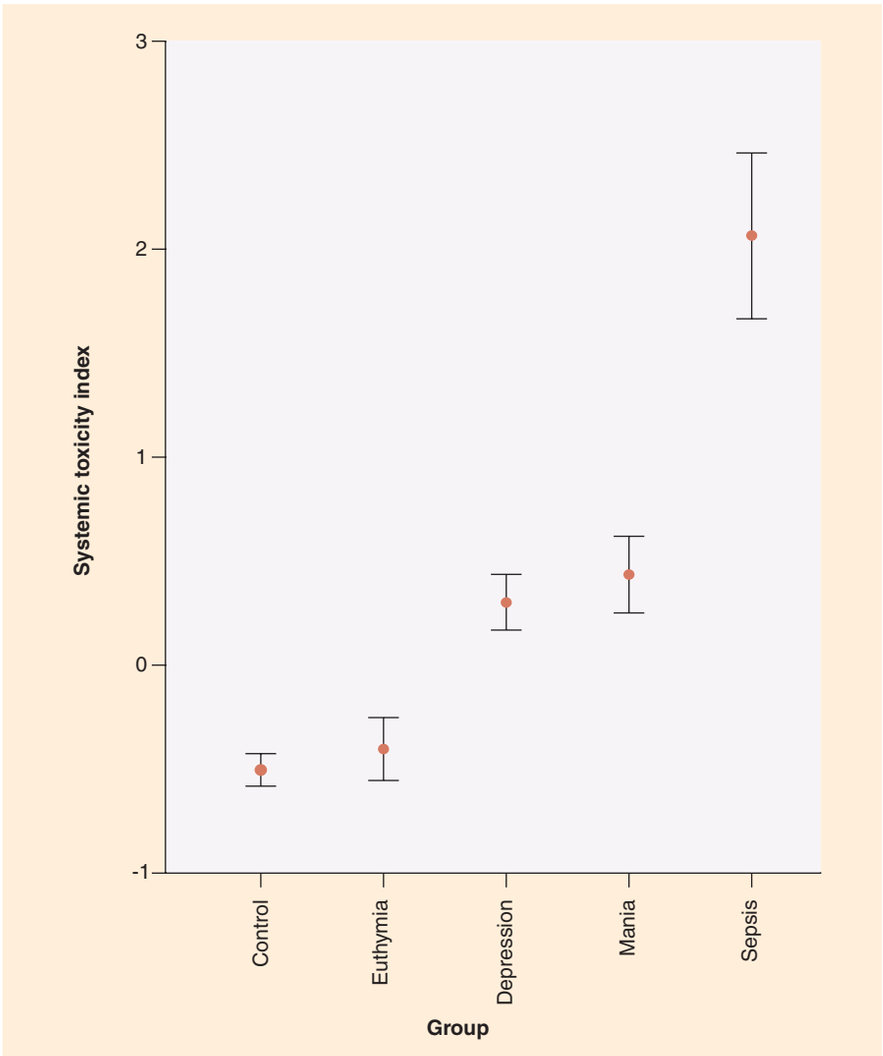


Figure 1. Systemic toxicity index to assess peripheral changes in mood episodes. To evaluate the peripheral toxicity associated with illness activity in patients with bipolar disorder, Kapczinski et al. conducted an en bloc assessment of a set of targets related to oxidative stress, neurotrophins and inflammation, all previously described as individual biomarkers of mood episodes. These peripheral biomarkers were measured in different acute mood states and in healthy subjects. Moreover, they also were evaluated in patients with sepsis ('positive' control group for extreme peripheral illness) with the aim of highlighting the relevance of potential changes between groups. More specifically, the biomarkers assessed were neurotrophins (brain-derived neurotrophic factor, neurotrophin-3), oxidative stress markers (protein carbonyl content, thiobarbituric acid reactive substances, and total reactive antioxidant potentials) and inflammatory markers (IL-6, IL-10 and TNF-α). The results demonstrated significant correlations among most biomarkers, which were then used to extract a systemic toxicity index. Patients in manic and depressive episodes showed higher systemic toxicity than euthymic patients and healthy controls; however, it was lower when compared with patients with sepsis. Figure reproduced with permission from [15].

turn, may be mechanistically associated with the reduced levels of BDNF observed in patients during acute mood episodes [164].

In summary, peripheral biomarkers have been consistently demonstrated to differentiate between patients with BD in manic or depression episodes and euthymic subjects. Whereas changes in one single biomarker usually have small effect sizes, the assessment of multiple biomarkers, especially primary mediators, could be a practical approach to improving diagnostic strategies and promoting earlier interventions [16,18].

Expert commentary

This review highlights the systemic toxicity related to acute mood episodes in BD and discusses possible mechanisms underlying these processes. Clinical and preclinical research gives overall support to the view of illness episodes as toxic to multiple elements in the body. Regarding the use of peripheral biomarkers as means for assessing illness activity, promising candidates at the moment can be subsumed in three main general areas: oxidative stress, inflammation and neurotrophins, in particular BDNF. These three groups do not yet meet the empirical characteristics of a traditional biomarker; however, they are relevant inasmuch as they provide information on the pathophysiology of BD and on illness activity. The assessment of systemic toxicity through a set of peripheral biomarkers may facilitate understanding of the body and brain damage associated with recurrent mood episodes and of the way it affects illness management.

Perhaps the most relevant upshot of having validated illness activity biomarkers would be the identification of biological features that indicate either the onset of an episode before specific symptoms occur or the lingering of illness activity despite an apparent response. Another potential application could be the detection of early response, before symptom resolution. Peripheral biomarker alterations in an acute mood episode could follow three different patterns, all of which would be powerful tools in guiding therapy (Figure 3).

Regarding new therapeutic strategies, preliminary evidence supports a role for novel adjunctive therapies in modulating neurotrophic, inflammatory, oxidative and apoptotic processes. Potential neuroprotective agents are currently available, but further clinical trial data are needed, as is information regarding which subgroups would benefit most from such interventions. Furthermore, in view of the high comorbidity rates observed in BD, there has been a push towards understanding the mechanisms underlying acute toxicity and illness activity. This perspective is the rationale behind an approach where the validation of

novel biological indicators will enhance the clinical strategies traditionally employed.

Five-year view

Well-documented studies evaluating potential peripheral biomarkers in BD have reported disturbances in inflammatory, neurotrophic, and oxidative stress markers in patients versus healthy individuals. However, pertinent questions remain about the translational applications of biomarkers in BD within the next years. It is expected that, in the future, translational approaches will be applied to the diagnosis and treatment of BD, using peripheral biomarkers to predict outcomes and identify high-risk individuals. This could guide the planning of more personalized clinical strategies and help monitor treatment interventions. In addition, a better understanding of illness activity mechanisms could advance the development of novel and more effective treatments. If changes in biomarkers can be reversed with treatment, we could ultimately consider that some pathological mechanisms are alterable, and thus allow interventions and secondary prevention (especially of the deleterious effects associated with multiple episodes).

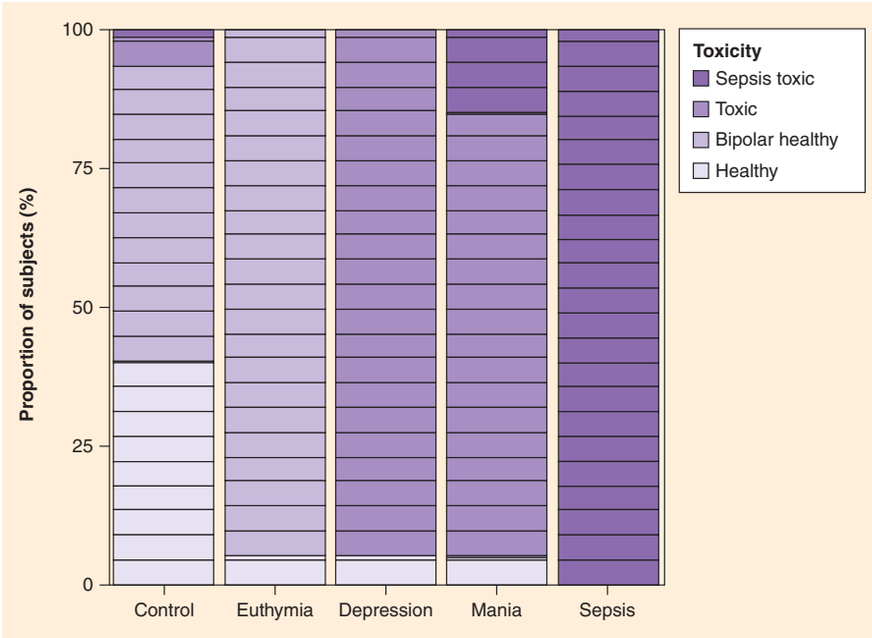


Figure 2. Peripheral biomarkers and illness activity in bipolar disorder. The graph shows the proportion of study subjects classified into four different categories: the category deemed most toxic, including all septic patients, was named 'sepsis toxic'. The less toxic category, which contained mostly healthy controls, was termed 'healthy'. The category that had serum biomarker levels in the toxic direction and included the patients in mood episodes was called 'toxic'. The last category was intermediary in terms of serum biomarkers and mostly contained euthymic patients, and was termed 'bipolar healthy'. Figure reproduced with permission from [14].

Future studies assessing biomarkers in large-scale, prospective cohorts (for an increased statistical power) and testing candidate biomarkers for sensitivity and specificity (to address overlaps with related disorders) will be quite valuable in determining the applicability of these biological markers in different rigorous scientific approaches [22]. In the past few years, much research has been undertaken to better understand individual biological markers related to the pathophysiology of BD; in the 5 years to come, an important direction will be measuring and comparing several biomarkers together, that is, not only the levels of each biomarker but also the correlations between them. Of note, several of these systemic toxicity mechanisms do not seem to be unique to BD, but may also be present in other psychiatric disorders presenting alterations related to illness activity (in cases of acute episodes followed by euthymia, such as in major depressive disorder or schizophrenia). However, the combination of peripheral alterations may differ between pathologies. For instance, neurotrophic alterations seem to go in an opposite direction in schizophrenia compared with BD [165]. In addition, inflammatory markers are different among different diagnosis and populations, which suggests peculiar means of activation of inflammation associated with specific disorders [166–168].

The common limitations of clinical studies will become an even more pressing issue in the investigation of more rigorous biomarkers for BD. For instance, until now, most research has been conducted with chronic patients treated for BD at tertiary care centers. Therefore, further studies, with other groups of patients with BD and other medical and psychiatric conditions, are required to increase the representativeness of the findings and to evaluate the effects of long-term medication use. In addition, studies conducted in community samples will be interesting to study individuals that are not usually seeking treatment at these healthcare facilities, thus avoiding a selection bias. Finally, among the clinical samples to be investigated, children, adolescents and young adults with BD are a group of great interest: evaluation of peripheral biomarkers in these individuals could contribute to a better understanding of primary illness changes [74] and some neurodevelopmental aspects, focusing on early interventions and, especially, on prevention attitudes.

Longitudinal studies will be able to confirm mood state-related findings and the hypothesis that these indices of peripheral abnormalities are related to course of illness, cognitive/functional impairment, and medical burden. Prospective studies assessing a set of measures, in turn, will be relevant to determine whether these peripheral biomarkers of illness activity may predict course of illness or medication response. In either way, the utility of these biomarkers will have to be validated via assessment of peripheral biological changes following specific therapies, for example, with anti-inflammatory or antioxidant agents [70,169], and treatment efficacy will have be evaluated based on mental health outcomes. If the biological changes suspected to occur during mood episodes are confirmed, novel

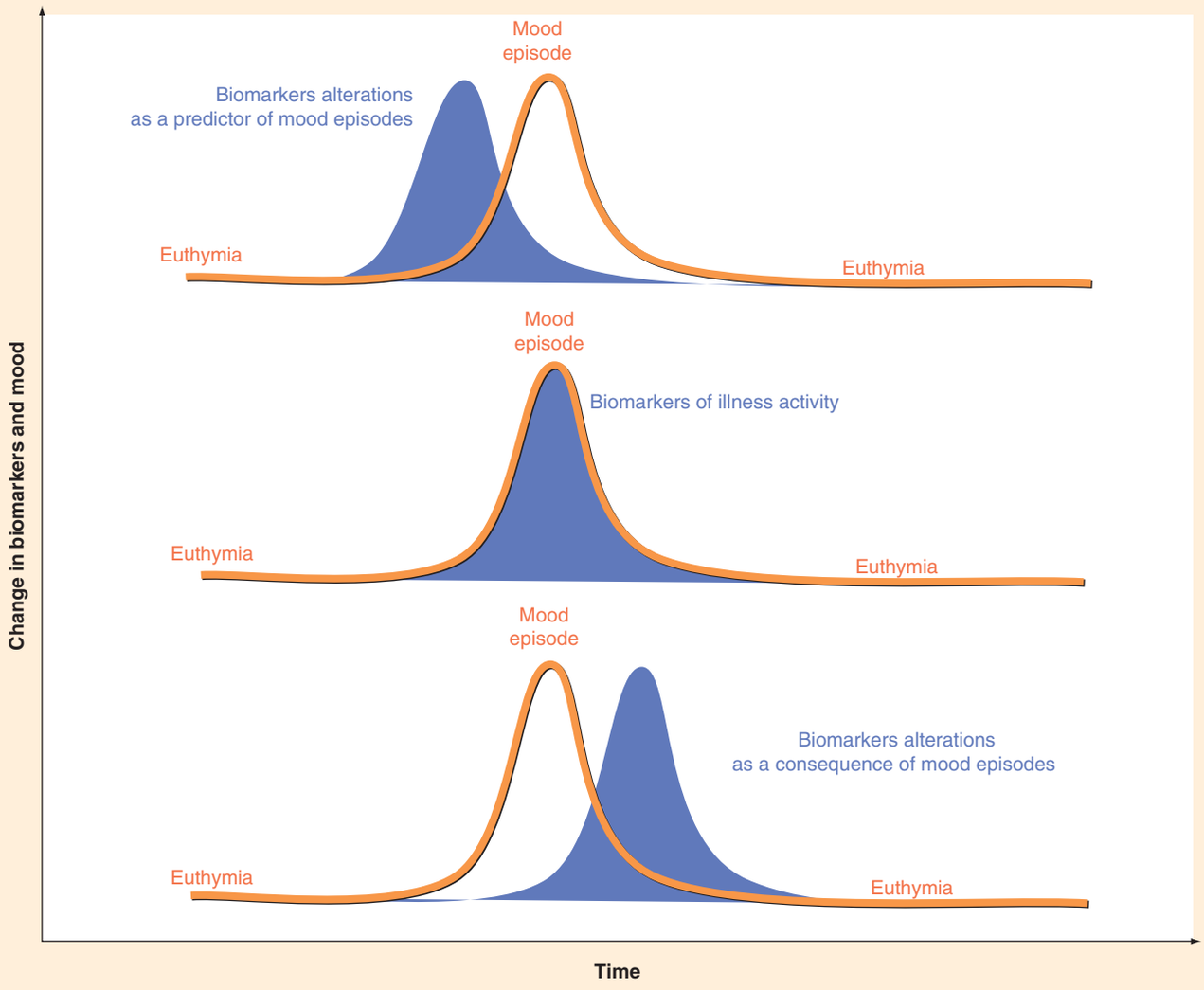


Figure 3. Peripheral biomarker alterations in an acute mood episode (mania or depression) could follow three different patterns. First, biomarkers could change before the beginning of a mood episode, showing a potential to predict these events. In this case, biomarkers would have a major therapeutic potential: they could help plan/implement early interventions and prevent/monitor treatment response. Second, biomarker changes could occur concomitantly with mood episodes, reflecting illness activity. In this case, they would be a useful tool in supporting clinical decisions for a better management of acute episodes. Finally, biomarkers could change after a mood episode, that is, because of it, which could contribute to improve our understanding of the pathophysiology of bipolar disorder. This assessment could be useful as a surrogate of pharmacological efficacy, predicting response to treatment of an acute episode after therapy initiation. The alteration patterns of biomarkers and their temporal relationship with mood episodes in bipolar disorder remain unknown.

treatment strategies should involve agents that act on pathways related with illness activity in BD. These findings could be useful not only to develop a more efficient, personalized approach to treat mood symptoms, but also to understand and perhaps revert biological changes associated with the illness, potentially bringing psychiatry into a new era of preventive psychopharmacology.

Financial & competing interests disclosure

F Kapczinski has received grant/research support from AstraZeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD and the Stanley Medical Research Institute; has been a member of the speakers' boards for AstraZeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier. B Pfaffenseller, GR Fries, B Wollenhaupt-Aguiar and L Stertz are supported by scholarships from Coordenacao de Aperfeicoamento de Pessoal de Nıvel Superior (CAPES), Brazil. PVS Magalhães is supported by a postdoctoral scholarship from Fapergs/CAPES, Brazil. GD Colpo and B Panizzutti are supported by scholarships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The recurrence of acute mood episodes in bipolar disorder (BD) often translates into several worse outcomes; for example, higher rates of clinical comorbidities, functional and cognitive impairments and decreased responsiveness to treatment.
- There is a growing interest in understanding the pathophysiological mechanisms that contribute to dysfunction resulting from multiple mood episodes in BD, especially those pathways involved in neuroprotection, oxidative stress and inflammation.
- Among neurothophins, consistent evidence suggests a possible role of brain-derived neurotrophic factor in the pathophysiology of BD: brain-derived neurotrophic factor levels are reduced during manic and depression episodes, and treatment with mood stabilizers is able to increase its levels.
- Mood episodes in BD have been characterized as pro-inflammatory states based on findings reporting alterations in the levels of cytokines and their receptors and an aberrant inflammatory gene expression.
- Several studies have demonstrated systemic alterations in diverse oxidative stress parameters in patients during mania or depression; for example, increased lipid peroxidation and nitric oxide levels and alterations in antioxidant enzymes superoxide dismutase and catalase.
- Mechanisms leading to reduced resilience associated with acute episodes probably involve organelles typically responsible for maintaining cellular homeostasis, for example, the mitochondrion and endoplasmic reticulum (ER), and could affect cells from both the periphery and the CNS, such as neurons and glia.
- A growing body of evidence suggests a key role of mitochondrial dysfunction in BD, including impaired energy metabolism, alterations in respiratory chain complex enzymes, altered levels of cytoplasmic calcium and downregulation of mitochondria-related genes. Patients with BD also seem to show a dysfunctional ER stress response, failing to stimulate an appropriate or sufficient response to maintain homeostasis under stress situations.
- Glial dysfunction and activation of a proinflammatory process by the release of damage-associated molecular patterns, as well as disruption of ER–mitochondria interactions, may be responsible for metabolic alterations and peripheral toxicity in BD.
- In light of the pathways known to be implicated in illness activity, novel therapies can be proposed for a better management of acute mood episodes and to prevent their recurrence. These could include adjuvant therapies with antioxidant, anti-inflammatory and neuroprotective agents.
- The assessment of systemic toxicity through a set of peripheral biomarkers may facilitate understanding of the body and brain damage associated with recurrent mood episodes and of the way how it impacts illness management.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Soreca I, Frank E, Kupfer DJ. The phenomenology of bipolar disorder: what drives the high rate of medical burden and determines long-term prognosis? *Depress. Anxiety* 26(1), 73–82 (2009).

• **Describes new evidence that the long-term course of bipolar disorder (BD) is associated with a high prevalence of psychiatric comorbidity and an increased mortality.**

2 Sanchez-Moreno J, Martinez-Aran A, Tabarés-Seisdedos R, Torrent C, Vieta E, Ayuso-Mateos JL. Functioning and disability in bipolar disorder: an extensive review. *Psychother. Psychosom.* 78(5), 285–297 (2009).

3 Magalhães PV, Kapczinski F, Nierenberg AA *et al.* Illness burden and medical comorbidity in the Systematic Treatment Enhancement Program for Bipolar Disorder. *Acta Psychiatr. Scand.* 125(4), 303–308 (2012).

• **Reports the prevalence and correlates of medical comorbidity in patients enrolled in the Systematic Treatment Enhancement Program for Bipolar Disorder with special interest in associations between illness chronicity and burden with comorbid medical conditions.**

4 Wingo AP, Harvey PD, Baldessarini RJ. Neurocognitive impairment in bipolar disorder patients: functional implications. *Bipolar Disord.* 11(2), 113–125 (2009).

5 Rosa AR, González-Ortega I, González-Pinto A *et al.* One-year psychosocial functioning in patients in the early vs. late stage of bipolar disorder. *Acta Psychiatr. Scand.* 125(4), 335–341 (2012).

6 Berk M, Hallam K, Malhi GS *et al.* Evidence and implications for early intervention in bipolar disorder. *J. Ment. Health* 19(2), 113–126 (2010).

7 Fiedorowicz JG, Palagummi NM, Forman-Hoffman VL, Miller DD, Haynes WG. Elevated prevalence of obesity, metabolic syndrome, and cardiovascular risk factors in bipolar disorder. *Ann. Clin. Psychiatry* 20(3), 131–137 (2008).

8 Kapczinski F, Vieta E, Andreazza AC *et al.* Allostatic load in bipolar disorder: implications for pathophysiology and treatment. *Neurosci. Biobehav. Rev.* 32(4), 675–692 (2008).

•• **Proposes the theory of allostatic load in patients with BD, which may be related to the higher rates of clinical comorbid and cognitive decline seen in these patients.**

9 Grande I, Magalhães PV, Kunz M, Vieta E, Kapczinski F. Mediators of allostasis and systemic toxicity in bipolar disorder. *Physiol. Behav.* 106(1), 46–50 (2012).

• **Explores how traditional mediators of allostasis relate to the systemic dysfunction and illness progression that takes place in bipolar disorder.**

10 Vieta E, Popovic D, Rosa AR *et al.* The clinical implications of cognitive impairment and allostatic load in bipolar disorder. *Eur. Psychiatry* 28(1), 21–29 (2013).

11 McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. *Horm. Behav.* 43(1), 2–15 (2003).

12 McEwen BS, Gianaros PJ. Stress- and allostasis-induced brain plasticity. *Annu. Rev. Med.* 62, 431–445 (2011).

• **Focuses on the allostasis and structural remodeling in different brain regions that play a major role in human psychiatric disorders.**

13 Berk M, Kapczinski F, Andreazza AC *et al.* Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors.

Neurosci. Biobehav. Rev. 35(3), 804–817 (2011).

•• **The pathways explored in this review appear to be common targets of BD. These findings support the construct of early intervention, which suggests that the initiation of therapy early in the trajectory of the disorder may reduce the impairment founded in patients with BD.**

14 Kapczinski F, Dal-Pizzol F, Teixeira AL *et al.* Peripheral biomarkers and illness activity in bipolar disorder. *J. Psychiatr. Res.* 45(2), 156–161 (2011).

•• **Evaluates potential peripheral markers associated with oxidative stress, inflammation and neurotrophins, highlighting the use of these biomarkers to assess illness activity in bipolar disorder.**

15 Kapczinski F, Dal-Pizzol F, Teixeira AL *et al.* A systemic toxicity index developed to assess peripheral changes in mood episodes. *Mol. Psychiatry* 15(8), 784–786 (2010).

•• **This article evidences the peripheral toxicity associated with acute mood episodes in patients with BD through evaluation of several biomarkers.**

16 Juster RP, McEwen BS, Lupien SJ. Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neurosci. Biobehav. Rev.* 35(1), 2–16 (2010).

17 Fries GR, Pfaffenseller B, Stertz L *et al.* Staging and neuroprogression in bipolar disorder. *Curr. Psychiatry Rep.* 14(6), 667–675 (2012).

18 Singh I, Rose N. Biomarkers in psychiatry. *Nature* 460(7252), 202–207 (2009).

19 Group BDW. Biomarkers and surrogate end points:preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 69, 89–95 (2001).

20 Puntmann VO. How-to guide on biomarkers: biomarker definitions, validation and applications with examples from cardiovascular disease. *Postgrad. Med. J.* 85(1008), 538–545 (2009).

• **Describes important definitions of a biomarker, describing types, main functions and also explaining how to perform a validation and illustrating their different applications in clinical research.**

21 Schwarz E, Bahn S. The utility of biomarker discovery approaches for the detection of disease mechanisms in psychiatric disorders. *Br. J. Pharmacol.* 153(Suppl. 1), S133–S136 (2008).

• **Discusses the biomarkers as a potential tool to discover disease mechanisms and to improve diagnosis and treatment of psychiatric disorders through technological advances, which facilitate the profiling of proteins and metabolites of a large sample numbers.**

22 Frey BN, Andreazza AC, Houenou J *et al.* Biomarkers in bipolar disorder: a positional paper from the International Society for Bipolar Disorders Biomarkers Task Force. *Aust. N. Z. J. Psychiatry* 47(4), 321–332 (2013).

23 Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood–brain barrier. *Neurobiol. Dis.* 37(1), 13–25 (2010).

24 Kastin AJ, Akerstrom V, Pan W. Neuregulin-1-β1 enters brain and spinal cord by receptor-mediated transport. *J. Neurochem.* 88(4), 965–970 (2004).

25 Pan W, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood–brain barrier. *Neuropharmacology* 37(12), 1553–1561 (1998).

26 Pillai A, Kale A, Joshi S *et al.* Decreased BDNF levels in CSF of drug-naive first-episode psychotic subjects: correlation with plasma BDNF and psychopathology. *Int. J. Neuropsychopharmacol.* 13(4), 535–539 (2010).

27 Chao MV, Rajagopal R, Lee FS. Neurotrophin signalling in health and disease. *Clin. Sci.* 110(2), 167–173 (2006).

28 Berk M. Neuroprogression: pathways to progressive brain changes in bipolar disorder. *Int. J. Neuropsychopharmacol.* 12(4), 441–445 (2009).

29 Lang UE, Hellweg R, Seifert F, Schubert F, Gallinat J. Correlation between serum brain-derived neurotrophic factor level and an *in vivo* marker of cortical integrity. *Biol. Psychiatry* 62(5), 530–535 (2007).

30 Schmidt HD, Duman RS. Peripheral BDNF produces antidepressant-like effects in cellular and behavioral models. *Neuropsychopharmacology* 35(12), 2378–2391 (2010).

31 Cunha AB, Frey BN, Andreazza AC *et al.* Serum brain-derived neurotrophic factor is decreased in bipolar disorder during depressive and manic episodes. *Neurosci. Lett.* 398(3), 215–219 (2006).

• **Landmark study that found decreased serum brain-derived neurotrophic factor (BDNF) levels in patients during manic and depressive episodes compared with euthymic patients and healthy controls.**

32 Goldstein BI, Collinger KA, Lotrich F *et al.* Preliminary findings regarding proinflammatory markers and brain-derived neurotrophic factor among adolescents with bipolar spectrum disorders. *J. Child Adolesc. Psychopharmacol.* 21(5), 479–484 (2011).

33 Machado-Vieira R, Andreazza AC, Viale CI *et al.* Oxidative stress parameters in unmedicated and treated bipolar subjects during initial manic episode: a possible role for lithium antioxidant effects. *Neurosci. Lett.* 421(1), 33–36 (2007).

34 de Oliveira GS, Ceresér KM, Fernandes BS *et al.* Decreased brain-derived neurotrophic factor in medicated and drug-free bipolar patients. *J. Psychiatr. Res.* 43(14), 1171–1174 (2009).

35 Pandey GN, Rizavi HS, Dwivedi Y, Pavuluri MN. Brain-derived neurotrophic factor gene expression in pediatric bipolar disorder: effects of treatment and clinical response. *J. Am. Acad. Child Adolesc. Psychiatry* 47(9), 1077–1085 (2008).

36 Monteleone P, Serritella C, Martiadis V, Maj M. Decreased levels of serum brain-derived neurotrophic factor in both depressed and euthymic patients with unipolar depression and in euthymic patients with bipolar I and II disorders. *Bipolar Disord.* 10(1), 95–100 (2008).

37 Barbosa IG, Rocha NP, Miranda AS *et al.* Increased BDNF levels in long-term bipolar disorder patients. *Rev. Bras. Psiquiatr.* 35(1), 67–69 (2013).

38 Dias VV, Brissos S, Frey BN, Andreazza AC, Cardoso C, Kapczinski F. Cognitive function and serum levels of brain-derived neurotrophic factor in patients with bipolar disorder. *Bipolar Disord.* 11(6), 663–671 (2009).

39 Huang TL, Hung YY, Lee CT, Chen RF. Serum protein levels of brain-derived neurotrophic factor and tropomyosin-related kinase B in bipolar disorder: effects of mood stabilizers. *Neuropsychobiology* 65(2), 65–69 (2012).

40 Fernandes BS, Gama CS, Ceresér KM *et al.* Brain-derived neurotrophic factor as a state-marker of mood episodes in bipolar disorders: a systematic review and meta-regression analysis. *J. Psychiatr. Res.* 45(8), 995–1004 (2011).

•• **Systematic review indicating that BDNF levels are reduced during mood episodes but not during euthymia and also shows that BDNF levels normalize following pharmacological treatment for a manic episode.**

41 Regenold WT, Phatak P, Marano CM, Sassan A, Conley RR, Kling MA. Elevated cerebrospinal fluid lactate concentrations in patients with bipolar disorder and schizophrenia: implications for the mitochondrial dysfunction hypothesis. *Biol. Psychiatry* 65(6), 489–494 (2009).

42 Kauer-Sant'Anna M, Kapczinski F, Andreazza AC *et al.* Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. *Int. J. Neuropsychopharmacol.* 12(4), 447–458 (2009).

43 Yatham LN, Kapczinski F, Andreazza AC, Trevor Young L, Lam RW, Kauer-Sant'anna M. Accelerated age-related decrease in brain-derived neurotrophic factor levels in bipolar disorder. *Int. J. Neuropsychopharmacol.* 12(1), 137–139 (2009).

44 Kapczinski F, Dias VV, Kauer-Sant'Anna M *et al.* The potential use of biomarkers as an adjunctive tool for staging bipolar disorder. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33(8), 1366–1371 (2009).

45 Tramontina JF, Andreazza AC, Kauer-Sant'anna M *et al.* Brain-derived neurotrophic factor serum levels before and after treatment for acute mania. *Neurosci. Lett.* 452(2), 111–113 (2009).

46 Rybakowski JK, Suwalska A. Excellent lithium responders have normal cognitive functions and plasma BDNF levels. *Int. J. Neuropsychopharmacol.* 13(5), 617–622 (2010).

47 de Sousa RT, van de Bilt MT, Diniz BS *et al.* Lithium increases plasma brain-derived neurotrophic factor in acute bipolar mania: a preliminary 4-week study. *Neurosci. Lett.* 494(1), 54–56 (2011).

48 Walz JC, Andreazza AC, Frey BN *et al.* Serum neurotrophin-3 is increased during manic and depressive episodes in bipolar disorder. *Neurosci. Lett.* 415(1), 87–89 (2007).

49 Fernandes BS, Gama CS, Walz JC *et al.* Increased neurotrophin-3 in drug-free subjects with bipolar disorder during manic and depressive episodes. *J. Psychiatr. Res.* 44(9), 561–565 (2010).

50 Walz JC, Magalhães PV, Giglio LM *et al.* Increased serum neurotrophin-4/5 levels in bipolar disorder. *J. Psychiatr. Res.* 43(7), 721–723 (2009).

51 Barbosa IG, Huguet RB, Sousa LP *et al.* Circulating levels of GDNF in bipolar disorder. *Neurosci. Lett.* 502(2), 103–106 (2011).

52 Rosa AR, Frey BN, Andreazza AC *et al.* Increased serum glial cell line-derived neurotrophic factor immunocontent during manic and depressive episodes in individuals with bipolar disorder. *Neurosci. Lett.* 407(2), 146–150 (2006).

53 Zhang X, Zhang Z, Sha W *et al.* Effect of treatment on serum glial cell line-derived neurotrophic factor in bipolar patients. *J. Affect. Disord.* 126(1–2), 326–329 (2010).

54 Takebayashi M, Hisaoka K, Nishida A *et al.* Decreased levels of whole blood glial cell line-derived neurotrophic factor (GDNF) in remitted patients with mood disorders. *Int. J. Neuropsychopharmacol.* 9(5), 607–612 (2006).

55 Lundborg C, Hahn-Zoric M, Biber B, Hansson E. Glial cell line-derived neurotrophic factor is increased in cerebrospinal fluid but decreased in blood during long-term pain. *J. Neuroimmunol.* 220(1–2), 108–113 (2010).

56 Leboyer M, Soreca I, Scott J *et al.* Can bipolar disorder be viewed as a multi-system inflammatory disease? *J. Affect. Disord.* 141(1), 1–10 (2012).

57 Barbosa IG, Rocha NP, Bauer ME *et al.* Chemokines in bipolar disorder: trait or state? *Eur. Arch. Psychiatry Clin. Neurosci.* 263(2), 159–165 (2013).

58 Nery FG, Monkul ES, Hatch JP *et al.* Celecoxib as an adjunct in the treatment of depressive or mixed episodes of bipolar disorder: a double-blind, randomized, placebo-controlled study. *Hum. Psychopharmacol.* 23(2), 87–94 (2008).

59 Dean OM, van den Buuse M, Berk M, Copolov DL, Mavros C, Bush AI. N-acetyl cysteine restores brain glutathione loss in combined 2-cyclohexene-1-one and D-amphetamine-treated rats: relevance to schizophrenia and bipolar disorder. *Neurosci. Lett.* 499(3), 149–153 (2011).

60 Brietzke E, Stertz L, Fernandes BS *et al.* Comparison of cytokine levels in depressed, manic and euthymic patients with bipolar disorder. *J. Affect. Disord.* 116(3), 214–217 (2009).

61 Ortiz-Domínguez A, Hernández ME, Berlanga C *et al.* Immune variations in bipolar disorder: phasic differences. *Bipolar Disord.* 9(6), 596–602 (2007).

62 Kim YK, Jung HG, Myint AM, Kim H, Park SH. Imbalance between pro-inflammatory and anti-inflammatory cytokines in bipolar disorder. *J. Affect. Disord.* 104(1–3), 91–95 (2007).

63 Munkholm K, Vinberg M, Berk M, Kessing LV. State-related alterations of gene expression in bipolar disorder: a systematic review. *Bipolar Disord.* 14(7), 684–696 (2012).

- **Highlights the state-related alterations in gene expression in patients with BD.**

64 Modabbernia A, Taslimi S, Brietzke E, Ashrafi M. Cytokine alterations in bipolar disorder: a meta-analysis of 30 studies. *Biol. Psychiatry* doi:10.1016/j.biopsych.2013.01.007 (2013) (Epub ahead of print).

- **Meta-analysis of studies comparing cytokine concentrations between patients with bipolar disorder and healthy controls, providing evidence for significant elevation of proinflammatory, anti-inflammatory and regulatory cytokines in this disorder.**

65 Padmos RC, Hillegers MH, Knijff EM *et al.* A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. *Arch. Gen. Psychiatry* 65(4), 395–407 (2008).

66 Padmos RC, Van Baal GC, Vonk R *et al.* Genetic and environmental influences on pro-inflammatory monocytes in bipolar disorder: a twin study. *Arch. Gen. Psychiatry* 66(9), 957–965 (2009).

67 Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC *et al.* Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *Int. J. Neuropsychopharmacol.* 13(10), 1369–1381 (2010).

68 Herberth M, Koethe D, Levin Y *et al.* Peripheral profiling analysis for bipolar disorder reveals markers associated with reduced cell survival. *Proteomics* 11(1), 94–105 (2011).

69 Rutten BP, Mill J. Epigenetic mediation of environmental influences in major psychotic disorders. *Schizophr. Bull.* 35(6), 1045–1056 (2009).

70 Goldstein BI, Kemp DE, Soczynska JK, McIntyre RS. Inflammation and the phenomenology, pathophysiology, comorbidity, and treatment of bipolar disorder: a systematic review of the literature. *J. Clin. Psychiatry* 70(8), 1078–1090 (2009).

71 Murray DP, Weiner M, Prabhakar M, Fiedorowicz JG. Mania and mortality: why the excess cardiovascular risk in bipolar disorder? *Curr. Psychiatry Rep.* 11(6), 475–480 (2009).

72 McIntyre RS, Danilewitz M, Liauw SS *et al.* Bipolar disorder and metabolic

syndrome: an international perspective. *J. Affect. Disord.* 126(3), 366–387 (2010).

73 Weiner M, Warren L, Fiedorowicz JG. Cardiovascular morbidity and mortality in bipolar disorder. *Ann. Clin. Psychiatry* 23(1), 40–47 (2011).

74 Berk M, Malhi GS, Hallam K *et al.* Early intervention in bipolar disorders: clinical, biochemical and neuroimaging imperatives. *J. Affect. Disord.* 114(1–3), 1–13 (2009).

75 Hamdani N, Tamouza R, Leboyer M. Immuno- inflammatory markers of bipolar disorder: a review of evidence. *Front. Biosci. (Elite Ed.)* 4, 2170–2182 (2012).

76 Stertz L, Magalhães PV, Kapczinski F. Is bipolar disorder an inflammatory condition? The relevance of microglial activation. *Curr. Opin. Psychiatry* 26(1), 19–26 (2013).

77 Knijff EM, Breunis MN, Kupka RW *et al.* An imbalance in the production of IL-1beta and IL-6 by monocytes of bipolar patients: restoration by lithium treatment. *Bipolar Disord.* 9(7), 743–753 (2007).

78 Guloksuz S, Altinbas K, Aktas Cetin E *et al.* Evidence for an association between tumor necrosis factor-α levels and lithium response. *J. Affect. Disord.* 143(1–3), 148–152 (2012).

79 Andreazza AC, Kauer-Sant'anna M, Frey BN *et al.* Oxidative stress markers in bipolar disorder: a meta-analysis. *J. Affect. Disord.* 111(2–3), 135–144 (2008).

• **Meta-analysis of studies that evaluated markers of oxidative stress in patients with bipolar disorder compared with healthy controls, suggesting that oxidative stress may play a role in the bipolar disorder pathophysiology.**

80 Dean OM, van den Buuse M, Bush AI *et al.* A role for glutathione in the pathophysiology of bipolar disorder and schizophrenia? Animal models and relevance to clinical practice. *Curr. Med. Chem.* 16(23), 2965–2976 (2009).

81 Zhang XY, Yao JK. Oxidative stress and therapeutic implications in psychiatric disorders. *Prog. Neuropsychopharmacol. Biol. Psychiatry* doi:10.1016/j.pnpbp.2013.03.003 (2013) (Epub ahead of print).

82 Halliwell B, Gutteridg J. *Radicals in Biology and Medicine (4th Edition).* Oxford University Press, NY, USA (2007).

83 Olmez I, Ozyurt H. Reactive oxygen species and ischemic cerebrovascular disease. *Neurochem. Int.* 60(2), 208–212 (2012).

84 Khairova R, Pawar R, Salvadore G *et al.* Effects of lithium on oxidative stress parameters in healthy subjects. *Mol. Med. Rep.* 5(3), 680–682 (2012).

85 Halliwell B. Free radicals and antioxidants – *quo vadis*? *Trends Pharmacol. Sci.* 32(3), 125–130 (2011).

86 Wood SJ, Berger GE, Wellard RM *et al.* Medial temporal lobe glutathione concentration in first episode psychosis: a 1H-MRS investigation. *Neurobiol. Dis.* 33(3), 354–357 (2009).

87 Dröse S, Brandt U. Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Adv. Exp. Med. Biol.* 748, 145–169 (2012).

88 Andreazza AC, Shao L, Wang JF, Young LT. Mitochondrial complex I activity and oxidative damage to mitochondrial proteins in the prefrontal cortex of patients with bipolar disorder. *Arch. Gen. Psychiatry* 67(4), 360–368 (2010).

89 Gawryluk JW, Wang JF, Andreazza AC, Shao L, Young LT. Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders. *Int. J. Neuropsychopharmacol.* 14(1), 123–130 (2011).

90 Andreazza AC, Cassini C, Rosa AR *et al.* Serum S100B and antioxidant enzymes in bipolar patients. *J. Psychiatr. Res.* 41(6), 523–529 (2007).

91 Kunz M, Gama CS, Andreazza AC *et al.* Elevated serum superoxide dismutase and thiobarbituric acid reactive substances in different phases of bipolar disorder and in schizophrenia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32(7), 1677–1681 (2008).

92 Selek S, Savas HA, Gergerlioglu HS, Bulbul F, Uz E, Yumru M. The course of nitric oxide and superoxide dismutase during treatment of bipolar depressive episode. *J. Affect. Disord.* 107(1-3), 89–94 (2008).

93 Gergerlioglu HS, Savas HA, Bulbul F, Selek S, Uz E, Yumru M. Changes in nitric oxide level and superoxide dismutase activity during antimanic treatment. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 31(3), 697–702 (2007).

94 Ranjekar PK, Hinge A, Hegde MV *et al.* Decreased antioxidant enzymes and membrane essential polyunsaturated fatty acids in schizophrenic and bipolar mood disorder patients. *Psychiatry Res.* 121(2), 109–122 (2003).

95 Raffa M, Barhoumi S, Atig F, Fendri C, Kerkeni A, Mechri A. Reduced antioxidant defense systems in schizophrenia and bipolar I disorder. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 39(2), 371–375 (2012).

96 Andreazza AC, Frey BN, Erdtmann B *et al.* DNA damage in bipolar disorder. *Psychiatry Res.* 153(1), 27–32 (2007).

97 Pillai A. Brain-derived neurotropic factor/TrkB signaling in the pathogenesis and novel pharmacotherapy of schizophrenia. *Neurosignals.* 16(2–3), 183–193 (2008).

98 Berk M, Dean OM, Cotton SM *et al.* Maintenance *N*-acetyl cysteine treatment for bipolar disorder: a double-blind randomized placebo controlled trial. *BMC Med.* 10, 91 (2012).

99 Berk M, Copolov DL, Dean O *et al.* *N*-acetyl cysteine for depressive symptoms in bipolar disorder – a double-blind randomized placebo-controlled trial. *Biol. Psychiatry* 64(6), 468–475 (2008).

100 Magalhães PV, Dean OM, Bush AI *et al.* *N*-acetyl cysteine add-on treatment for bipolar II disorder: a subgroup analysis of a randomized placebo-controlled trial. *J. Affect. Disord.* 129(1–3), 317–320 (2011).

101 Magalhães PV, Dean OM, Bush AI *et al.* *N*-acetylcysteine for major depressive episodes in bipolar disorder. *Rev. Bras. Psiquiatr.* 33(4), 374–378 (2011).

102 Manji H, Kato T, Di Prospero NA *et al.* Impaired mitochondrial function in psychiatric disorders. *Nat. Rev. Neurosci.* 13(5), 293–307 (2012).

• **Reviews several lines of evidence of mitochondrial dysfunction in psychiatric disorders, and suggests mechanisms by which this dysfunction can contribute to the atrophic changes that underlie the deteriorating long-term course of these illnesses.**

103 Cataldo AM, McPhie DL, Lange NT *et al.* Abnormalities in mitochondrial structure in cells from patients with bipolar disorder. *Am. J. Pathol.* 177(2), 575–585 (2010).

104 Regenold WT, Pratt M, Nekkalapu S, Shapiro PS, Kristian T, Fiskum G. Mitochondrial detachment of hexokinase 1 in mood and psychotic disorders: implications for brain energy metabolism and neurotrophic signaling. *J. Psychiatr. Res.* 46(1), 95–104 (2012).

105 Valvassori SS, Rezin GT, Ferreira CL *et al.* Effects of mood stabilizers on mitochondrial respiratory chain activity in brain of rats treated with d-amphetamine. *J. Psychiatr. Res.* 44(14), 903–909 (2010).

106 Bachmann RF, Wang Y, Yuan P *et al.* Common effects of lithium and valproate on mitochondrial functions: protection against methamphetamine-induced mitochondrial damage. *Int. J. Neuropsychopharmacol.* 12(6), 805–822 (2009).

107 Maurer IC, Schippel P, Volz HP. Lithium-induced enhancement of mitochondrial oxidative phosphorylation in human brain tissue. *Bipolar Disord.* 11(5), 515–522 (2009).

108 Post RM, Fleming J, Kapczinski F. Neurobiological correlates of illness progression in the recurrent affective disorders. *J. Psychiatr. Res.* 46(5), 561–573 (2012).

109 Daban C, Vieta E, Mackin P, Young AH. Hypothalamic–pituitary–adrenal axis and bipolar disorder. *Psychiatr. Clin. North Am.* 28(2), 469–480 (2005).

110 Tatro ET, Everall IP, Kaul M, Achim CL. Modulation of glucocorticoid receptor nuclear translocation in neurons by immunophilins FKBP51 and FKBP52: implications for major depressive disorder. *Brain Res.* 1286, 1–12 (2009).

111 Cervantes P, Gelber S, Kin FN, Nair VN, Schwartz G. Circadian secretion of cortisol in bipolar disorder. *J. Psychiatry Neurosci.* 26(5), 411–416 (2001).

112 Du J, Wang Y, Hunter R *et al.* Dynamic regulation of mitochondrial function by glucocorticoids. *Proc. Natl Acad. Sci. USA* 106(9), 3543–3548 (2009).

113 Gong Y, Chai Y, Ding JH, Sun XL, Hu G. Chronic mild stress damages mitochondrial ultrastructure and function in mouse brain. *Neurosci. Lett.* 488(1), 76–80 (2011).

114 Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM. The stressed CNS: when glucocorticoids aggravate inflammation. *Neuron* 64(1), 33–39 (2009).

115 Yamamoto BK, Raudensky J. The role of oxidative stress, metabolic compromise, and inflammation in neuronal injury produced by amphetamine-related drugs of abuse. *J. Neuroimmune Pharmacol.* 3(4), 203–217 (2008).

116 Rutkowski DT, Kaufman RJ. A trip to the ER: coping with stress. *Trends Cell Biol.* 14(1), 20–28 (2004).

117 Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334(6059), 1081–1086 (2011).

118 Kimata Y, Kohno K. Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. *Curr. Opin. Cell Biol.* 23(2), 135–142 (2011).

119 So J, Warsh JJ, Li PP. Impaired endoplasmic reticulum stress response in B-lymphoblasts from patients with bipolar-I disorder. *Biol. Psychiatry* 62(2), 141–147 (2007).

120 Hayashi A, Kasahara T, Kametani M, Toyota T, Yoshikawa T, Kato T. Aberrant endoplasmic reticulum stress response in lymphoblastoid cells from patients with bipolar disorder. *Int. J. Neuropsychopharmacol.* 12(1), 33–43 (2009).

121 Kakiuchi C, Ishigaki S, Oslowski CM, Fonseca SG, Kato T, Urano F. Valproate, a mood stabilizer, induces WFS1 expression and modulates its interaction with ER stress protein GRP94. *PLoS ONE* 4(1), e4134 (2009).

122 Kim B, Kim CY, Lee MJ, Joo YH. Preliminary evidence on the association between XBP1-116C/G polymorphism and response to prophylactic treatment with valproate in bipolar disorders. *Psychiatry Res.* 168(3), 209–212 (2009).

123 Zhang Z, Tong N, Gong Y *et al.* Valproate protects the retina from endoplasmic reticulum stress-induced apoptosis after ischemia-reperfusion injury. *Neurosci. Lett.* 504(2), 88–92 (2011).

124 Pfaffenseller B, Aguiar BW, Fries GR. Impaired endoplasmic reticulum stress response in lymphocytes from patients with bipolar diosrder. In: *10th International Congress on Cell Biology and the 16th Meeting of the Brazilian Society for Cell Biology.* Rio de Janeiro, Brazil, 25–28 July 2012.

125 Vandenberghe W, Nicoll RA, Bredt DS. Interaction with the unfolded protein response reveals a role for stargazin in biosynthetic AMPA receptor transport. *J. Neurosci.* 25(5), 1095–1102 (2005).

126 Verkhratsky A. The endoplasmic reticulum and neuronal calcium signalling. *Cell Calcium* 32(5–6), 393–404 (2002).

127 Weng WC, Lee WT, Hsu WM, Chang BE, Lee H. Role of glucose-regulated Protein 78 in embryonic development and neurological disorders. *J. Formos. Med. Assoc.* 110(7), 428–437 (2011).

128 Giorgi C, De Stefani D, Bononi A, Rizzuto R, Pinton P. Structural and functional link between the mitochondrial network and the endoplasmic reticulum. *Int. J. Biochem. Cell Biol.* 41(10), 1817–1827 (2009).

129 Wang X, Eno CO, Altman BJ *et al.* ER stress modulates cellular metabolism. *Biochem. J.* 435(1), 285–296 (2011).

130 Uemura T, Green M, Corson TW, Perova T, Li PP, Warsh JJ. *Bcl-2* SNP rs956572 associates with disrupted intracellular calcium homeostasis in bipolar I disorder. *Bipolar Disord.* 13(1), 41–51 (2011).

131 Csordás G, Hajnóczky G. SR/ER-mitochondrial local communication: calcium and ROS. *Biochim. Biophys. Acta* 1787(11), 1352–1362 (2009).

132 Chen G, Fan Z, Wang X *et al.* Brain-derived neurotrophic factor suppresses tunicamycin-induced upregulation of CHOP in neurons. *J. Neurosci. Res.* 85(8), 1674–1684 (2007).

133 Hashimoto K. Sigma-1 receptor chaperone and brain-derived neurotrophic factor: emerging links between cardiovascular disease and depression. *Prog. Neurobiol.* 100, 15–29 (2013).

134 Malhotra JD, Miao H, Zhang K *et al.* Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc. Natl Acad. Sci. USA* 105(47), 18525–18530 (2008).

135 Adolph TE, Niederreiter L, Blumberg RS, Kaser A. Endoplasmic reticulum stress and inflammation. *Dig. Dis.* 30(4), 341–346 (2012).

136 Parpura V, Heneka MT, Montana V *et al.* Glial cells in (patho)physiology. *J. Neurochem.* 121(1), 4–27 (2012).

137 Altshuler LL, Abulseoud OA, Foland-Ross L *et al.* Amygdala astrocyte reduction in subjects with major depressive disorder but not bipolar disorder. *Bipolar Disord.* 12(5), 541–549 (2010).

138 Pantazopoulos H, Woo TU, Lim MP, Lange N, Berretta S. Extracellular matrix-glial abnormalities in the amygdala and entorhinal cortex of subjects diagnosed with schizophrenia. *Arch. Gen. Psychiatry* 67(2), 155–166 (2010).

139 Silvestroff L, Franco PG, Pasquini JM. ApoTransferrin: dual role on adult subventricular zone-derived neurospheres. *PLoS ONE* 7(3), e33937 (2012).

140 Barley K, Dracheva S, Byne W. Subcortical oligodendrocyte- and astrocyte-associated gene expression in subjects with schizophrenia, major depression and bipolar disorder. *Schizophr. Res.* 112(1–3), 54–64 (2009).

141 Rao JS, Harry GJ, Rapoport SI, Kim HW. Increased excitotoxicity and neuroinflammatory markers in postmortem frontal cortex from bipolar disorder patients. *Mol. Psychiatry* 15(4), 384–392 (2010).

142 Harry GJ, Kraft AD. Microglia in the developing brain: a potential target with

lifetime effects. *Neurotoxicology* 33(2), 191–206 (2012).

143 Taveggia C, Thaker P, Petrylak A *et al.* Type III neuregulin-1 promotes oligodendrocyte myelination. *Glia* 56(3), 284–293 (2008).

144 Ongür D, Jensen JE, Prescot AP *et al.* Abnormal glutamatergic neurotransmission and neuronal–glial interactions in acute mania. *Biol. Psychiatry* 64(8), 718–726 (2008).

145 Fries GR, Kapczinski F. *N*-acetylcysteine as a mitochondrial enhancer: a new class of psychoactive drugs? *Rev. Bras. Psiquiatr.* 33(4), 321–322 (2011).

146 Ferreira AP, Pasin JS, Saraiva AL *et al.* *N*-acetylcysteine prevents baker's-yeast-induced inflammation and fever. *Inflamm. Res.* 61(2), 103–112 (2012).

147 Guloksuz S, Cetin EA, Cetin T, Deniz G, Oral ET, Nutt DJ. Cytokine levels in euthymic bipolar patients. *J. Affect. Disord.* 126(3), 458–462 (2010).

148 Rao JS, Rapoport SI. Mood-stabilizers target the brain arachidonic acid cascade. *Curr. Mol. Pharmacol.* 2(2), 207–214 (2009).

149 Kim HW, Rapoport SI, Rao JS. Altered arachidonic acid cascade enzymes in postmortem brain from bipolar disorder patients. *Mol. Psychiatry* 16(4), 419–428 (2011).

150 Dean OM, Data-Franco J, Giorlando F, Berk M. Minocycline: therapeutic potential in psychiatry. *CNS Drugs* 26(5), 391–401 (2012).

151 Hailer NP. Immunosuppression after traumatic or ischemic CNS damage: it is neuroprotective and illuminates the role of microglial cells. *Prog. Neurobiol.* 84(3), 211–233 (2008).

152 Pae CU, Marks DM, Han C, Patkar AA. Does minocycline have antidepressant effect? *Biomed. Pharmacother.* 62(5), 308–311 (2008).

153 Savitz J, Preskorn S, Teague TK, Drevets D, Yates W, Drevets W. Minocycline and aspirin in the treatment of bipolar depression: a protocol for a proof-of-concept, randomised, double-blind, placebo-controlled, 2 × 2 clinical trial. *BMJ Open* 2(1), e000643 (2012).

154 Wu A, Ying Z, Gomez-Pinilla F. Dietary ω-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract learning disability after traumatic brain injury in rats. *J. Neurotrauma* 21(10), 1457–1467 (2004).

155 Sarris J, Mischoulon D, Schweitzer I. ω-3 for bipolar disorder: meta-analyses of use in mania and bipolar depression. *J. Clin. Psychiatry* 73(1), 81–86 (2012).

156 Balanzá-Martínez V, Fries GR, Colpo GD *et al.* Therapeutic use of ω-3 fatty acids in bipolar disorder. *Expert Rev. Neurother.* 11(7), 1029–1047 (2011).

157 Soeiro-de-Souza MG, Dias VV, Figueira ML *et al.* Translating neurotrophic and cellular plasticity: from pathophysiology to improved therapeutics for bipolar disorder. *Acta Psychiatr. Scand.* 126(5), 332–341 (2012).

158 Hosseinkhani H, Hosseinkhani M. Biodegradable polymer-metal complexes for gene and drug delivery. *Curr. Drug Saf.* 4(1), 79–83 (2009).

159 Mohtaram NK, Montgomery A, Willerth SM. Biomaterial-based drug delivery systems for the controlled release of neurotrophic factors. *Biomed. Mater.* 8(2), 022001 (2013).

160 Chiu CT, Wang Z, Hunsberger JG, Chuang DM. Therapeutic potential of mood stabilizers lithium and valproic acid: beyond bipolar disorder. *Pharmacol. Rev.* 65(1), 105–142 (2013).

161 Gigante AD, Young LT, Yatham LN *et al.* Morphometric post-mortem studies in bipolar disorder: possible association with oxidative stress and apoptosis. *Int. J. Neuropsychopharmacol.* 14(8), 1075–1089 (2011).

162 Chaudhary M, Rao PV. Brain oxidative stress after dermal and subcutaneous exposure of T-2 toxin in mice. *Food Chem. Toxicol.* 48(12), 3436–3442 (2010).

163 de Gonzalo-Calvo D, Neitzert K, Fernández M *et al.* Differential inflammatory responses in aging and disease: TNF-α and IL-6 as possible biomarkers. *Free Radic. Biol. Med.* 49(5), 733–737 (2010).

164 Kapczinski F, Frey BN, Andreazza AC, Kauer-Sant'Anna M, Cunha AB, Post RM. Increased oxidative stress as a mechanism for decreased BDNF levels in acute manic episodes. *Rev. Bras. Psiquiatr.* 30(3), 243–245 (2008).

165 Gama CS, Andreazza AC, Kunz M, Berk M, Belmonte-de-Abreu PS, Kapczinski F. Serum levels of brain-derived neurotrophic factor in patients with schizophrenia and bipolar disorder. *Neurosci. Lett.* 420(1), 45–48 (2007).

166 Hope S, Dieset I, Agartz I *et al.* Affective symptoms are associated with markers of inflammation and immune activation in bipolar disorders but not in schizophrenia. *J. Psychiatr. Res.* 45(12), 1608–1616 (2011).

167 Maes M, Delange J, Ranjan R *et al.* Acute phase proteins in schizophrenia, mania and major depression: modulation by psychotropic drugs. *Psychiatry Res.* 66(1), 1–11 (1997).

168 Kim YK, Suh IB, Kim H *et al.* The plasma levels of interleukin-12 in schizophrenia, major depression, and bipolar mania: effects of psychotropic drugs. *Mol. Psychiatry* 7(10), 1107–1114 (2002).

169 Dodd S, Dean O, Copolov DL, Malhi GS, Berk M. *N*-acetylcysteine for antioxidant therapy: pharmacology and clinical utility. *Expert Opin. Biol. Ther.* 8(12), 1955–1962 (2008).

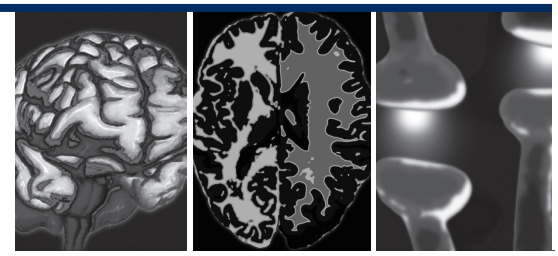
3.2 CAPÍTULO 2

Anatomical faces of neuroprogression in bipolar disorder

Bianca Pfaffenseller, Clarissa Severino Gama, Flavio Kapczinski, Juliana Avila Duarte & Mauricio Kunz

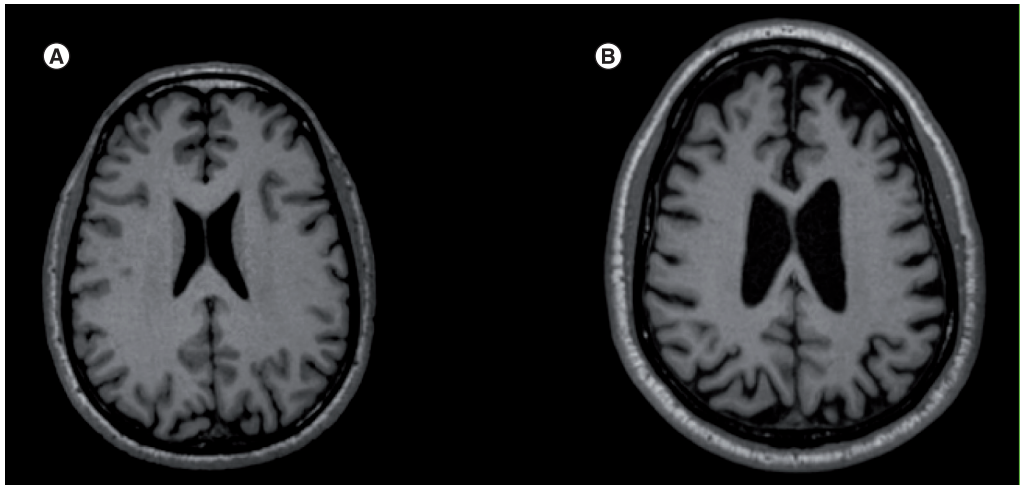
Artigo publicado na revista *Neuropsychiatry* em 2012.

CLINICAL SNAPSHOT



Anatomical faces of neuroprogression in bipolar disorder

Bianca Pfaffenseller[1,2], Clarissa Severino Gama[1,3,4], Flavio Kapczinski*[1,3], Juliana Avila Duarte[5,6] & Mauricio Kunz[1,3]



These two MRI images illustrate the anatomical changes associated with the progression of illness in bipolar disorder (BD). There is significant cortical atrophy and enlargement of ventricles in a patient with a history of multiple mood episodes **(B)** compared with a patient of the same gender and similar age that only experienced a few episodes and a shorter length of illness **(A)**. Alterations in brain structures have been reported in BD patients [1–4], and in these images it is possible to observe that such neuroanatomical changes are more pronounced after repeated episodes. In BD, neural substrate reactivity is changed by repeated mood episodes, ultimately promoting brain rewiring associated with increased vulnerability to life stress [5]. The neurobiological mechanisms of more pronounced neuroanatomical brain changes in patients with multiple mood episodes of BD appear to include increased oxidative stress, increased proinflammatory markers and a deficit in neuroprotection [6]. Acute mood episodes have been associated with significant systemic toxicity, cognitive and functional impairment and biological changes [7,8]. These effects are cumulative, being much more prominent after multiple episodes [9–12]. This suggests that mood episodes function as allostatic states, generating a load that accumulates

[1]Laboratório de Psiquiatria Molecular, Bipolar Disorder Program & INCT for Translational Medicine, Hospital de Clínicas de Porto Alegre (HCPA), Universidade Federal do Rio Grande do Sul (UFRGS), Ramiro Barcelos, 2350, 90035-903, Porto Alegre, RS, Brazil
[2]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[3]Programa de Pós-Graduação em Medicina: Psiquiatria, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[4]Schizophrenia Program, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, RS, Brazil
[5]Magnetic Resonance, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, RS, Brazil
[6]Magnetic Resonance, Tomoclinica, Canoas, RS, Brazil
*Author for correspondence: Tel.: +55 51 21018845; kapcz@terra.com.br

Future Medicine part of fsg

and compromises regulatory systems, ultimately being responsible for the illness progression seen in BD. These changes in brain anatomy support the notion of neuroprogressive changes over time in patients with BD [6]. Neuroprogression can have important clinical implications, given that early and late stages of the disorder appear to present different biological features and therefore may require different treatment strategies. This image highlights the importance of longitudinal studies in evaluating the effects of illness progression on neurostructures and neurofunctions in BD. These findings may potentially have clinical implications by establishing means for monitoring the impact of treatments, as well as supporting more aggressive and earlier therapeutic interventions to minimize affective symptomatology and clinical deterioration. The possibility to identify neuroanatomical abnormalities with methods, such as MRI, will provide better insights into some of the potential pathophysiological mechanisms involved in illness progression, as well as better diagnosis, prognosis and long-term prophylaxis in BD.

Informed consent

Patients were recruited at the Bipolar Disorder Program, an outpatient program of Hospital de Clínicas de Porto Alegre, Brazil. This protocol was approved by the local ethics committee and the subjects provided their written informed consent for the collection and use of MRI images.

Financial & competing interests disclosure

F Kapczinski is a NARSAD Independent Investigator and is supported by the Stanley Medical Research Institute, CNPq, INCT-TM, CAPES and FIPE-HCPA. In addition, F Kapczinski is a speaker and/or advisor and/or receives research support from Lilly, Lundbeck, AstraZeneca, Servier and Janssen. CS Gama has received grant/research support from CNPq, FIPE/HCPA and FAPERGS and has been a speaker/advisory for Lundbeck, Pfizer and Actelion. These agencies had no role in the study design, in the acquisition or interpretation of the data or in writing the report. This study was supported by grants from CNPq (Universal 470326/2011-5 and PQ 302195/2011-4), FAPERGS (PqG 1009340-06/2010) and FAPERGS/CNPq (PRONEM 11/2057-2), Brazil. These agencies had no role in the study design, in the acquisition or interpretation of the data or in writing the report. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

1 Fornito A, Mahli GS, Lagopoulos J *et al.* Anatomical abnormalities of the anterior cingulate and paracingulate cortex in patients with bipolar I disorder. *Psychiatry Res.* 62, 123–132 (2008).

2 Konarski JZ, McIntyre RS, Kennedy SH, Rafi-Tari S, Soczynska JK, Ketter TA. Volumetric neuroimaging investigations in mood disorders: bipolar disorder versus major depressive disorder. *Bipolar Disord.* 10(1), 1–37 (2008).

3 Hallahan B, Newell J, Soares JC *et al.* Structural magnetic resonance imaging in bipolar disorder: an international collaborative mega-analysis of individual adult patient data. *Biol. Psychiatry* 69(4), 326–335 (2011).

4 Lisy ME, Jarvis KB, DelBello MP *et al.* Progressive neurostructural changes in adolescent and adult patients with bipolar disorder. *Bipolar Disord.* 13(4), 396–405 (2011).

5 Kapczinski F, Vieta E, Andreazza AC *et al.* Allostatic load in bipolar disorder: implications for pathophysiology and treatment. *Neurosci. Biobehav. Rev.* 32(4), 675–692 (2008).

6 Berk M, Kapczinski F, Andreazza AC *et al.* Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors. *Neurosci. Biobehav. Rev.* 35(3), 804–817 (2011).

7 Kapczinski F, Dal-Pizzol F, Teixeira AL *et al.* A systemic toxicity index developed to assess peripheral changes in mood episodes. *Mol. Psychiatry* 15(8), 784–786 (2010).

8 Kapczinski F, Dal-Pizzol F, Teixeira AL *et al.* Peripheral biomarkers and illness activity in bipolar disorder. *J. Psychiatr. Res.* 45(2), 156–161 (2011).

9 Kauer-Sant'Anna M, Kapczinski F, Andreazza AC *et al.* Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs late-stage bipolar disorder. *Int. J. Neuropsychopharmacol.* 12(4), 447–458 (2009).

10 Andreazza AC, Kapczinski F, Kauer-Sant'Anna M *et al.* 3-Nitrotyrosine and glutathione antioxidant system in patients in the early and late stages of bipolar disorder. *J. Psychiatry Neurosci.* 34(4), 263–271 (2009).

11 Magalhães PV, Jansen K, Pinheiro RT *et al.* Peripheral oxidative damage in early-stage mood disorders: a nested population-based case–control study. *Int. J. Neuropsychopharmacol.* 19, 1–8 (2011).

12 Lewandowski KE, Cohen BM, Ongur D. Evolution of neuropsychological dysfunction during the course of schizophrenia and bipolar disorder. *Psychol. Med.* 41(2), 225–241 (2011).

3.3 CAPÍTULO 3

Differential expression of transcriptional regulatory units in the prefrontal cortex of patients with bipolar disorder: potential role of early growth response gene 3

Bianca Pfaffenseller, Pedro V.S. Magalhães, Marco Antônio De Bastiani, Mauro A.A. Castro, Amelia L. Gallitano, Flávio Kapczinski e Fábio Klamt

Artigo publicado na revista *Translational Psychiatry* em 2016.

OPEN

Citation: Transl Psychiatry (2016) 6, e805; doi:10.1038/tp.2016.78

www.nature.com/tp

ORIGINAL ARTICLE

Differential expression of transcriptional regulatory units in the prefrontal cortex of patients with bipolar disorder: potential role of early growth response gene 3

B Pfaffenseller[1,2], PV da Silva Magalhães[1,3], MA De Bastiani[2], MAA Castro[4], AL Gallitano[5], F Kapczinski[1,3] and F Klamt[2]

Bipolar disorder (BD) is a severe mental illness with a strong genetic component. Despite its high degree of heritability, current genetic studies have failed to reveal individual loci of large effect size. In lieu of focusing on individual genes, we investigated regulatory units (regulons) in BD to identify candidate transcription factors (TFs) that regulate large groups of differentially expressed genes. Network-based approaches should elucidate the molecular pathways governing the pathophysiology of BD and reveal targets for potential therapeutic intervention. The data from a large-scale microarray study was used to reconstruct the transcriptional associations in the human prefrontal cortex, and results from two independent microarray data sets to obtain BD gene signatures. The regulatory network was derived by mapping the significant interactions between known TFs and all potential targets. Five regulons were identified in both transcriptional network models: early growth response 3 (*EGR3*), TSC22 domain family, member 4 (*TSC22D4*), interleukin enhancer-binding factor 2 (*ILF2*), Y-box binding protein 1 (*YBX1*) and MAP-kinase-activating death domain (*MADD*). With a high stringency threshold, the consensus across tests was achieved only for the *EGR3* regulon. We identified *EGR3* in the prefrontal cortex as a potential key target, robustly repressed in both BD signatures. Considering that *EGR3* translates environmental stimuli into long-term changes in the brain, disruption in biological pathways involving *EGR3* may induce an impaired response to stress and influence on risk for psychiatric disorders, particularly BD.

Translational Psychiatry (2016) **6,** e805; doi:10.1038/tp.2016.78; published online 10 May 2016

INTRODUCTION

Bipolar disorder (BD) is a severe mental illness with a strong genetic component. Heritability is high, as evaluated by monozygotic and dizygotic twin concordance, albeit not perfect.[1,2] Nevertheless, current molecular genetic studies indicate that no particular locus of large effect is involved in its etiology. The candidate gene approach has not delivered robust results and genome-wide association studies also often fail to show strong positive signals.[2] Current polygenic analyses are consistent with hundreds or thousands of susceptibility variants of weak effect, with the variation in single-nucleotide polymorphisms explaining 20 to 30% of the heritability shown in family and twin studies.[3,4]

The generally low yield of pure genetic association studies has generated interest in alternative approaches.[5] Gene expression studies move the discussion beyond statistical associations into the realm of neurobiology. RNA analysis can be more informative of the status of the cell, as it reflects a functional state not only influenced by genetic polymorphisms, but also by transcriptional modulation. Using high-throughput technologies, such as microarrays, the differential expression of genomic DNA in the form of mRNA has the potential to lead to disease signatures.[6] One major caveat is that gene expression is tissue specific. For BD studies, this means obtaining brain tissue from postmortem donors, and few brain bank collections exist for this illness.[7]

Given the difficulty of obtaining brain tissue, the data on gene expression in BD is quite limited (compared with cancer research, for example). To get an idea of the sparseness of existing data in BD, a recent systematic review of gene expression studies revealed publicly available data from only 57 unique BD cases.[8] Furthermore, gene expression varies depending on the area and structure under study.[9] This underscores the importance of selecting the appropriate brain regions and using a methodological framework to extract biologically meaningful information from large-scale data. Although the neurocircuitry involved in the mood disorders is expected to be complex, there are major areas of interest that could be fruitfully explored in postmortem studies. Overall, interest has focused on the limbic–cortical–striato–pallido–thalamic circuits. The prefrontal cortex is a relevant nexus, where recent research points to areas in the medial prefrontal and orbitofrontal cortex.[10]

In the field of systems biology, approaches to identify candidate master regulators (MRs) have focused on transcription factors (TFs) that exert large influences on a phenotype. Recently, a number of computational methods have been developed to identify groups of genes, and even entire pathways, coordinated by a small number of TFs. These approaches have successfully identified gene units that are impaired in diseases such as cancer and diabetes.[11–15] As in other traditional medical fields, psychiatry is

[1]Bipolar Disorder Program, Laboratory of Molecular Psychiatry, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil; [2]Laboratory of Cellular Biochemistry, Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [3]Department of Psychiatry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [4]Bioinformatics and Systems Biology Laboratory, Federal University of Paraná, Polytechnic Center, Curitiba, Brazil and [5]Department of Basic Medical Sciences, University of Arizona College of Medicine, Phoenix, AZ, USA. Correspondence: Professor PV da Silva Magalhães, Department of Psychiatry, Universidade Federal do Rio Grande do Sul, 2350 Ramiro Barcelos Street, Porto Alegre 90035 903, Brazil.
E-mail: pedromaga2@gmail.com
Received 16 March 2016; accepted 23 March 2016

currently focusing on the study of biological pathways. Reverse engineering algorithms are used to reconstruct cell type-specific regulatory networks from high-throughput data. This approach efficiently reduces the complexity of the network, allowing the identification of MR TFs with tissue-specific signatures.

Here we query the genetic regulatory signature of BD through a series of steps. First, we analyze a large gene expression data set from healthy human prefrontal cortex across the lifespan[16] to construct a regulatory network of known TFs and all potential targets. As gene expression is tissue specific, this empirical approach has the benefit of being a more realistic representation of prefrontal cortex functioning. Next, we identify genes that are differentially expressed in the prefrontal cortex of patients and healthy controls from two separate data sets of postmortem tissue samples. We reasoned that if the BD gene expression signature in the prefrontal cortical regulatory units is influenced by the activation (or repression) of specific TFs, then both the downstream targets, as well as the upstream regulators, of these TFs should be among the most differentially expressed genes in the BD phenotype. The use of network-based approaches to elucidate biological mechanisms of complex diseases may allow a clearer view of the molecular networks governing the pathophysiology and reveal potential targets for drug design and therapeutic intervention.

MATERIALS AND METHODS

Microarray data

The data used to reconstruct the transcriptional associations in the human prefrontal cortex was obtained from a large-scale microarray study describing an extensive series ($n = 269$) of brain tissue samples from fetal development through aging,[16] downloaded from GEO (accession number GSE30272). Two studies using independent microarray platforms (Affymetrix Human Genome 133A GeneChips and Codelink Expression Assay arrays) were used to obtain the BD gene expression signatures (accession numbers GSE12679 and GSE5388). The two selected data sets were the best and most interesting sets available to our goal. As we aimed to evaluate differential expression of transcriptional regulatory units in the prefrontal cortex of bipolar disorder patients, we sought the most specific samples available (data set 1—data from pyramidal neurons) and other presenting more sensibility (data set 2—data from all cortical cells), both data sets from the same prefrontal area (Brodmann area 9—BA9). As such, the samples and raw data were independently processed and generated, contributing to check the consistency of the regulatory units characterized in our study.

For the first data set, the samples were obtained using laser-capture microdissection[17] and included 1000 pyramidal neurons isolated from region BA9 of prefrontal cortex from five individuals with BD and six control subjects. For the second data set, fresh-frozen prefrontal cortex tissue (region BA9) was obtained from the Neuropathology Consortium of the Stanley brain collection (Stanley Medical Research Institute, Chevy Chase, MD, USA)[18] and included 30 BD and 31 control samples. The demographic variables for the samples have been scrutinized in the original studies that produced the public data. For the first data set, the information available includes age (years): patients—43.4 ± 6.3, controls—41.2 ± 7.2; gender (F/M): patients—1/4, controls—2/4; postmortem interval (hours): patients—28.2 ± 5.4, controls—30 ± 18.6. The study related to the second data set presents more detailed information that is summarized below. Age (years): patients—44.5 ± 10.7, controls—43.8 ± 7.3; gender (F/M): patients—14/16, controls—7/24; brain pH: patients—6.48 ± 0.27, controls—6.62 ± 0.27; postmortem interval (hours): patients—37.2 ± 17.7, controls—29.1 ± 13.1; suicide (Y/N): patients—12/18, controls—0/31; alcohol abuse (none or light/moderate to severe): patients—13/17, controls—27/4. Regarding treatment of patients, lithium (Y/N): 8/22; valproate (Y/N): 10/20; electroconvulsive therapy history (Y/N): 2/28. Some potential confounding issues are not available to be controlled; however, they are addressed in the discussion.

Transcriptional network inference

The transcriptional networks were constructed using the R package RTN.[19] Regarding the code availability used in this study, it is publicly available from Bioconductor in the R packages RTN (http://bioconductor.org/packages/RTN/). Gene probes (complementary sequences to the target mRNAs used in microarray to assay gene expression) were filtered based on their coefficient of variation and mutual information was calculated in the R package *minet*.[20] The regulatory structure of the network is derived by mapping the significant interactions between known TFs and all potential targets in the gene expression matrix. The interactions that are below a minimum mutual information threshold are eliminated by permutation analysis. Unstable interactions are additionally removed by bootstrap analysis using 1000 bootstrap samples to create the consensus bootstrap network (that is, the relevance network). In an additional step, the Data Processing Inequality (DPI) algorithm is applied with tolerance = 0.0 to eliminate interactions that are likely to be mediated by another TF.[11] As the DPI removes the weakest edge of each network triplet, the vast majority of the interactions that are likely to be indirect are eliminated in this step. The resulting DPI-filtered transcriptional network is subsequently interrogated in the enrichment analysis. Both DPI-filtered and unfiltered transcriptional networks are used to visualize the final results. The analysis pipeline, resampling procedures and methods used to reconstruct the transcriptional networks are summarized in Supplementary Figure 1.

MR and gene set enrichment analysis

The MR analysis is described elsewhere.[15] Briefly, the gene set enrichment analysis (GSEA) is used to assess whether a given transcriptional regulatory unit (regulon) is enriched for genes that are differentially expressed among two classes of microarrays. The GSEA uses a rank-based scoring metric obtained from the differentially expressed signatures to test the association between gene sets and the ranked phenotypic difference. The current analysis treats regulons as gene sets, and the BD signatures as the phenotype, in an extension of the GSEA analysis as previously described.[21] The GSEA was performed in the R package RTN using 1000 permutations.

Two-tailed GSEA

The two-tailed GSEA assesses the direction of inferred connection between a given MR and the differentially expressed signatures, a proxy for induced or repressed associations. The method is based on the Connectivity Map procedure.[22] The regulon is split into two subgroups, positive targets (*A*) and negative targets (*B*) using Pearson's correlation, whereas genes in the phenotype are ranked using the differentially expressed signatures (that is, top-down phenotype). The distribution of *A* and *B* are then tested by the GSEA statistics in the ranked phenotype, producing independent enrichment scores (ES) for each subgroup. A good separation of the two distributions and maximum deviation from zero near opposite extremes is desirable for a clear association. Therefore, an additional step is executed testing the differential enrichment ($ES_A - ES_B$). A high positive differential score indicates that the phenotype induced the regulon, whereas a high negative differential score indicates that the phenotype repressed the regulon. The two-tailed GSEA was performed in R using the function *tni.gsea2* in the RTN package with 1000 permutations.

Analysis of gene expression data

The Bioconductor package *limma*[23] was used to call differentially expressed genes, and the log fold change (logFC) metric was used to obtain the ranked phenotypes required for the GSEA analysis.

RESULTS

A tissue-specific regulatory network for the human prefrontal cortex

We first established a tissue-specific transcriptional network model computed from a large-scale human prefrontal microarray data set (transcriptional network reconstruction summarized in Figure 1a). The microarray data were pre-processed and probes with low variation were removed from the analysis. Two transcriptional networks (*TN1* and *TN2*) were then derived by computing the mutual information between annotated TFs and all potential targets in the data set. *TN1* represents the totality of the 269-microarray samples in the study, whereas *TN2* is derived from a subsample with adult human prefrontal cortex only (see Supplementary Figure 1 for additional details of the resampling

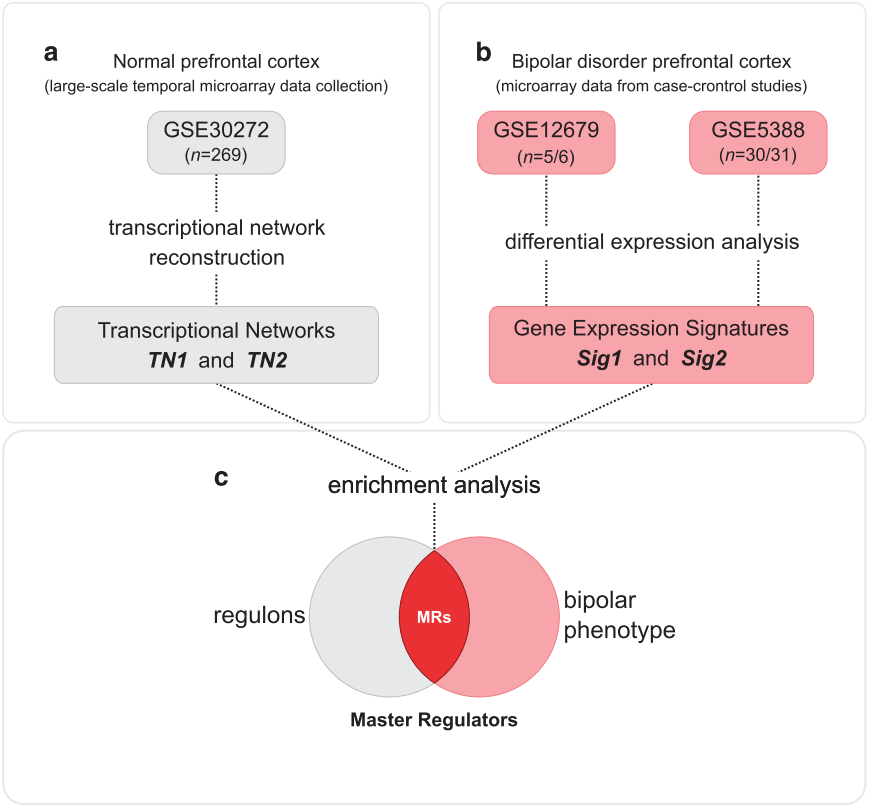


Figure 1. Master regulator (MR) analysis flowchart. (**a**) Data source used to reconstruct the transcriptional regulatory units in normal human prefrontal cortex. A large-scale microarray study (GSE30272) describing an extensive series of brain tissue from fetal development through aging was used to compute the transcription factor-centric regulatory networks (regulons). The transcriptional network *TN1* represents the totality of the 269-microarray samples in the study, whereas the *TN2* derives from a subsample with adult human prefrontal cortex only. The analysis pipeline, resampling procedures and methods used to reconstruct the transcriptional networks are further detailed in the Supplementary Figure 1. (**b**) Flowchart summarizing the microarray data used to obtain two independent bipolar disorder gene expression signatures (bipolar phenotypes). Signature 1 (*Sig1*; GSE12679) is derived from laser-capture microdissected human neurons isolated from postmortem dorsolateral prefrontal cortex, whereas signature 2 (*Sig2*; GSE5388) is derived from human postmortem brain tissue from adult subjects. (**c**) The enrichment analysis aims to identify transcriptional regulatory units associated with the gene expression signatures.

procedures). The association map in Figure 2a summarizes the transcriptional network *TN1* and shows the degree of similarity among the inferred regulatory units (regulons). The node size represents the number of targets in a given regulon, whereas edge width corresponds to the number of common targets between any two regulons assessed by the Jaccard coefficient (JC). In this reference network, each target can be linked to multiple TFs and regulation can occur as a result of both direct (TF–target) and indirect interactions (TF–TF–target). To preserve the dominant TF–target pairs for the subsequent enrichment analyses, we additionally applied the DPI algorithm, which removes the weakest interaction in any eventual triplet formed by two TFs and a common target gene (see methods and computational pipeline summarized in Supplementary Figure 1).

MRs in BD prefrontal cortex

The inferred transcriptional network model was next used to query regulons enriched for the BD gene expression signatures. These signatures were obtained by differential expression analysis using microarray data from the two independent case–control sets (Figure 1b). Signature 1 (*Sig1*) is derived from laser-capture microdissected human neurons isolated from postmortem dorsolateral prefrontal cortex, whereas signature 2 (*Sig2*) is derived from human postmortem brain tissue from adult subjects. The MR analysis[13] aims to identify regulons associated with the gene expression signatures (Figure 1c). Therefore, our primary goal here is to generate hypotheses regarding the transcriptional regulation in BD, identifying the MRs responsible for coordinating the activity of the signature genes. We used the GSEA statistics to test the enrichment of the signature genes in each regulon. Figure 2b presents the results of the GSEA analysis using *TN1* and *Sig1* and shows the distribution of the BD phenotype onto the transcriptional association map.

Among the several candidates identified, 10 regulons were significantly enriched for both gene expression signatures (Figure 3a), five of which are consensus in both *TN1* and *TN2* transcriptional network models: early growth response protein 3 (*EGR3*), TSC22 domain family, member 4 (*TSC22D4*), interleukin enhancer-binding factor 2 (*ILF2*), Y-box binding protein 1 (*YBX1*) and MAP-kinase-activating death domain (*MADD*). When a high stringent threshold is applied, the overall consensus across all the tests is only obtained for the regulon of the *EGR3*. The GSEA plots in Figure 3b shows the distribution of the top-five consensus MRs in the BD phenotype.

Mode of action of the computationally defined regulons

To visualize the five MRs, we show in Figure 4a the correlation pattern observed between the TFs (square nodes) and its inferred targets (round nodes) assessed by the Pearson's correlation on *TN1*. This network graph shows all interactions inferred for each

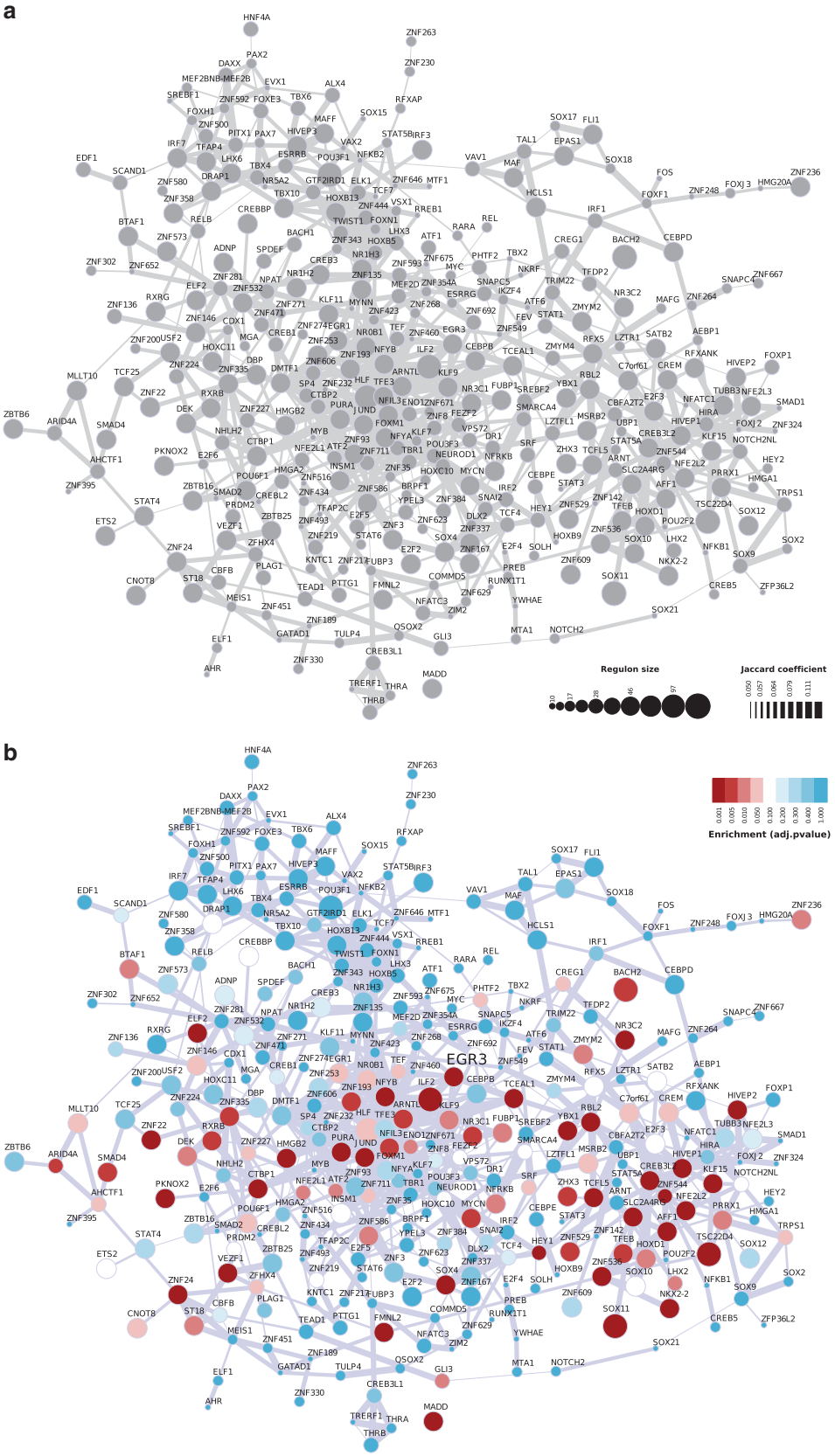


Figure 2. A systems model of the human prefrontal transcriptional network. (**a**) Association map showing the degree of similarity among regulons in the transcriptional network *TN1*. The node size represents the number of transcription factor (TF)–targets in the relevance network, whereas edge width corresponds to the overlap between regulons assessed by the Jaccard coefficient (JC). Unconnected regulons are not shown. (**b**) Enrichment analysis using gene expression signature 1 (*Sig1*) showing the distribution of the bipolar phenotype onto the association map (adjusted *P*-value < 0.05 are shown in red-color scale).

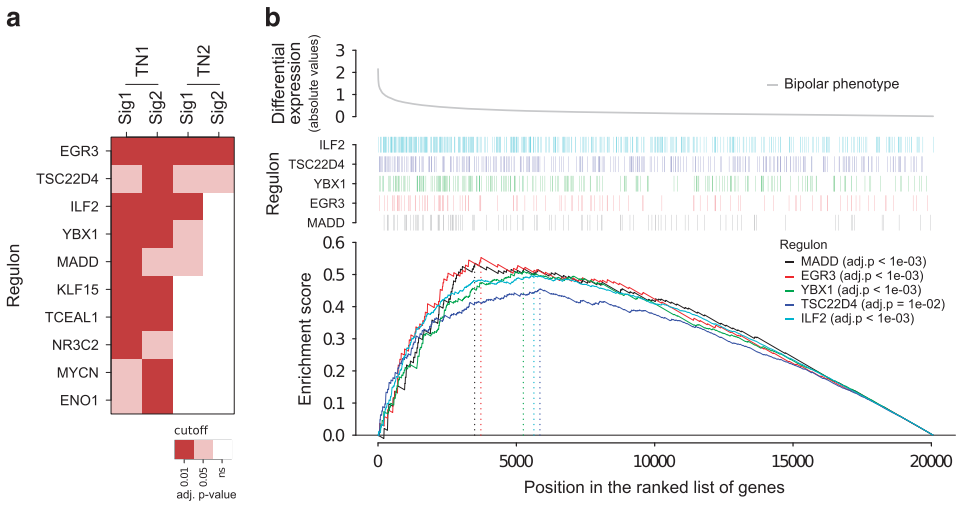


Figure 3. Consensus master regulators enriched for the bipolar disorder signatures. (**a**) Gene set enrichment statistics showing the regulatory units consistently enriched for the expression signatures *Sig1* and *Sig2* on the transcriptional network *TN1*, together with the results obtained for the same regulons on *TN2*. (**b**) Gene set enrichment plots showing the distribution of the top-five master regulators (that is, the consensus regulatory units) across the ranked bipolar phenotype represented by the absolute differential expression values (absolute logFC) derived from *Sig1*. The enrichment score is obtained based on the distribution of the hits: the *x* axis indicates the position of all genes ranked by the phenotype, and the hits indicate the position of each gene of a given regulon (see methods for additional description on the GSEA statistics). ns, not significant.

regulon, whether positive (red targets) or negative (blue targets). Using this information, we extended the gene set enrichment analysis in order to access how the mode of action of these regulons are connected with the BD gene signatures. We used a two-tailed GSEA statistics on regulons split into positive (red) and negative (blue) targets (Figure 4b), and the resulting distributions were tested against the BD phenotype ranked from the highest (+) to the lowest (−) differential expression values.

Accordingly, *EGR3* and *MADD* negative targets are associated with the positive phenotype (that is, most induced genes), whereas the positive targets are associated with negative phenotype (that is, most repressed genes), providing a high negative differential score with adjusted *P*-value < 0.001. It suggests that both *EGR3* and *MADD* regulons are repressed in the BD gene signature, whereas the other three regulons appear to be increased.

DISCUSSION

The primary goal of this study was to generate hypotheses regarding transcriptional regulation in BD, and to identify putative regulatory units that are dysfunctional in the prefrontal cortex of patients. To that end, we sought differentially expressed signatures that converged from two available gene expression data sets with distinctive strengths and weaknesses. Using MR analysis, our major finding was that the *EGR3* regulon was robustly repressed in both BD gene expression signatures. Four additional MRs showed a lower level of association with the BD phenotype.

EGR3 is a member of the *EGR* gene family of immediate early genes transcription factors. These genes are expressed at basal levels throughout the brain, including the cortex, hippocampus and other limbic areas, and the basal ganglia.[24] *EGR* expression is induced at high levels in response to environmental events and stressful stimuli across a range of intensities. In the brain, this activation is triggered by neurotransmitter-receptor stimulation or depolarization.[24] Numerous behavioral and electrophysiologic studies in animals have shown that the *EGR* family has a role in memory acquisition and consolidation and hippocampal synaptic plasticity.[24–29] *EGR3*, in particular, is required for the normal response to stress as well as in the neuroplasticity induced by this responsivity, ultimately regulating neuronal gene expression.[27,30,31]

Of particular relevance are studies demonstrating that mice lacking functional *EGR3* display behavioral and physiologic abnormalities consistent with models of mental illness. These include a heightened response to stress (evidenced behaviorally and by elevated release of corticosterone), hyperactivity and failure to habituate to environmental stimuli and social cues.[27] The hyperactivity, a rodent psychosis phenotype, is reversible with antipsychotic medications used to treat BD.[32,33] *EGR3* regulates expression of important plasticity associated genes, such as those encoding the activity regulated cytoskeletal associated gene (*Arc*)[30,34] and GABA receptor subunit 4 (*GABRA4*),[35] and other member of the *EGR* family regulates the synaptic vesicle associated proteins *synapsin 1*[36] and *synapsin 2*.[37] Thus, requirement of *EGR3* in processes of memory, learning and synaptic plasticity is likely to be mediated by these, and presumably other as-yet unidentified, target effector genes.

The neuronal expression of *EGR3* is regulated by synaptic activity and is coupled to MAPK-ERK signaling.[24,28] Gallitano-Mendel and colleagues noted that *EGR3* is activated downstream of numerous proteins associated with risk for psychotic illness, including neuregulin 1 (*NRG1*), calcineurin (*CN*) and *N*-methyl-D-aspartate (*NMDA*) receptors.[27,32,38–41] Moreover, drugs that induce psychosis via serotonin 2A receptors (5-HT2ARs) regulate expression of *EGR3*.[42] They have hypothesized that these genes, together with targets of *EGR3*, comprise a pathway of proteins which, when disrupted at any level, increases risk for psychotic illness. In addition, brain-derived neurotrophic factor (*BDNF*) has been shown to induce *EGR3* expression via a PKC/MAPK-dependent pathway.[35] These are all interesting links, as *BDNF* has been proposed as a critical factor in the reduced cellular resilience associated to BD.[43,44] A growing body of data has shown that peripheral *BDNF* levels are decreased during BD episodes and with the illness progression.[45,46] Despite limitations in the studies and conflicting results in this area, it is intriguing to speculate that reduced peripheral *BDNF* levels, whether related to its decreased expression in the brain, may influence the *EGR3* repression as this

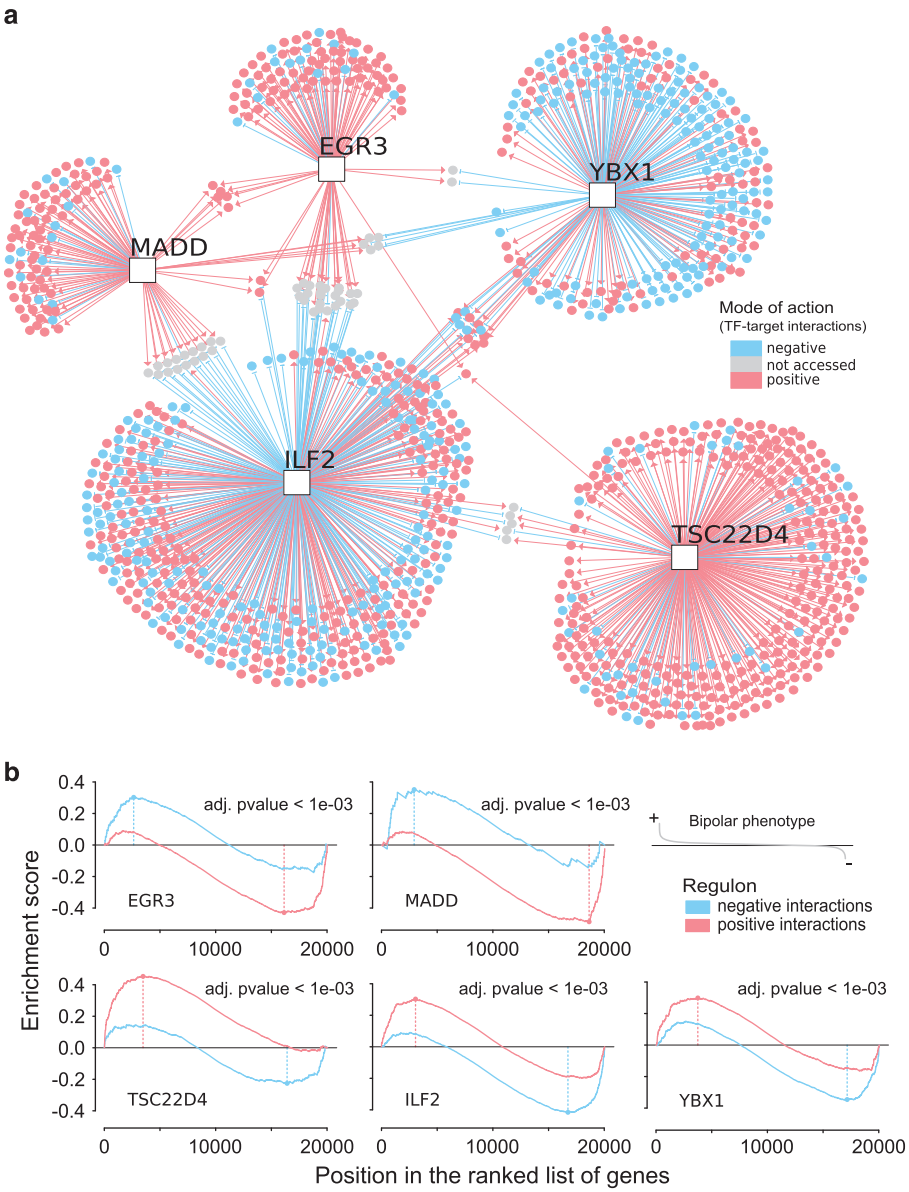


Figure 4. Regulatory units associated with the bipolar disorder phenotype. (**a**) The regulatory network shows the transcription factor (TF)–target interactions of the five master regulators, each one comprising one TF (square nodes) and all inferred targets (round nodes). The mode of action represented in red/blue colors corresponds to the correlation pattern observed between a given transcription factor and its targets, assessed by the Pearson's correlation on *TN1*. (**b**) Two-tailed gene set enrichment analysis. The enrichment plots show the distribution of the genes in each regulon across the ranked phenotype derived from *Sig1*. Regulons are split in positive (red) and negative (blue) targets, whereas the phenotype is ranked from the highest (+) to the lowest (−) differential expression values (logFC), that is, from the most increased to the most decreased gene expression values.

TF is regulated by *BDNF*. Obviously, further research is warranted for substantially improving the knowledge regarding the link between *BDNF* and *EGR3* in a shared biological pathway and their role in BD.

Downstream, *EGR3* targets the promoter region of genes involved in neuroplasticity or stimuli response. So far, experimental studies show effects on NMDA receptor subunits NR1 and NR2B, and type A GABA receptor, and possibly on genes involved in microglia deregulation associated with psychiatric disorders, such as the triggering receptor expressed on myeloid cells 1 (*TREM-1*).[27,35,47,48] *EGR3* also regulates the expression of NGFR (*p75NTR*),[49] a receptor for neurotrophins that is involved in the regulation of axonal elongation.[50] Perhaps most intriguing is the recent findings that the *EGR3* target gene *ARC*, which modifies synapses in response to environmental stimuli,[51] is implicated in risk for psychotic disorders.[41,52–54] Altogether, *EGR3* targets trigger different downstream genes and pathways involved in processes such as synaptic plasticity, axon extension, regulation of *BDNF* and receptors expression, among others.[55]

EGRs translate environmental events into long-term changes in neural gene expression. This has led to the hypothesis that dysfunction in EGRs may account for both the genetic and environmental influences on risk for psychiatric illnesses.[32,41,56] The *EGR* family has been more closely scrutinized in schizophrenia, with fewer and less-consistent studies in BD.[47,56–58] *EGR3* has been significantly associated with schizophrenia in the Japanese,[40] Korean and Han Chinese populations,[59,60] and recently in a population of European descent.[41] Although not all studies have

found significant associations,[57,61,62] a meta-analysis of the studies in Asian populations supported association between *EGR3* and schizophrenia.[60] In addition, the AA genotype of the *rs35201266* SNP was recently associated with the hemodynamic state of the prefrontal cortex in both patients with schizophrenia and healthy participants, possibly suggesting a pathway from neurodevelopment to brain function.[63] Furthermore, a study involving an entire network of TFs and microRNAs related to schizophrenia identified *EGR3* as the central gene in the regulatory network.[55]

Studies examining a potential role for *EGR3* in BD identified nominal associations that did not meet the threshold for significance following the strict Bonferroni correction for multiple comparisons. In the first study, examining association of genes involved in circadian rhythms with BD, *EGR3* was to sole gene that achieved a significance level of $P < 0.05$.[57] A second investigation, a family-based association study identified a nominal association of *EGR3* with risk for child with BD I.[56] These findings suggest that *EGR3* may be a fruitful gene for future genetics studies to identify mechanisms by which environment and genetic predisposition interact to influence BD. Although the statistically significant findings supporting an association between *EGR3* and psychiatric illness have been in schizophrenia, research has increasingly demonstrated that the molecular and genetic processes underlying BD and schizophrenia are highly coincident.[64]

The possible effects of mood stabilizers, psychotropic medications and substance use on the *EGR3* regulon are also an interesting point to be discussed. However, there are few studies on this matter, most of them evaluating other *EGR* genes. To our knowledge, there are no studies showing association between lithium or valproate effects and *EGR3* expression. In this context, studies observed that the expression of *EGR1* was increased by lithium in mouse frontal cortex,[65] and by valproate in neural stem cells.[66] Considering that lithium has been associated with neurogenesis, it is conceivable that it induces *EGR* genes as well. Nevertheless, our results point to the repression of this regulon in bipolar disorder, suggesting that lithium treatment did not influence our findings. Studies in rodents have shown that other psychoactive medications induce immediate early genes in the brain. For instance, chronic treatment with aripiprazole induces differential gene expression of *EGR1*, *EGR2* and *EGR4* in the rat frontal cortex;[67] *EGR1* is differentially expressed also in rat striatum after haloperidol and clozapine treatments.[68] Though less studied than *EGR1*, expression of *EGR3* is induced by several of the same stimuli of *EGR1*, including antipsychotic medications or drugs that induce psychosis.[25,42] Other factors that might possibly affect the *EGR3* regulon is alcohol or substance use; a relationship between drug intake or withdrawal and induction of *EGR* genes has been reported. For instance, amphetamine and cocaine increase *EGR1* mRNA expression in the striatum,[69] cocaine also induce *EGR3* in the striatum[25] and amphetamine or alcohol withdrawal induce *EGR1* expression.[70] Considering these observations, it seems unlikely that antipsychotic treatment, alcohol or other substances are responsible for our findings, as they induce *EGR3* and other growth response genes, whereas our results pointed to the repression of the *EGR3* regulon in BD signatures.

Our data suggest that decreased function of *EGR3* may be involved in BD. As a MR of a network of genes and pathways that mediate critical neurobiological processes, dysfunction in *EGR3* indicates a possible explanation for both the influence of environment, as well as the role of numerous genes in the pathogenesis of BD. The identified network thus provides potential targets for follow-up experimental evaluation and development of novel therapeutics for this severe mental illness. The results presented here are both innovative and exploratory, and are therefore in need of confirmation before more definitive assertions regarding the relevance of *EGR3* in BD can be made. A new generation of bioinformatics methods has been developed to deal with the notorious limitations of functional genomics data.[12] Nevertheless, further validation through basic science laboratory approaches, including mRNA expression of *EGR3* and key interacting genes in BD postmortem PFC tissue using PCR with reverse transcription, is an important step towards firmly confirming our results. However, studies in *EGR3*-deficient mice demonstrating psychosis-like phenotypes and hyperactivity that can be reversed with antipsychotic medications that are used in treatment of BD already provide important support for our findings.[27,32,33]

Limitations of this study include the fact that only two microarray sets were used to obtain the signatures. Although we do not intend to perform an exhaustive analysis of all regions and all available data sets, our bioinformatics approach is constrained by the availability of a unique cohort study with a large sample size (to compute the regulatory unities) and the gene expression signatures interrogating the tissue under study. The starting point for our analysis was the public availability of a unique study, which sampled prefrontal cortex from people with bipolar disorder obtained using laser-capture microdissection. As this study was limited by sample size, and there are no other analyses using this technique, we next sought the largest available data set that used brain homogenates from the same prefrontal area (BA9), and we found just one study with these criteria. Hence the two data sets were formed not by all sets, but by the best and most interesting sets available. Other limitation is that the analysis was restricted to prefrontal cortex (BA9) and gene expression profiles might look different in other laminar and brain regions. Future studies should aim at evaluating laminar and regional specificity of our results, and validating our findings with biochemical/molecular analyses in independent biological samples, as well as studying *EGR3* targets, their role in BD and in the mechanisms of action of drugs. When it comes to the means of modulation of *EGR3*, it is likely that genetic and epigenetic mechanisms might be underlying the alterations seen in BD, which warrants further studies on this matter as well.

In conclusion, we have used an innovative approach based on MR analysis to study transcriptional regulation in BD. This method identified the *EGR3* gene as a potential key target, with the *EGR3* regulon robustly repressed in both of the two BD gene expression data sources we examined from postmortem prefrontal cortex. Considering that *EGR3* is activated throughout the brain in response to stressful environmental stimuli, the possible disruption in biological pathways involving *EGR3* may result in an impaired response and adaptation to stress. This could result in reduced neurobiological resilience and ultimately lead to the symptoms of executive and cognitive dysfunction seen in BD. The bioinformatics approach used in this work may give insights for identifying targets possibly involved with the risk for psychiatric disorders and inspire drug-discovery programs that can affect these disorders.

CONFLICT OF INTEREST

Professor Kapczinski has received grant/research support from Astra-Zeneca, Eli Lilly, the 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. The remaining authors declare no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by the Brazilian funds CNPq/MS/SCTIE/DECIT—Pesquisas Sobre Doenças Neurodegenerativas (466989/2014-8) and MCT/CNPq INCT-TM (573671/2008-7), without interference in the ongoing work. BP received a fellowship from CAPES, Brazil; and MADB received a fellowship from CNPq, Brazil. We thank Gabriel R Fries for helpful discussions and critical reading of the manuscript.

REFERENCES

1 Kieseppa T, Partonen T, Haukka J, Kaprio J, Lönnqvist J. High concordance of bipolar I disorder in a nationwide sample of twins. *Am J Psychiatry* 2004; **161**: 1814–1821.
2 Craddock N, Sklar P. Genetics of bipolar disorder. *Lancet* 2013; **381**: 1654–1662.
3 International Schizophrenia Consortium, Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC *et al.* Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* 2009; **460**: 748–752.
4 Lee SH, Wray NR, Goddard ME, Visscher PM. Estimating missing heritability for disease from genome-wide association studies. *Am J Hum Genet* 2011; **88**: 294–305.
5 Gershon ES, Alliey-Rodriguez N, Liu C. After GWAS: searching for genetic risk for schizophrenia and bipolar disorder. *Am J Psychiatry* 2011; **168**: 253–256.
6 Altar CA, Vawter MP, Ginsberg SD. Target identification for CNS diseases by transcriptional profiling. *Neuropsychopharmacology* 2009; **34**: 18–54.
7 McCullumsmith RE, Meador-Woodruff JH. Novel approaches to the study of postmortem brain in psychiatric illness: old limitations and new challenges. *Biol Psychiatry* 2011; **69**: 127–133.
8 Seifuddin F, Pirooznia M, Judy JT, Goes FS, Potash JB, Zandi PP. Systematic review of genome-wide gene expression studies of bipolar disorder. *BMC Psychiatry* 2013; **13**: 213.
9 Hawrylycz M, Ng L, Page D, Morris J, Lau C, Faber S *et al.* Multi-scale correlation structure of gene expression in the brain. *Neural Netw* 2011; **24**: 933–942.
10 Price JL, Drevets WC. Neural circuits underlying the pathophysiology of mood disorders. *Trends Cogn Sci* 2012; **16**: 61–71.
11 Margolin AA, Wang K, Lim WK, Kustagi M, Nemenman I, Califano A. Reverse engineering cellular networks. *Nat Protoc* 2006; **1**: 662–671.
12 Castro MA, de Santiago I, Campbell TM, Vaughn C, Hickey TE, Ross E *et al.* Regulators of genetic risk of breast cancer identified by integrative network analysis. *Nat Genet* 2016; **48**: 12–21.
13 Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY *et al.* The transcriptional network for mesenchymal transformation of brain tumours. *Nature* 2010; **463**: 318–325.
14 Piao G, Saito S, Sun Y, Liu ZP, Wang Y, Han X *et al.* A computational procedure for identifying master regulator candidates: a case study on diabetes progression in Goto-Kakizaki rats. *BMC Syst Biol* 2012; **6**(Suppl 1): S2.
15 Fletcher MN, Castro MA, Wang X, de Santiago I, O'Reilly M, Chin SF *et al.* Master regulators of FGFR2 signaling and breast cancer risk. *Nat Commun* 2013; **4**: 2464.
16 Colantuoni C, Lipska BK, Ye T, Hyde TM, Tao R, Leek JT *et al.* Temporal dynamics and genetic control of transcription in the human prefrontal cortex. *Nature* 2011; **478**: 519–523.
17 Harris LW, Wayland M, Lan M, Ryan M, Giger T, Lockstone H *et al.* The cerebral microvasculature in schizophrenia: a laser capture microdissection study. *PLoS One* 2008; **3**: e3964.
18 Ryan MM, Lockstone HE, Huffaker SJ, Wayland MT, Webster MJ, Bahn S. Gene expression analysis of bipolar disorder reveals downregulation of the ubiquitin cycle and alterations in synaptic genes. *Mol Psychiatry* 2006; **11**: 965–978.
19 Castro MA, Wang X, Fletcher MN, Meyer KB, Markowetz F. RedeR: R/Bioconductor package for representing modular structures, nested networks and multiple levels of hierarchical associations. *Genome Biol* 2012; **13**: R29.
20 Meyer PE, Lafitte F, Bontempi G. minet: A R/Bioconductor package for inferring large transcriptional networks using mutual information. *BMC Bioinformatics* 2008; **9**: 461.
21 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005; **102**: 15545–15550.
22 Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ *et al.* The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* 2006; **313**: 1929–1935.
23 Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article3.
24 O'Donovan KJ, Tourtellotte WG, Millbrandt J, Baraban JM. The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci* 1999; **22**: 167–173.
25 Yamagata K, Kaufmann WE, Lanahan A, Papapavlou M, Barnes CA, Andreasson KI *et al.* Egr3/Pilot, a zinc finger transcription factor, is rapidly regulated by activity in brain neurons and colocalizes with Egr1/zif268. *Learn Mem* 1994; **1**: 140–152.
26 Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S *et al.* A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci* 2001; **4**: 289–296.
27 Gallitano-Mendel A, Izumi Y, Tokuda K, Zorumski CF, Howell MP, Muglia LJ *et al.* The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. *Neuroscience* 2007; **148**: 633–643.
28 Li L, Yun SH, Keblesh J, Trommer BL, Xiong H, Radulovic J *et al.* Egr3, a synaptic activity regulated transcription factor that is essential for learning and memory. *Mol Cell Neurosci* 2007; **35**: 76–88.
29 Cheval H, Chagneau C, Levasseur G, Veyrac A, Faucon-Biguet N, Laroche S *et al.* Distinctive features of Egr transcription factor regulation and DNA binding activity in CA1 of the hippocampus in synaptic plasticity and consolidation and reconsolidation of fear memory. *Hippocampus* 2012; **22**: 631–642.
30 Li L, Carter J, Gao X, Whitehead J, Tourtellotte WG. The neuroplasticity-associated arc gene is a direct transcriptional target of early growth response (Egr) transcription factors. *Mol Cell Biol* 2005; **25**: 10286–10300.
31 Maple AM, Zhao X, Elizalde DI, McBride AK, Gallitano AL. Htr2a expression responds rapidly to environmental stimuli in an Egr3-dependent manner. *ACS Chem Neurosci* 2015; **6**: 1137–1142.
32 Gallitano-Mendel A, Wozniak DF, Pehek EA, Milbrandt J. Mice lacking the immediate early gene Egr3 respond to the anti-aggressive effects of clozapine yet are relatively resistant to its sedating effects. *Neuropsychopharmacology* 2008; **33**: 1266–1275.
33 Williams AA, Ingram WM, Levine S, Resnik J, Kamel CM, Lish JR *et al.* Reduced levels of serotonin 2 A receptors underlie resistance of Egr3-deficient mice to locomotor suppression by clozapine. *Neuropsychopharmacology* 2012; **37**: 2285–2298.
34 Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG *et al.* Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 1995; **14**: 433–445.
35 Roberts DS, Hu Y, Lund IV, Brooks-Kayal AR, Russek SJ. Brain-derived neurotrophic factor (BDNF)-induced synthesis of early growth response factor 3 (Egr3) controls the levels of type A GABA receptor alpha 4 subunits in hippocampal neurons. *J Biol Chem* 2006; **281**: 29431–29435.
36 Thiel G, Schoch S, Petersohn D. Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. *J Biol Chem* 1994; **269**: 15294–15301.
37 Petersohn D, Schoch S, Brinkmann DR, Thiel G. The human synapsin II gene promoter. Possible role for the transcription factor zif268/egr-1, polyoma enhancer activator 3, and AP2. *J Biol Chem* 1995; **270**: 24361–24369.
38 Hippenmeyer S, Shneider NA, Birchmeier C, Burden SJ, Jessell TM, Arber S. A role for neuregulin1 signaling in muscle spindle differentiation. *Neuron* 2002; **36**: 1035–1049.
39 Gerber DJ, Hall D, Miyakawa T, Demars S, Gogos JA, Karayiorgou M *et al.* Evidence for association of schizophrenia with genetic variation in the 8p21.3 gene, PPP3CC, encoding the calcineurin gamma subunit. *Proc Natl Acad Sci USA* 2003; **100**: 8993–8998.
40 Yamada K, Gerber DJ, Iwayama Y, Ohnishi T, Ohba H, Toyota T *et al.* Genetic analysis of the calcineurin pathway identifies members of the EGR gene family, specifically EGR3, as potential susceptibility candidates in schizophrenia. *Proc Natl Acad Sci USA* 2007; **104**: 2815–2820.
41 Huentelman MJ, Muppana L, Corneveaux JJ, Dinu V, Pruzin JJ, Reiman R *et al.* Association of SNPs in EGR3 and ARC with schizophrenia supports a biological pathway for schizophrenia risk. *PLoS One* 2015; **10**: e0135076.
42 González-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M *et al.* Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *J Neurosci* 2003; **23**: 8836–8843.
43 Manji HK, Quiroz JA, Payne JL, Singh J, Lopes BP, Viegas JS *et al.* The underlying neurobiology of bipolar disorder. *World Psychiatry* 2003; **2**: 136–146.
44 Berk M, Kapczinski F, Andreazza AC, Dean OM, Giorlando F, Maes M *et al.* Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors. *Neurosci Biobehav Rev* 2011; **35**: 804–817.
45 Fernandes BS, Gama CS, Ceresér KM, Yatham LN, Fries GR, Colpo G *et al.* Brain-derived neurotrophic factor as a state-marker of mood episodes in bipolar disorders: a systematic review and meta-regression analysis. *J Psychiatr Res* 2011; **45**: 995–1004.
46 Frey BN, Andreazza AC, Houenou J, Jamain S, Goldstein BI, Frye MA *et al.* Biomarkers in bipolar disorder: a positional paper from the International Society for Bipolar Disorders Biomarkers Task Force. *Aust N Z J Psychiatry* 2013; **47**: 321–332.
47 Weigelt K, Carvalho LA, Drexhage RC, Wijkhuijs A, de Wit H, van Beveren NJ *et al.* TREM-1 and DAP12 expression in monocytes of patients with severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors. *Brain Behav Immun* 2011; **25**: 1162–1169.
48 Kim JH, Roberts DS, Hu Y, Lau GC, Brooks-Kayal AR, Farb DH *et al.* Brain-derived neurotrophic factor uses CREB and Egr3 to regulate NMDA receptor levels in cortical neurons. *J Neurochem* 2012; **120**: 210–219.
49 Gao X, Daugherty RL, Tourtellotte WG. Regulation of low affinity neurotrophin receptor (p75(NTR)) by early growth response (Egr) transcriptional regulators. *Mol Cell Neurosci* 2007; **36**: 501–514.

50 Dechant G, Barde YA. The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* 2002; **5**: 1131–1136.
51 Farris S, Lewandowski G, Cox CD, Steward O. Selective localization of arc mRNA in dendrites involves activity- and translation-dependent mRNA degradation. *J Neurosci* 2014; **34**: 4481–4493.
52 Kirov G, Pocklington AJ, Holmans P, Ivanov D, Ikeda M, Ruderfer D *et al. De novo* CNV analysis implicates specific abnormalities of postsynaptic signalling complexes in the pathogenesis of schizophrenia. *Mol Psychiatry* 2012; **17**: 142–153.
53 Fromer M, Pocklington AJ, Kavanagh DH, Williams HJ, Dwyer S, Gormley P *et al. De novo* mutations in schizophrenia implicate synaptic networks. *Nature* 2014; **506**: 179–184.
54 Purcell SM, Moran JL, Fromer M, Ruderfer D, Solovieff N, Roussos P *et al.* A polygenic burden of rare disruptive mutations in schizophrenia. *Nature* 2014; **506**: 185–190.
55 Guo AY, Sun J, Jia P, Zhao Z. A novel microRNA and transcription factor mediated regulatory network in schizophrenia. *BMC Syst Biol* 2010; **4**: 10.
56 Gallitano AL, Tillman R, Dinu V, Geller B. Family-based association study of early growth response gene 3 with child bipolar I disorder. *J Affect Disord* 2012; **138**: 387–396.
57 Mansour HA, Talkowski ME, Wood J, Chowdari KV, McClain L, Prasad K *et al.* Association study of 21 circadian genes with bipolar I disorder, schizoaffective disorder, and schizophrenia. *Bipolar Disord* 2009; **11**: 701–710.
58 Byrne EM, Heath AC, Madden PA, Pergadia ML, Hickie IB, Montgomery GW *et al.* Testing the role of circadian genes in conferring risk for psychiatric disorders. *Am J Med Genet B Neuropsychiatr Genet* 2014; **165B**: 254–260.
59 Kim SH, Song JY, Joo EJ, Lee KY, Ahn YM, Kim YS. EGR3 as a potential susceptibility gene for schizophrenia in Korea. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**: 1355–1360.
60 Zhang R, Lu S, Meng L, Min Z, Tian J, Valenzuela RK *et al.* Genetic evidence for the association between the early growth response 3 (EGR3) gene and schizophrenia. *PLoS One* 2012; **7**: e30237.
61 Liu BC, Zhang J, Wang L, Li XW, Wang Y, Ji J *et al.* No association between EGR gene family polymorphisms and schizophrenia in the Chinese population. *Prog Neuropsychopharmacol Biol Psychiatry* 2010; **34**: 506–509.
62 Kyogoku C, Yanagi M, Nishimura K, Sugiyama D, Morinobu A, Fukutake M *et al.* Association of calcineurin A gamma subunit (PPP3CC) and early growth response 3 (EGR3) gene polymorphisms with susceptibility to schizophrenia in a Japanese population. *Psychiatry Res* 2011; **185**: 16–19.
63 Nishimura Y, Takizawa R, Koike S, Kinoshita A, Satomura Y, Kawasaki S *et al.* Association of decreased prefrontal hemodynamic response during a verbal fluency task with EGR3 gene polymorphism in patients with schizophrenia and in healthy individuals. *Neuroimage* 2014; **85**(Pt 1): 527–534.
64 Shinozaki G, Potash JB. New developments in the genetics of bipolar disorder. *Curr Psychiatry Rep* 2014; **16**: 493.
65 Kim SH, Yu HS, Park HG, Ahn YM, Kim YS, Lee YH *et al.* Egr1 regulates lithium-induced transcription of the Period 2 (PER2) gene. *Biochim Biophys Acta* 2013; **1832**: 1969–1979.
66 Almutawaa W, Kang NH, Pan Y, Niles LP. Induction of neurotrophic and differentiation factors in neural stem cells by valproic acid. *Basic Clin Pharmacol Toxicol* 2014; **115**: 216–221.
67 Cheng MC, Liao DL, Hsiung CA, Chen CY, Liao YC, Chen CH. Chronic treatment with aripiprazole induces differential gene expression in the rat frontal cortex. *Int J Neuropsychopharmacol* 2008; **11**: 207–216.
68 Nguyen TV, Kosofsky BE, Birnbaum R, Cohen BM, Hyman SE. Differential expression of c-fos and zif268 in rat striatum after haloperidol, clozapine, and amphetamine. *Proc Natl Acad Sci USA* 1992; **89**: 4270–4274.
69 Moratalla R, Robertson HA, Graybiel AM. Dynamic regulation of NGFI-A (zif268, egr1) gene expression in the striatum. *J Neurosci* 1992; **12**: 2609–2622.
70 Beckmann AM, Wilce PA. Egr transcription factors in the nervous system. *Neurochem Int* 1997; **31**: 477–510.

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

3.4 CAPÍTULO 4

Caracterização neuronal do modelo de células de neuroblastoma humano SH-SY5Y diferenciadas para estudo de alvos relacionados ao transtorno bipolar

INTRODUÇÃO

Vários métodos de pesquisa são necessários na busca por um melhor entendimento dos processos biológicos, fisiológicos e clínicos que contribuem para o transtorno bipolar e outras doenças psiquiátricas. Neste contexto de estratégias complementares, atualmente tem aumentado o interesse no estudo de vias moleculares utilizando ferramentas de bioinformática e dados em larga escala disponíveis publicamente. Esse método tem o potencial de identificar alvos possivelmente envolvidos nestas doenças, seja auxiliando no entendimento do diagnóstico, tratamento ou fisiopatologia, propondo experimentos mais focados em certos alvos, para validação destes por outras abordagens experimentais (Margolin *et al*., 2006a; 2006b). Uma forma de validar experimentalmente os resultados obtidos por abordagens de bioinformática é avaliar os alvos propostos (através dos níveis de expressão gênica e proteica, por exemplo) em amostras biológicas independentes (tais como, células/tecido oriundos de pacientes ou modelos animais).

As doenças que afetam o sistema nervoso central continuam sendo um desafio para os cientistas, considerando tanto a complexidade destas doenças como a escassa disponibilidade e difícil acesso a amostras de origem neural dos pacientes para estudo. Além disso, há muitas limitações nos modelos animais para doenças psiquiátricas pela

dificuldade em mimetizar alterações de comportamento em animais (Kaiser e Feng, 2015). A busca por modelos experimentais adequados que possibilitem correlações biológicas/moleculares com eventos clínicos é fundamental no desenvolvimento dessa área de pesquisa. Neste contexto, tem sido proposto que o modelo de diferenciação das células de neuroblastoma humano da linhagem celular SH-SY5Y em células com perfil neuronal pode ser uma ferramenta interessante para estudar doenças que afetam o sistema nervoso central, como doenças neurodegenerativas e transtornos psiquiátricos.

As vantagens da linhagem do neuroblastoma humano SH-SY5Y é a sua origem humana e a capacidade de desenvolver características neuronais a partir de um processo de diferenciação *in vitro*. Previamente nosso grupo de pesquisa estabeleceu as condições experimentais para a diferenciação da linhagem SH-SY5Y em neurônios dopaminérgicos a partir da adição de ácido retinóico (AR). O AR promove *in vitro* a diferenciação neuronal e inibe a divisão celular, regulando a transição das células precursoras para células diferenciadas. Este processo resulta no surgimento de neuritos, modificação de sua morfologia epitelial para uma forma estrelada e expressão de marcadores neuronais, como a tirosina hidroxilase, enolase específica de neurônio, proteína de núcleo de neurônio e o transportador de dopamina (Lopes *et al*., 2010). Com base em dados da literatura que demonstram que a diferenciação por AR induz a expressão do receptor TrkB tornando as células responsivas ao BDNF (Kaplan *et al*., 1993, Edsjo *et al*., 2003), nosso grupo também avaliou a diferenciação da SH-SY5Y em neurônios colinérgicos com o tratamento das células com AR e BDNF. O cotratamento com BDNF resultou em células neuronais, apresentando aumento na densidade e comprimento de neuritos e em marcadores colinérgicos (atividade da acetilcolinesterase e atividade e imunoconteúdo da colina acetiltransferase) (Medeiros *et al*., 2015).

Assim, nosso grupo de pesquisa estabeleceu as melhores condições experimentais para a diferenciação do neuroblastoma humano SH-SY5Y em neurônios dopaminérgicos e colinérgicos e tem proposto a utilização deste modelo em neurociências (Lopes *et al*., 2010; 2012; Schönhofen *et al*., 2015). Nessa linha, este modelo apresenta potencial de ser uma ferramenta para estudo do transtorno bipolar, incluindo seu uso para complementar e validar dados obtidos por outras abordagens experimentais. No entanto, ainda é restrito o conhecimento sobre os mecanismos ou as vias envolvidas no processo de diferenciação das células SH-SY5Y, o que indica a necessidade de mais estudos com estratégias experimentais diferentes e inovadoras para aprimorar a caracterização deste modelo celular. Nesse sentido, a tecnologia de microarranjo tornou-se uma ferramenta muito útil no estudo de vias complexas, uma vez que permite avaliar a expressão de milhares de transcritos simultaneamente e assim contribuir no entendimento de processos biológicos já conhecidos e na elucidação de novos processos.

Um dos objetivos deste trabalho foi realizar uma análise de microarranjo nas células SH-SY5Y utilizadas como modelo neural para avaliar o perfil gênico e os processos biológicos relevantes de cada fenótipo (células indiferenciadas e células diferenciadas). A caracterização deste modelo conforme seu perfil de expressão gênica foi fundamental para o objetivo primário deste estudo, que consistiu em avaliar se esse modelo celular seria apropriado para estudar a fisiopatologia do transtorno bipolar e validar os dados de bioinformática relacionados ao capítulo 3 desta tese. Para isso, mais especificamente, avaliou-se o envolvimento da unidade regulatória (regulon) do *EGR3* no processo de diferenciação das SH-SY5Y, ou seja, se o regulon está envolvido com a diferenciação e se alvos do *EGR3* são diferencialmente expressos neste processo.

Além disso, a análise de microarranjo nas células SH-SY5Y permitiu disponibilizar dados para elucidar as redes reguladoras deste modelo celular e extrair assinaturas moleculares deste processo. Como exemplo dessa aplicação, destaca-se um estudo (Anexo 4) relacionado com a análise de potenciais diferenças entre células indiferenciadas e células diferenciadas com ácido retinóico em relação a vários parâmetros celulares, que será abordado a seguir.

Outro objetivo deste capítulo da presente tese consistiu em avaliar moléculas com potencial de atuar nos regulons enriquecidos no transtorno bipolar. Através de uma ferramenta de bioinformática chamada de Mapa de Conectividade, é possível comparar vários compostos e observar perfis similares entre eles e a assinatura gênica de doenças, fornecendo candidatos interessantes para testar em modelos experimentais (Lamb *et al*., 2006).

MÉTODOS

Cultivo celular e protocolo de diferenciação

As células da linhagem de neuroblastoma humano SH-SY5Y proliferativas foram cultivadas em meio DMEM: F12 (1:1) contendo 10 % de soro fetal bovino (SFB), em incubadora úmida a 37°C e 5 % de CO_2. O protocolo de diferenciação das células proliferativas em modelo neural envolve a redução da concentração de SFB para 1 % e adição de 10 μM de ácido retinóico durante sete dias, com troca de meio a cada três dias. Depois desse protocolo, as células adquirem características morfológicas e bioquímicas de neurônios dopaminérgicos, conforme descrito anteriormente (Lopes *et al*., 2010). Esse protocolo de diferenciação é o protocolo inicialmente estabelecido

como diferenciação dopaminérgica, e é o mais utilizado até o momento no grupo de pesquisa. Além deste, mais recentemente, padronizou-se também o protocolo de diferenciação colinérgica, que difere do primeiro pela adição da neurotrofina BDNF (10 nM) ao meio de cultura a partir do quarto dia de diferenciação, e que também foi utilizado neste trabalho (Figura 4). O processo de diferenciação em células 'tipo neuronal' foi confirmado pela diminuição da proliferação celular, alteração da morfologia da célula para um formato mais estrelado e aumento da densidade de neuritos.

Isolamento de RNA e Microarranjo

Para a realização do microarranjo para avaliação do perfil de expressão gênica das células SH-SY5Y proliferativas e diferenciadas, realizou-se quatro grupos experimentais (Figura 4):

1) células proliferativas ('*Proliferative*');

2) células diferenciadas com ácido retinóico por 4 dias ('*4d Differentiation'*);

3) células diferenciadas com ácido retinóico por 7 dias ('*7d Differentiation'*);

4) células diferenciadas com ácido retinóico por 7 dias e com adição de BDNF a partir do 4º dia de diferenciação ('*BDNF Differentiation'*).

A partir destas células SH-SY5Y não tratadas (indiferenciadas) ou submetidas aos protocolos de diferenciação neuronal com ácido retinóico somente ou ácido retinóico+BDNF, obteve-se 16 amostras para o microarranjo. Para cada amostra, utilizou-se 10 milhões de células para a extração de RNA. Para isso, o isolamento de

RNA das amostras seguiu o protocolo de extração por *TRIzol Reagent* (Life Technologies) e purificação e tratamento com DNAse com kits comerciais (*Qiagen RNeasy Mini Kit* e *Qiagen RNase-Free DNase Set*, respectivamente). As amostras de RNA foram quantificadas com o uso de NanoDrop (Thermo) com rendimento final mínimo de 100 ng/µL e armazenadas a – 80°C para posterior análise por microarranjo.

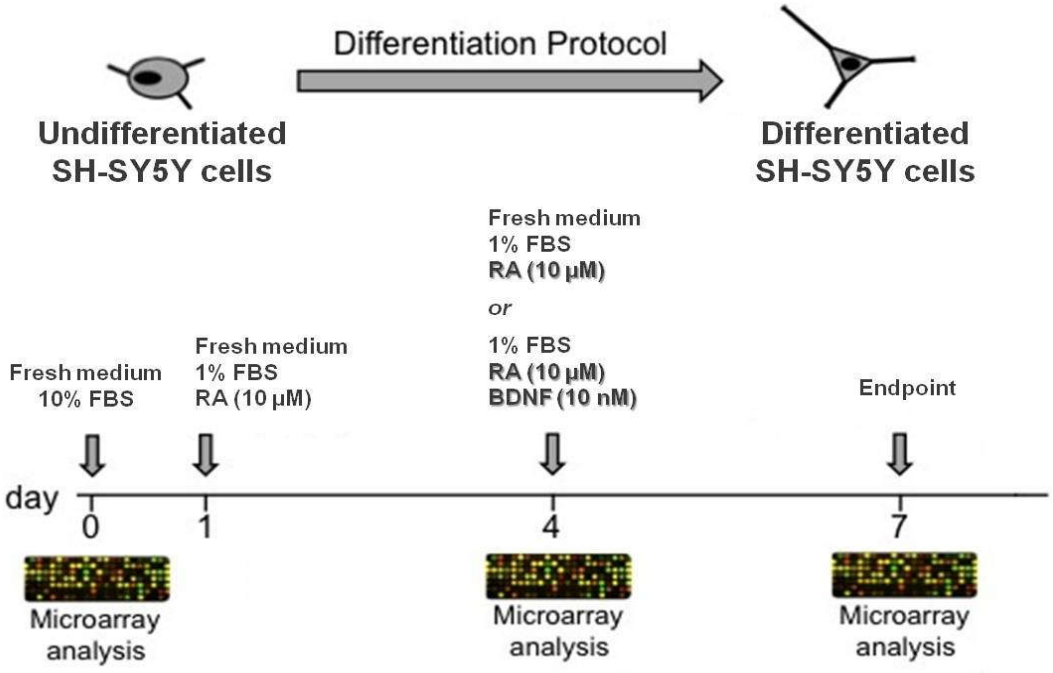


Figura 4. Esquema do protocolo de diferenciação das células SH-SY5Y. No dia 0, as células indiferenciadas foram cultivadas em meio contendo 10% de soro fetal bovino (FBS). Após 24 horas (dia 1), o meio de cultivo anterior foi removido e adicionado meio fresco contendo 1% de FBS e 10 µM de ácido retinóico (RA) (meio de diferenciação). Após três dias (dia 4), o meio de diferenciação foi substituído por meio fresco, que pode ser meio contendo somente RA (para diferenciação dopaminérgica) ou meio contendo também 10 nM de BDNF (para diferenciação colinérgica). No dia 7, as células SH-SY5Y estão diferenciadas e prontas para a realização dos experimentos de interesse. Neste caso, as células foram coletadas para as análises de microarranjo em quatro condições diferentes do protocolo (dia 0 = células proliferativas; dia 4 = células diferenciadas com RA por quatro dias; dia 7 = células diferenciadas com RA por sete dias, ou diferenciadas com RA/BDNF).

A técnica de microarranjo consiste na hibridização entre sondas (sequências complementares aos mRNAs alvo que compõe o chip) e o RNA das amostras, para a análise de expressão gênica. É uma ferramenta que permite realizar análises globais e

prover informações sobre mudanças nos níveis e na dinâmica de componentes celulares, para a avaliação sistêmica de vias/processos biológicos em amostras de ensaios celulares. O microarranjo foi realizado utilizando o chip *GeneChip® PrimeView™ Human Gene Expression Array* (Affymetrix), conforme orientações do fabricante. Resumidamente, a partir de 100 ng de RNA total, é gerado RNA complementar (RNAc) amplificado e biotinilado. Após purificação e fragmentação, 10 µg de cRNA foram hibridizados por 16 horas a 45 ºC no *GeneChip Human PrimeView* (Affymetrix), e estes então foram lavados e marcados na estação *Affymetrix Fluidics Station 450*. Para o passo final, os chips foram escaneados usando *o GeneChip® Scanner 3000 7G* (Affymetrix).

Análise dos dados do microarranjo

Para análise do microarranjo, primeiramente o controle de qualidade da extração de RNA, o processamento para hibridização, a marcação com fluorescência e a aquisição dos dados foram avaliados e os dados brutos processados por bioinformata utilizando o pacote do *Bioconductor* em ambiente R. Os dados processados foram submetidos a um repositório público de dados de genômica funcional (*Gene Expression Omnibus* - GEO).

Análise de componentes principais (PCA analysis)

Análise de Componentes Principais (PCA) é uma técnica matemática utilizada para enfatizar as variações entre as amostras. Consiste em uma transformação linear ortogonal que transforma os dados para um novo sistema de coordenadas de forma que a maior variância dos dados fica ao longo da primeira coordenada (chamado

de primeiro componente – PC1), a segunda maior variância fica ao longo da segunda coordenada (PC2), e assim por diante. Essa análise visa encontrar padrões em dados de elevada dimensão para expressar estes dados de modo a realçar as suas semelhanças e diferenças, facilitando a visualização.

Análise de expressão diferencial e análise de enriquecimento de processos biológicos

A análise dos genes diferencialmente expressos foi realizada no pacote *limma* do software Bioconductor (Smyth, 2004), para avaliar as vias que foram moduladas diferentemente pelo processo de diferenciação das células. Essa análise foi realizada na comparação entre o fenótipo das células proliferativas e o fenótipo das células diferenciadas com ácido retinóico por sete dias (diferenciação dopaminérgica - *7d Differentiation*) visto que este é o modelo que tem sido mais utilizado. Nessa comparação, os grupos de genes diferencialmente expressos em cada fenótipo foram divididos usando uma métrica logarítimica (logFC) conforme os níveis de expressão.

Então, os grupos de genes separados dessa maneira foram utilizados para identificar os processos biológicos enriquecidos por fenótipo, pela análise de enriquecimento de grupos de genes (GSEA) usando o *Gene Ontology* (GO). GO é um projeto de bioinformática colaborativo criado para unificar a representação de genes e produtos gênicos em todas as espécies (The Gene Ontology Consortium, 2008) que permite, por exemplo, a interpretação funcional de dados experimentais através de análise de enriquecimento. O domínio do GO utilizado neste estudo refere-se aos processos biológicos, que podem ser definidos como conjuntos de eventos moleculares com início e fim definidos. Assim, considerando o grupo de genes diferencialmente expressos em cada fenótipo (células proliferativas e diferenciadas), a análise de

enriquecimento identificou quais processos biológicos do GO estão significativamente representados neste conjunto de genes, e assim enriquecidos para um determinado fenótipo.

Avaliação do regulon do EGR3 no modelo celular

Análise de expressão diferencial e análise de enriquecimento

A análise dos genes pertencentes ao regulon do *EGR3* diferencialmente expressos com o processo de diferenciação celular foi realizada no pacote *limma* do software Bioconductor (Smyth, 2004). Essa análise foi realizada entre todos os fenótipos (células proliferativas, células diferenciadas com ácido retinóico por quatro dias e sete dias e células diferenciadas com ácido retinóico+BDNF), com comparação dois a dois.

Então, a partir desta lista de genes diferencialmente expressos representada por uma métrica baseada em ranques, realizou-se a análise de enriquecimento (GSEA) para testar a associação entre um conjunto de genes (genes do regulon do *EGR3*) e os fenótipos celulares. Ou seja, avaliar se o regulon do *EGR3* está enriquecido para genes que são expressos diferencialmente entre dois fenótipos avaliados no microarranjo.

Análise do Mapa de Conectividade

A abordagem utilizando mapa de conectividade (CMAP) é descrita no trabalho de Lamb e colaboradores (2006). Consiste em um banco de dados de expressão gênica de células humanas em cultura tratadas com pequenas moléculas bioativas, analisado a

partir de algoritmos de reconhecimento de padrões que permitem a descoberta de conexões funcionais entre fármacos, genes e doenças através de alterações de expressão gênica. Resumidamente, a similaridade de uma assinatura-teste com um perfil de expressão de um banco de linhagens de referência é avaliada utilizando uma estratégia não-paramétrica de ranqueamento com base na estatística de *Kolmogorov-Smirnov*. A assinatura-teste é qualquer lista de genes cuja expressão está correlacionada com um estado biológico de interesse (neste caso, os regulons enriquecidos no transtorno bipolar).

Os perfis de expressão do banco de linhagens de referência são oriundos de microarranjos de células tratadas com 1.309 compostos diferentes. Os genes nos bancos de dados de expressão de referência são ordenados em um ranque de acordo com a sua expressão diferencial em relação ao controle (células não tratadas). Assim, cada tratamento dá origem a uma lista ordenada com um ranque de aproximadamente 22.000 genes relacionados com a molécula testada. A assinatura-teste é então comparada com cada lista ranqueada para determinar se os genes *up-regulated* da assinatura-teste tendem a aparecer perto do topo da lista ranqueada e os genes *down-regulated* na assinatura-teste, perto do fim da lista ranqueada. Isto resulta em um "escore de conectividade", que associa o estado biológico vinculado à assinatura-teste com a molécula testada nas linhagens celulares, identificando moléculas potencialmente relacionadas (positiva ou negativamente) com o estado biológico de interesse e assim propondo alvos terapêuticos a serem estudados experimentalmente.

Neste estudo, a assinatura-teste é a lista de genes (*up-regulated e down-regulated* no transtorno bipolar) pertencentes a um determinado regulon que foi enriquecido no transtorno, conforme resultados discutidos no capítulo 3 desta tese. São

eles: early growth response 3 (*EGR3*), TSC22 domain family, member 4 (*TSC22D4*), interleukin enhancer-binding factor 2 (*ILF2*), Y-box binding protein 1 (*YBX1*) e MAP-kinase activating death domain (*MADD*). Para obtenção da assinatura-teste da doença, utilizamos quatro estudos (GSE5388, GSE12679, GSE12654, GSE12649) com dados de microarranjo de córtex pré-frontal de pacientes com transtorno bipolar e controles e, a partir deles, realizamos a análise de expressão diferencial de genes pertencentes aos regulons acima. O objetivo, então, foi identificar moléculas/fármacos que potencialmente mimetizam ou antagonizam a assinatura da doença em relação aos genes de cada regulon analisado.

RESULTADOS

Análises do microarranjo

Depósito no GEO

Os dados de expressão gênica no modelo de diferenciação das células SH-SY5Y, obtidos a partir do microarranjo, submetidos no GEO foram aceitos para depósito nesse repositório sob o número de acesso GSE71817 (Figura 5). O acesso está configurado para privado por enquanto.

Figura 5. Página da internet do GEO com a visualização das informações referente ao depósito dos dados do microarranjo do modelo celular de diferenciação das células SH-SY5Y, sob o número de acesso GSE71817.

Análise de componentes principais (PCA analysis)

Essa análise identifica padrões nos dados analisados representando as suas semelhanças e diferenças. Esse método foi utilizado como um controle de qualidade da técnica de microarranjo e do modelo experimental, visto que foi observada uma nítida diferença no perfil das amostras: as células proliferativas (representadas por círculos vermelhos) estão distribuídas à esquerda do gráfico e as células diferenciadas (representadas por círculos azuis e verdes, de acordo com a etapa da diferenciação) se distribuem ao longo do outro extremo do gráfico (Figura 6).

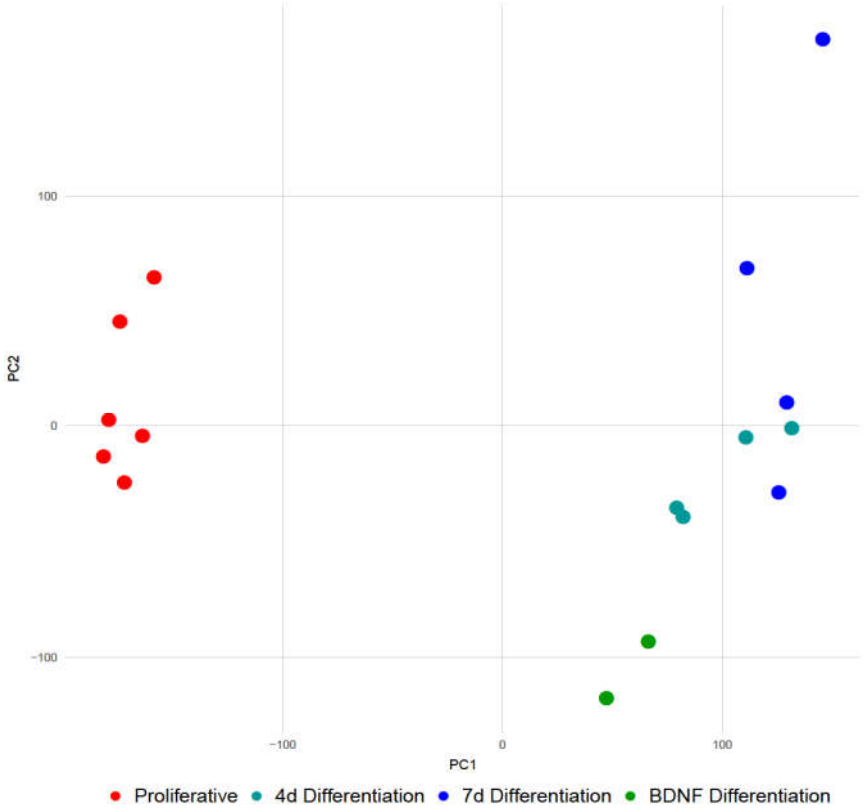


Figura 6. Análise de componentes principais (PCA). Técnica utilizada para enfatizar as variações entre as amostras, através da transformação dos dados para um novo sistema de coordenadas no qual a maior variância está representada pela primeira coordenada (PC1) e a segunda maior variância pela segunda coordenada (PC2).

Análise da expressão diferencial no modelo de diferenciação dopaminérgica

A análise de genes diferencialmente expressos foi realizada na comparação entre as células proliferativas e as células diferenciadas com ácido retinóico por sete dias (*7d Differentiation* - modelo mais utilizado no nosso grupo de pesquisa) para avaliar quais são os genes mais expressos em cada fenótipo (Tabela 1) e utilizar estes genes para validar por PCR quantitativo o experimento do microarranjo e assim confirmar o resultado de expressão diferencial.

Tabela 1. Lista de genes diferencialmente expressos na comparação entre as células SH-SY5Y proliferativas e as células diferenciadas com ácido retinóico por sete dias.

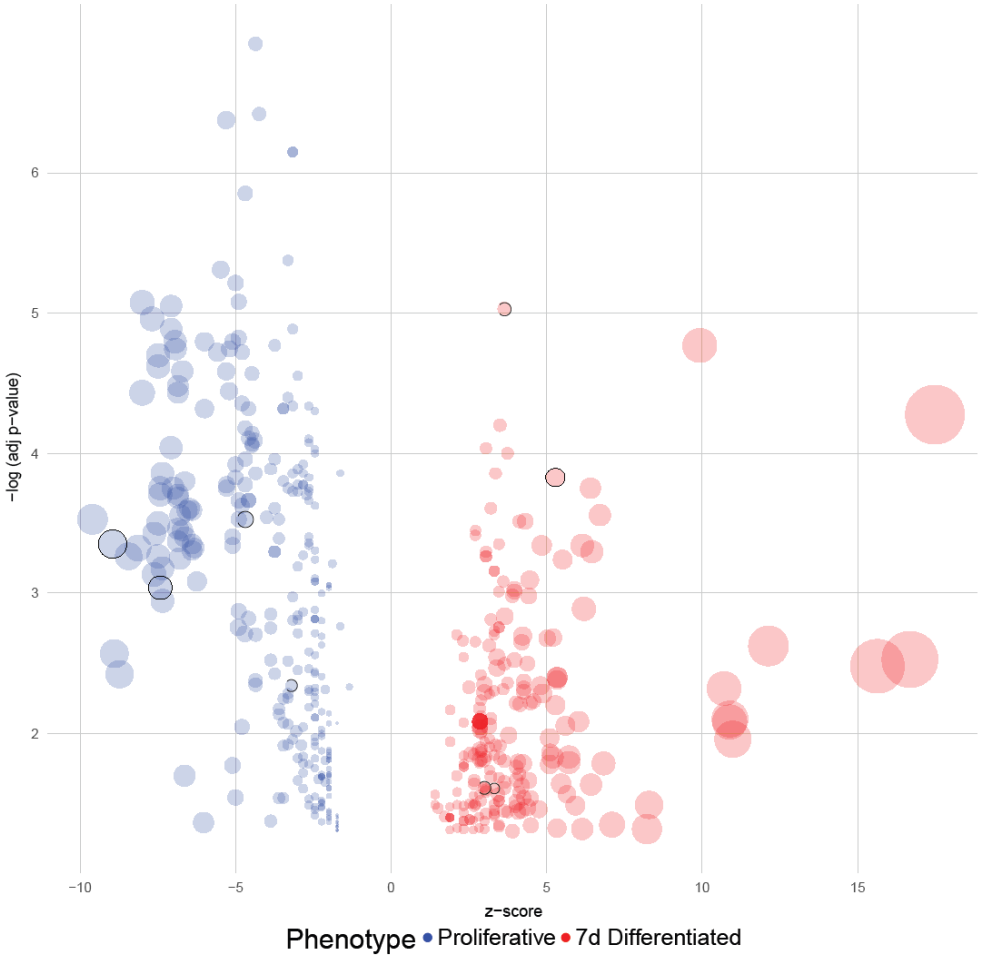
Phenotype	Gene Symbol	Gene Name
proliferative cells	*AGTR1*	angiotensin II receptor type 1
	NR4A3	nuclear receptor subfamily 4 group A member 3
	NELL1	neural EGFL like 1
	SOX6	SRY-box 6
	TMEM100	transmembrane protein 100
	RORB	RAR related orphan receptor B
	LMO4	LIM domain only 4
	IGFBP5	insulin like growth factor binding protein 5
	LRRN3	leucine rich repeat neuronal 3
	SAMD11	sterile alpha motif domain containing 11
7d differentiated cells		
	CYP26A1	cytochrome P450 family 26 subfamily A member 1
	CYP26B1	cytochrome P450 family 26 subfamily B member 1

GDF10	growth differentiation factor 10
TGM2	transglutaminase 2
NTRK2 (TRKB)	neurotrophic tyrosine kinase receptor type
TNFRSF19	tumor necrosis factor receptor superfamily member 19
SNCAIP	synuclein alpha interacting protein
DLG2	discs large homolog 2
NOS1	nitric oxide synthase 1
HTR2B	5-hydroxytryptamine receptor 2B

Representados os dez genes mais diferencialmente expressos em cada fenótipo ($p<0.001$).

Análise de enriquecimento de processos biológicos no modelo de diferenciação dopaminérgica

Com base no grupo de genes diferencialmente expressos em cada fenótipo (células proliferativas e diferenciadas), identificamos os processos biológicos contidos no GO que estão significativamente representados nestes grupos de genes, ou seja, os processos biológicos enriquecidos em cada fenótipo celular. O padrão de enriquecimento apresentou diferenças marcantes entre as células proliferativas e as células diferenciadas (Figura 7). Entre vários processos enriquecidos, nas células proliferativas se destacam os processos relacionados à proliferação celular, metabolismo, biossíntese e regulação do ciclo celular. Já nas células diferenciadas, os processos mais enriquecidos estão envolvidos na sinapse, regulação dos níveis de neurotransmissores e do potencial de membrana.



ID	*Description*
GO:0050808	Synapse organization
GO:0007268	Synaptic transmission
GO:0042391	Regulation of membrane potential
GO:0001505	Regulation of neurotransmitter levels
GO:0008283	Cell proliferation
GO:0044237	Cellular metabolic process
GO:0009058	Biosynthetic process
GO:0051726	Regulation of cell cycle

Figura 7. Processos biológicos enriquecidos nos fenótipos celulares. No grafo acima, os processos biológicos estão representados por círculos azuis quando enriquecidos nas células proliferativas e por círculos vermelhos quando o enriquecimento ocorre nas células diferenciadas. Alguns processos de destaque em cada fenótipo estão descritos abaixo do grafo com as respectivas identificações no GO, e estão representadas por círculos contornados no grafo acima.

Dados complementares de caracterização do modelo de diferenciação

O microarranjo no modelo de diferenciação das SH-SY5Y permitiu uma melhor caracterização deste modelo, com a identificação de características neuronais relevantes para discriminar entre células indiferenciadas e células diferenciadas com ácido retinóico. Resultados desta caracterização do modelo encontram-se descritos no trabalho pertencente ao Anexo 4[#] desta tese. Nesse estudo, entre outras análises, avaliou-se a caracterização neuronal, caracterização dopaminérgica e caracterização redox a partir dos dados de microarranjo das SH-SY5Y. Entre os dados deste trabalho, por exemplo, foi observado um significativo aumento nos níveis de expressão gênica de componentes do ciclo da vesícula sináptica nas células diferenciadas com ácido retinóico comparado às células proliferativas, por análise de enriquecimento (GSEA; P<0.05, Figura 8).

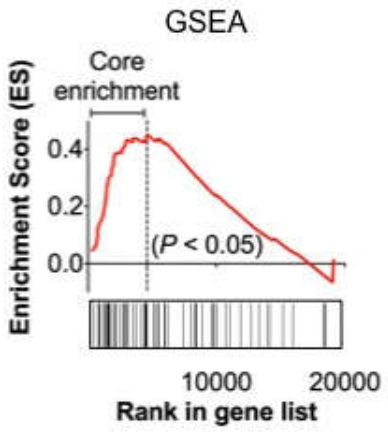


Figura 8. Enriquecimento da rede de ciclo da vesícula sináptica nas células diferenciadas com ácido retinóico. Pela análise de GSEA, foi observado um enriquecimento significativo de genes dessa rede essencial para a sinapse nas células diferenciadas.

[#]Artigo pertencente ao Anexo 4 desta tese (Lopes FM, Motta LL, De Bastiani MA, Pfaffenseller B, Aguiar BW, Souza LF et al. Dissimilar Mechanism of Action and Dopamine Transporter Dependency of 6-Hydroxydopamine-Induced Toxicity in Undifferentiated and RA-Differentiated SH-SY5Y Human Neuroblastoma Cells. 2016. Submetido na Cell Death & Disease).

Para a caracterização dopaminérgica, realizou-se análise de enriquecimento (GSEA) para identificar os genes que contribuem individualmente com as alterações globais na expressão de genes da rede de sinapse dopaminérgica nas células diferenciadas. Através de análise de expressão gênica diferencial, alguns marcadores dopaminérgicos de neurônios pré-sinápticos apresentaram sua expressão significantemente aumentada nas células diferenciadas comparadas às células proliferativas (Figura 9).

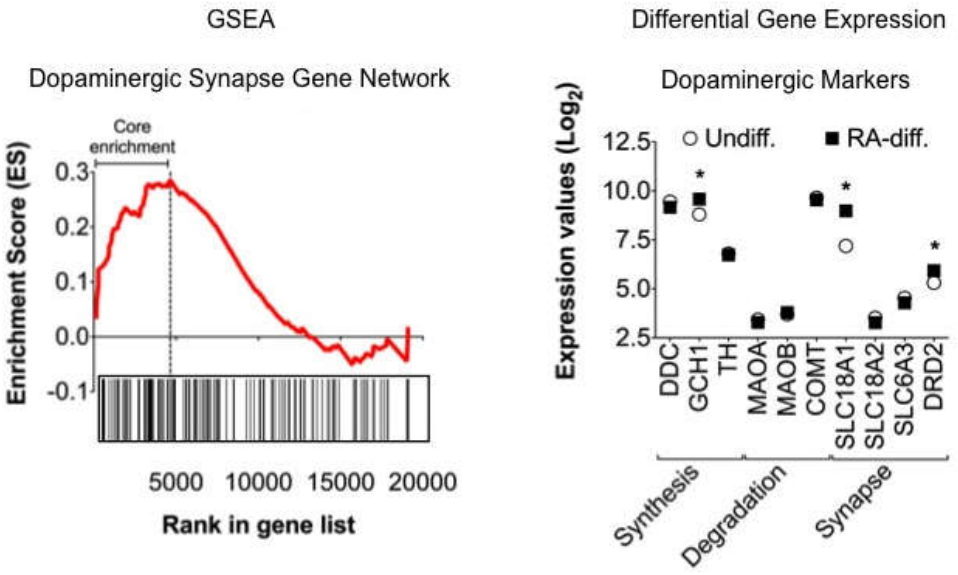


Figura 9. Análise de enriquecimento (GSEA) da rede de sinapse dopaminérgica nas células diferenciadas e análise de expressão diferencial de marcadores dopaminérgicos pré-sinápticos nas células proliferativas e diferenciadas.

Avaliação do regulon do EGR3 no modelo celular

Como o modelo de células SH-SY5Y diferenciadas tem sido proposto para estudo em neurociências e as análises de caracterização deste modelo tem reforçado essa aplicação demonstrando que as células diferenciadas apresentam um perfil neuronal interessante, há o interesse também em utilizar este modelo celular para avaliar vias e alvos que têm sido associados ao transtorno bipolar, como o regulon do *EGR3* (conforme discutido no capítulo 3 desta tese). Com os dados de microarranjo das SH-SY5Y disponíveis, foi possível avaliar como este regulon é modulado pela diferenciação.

Análise de expressão diferencial

Os genes integrantes do regulon do *EGR3* (alvos deste fator de transcrição) que foram diferencialmente expressos, de forma significativa, com o processo de diferenciação das células SH-SY5Y estão representados na Tabela 2. Não houve muita diferença entre os fenótipos de células diferenciadas (4d, 7d e BDNF), mas sim entre as diferenciadas de um modo geral e as proliferativas. Dois genes (*SLC7A14* e *EGR1)* tiveram expressão diferencial apenas nas células que já completaram o processo de diferenciação (7d e BDNF) comparado com as células indiferenciadas.

Tabela 2. Lista de genes integrantes do regulon do *EGR3* diferencialmente expressos nas células SH-SY5Y diferenciadas comparadas com as células proliferativas.

Phenotype	Gene Symbol	Gene Name
	CYP26B1	cytochrome P450 family 26 subfamily B member 1
4d Differentiation	*WSCD2*	WSC domain containing 2
7d Differentiation	*RGS7*	regulator of G-protein signaling 7

BDNF Differentiation		
	SCG5	secretogranin V
	C19orf66	chromosome 19 open reading frame 66
	SLC6A17	solute carrier family 6 member 17
7d Differentiation	*SLC7A14*	solute carrier family 7 member 14
BDNF Differentiation		
	EGR1	early growth response 1

Análise de enriquecimento

A partir desta lista de genes diferencialmente expressos, realizou-se a análise de GSEA para testar a associação entre os genes do regulon do *EGR3* e os fenótipos celulares do modelo de diferenciação. Ou seja, avaliamos se o regulon do *EGR3* está enriquecido para genes que são expressos diferencialmente entre dois fenótipos avaliados no microarranjo.

Observamos que o regulon de *EGR3* está enriquecido nos fenótipos de células diferenciadas (4d, 7d e BDNF) conforme Figuras 10 e 11. No caso das células que já completaram o protocolo de diferenciação com BDNF, houve enriquecimento de *EGR3* comparado às células no momento em que se adiciona BDNF no protocolo de diferenciação (após 4 dias com ácido retinóico) (Figura 11). O enriquecimento do regulon de *EGR3* também foi observado nas células diferenciadas com BDNF comparado às células diferenciadas somente com ácido retinóico (Figura 12), indicando que o regulon foi modulado por BDNF.

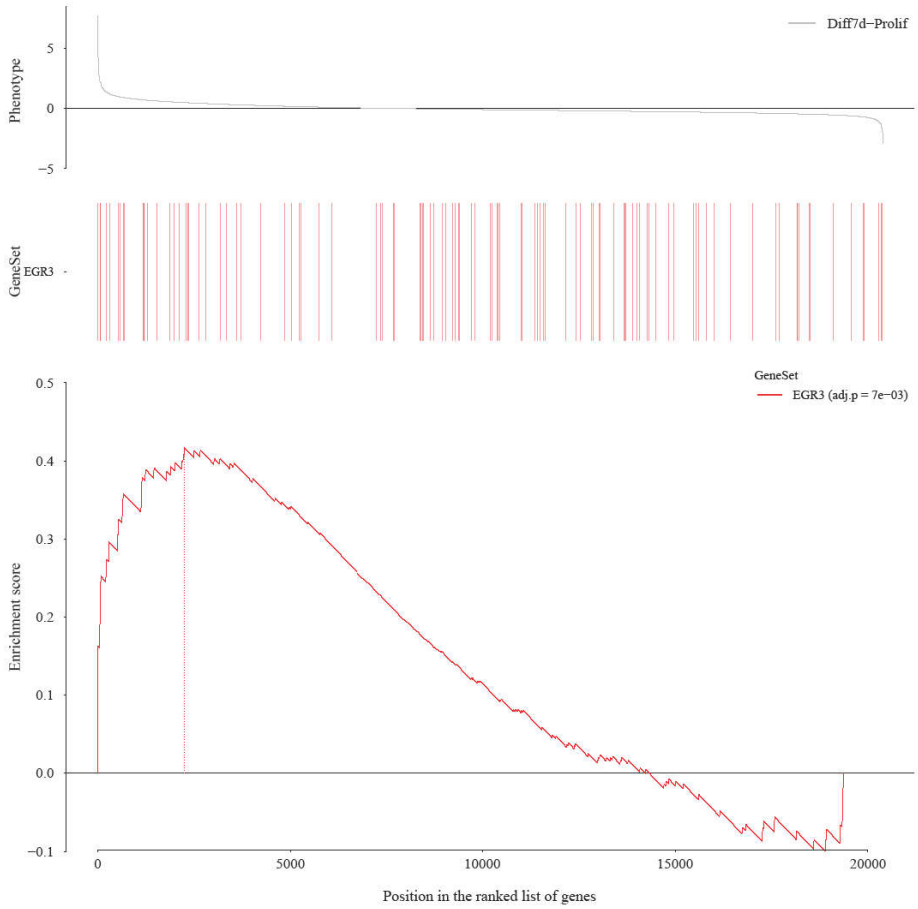


Figura 10. Enriquecimento do regulon de *EGR3* nas células diferenciadas por 7 dias com ácido retinóico comparado às células proliferativas.

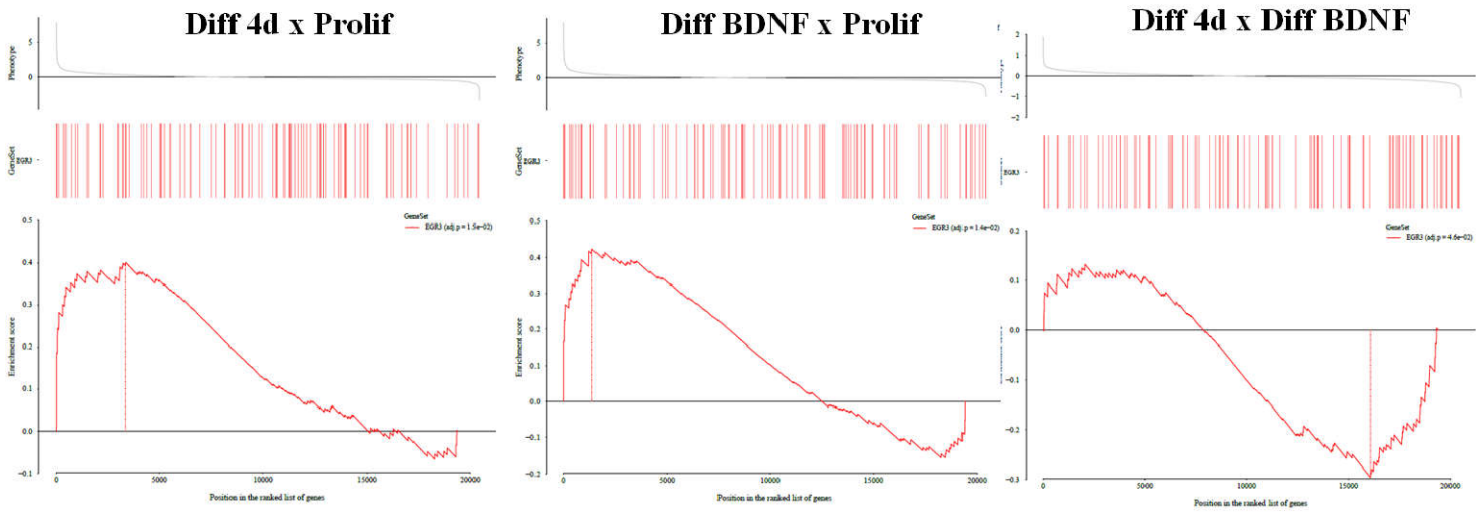


Figura 11. Enriquecimento do regulon de *EGR3* nas células diferenciadas por 4 dias com ácido retinóico e por 7 dias com ácido retinóico + BDNF comparado às células proliferativas. As células que já completaram o protocolo de diferenciação com BDNF apresentam maior enriquecimento de *EGR3* comparado às células nas quais ainda não se adicionou BDNF (4 dias de diferenciação com ácido retinóico).

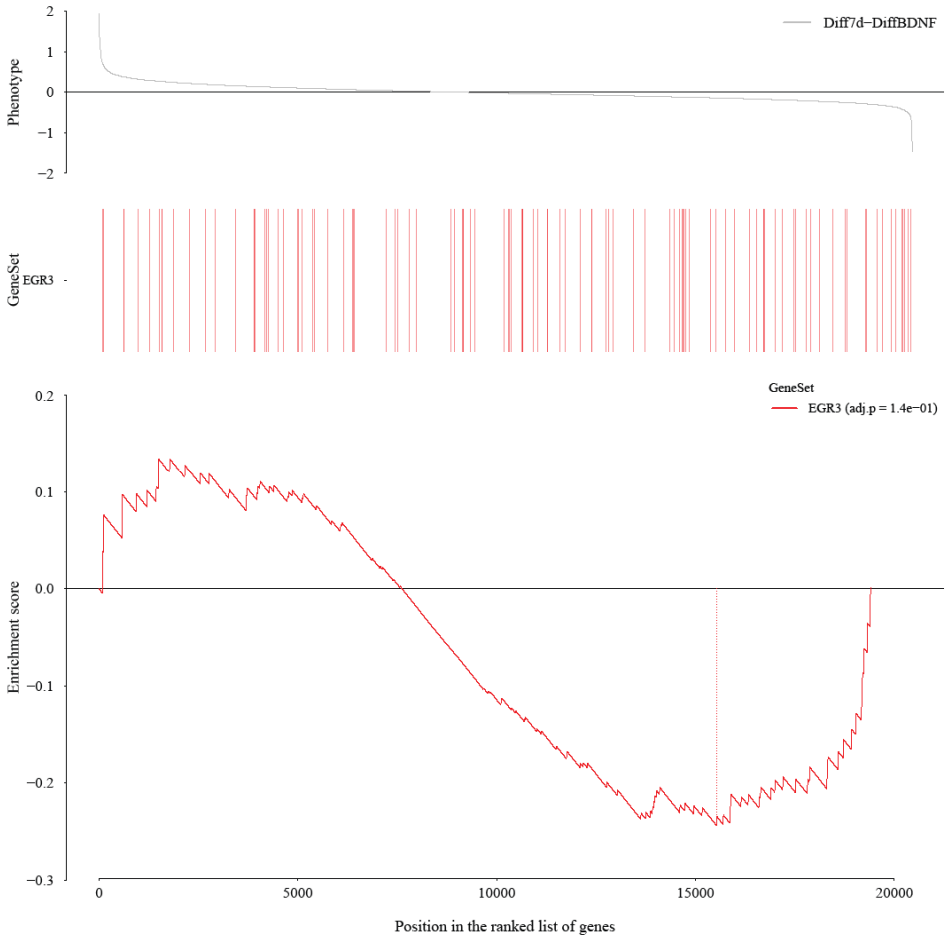


Figura 12. Enriquecimento do regulon de *EGR3* nas células diferenciadas com BDNF comparado às células diferenciadas com ácido retinóico por sete dias.

Análise do Mapa de Conectividade

Através desta análise, avaliamos as moléculas que poderiam estar associadas com a assinatura do transtorno bipolar relacionada aos cinco regulons enriquecidos (*EGR3*, *TSC22D4*, *ILF2*, *YBX1* e *MADD)* conforme discutido no capítulo 3, selecionando apenas aquelas que apresentaram associação em pelo menos três (Figura 13) dos quatro estudos utilizados para obtenção da assinatura-teste da doença (GSE5388, GSE12679, GSE12654, GSE12649). Em seguida, focamos no regulon do *EGR3* avaliando neste caso também a assinatura de *EGR3* obtida a partir da rede

transcricional do córtex pré-frontal somente da fase adulta (TN2) além daquela obtida pela rede inteira (TN1) (Figura 14).

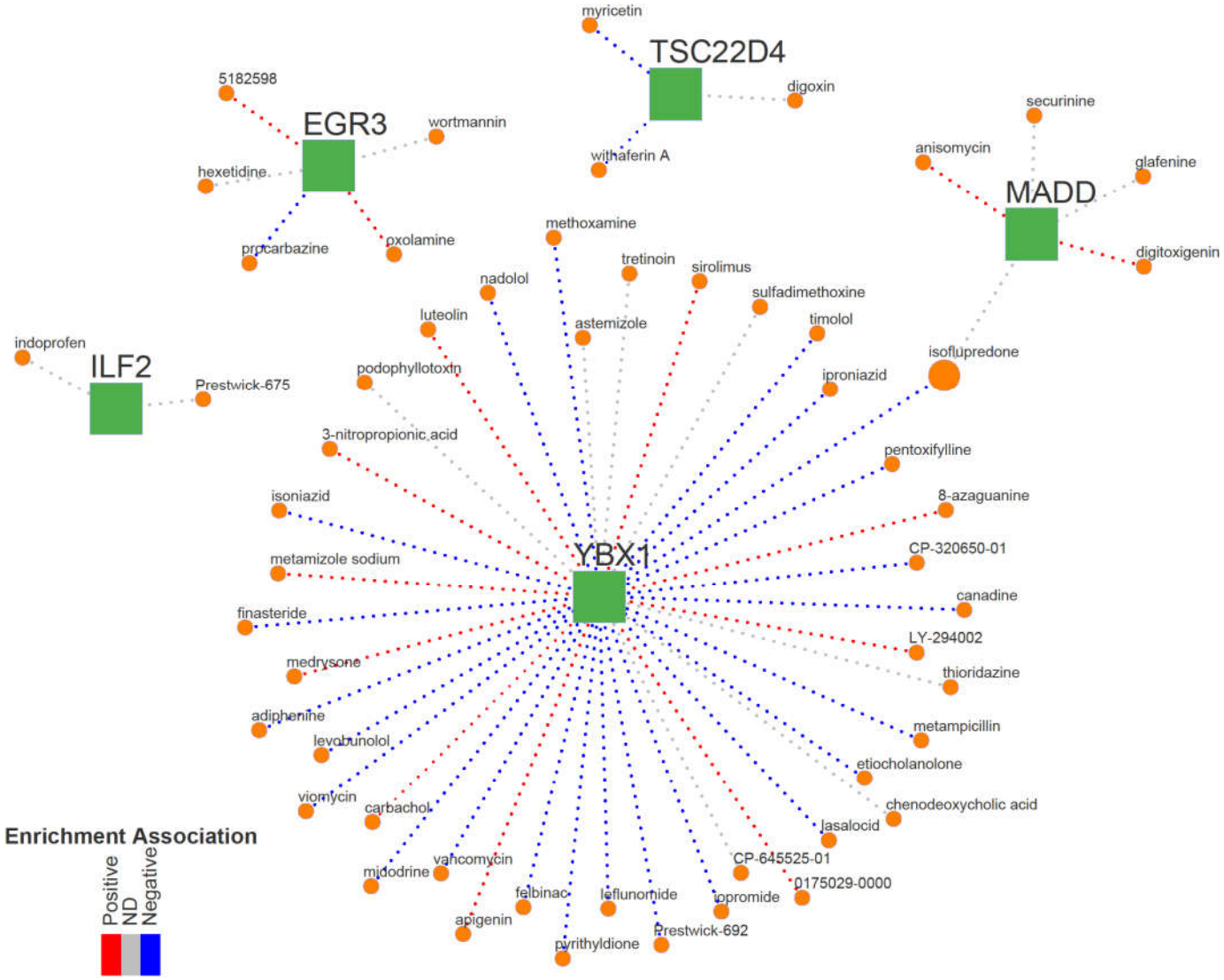


Figura 13. Mapa de conectividade para os regulons enriquecidos no transtorno bipolar. Cada círculo representa uma molécula associada significativamente à assinatura gênica dos regulons enriquecidos no transtorno bipolar. Associação positiva (vermelho) significa que a molécula tem potencial de mimetizar a assinatura; associações negativas (azul) representam o potencial das moléculas antagonizarem a assinatura. Nesta análise, utilizou-se como base a rede TN1 (rede transcricional do córtex pré-frontal ao longo do desenvolvimento).

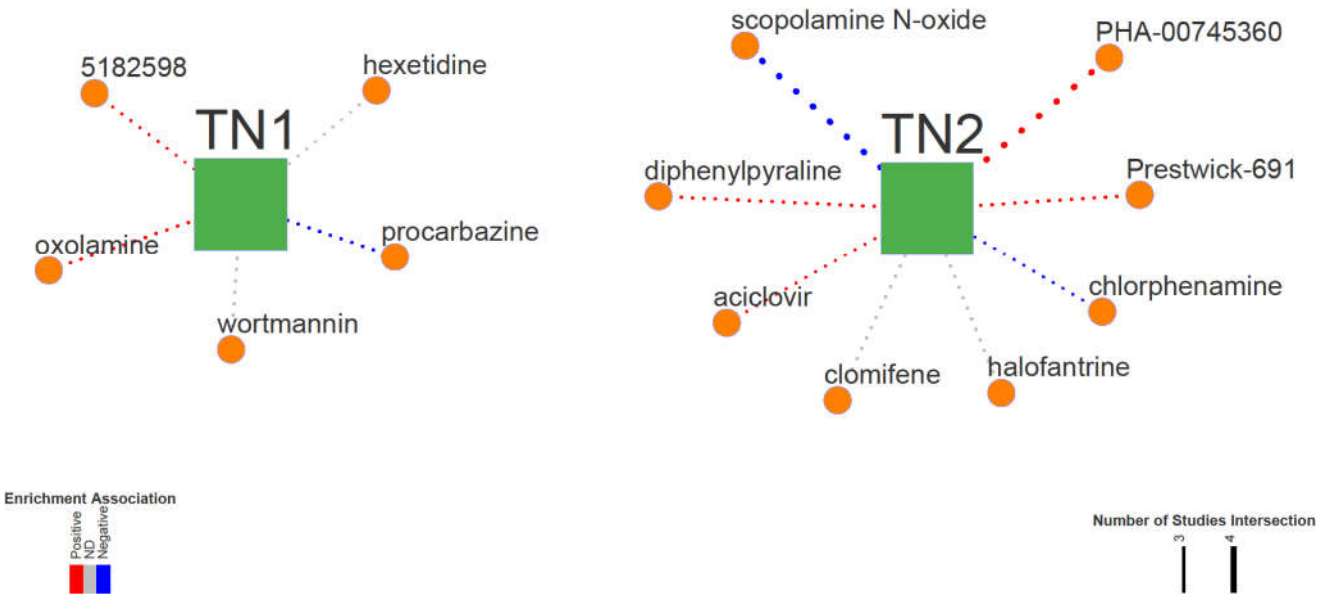


Figura 14. Mapa de conectividade para o regulon *EGR3* em duas redes transcricionais. Cada círculo representa uma molécula associada significativamente à assinatura gênica do regulon de *EGR3* enriquecido no transtorno bipolar. Associação positiva (vermelho) significa que a molécula tem potencial de mimetizar a assinatura; associações negativas (azul) representam o potencial das moléculas antagonizarem a assinatura. TN1 = rede transcricional do córtex pré-frontal ao longo do desenvolvimento; TN2 = rede transcricional do córtex pré-frontal somente da fase adulta.

As moléculas associadas significativamente à assinatura do transtorno bipolar considerando os regulons enriquecidos no fenótipo da doença e as suas atividades estão descritas na Tabela 3.

Tabela 3. Moléculas associadas à assinatura do transtorno bipolar identificadas pela análise de CMAP.

Molecule	Activities	Regulon
Aciclovir	Antiviral	*EGR3*[#]
Chlorphenamine	Antihistamine	*EGR3*[#]
Clomifene	Selective estrogen receptor modulator	*EGR3*[#]
Diphenylpyraline	Antihistamine with anticholinergic effects Act as a dopamine reuptake inhibitor and produces hyperactivity in rodents	*EGR3*[#]
Halofantrine	Treatment of malaria	*EGR3*[#]
PHA-00745360	Undefined	*EGR3*[#]
Prestwick-691	Undefined	*EGR3*[#]
Scopolamine N-oxide	Competitive antimuscarinic agent that blocks binding at the acetylcholine receptor	*EGR3*[#]
5182598	Undefined	*EGR3*
Hexetidine	Bactericidal and fungicidal antiseptic	*EGR3*
Oxolamine	Analgesic, anti-inflammatory, local anaesthetic and antisposmodic properties Posseses an antitussive activity	*EGR3*
Procarbazine	Alkylating drug used as an antineoplasic agent in the treatment of Hodgkin's disease	*EGR3*
Wortmannin	Selective protein kinase inhibitor, including PI3K Phosphodiesterase inhibitor Immunosuppressive agent Serotonin antagonist Insulin antagonist Antifungal agent	*EGR3*
Indoprofen	Anti-inflammatory - Cyclooxygenase inhibitor	*ILF2*
Prestwick-675/Albendazole	Antiprotozoal and anthelmintic agent	*ILF2*

Molecule	Activities	Regulon
Anisomycin	Nucleic acid synthesis inhibitor Protein synthesis inhibitor Anti-bacterial and antiprotozoal agent	*MADD*
Digitoxigenin	Cardenolide, a type of steroid	*MADD*
Glafenine	Analgesic agent that typically do not bind to opioid receptors	*MADD*
Isoflupredone	Glucocorticoid	*MADD*
Securinine	Selective GABA receptor antagonist	*MADD*
Digoxin	Specific Na-K ATPase inhibitor Cardiac glycoside that acts as anti-arrhythmia agent	*TSC22D4*
Myricetin	Belongs to the flavonol class of polyphenols Acts as a redox-dependent topoisomerase II poison	*TSC22D4*
Withaferin A	Steroidal lactone that binds to and inhibits vimentin Antiangiogenesis compound and proteasome inhibitor	*TSC22D4*
0175029-0000	Cyclin-dependent kinase inhibitor (CDKi)	*YBX1*
3-nitropropionic acid	Specific inhibitor of mitochondrial respiratory complex II	*YBX1*
8-azaguanine	Purine analogue with potential antineoplastic activity	*YBX1*
Adiphenine	Parasympatholytic Inhibitor of cholinergic receptors and antispamodic	*YBX1*
Apigenin	Bioactive flavonoid with anti-inflammatory, antioxidant and anticancer properties Inhibitory effect on proinflammatory cytokine production	*YBX1*
Astemizole	Second generation H1-receptor antagonist Long-acting and non-sedative antihistaminic	*YBX1*

Molecule	Activities	Regulon
Canadine	Anti-arrhythmia agent Platelet aggregation inhibitor Calcium channel blocker	*YBX1*
Carbachol	Parasympathomimetic that binds on both muscarinic and nicotinic receptors Cholinergic agonist Non-narcotic analgesic Cardiotonic agent	*YBX1*
Chenodeoxycholic acid	Cathartic and gastrointestinal agent; supresses hepatic synthesis of both cholesterol and cholic acid	*YBX1*
CP-320650-01	Undefined	*YBX1*
CP-645525-01	Undefined	*YBX1*
Etiocholanolone	Inhibitory androstane neurosteroid, acting as a positive allosteric modulator of the GABA-A receptor and possesses anticonvulsant effects	*YBX1*
Felbinac	Non-steroidal anti-inflammatory agent Possesses analgesic, antipyretic, and platelet-inhibitory actions	*YBX1*
Finasteride	5-alpha reductase inhibitor	*YBX1*
Iopromide	Molecule used as a contrast medium. It is a low osmolar, non-ionic contrast agent for intravascular use	*YBX1*
Iproniazid	Monoamine oxidase inhibitor Antidepressive agent	*YBX1*
Isoflupredone	Glucocorticoid Steroidal anti-inflammatory agent	*YBX1*
Isoniazid	Fatty acid synthesis inhibitor Antitubercular agent	*YBX1*
Lasalocid	Ionophores Anti-bacterial agent Coccidiostat	*YBX1*
Leflunomide	Pyrimidine synthesis inhibitor Possesses immunosuppressive and anti-inflammatory properties Antirheumatic agent	*YBX1*

Molecule	Activities	Regulon
Levobunolol	Adrenergic beta-antagonist	*YBX1*
Luteolin	Flavonoid. Presents anti-inflammatory, antiallergic and neuroprotective properties Potent anticancer action	*YBX1*
LY-294002	Acts in vivo as a highly selective inhibitor of phosphatidylinositol 3 kinase (PI3K)	*YBX1*
Medrysone	Synthetic glucocorticoid with metabolic, anti-inflammatory and antiallergic properties	*YBX1*
Metamizole sodium	Non-steroidal anti-inflammatory drug that possesses also analgesic and antipyretic properties	*YBX1*
Metampicillin	Penicillin antibiotic; anti-bacterial agent	*YBX1*
Methoxamine	Adrenergic alpha-1 receptor agonist that causes prolonged peripheral vasoconstriction	*YBX1*
Midodrine	Ethanolamine derivative that is an adrenergic alpha-1 receptor agonist Vasoconstrictor agent	*YBX1*
Nadolol	Nonselective beta-adrenergic receptor antagonist Anti-arrhythmia and antihypertensive agent	*YBX1*
Pentoxifylline	Inhibits Th-1 mediated inflammatory and autoimmune responses, while it induces Th2-like cytokine production Phosphodiesterase inhibitor Platelet aggregation inhibitor Free radical scavenger Vasodilator agent	*YBX1*
Podophyllotoxin	Tubulin modulator and keratolytic agent It may have some antineoplastic properties	*YBX1*
Prestwick-692	Undefined	*YBX1*
Pyrithyldione	Psychoactive drug formerly used as a sedative	*YBX1*

Molecule	Activities	Regulon
Sirolimus/Rapamycin	Macrolide that is a mTOR pathway inhibitor and restores the Akt function It inhibits cytokine production Immunosuppressant, antifungal, anti-bacterial and antineoplastic properties	*YBX1*
Sulfadimethoxine	Long-acting sulfonamide antibiotic	*YBX1*
Thioridazine	Dopamine antagonist Antipsychotic used in the management of schizophrenia and others psychosis	*YBX1*
Timolol	Beta-adrenergic antagonist Antihypertensive and anti-arrhythmia agent	*YBX1*
Tretinoin	Regulator of cell proliferation and differentiation Used to treat acne and photodamaged skin and to manage keratinization disorders Antineoplastic agent	*YBX1*
Vancomycin	Tricyclic glycosylated nonribosomal peptide with bactericidal activity	*YBX1*
Viomycin	Anti-bacterial and antitubercular agent	*YBX1*

Análise feita a partir da rede transcricional TN2 (rede transcricional do córtex pré-frontal somente da fase adulta); as demais foram realizadas com a rede TN1 (rede transcricional do córtex pré-frontal ao longo do desenvolvimento)

DISCUSSÃO

Em conjunto, os dados apresentados nesse capítulo representam algumas etapas necessárias para estabelecer um modelo celular para estudo de uma doença. Neste caso, o modelo de células de neuroblastoma humano SH-SY5Y diferenciadas em células com perfil neuronal que tem sido proposto como um modelo experimental em neurociências. Para usar este modelo com este propósito, é importante se certificar de que ele possui características apropriadas para o que se quer avaliar (no nosso caso, vias e alvos que têm sido associados com o transtorno bipolar). A grande vantagem das células SH-SY5Y, além de ser de origem humana, é a capacidade de se diferenciar em células com fenótipo neuronal, conforme já descrito pela literatura e pelo nosso grupo de pesquisa (Lopes *et al*., 2010). Como o perfil neuronal destas células é o nosso grande interesse, é essencial confirmar esse perfil por diferentes metodologias e investigar com mais detalhes as diferenças entre as células proliferativas e diferenciadas. Neste sentido, a análise de microarranjo realizada neste trabalho foi essencial para fornecer dados de expressão gênica do modelo celular em questão e assim permitir uma melhor caracterização dessas células. Uma vez que os dados foram depositados em repositório público (GEO), poderão ser utilizados pela comunidade científica em vários outros estudos futuros em que a expressão gênica dessas células for uma informação relevante, contribuindo para o conhecimento nessa área.

Primeiramente, através da *análise de PCA*, foi possível confirmar a qualidade de execução da técnica de microarranjo e do modelo experimental. Como era esperado, as amostras de células proliferativas ficaram visivelmente distantes das amostras referentes às células diferenciadas no gráfico de PCA (Figura 6), o qual demonstra a variação entre as amostras na técnica. Isso quer dizer que o perfil das células proliferativas é bem distinto das diferenciadas de um modo geral. Entre as células diferenciadas (4d, 7d,

BDNF), houve uma variação mais sutil. Observamos também baixa variabilidade entre as amostras de um mesmo fenótipo, o que é importante quando se trabalha com replicatas biológicas. Assim, esta análise inicial reforçou o interesse em seguir com as análises de microarranjo para avaliar estas diferenças no perfil das células proliferativas e diferenciadas.

A *análise de expressão diferencial* nos dados de microarranjo (proliferativa x diferenciadas 7d) foi coerente com o fenótipo celular e alguns genes diferencialmente expressos (Tabela 1) terão seus níveis de mRNA avaliados por PCR quantitativo para validar a técnica de microarranjo. Observamos que a lista de genes diferencialmente expressos nas células proliferativas inclui genes envolvidos na regulação do crescimento celular (*NELL1*) e em processos de metabolismo proteico (*IGFBP5*), no desenvolvimento normal do sistema nervoso central (*SOX6*) e outros que agem como ativadores/reguladores da transcrição (*NR4A3*, *LMO4*). No caso de *LMO4*, esse gene também pode atuam como um oncogene, fazendo sentido sua alta expressão nas células proliferativas (tumorais). Já as células diferenciadas tiveram aumento considerável na expressão de genes que controlam o processo de diferenciação (*GDF10*, *NTRK2*), genes relacionados com o ácido retinóico (*CYP26A1*, *CYP26B1*, *TGM2*) e com o processo de neurotransmissão (*NOS1*, *HTR2B*). Os dois genes mais diferencialmente expressos foram *CYP26A1* e *CYP26B1*, enzimas do citocromo P450 que regulam os níveis de retinóides (como o ácido retinóico), os quais estão envolvidos com a regulação da expressão gênica. Destaca-se entre os mais expressos, o gene *NTRK2* (também conhecido com *TRKB*) que é um receptor de neurotrofinas e está associado à diferenciação celular, e o gene *DLG2* que parece interagir em locais pós-sinápticos para facilitar o agrupamento de receptores, canais iônicos, e proteínas sinalizadoras (*Gene Bank*, NCBI).

Desta forma, os genes diferencialmente expressos foram genes associados com processos importantes para cada fenótipo: células indiferenciadas com características tumorais, perfil proliferativo, metabolismo acelerado x células diferenciadas com características voltadas ao perfil neuronal e responsivas ao ácido retinóico (como era esperado), reforçando o modelo de diferenciação que gera dois distintos fenótipos das células SH-SY5Y. Para complementar essa caracterização dos fenótipos, realizamos uma análise de enriquecimento considerando o grupo de genes diferencialmente expressos, para identificar quais processos biológicos estão significativamente representados neste conjunto de genes e assim 'enriquecidos' para um determinado fenótipo (ou seja, associados a um determinado fenótipo). Os processos biológicos mais representativos nas células proliferativas foram processos relacionados à proliferação celular, ciclo celular, metabolismo celular, biogênese e processos de regulação destes mecanismos (Figura 7). Já nas células diferenciadas, além dos processos associados à resposta ao ácido retinóico, os processos biológicos que se destacaram estão envolvidos no desenvolvimento de neuritos, organização da sinapse e transmissão sináptica, liberação e transporte de neurotransmissores e regulação dos seus níveis (Figura 7), processos típicos de células neuronais. Também por análises de expressão diferencial e enriquecimento, verificamos aumento nos níveis de expressão gênica de componentes do ciclo da vesícula sináptica (Figura 8) e de alguns marcadores dopaminérgicos de neurônios pré-sinápticos (Figura 9) nas células diferenciadas comparadas às células proliferativas.

Assim, a caracterização do modelo celular através de análises de expressão gênica a partir dos dados de microarranjo corrobora com os dados morfológicos e bioquímicos avaliados anteriormente pelo nosso grupo (Lopes *et al*., 2010), o que reforça o uso das células SH-SY5Y diferenciadas como modelo celular de fenótipo

neuronal e, assim, com potenciais aplicações na área de neurociências. Entre estas aplicações, encontra-se o uso deste modelo celular para validar algum alvo, testar algum fator associado à fisiopatologia do transtorno bipolar e também investigar os efeitos de moléculas com potencial terapêutico.

No caso da primeira aplicação, foi justamente um dos objetivos deste capítulo: avaliar a adequabilidade de usar o modelo de células SH-SY5Y diferenciadas para validar os dados relacionados ao capítulo 3 desta tese, mais especificamente, estudar o alvo encontrado (regulon do *EGR3*) neste modelo celular. Para isso, após caracterizar o modelo pela expressão gênica e reforçar o seu perfil neuronal, avaliamos a possível associação do regulon do *EGR3* com algum dos fenótipos celulares. Nesta análise, consideramos, além das células proliferativas e diferenciadas por 7 dias com ácido retinóico, também células diferenciadas com ácido retinóico+BDNF (visto que o *EGR3* é responsivo ao BDNF) e células diferenciadas com ácido retinóico por 4 dias, pois é no quarto dia que o BDNF é adicionado às células no processo de diferenciação (Figura 4).

Entre os genes integrantes do regulon do *EGR3* (ou seja, alvos deste fator de transcrição) que foram diferencialmente expressos com o processo de diferenciação das células SH-SY5Y (Tabela 2), destacam-se os genes *SLC7A14* e *EGR1* que tiveram expressão aumentada nas células que já completaram o processo de diferenciação (7d e BDNF). O primeiro é um transportador transmembrana de aminoácidos e o segundo é um fator de transcrição que regula alvos envolvidos com a diferenciação. Para avaliar o regulon como um todo no modelo celular, além dos genes individuais diferencialmente expressos, realizamos a análise de enriquecimento e os resultados foram bem interessantes. O regulon do *EGR3* foi enriquecido em todos os fenótipos de células diferenciadas (4d, 7d, BDNF) quando comparado às célula proliferativas (Figura 10),

indicando que os genes do regulon do *EGR3* em conjunto são importantes para a diferenciação das SH-SY5Y. Comparando entre os grupos de células diferenciadas, o regulon se mostrou enriquecido nas células diferenciadas com BDNF comparado às células diferenciadas 4d e às células diferenciadas 7 d com AR (Figuras 11 e 12), sugerindo que o regulon seja modulado pelo BDNF conforme outros estudos têm demonstrado (Roberts *et al*., 2006).

Desta forma, o regulon do *EGR3* parece ser modulado pelo processo de diferenciação das SH-SY5Y, o que indica que os genes alvos regulados por este fator de transcrição são relevantes para a diferenciação. Considerando o envolvimento do *EGR3* no desenvolvimento neuronal e o fenótipo neuronal demonstrado pelas células SH-SY5Y diferenciadas, essas associações parecem ser contundentes e esses dados sugerem que este modelo celular é adequado para estudar o regulon do *EGR3* e sua regulação.

Nesse contexto, seria interessante utilizar o modelo celular de células SH-SY5Y diferenciadas para avaliar experimentalmente as moléculas identificadas pela análise realizada pelo Mapa de Conectividade (CMAP), que apresentam potencial de mimetizar ou antagonizar a assinatura gênica do transtorno bipolar referente aos regulons enriquecidos no transtorno (conforme capítulo 3). Relembrando que esta análise avalia a similaridade de uma assinatura-teste (neste caso, expressão diferencial de genes pertencentes aos regulons enriquecidos no transtorno bipolar) com um perfil de expressão gênica de um banco de linhagens celulares de referência tratadas com pequenas moléculas bioativas. O CMAP é uma ferramenta para gerar hipóteses, fornecendo uma associação positiva ou negativa entre a assinatura-teste da doença e a assinatura gênica da molécula avaliada. Uma associação de conectividade positiva indica que a molécula correspondente mimetiza a assinatura da doença enquanto que

uma associação negativa reverte o estado biológico representado pela assinatura da doença.

Primeiramente, avaliamos as moléculas que poderiam estar associadas com a assinatura do transtorno, nos cinco regulons enriquecidos (*EGR3*, *TSC22D4*, *ILF2*, *YBX1* e *MADD),* selecionando apenas aquelas que apresentaram associação em pelo menos três (Figura 13) dos quatro estudos utilizados para obtenção da assinatura-teste da doença (GSE5388, GSE12679, GSE12654, GSE12649). Em seguida, focamos no regulon do *EGR3* avaliando neste caso também a assinatura de *EGR3* obtida a partir da rede transcricional do córtex pré-frontal somente da fase adulta (TN2) além daquela obtida pela rede inteira (TN1) (Figura 14). Observamos associações positivas e negativas entre algumas moléculas e a assinatura dos regulons, o que nos sugere vários potenciais fármacos para estudo. Se o objetivo for testar estas moléculas experimentalmente visando um potencial uso clínico delas (no tratamento do transtorno bipolar, por exemplo), devemos nos focar naquelas que apresentaram associações negativas com a assinatura, ou seja, que tem o potencial de reverter o fenótipo bipolar. Entretanto, pensando em modelos experimentais, as moléculas com associações positivas também são interessantes, pois tem potencial de mimetizar a expressão gênica da doença em modelos animais ou celulares. De qualquer forma, ambos os tipos de associações podem ser relevantes uma vez que levantam hipóteses sobre a modulação dos regulons, sugerindo moléculas para estudos experimentais que podem auxiliar a entender a fisiopatologia da doença já que os mecanismos de ação de fármacos (vias que atuam) podem estar relacionados com as vias envolvidas na doença.

Entre as moléculas identificadas pela análise de CMAP, destaca-se a escopolamina, que apresentou associação negativa com a assinatura bipolar para o

regulon do *EGR3* (Figura 14) nos quatro estudos avaliados, sugerindo que esta molécula tem potencial de antagonizar a assinatura da doença. A escopolamina atua como anticolinérgico e estudos têm lhe atribuído um consistente efeito antidepressivo (Furey e Drevets, 2006; Drevets e Furey, 2010). Uma revisão sistemática demonstrou que a escopolamina é um antidepressivo rápido e eficiente tanto na depressão unipolar como bipolar comparada com a resposta terapêutica de antidepressivos e estabilizadores de humor convencionais (Jaffe, Novakovic e Peselow, 2013). Nesse sentido, estudos genéticos, *postmortem* e de neuroimagem também reforçam o envolvimento de receptores colinérgicos muscarínicos na fisiopatologia da depressão e do transtorno bipolar (Jeon e Dean, 2015). Outra molécula associada negativamente com a assinatura do *EGR3* no transtorno bipolar foi a clorfenamina, anti-histamínico que, além de ser um antagonista do receptor de histamina H1, parece atuar também como inibidor da captação de serotonina (Hellbom, 2006), com potencial propriedade antidepressiva e ansiolítica (Faganello e Mattioli, 2007).

Entre as moléculas associadas positivamente com a assinatura da doença (potencial para mimetizar o fenótipo bipolar), está o anti-histamínico difenilpiralina que atua também como inibidor da recaptação de dopamina com efeito similar a cocaína e outros estimulantes, produzindo hiperatividade em roedores (Lapa *et al*., 2005; Oleson *et al.,* 2012). Também se encontra nesse grupo o antiviral aciclovir, que já foi associado, em estudos de caso, com a indução de hipomania (Jayaram *et al*., 2011). Outra molécula similar, valaciclovir, também já foi associada com psicose e sintomas maníacos (Aslam *et al*., 2009), o que sugere que o tratamento com estas moléculas pode estar relacionado com episódios de humor em certos indivíduos. Já a molécula PHA-00745360 apresentou associação positiva com a assinatura da doença nos quatro estudos avaliados, mas há pouca informação disponível sobre ela. Pela predição no software PASS online,

a atividade da molécula PHA-00745360 está relacionada com a inibição de vias de transdução de sinal, como inibição da angiogênese e de proteínas cinases.

Embora a análise de CMAP seja hipotética, é interessante observar que muitas associações são coerentes com dados e relatos já disponíveis na literatura, reforçando o potencial uso destas moléculas no tratamento ou para a melhor compreensão da fisiopatologia do transtorno bipolar. Além disso, essa análise sugere alvos para estudos que almejem identificar aplicações novas para moléculas conhecidas.

3.5 CAPÍTULO 5

Reduced Neurite Density in Neuronal Cell Cultures Exposed to Serum of Patients with Bipolar Disorder

Bianca Wollenhaupt-Aguiar, Bianca Pfaffenseller, Vinicius de Saraiva Chagas, Mauro A. A. Castro, Ives Cavalcante Passos, Márcia Kauer-Sant'Anna, Flavio Kapczinski, Fábio Klamt

Artigo publicado na revista *The International Journal of Neuropsychopharmacology* em 2016.

OXFORD

International Journal of Neuropsychopharmacology (2016) 00(00): 1–5

doi:10.1093/ijnp/pyw051
Advance Access Publication: May 13, 2016
Rapid Communication

RAPID COMMUNICATION

Reduced Neurite Density in Neuronal Cell Cultures Exposed to Serum of Patients with Bipolar Disorder

Bianca Wollenhaupt-Aguiar, MSc; Bianca Pfaffenseller, MSc; Vinicius de Saraiva Chagas, BSc; Mauro A A Castro, MD, PhD; Ives Cavalcante Passos, MD, PhD; Márcia Kauer-Sant'Anna, MD, PhD; Flavio Kapczinski, MD, PhD; Fábio Klamt, PhD

Bipolar Disorder Program, Laboratory of Molecular Psychiatry, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil (Drs Wollenhaupt-Aguiar, Pfaffenseller, Passos, Kauer-Sant'Anna, and Kapczinski); Laboratory of Cellular Biochemistry, Post-Graduate Program in Biochemistry, Biochemistry Department, ICBS/UFRGS, Porto Alegre, Brazil (Drs Wollenhaupt-Aguiar, Pfaffenseller, and Klamt); Bioinformatics and Systems Biology Laboratory, Post-Graduate Program in Bioinformatics, Federal University of Paraná (UFPR), Polytechnic Center, Curitiba, Brazil (Drs de Saraiva Chagas and Castro); Graduation Program in Psychiatry and Department of Psychiatry, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil (Drs Passos, Kauer-Sant'Anna, and Kapczinski)

Correspondence: Professor Fábio Klamt, PhD, Laboratory of Cellular Biochemistry, Federal University of Rio Grande do Sul, Avenida Ramiro Barcelos, 2600, Porto Alegre (RS) 90035–903 Brazil (fabio.klamt@ufrgs.br)

Abstract

Background: Increased inflammatory markers and oxidative stress have been reported in serum among patients with bipolar disorder (BD). The aim of this study is to assess whether biochemical changes in the serum of patients induces neurotoxicity in neuronal cell cultures.
Methods: We challenged the retinoic acid-differentiated human neuroblastoma SH-SY5Y cells with the serum of BD patients at early and late stages of illness and assessed neurite density and cell viability as neurotoxic endpoints.
Results: Decreased neurite density was found in neurons treated with the serum of patients, mostly patients at late stages of illness. Also, neurons challenged with the serum of late-stage patients showed a significant decrease in cell viability.
Conclusions: Our findings showed that the serum of patients with bipolar disorder induced a decrease in neurite density and cell viability in neuronal cultures.

Keywords: bipolar disorder, neurite density, RA-differentiated SH-SY5Y cells, systemic toxicity

Introduction

Bipolar disorder (BD) affects about 2% of the world's population, with sub-threshold forms affecting up to a further 2% (Merikangas et al., 2007). The course of BD is highly variable, and a subset of patients seem to present a progressive course associated with brain changes (Cao et al., 2016) and functional impairment (Rosa et al., 2014). Nonetheless, the molecular foundations for this illness progression are just beginning to be explained. It is known that brain-derived neurotrophic factor

Received: April 13, 2016; **Revised:** May 7, 2016; **Accepted:** May 9, 2016

(BDNF) serum levels were decreased in the late-stage of BD when compared to those at an early stage of the illness (Kauer-Sant'Anna et al., 2009). Moreover, altered cellular resilience was reported in late-stage patients (Pfaffenseller et al., 2014). In addition, patients at a late stage of illness present increased levels of C-C motif ligand 11 (CCL11) and decreased levels of CXCL827 chemokines (Panizzutti et al., 2015). However, what is not known is how these abnormal peripheral blood markers may lead to, or be involved with, brain changes in BD.

In 2011, our group hypothesized that impairments in neuroplasticity of BD patients may be translated into shrinkage of the brain structures by reducing neurites and intercellular connections in the neuronal network (Berk et al., 2011). We also suggested that the aforementioned biochemical changes may play a causal role in this scenario, which has been called the systemic toxicity (Kapczinski et al., 2010). Recently, several studies reported reductions in the volume of the left hippocampi (Cao et al., 2016) and frontal cortices of patients with late-stage BD (Abe et al., 2015). These findings are in line with the pioneering work of Strakowski and colleagues (2002), which reports increased ventricle volumes in multiple-episode patients with BD compared to those who had only one episode. However, the causal role of the abnormal peripheral blood markers on neuronal cells of the patients with late-stage BD has not been investigated yet.

In the present study, we used an *in vitro* approach with the retinoic acid (RA)-differentiated human cell line SH-SY5Y exposed to serum of bipolar patients. The differentiated human neuroblastoma cell line, SH-SY5Y, has been used as an experimental model to assess molecular and biochemical pathways involved in the pathophysiology of brain disorders (Lopes et al., 2010) and for neurotoxicological experiments in developmental and mature neurons (Schonhofen et al., 2015). This model has the advantage of being derived of human cells, displaying neuronal morphology, and neuronal markers (as high neurite density, tyrosine hydroxylase, dopamine transporter) during RA-differentiation (Lopes et al., 2010).

Therefore, the aim of the present study was to assess whether biochemical changes in serum of patients with BD could induce neurotoxicity in neuronal cell cultures.

Methods

The Ethical Committee of the Hospital de Clínicas de Porto Alegre (HCPA) approved the study (application number: 12–0102). All subjects had signed the informed consent.

Subjects

We recruited 12 patients with BD from the Bipolar Disorders outpatient clinic of the HCPA. We also selected six healthy controls matched by age and gender from the blood donation center of HCPA. They had no previous history of psychiatric illness as well as no history of psychiatric or neurologic disorders in first-degree relatives. Inclusion criteria were euthymic subjects with BD type 1 according to the DSM-IV and aged between 18 and 60 years. Exclusion criteria were a history of autoimmune diseases or a history of chronic infection/inflammatory disorders, as well as any severe systemic disease or use of immunosuppressive therapy.

Assessments

Subjects were evaluated through a socio-demographic history form. Axis-I diagnoses and clinical and functioning characteristics were assessed using the Structured Clinical Interview for DSM-IV axis-I Disorders (SCID-I) and Functioning Assessment Short Test (FAST), respectively, which were administered by trained staff. Current dimensional mood symptoms were assessed with the Hamilton Depression Rating Scale (HDRS; Hamilton, 1960) and the Young Mania Rating Scale (YMRS; Young et al., 1978). Euthymia was defined by the HDRS score < 8 and YMRS score < 5.

The patients were classified in stages I to IV, based on functional impairment, as well as patterns of episode recurrences and severity of clinical features (Kapczinski et al., 2009). Patients were stratified in early-stage (stage I or II) or late-stage (stage III or IV) of BD. Of note, we used the same staging criteria of previous studies from our group (Fries et al., 2013; Pfaffenseller et al., 2014).

Sample Collection

Four milliliters of blood were collected from each subject by venipuncture into a free-anticoagulant vacuum tube. After withdrawal, the blood was centrifuged at 3000 g for 10 minutes and the serum was stored at -80°C until assayed.

Cell Culture and Treatment

The neuronal differentiation of human neuroblastoma SH-SY5Y cells were performed in accordance with the protocol established by Lopes and colleagues (2010). The neuronal differentiation is induced by reducing the fetal bovine serum (FBS) in the culture medium at 1% plus 10 µM all-trans retinoic acid (Enzo Life Sciences, Inc.) for seven days. At the end of this protocol, the cells acquire the morphological and biochemical characteristics of mature, differentiated neurons. After the neuronal differentiation protocol, the cells were treated with inactivated serum (56°C for 30 min) of controls and bipolar patients (1%) for 24 h. The cells treated with FBS were used as the control group for the experiment.

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). The cells were seeded in 12-well plates and 24-well plates at a density of 160 000 and 80 000 cells/well, respectively. After treatment, the medium was discarded and MTT (0,5 mg/mL; Sigma-Aldrich) was added to each well and the plate were incubated for 1 h at 37°C. Then, the MTT 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). Data were expressed as percentage of experimental control group of three independent experiments.

Immunofluorescence

The immunofluorescence was performed using anti-βIII tubulin antibody (Alexa 488-conjugated; Sigma-Aldrich) and with Nuclear dye Hoechst 33342 (0.25 µg/µL; Sigma-Aldrich). Randomly selected images were captured using an EVOS Floid Cell Imaging Station (Thermo Fisher Scientific Inc.) and analyzed with Nikon Imaging Software (NIS), the NIS- elements. The neurite density was assessed using the AutoQuant Neurite software (implemented in R program) and expressed as arbitrary units (AU) as described previously (Schonhofen et al., 2015).

Statistical Analysis

Statistical analyses were performed using the SPSS 18.0 software (SPSS Inc.). The normality of data distribution was assessed using

the Shapiro–Wilk test. Data are expressed as mean ± standard deviation (SD) with a *t*-test and one-way ANOVA used where appropriate (n = 6). *P* values < 0.05 were considered significant.

Results

There was no difference in age and gender among patients at early- and late-stage BD and healthy controls. Moreover, illness duration and medication status were not different between patients at early and late stages (Table 1). We performed a RA-differentiation protocol of SH-SY5Y cells and replaced the fetal bovine serum (FBS) with the serum of bipolar patients and control subjects (Figure 1A) and assessed neurite density and cell morphology using immunofluorescence (Figure 1B). We found a reduction in the neurite density of RA-differentiated SH-SY5Y cells treated with the serum of patients with BD compared to healthy controls (p = 0.0153). Furthermore, when the effect of the serum of patients at a late stage were compared to the serum of the control group, we also found a significant reduction in neurite density (p = 0.0089). There was no difference when the serum of patients at the early stages was compared to healthy controls (Figure 1C). There was no difference between the serums of patients and controls in cell viability analysis. However, higher serum neurotoxicity was found to be attributed to late-stage patients, leading to a significant decrease in cell viability compared to the serum of both early-stage patients (p = 0.0290) and healthy control subjects (p = 0.0075; Figure 1D).

Discussion

The present study showed that the serum of patients at a late stage induced a significant reduction of neurite density and a decrease in the cell viability compared to the serum of healthy controls. In addition, the serum of patients at a late stage caused a significant decrease in the cell viability compared to those at an early stage. Besides, we presented a potential new model for the study of illnesses that affect the central nervous system as BD, where it would be possible to evaluate the molecular and biochemical changes featured in BD.

Previously, a study reported the neurotoxic effect of the serum of patients with BD in human endothelial cells by inducing apoptosis (Politi et al., 2008). Moreover, another study showed that the serum of euthymic patients with BD had detrimental effects on peripheral blood mononuclear cells function (Herberth et al., 2011). To our knowledge, however, our work was the first to evaluate the effect of serum of patients at different stages of BD in cellular parameters using human neuronal cells. Our findings corroborate the hypothesis that patients with BD might have a loss of neuronal connectivity leading to neuroplasticity and cellular resilience impairments (Rajkowska, 2002). Thus, they suggest that the serum of patients with BD, mainly those at the late stage of the illness, may contain chemicals that could be toxic and alter neural cells, as proposed by the systemic toxicity hypothesis (Kapczinski et al., 2010). Specifically, our previous work showed that mood episodes are associated with peripheral changes in inflammation, oxidative stress, and neurotrophin markers (Kapczinski et al., 2010). In this sense, the cumulative damage caused by the recurrent mood episodes may explain why the serum of patients at a late stage is more neurotoxic than of patients at an early stage.

Moreover, these findings add to the notion of neuroprogression. The term neuroprogression has been proposed as the pathological rewiring of the brain that takes place in parallel with the clinical and neurocognitive deterioration in the course of BD (Berk et al., 2011). This hypothesis may explain why some patients with BD have a progressive course associated with a shortening of inter-episodic intervals, functional and cognitive impairment, treatment refractoriness, and suicide attempts (Merikangas et al., 2007; Rosa et al., 2014).

In addition, there was no difference between groups in illness duration (Table 1). This finding corroborates current staging models in BD, where the number of episodes and functioning impairments are more relevant to the definition of stages than length of illness (Kapczinski et al., 2014).

Our study has some limitations. First, there is a question as to what extent neuronal cell line experiments reflect what actually happens *in vivo*. Addressing this issue, a recent study proposed a model wherein transient or persistent disruption of blood-brain barrier integrity is associated with decreased central nervous system protection and increased permeability of proinflammatory and oxidative stress substances from the peripheral blood into the brain in patients with BD (Patel and Frey, 2015). Also, a positron emission tomography scan study reported that there is neuroinflammation in the brain of patients with BD (Haarman et al., 2014). Second, the sample size is small. Third, the patients were on medication, which could potentially change the serum biological markers. However, we observed that 24 h treatment with different medications has no detrimental effects in the cell viability in our study (data not shown). Moreover, there was no difference between medication status between patients at the early and late stages (see Table 1). Future research with drug-free patients would be important to evaluate the effect of drugs in this model. Finally, studies with large sample sizes are also needed to replicate our findings.

Table 1. Clinical and Demographic Characteristics of Controls and Euthymic Patients at Early vs. Late Stages of Bipolar Disorder

Euthymic patients	Early (n = 6)	Late (n = 6)	Control (n = 6)	*p*
Age (years)	48.2 ± 4.7	49.0 ± 5.0	48.8 ± 5.1	0.904[a]
Gender (male/female)	2/4	2/4	2/4	1.00[b]
Duration of illness	24.3 ± 11.29	21.5 ± 5.6	n/a	0.595[c]
Number of episodes	5.67 ± 3.5	15.83 ± 7.02	n/a	0.010[c]
Medications (%)				
Mood stabilizers	83.3%	50%	n/a	0.545[d]
Antidepressants	16.6%	0%	n/a	1.00[d]
Atypical antipsychotics	16.6%	33.3%	n/a	1.00[d]
Typical antipsychotics	0%	16,6%	n/a	1.00[d]
Benzodiazepines	16.6%	0%	n/a	1.00[d]

[a]analysis of variance, data expressed as mean ± standard deviation; [b]chi-square test; [c] independent-samples *t*-test, data expressed as mean ± standard deviation; [d]Fisher's Exact Test. n/a = not applicable.

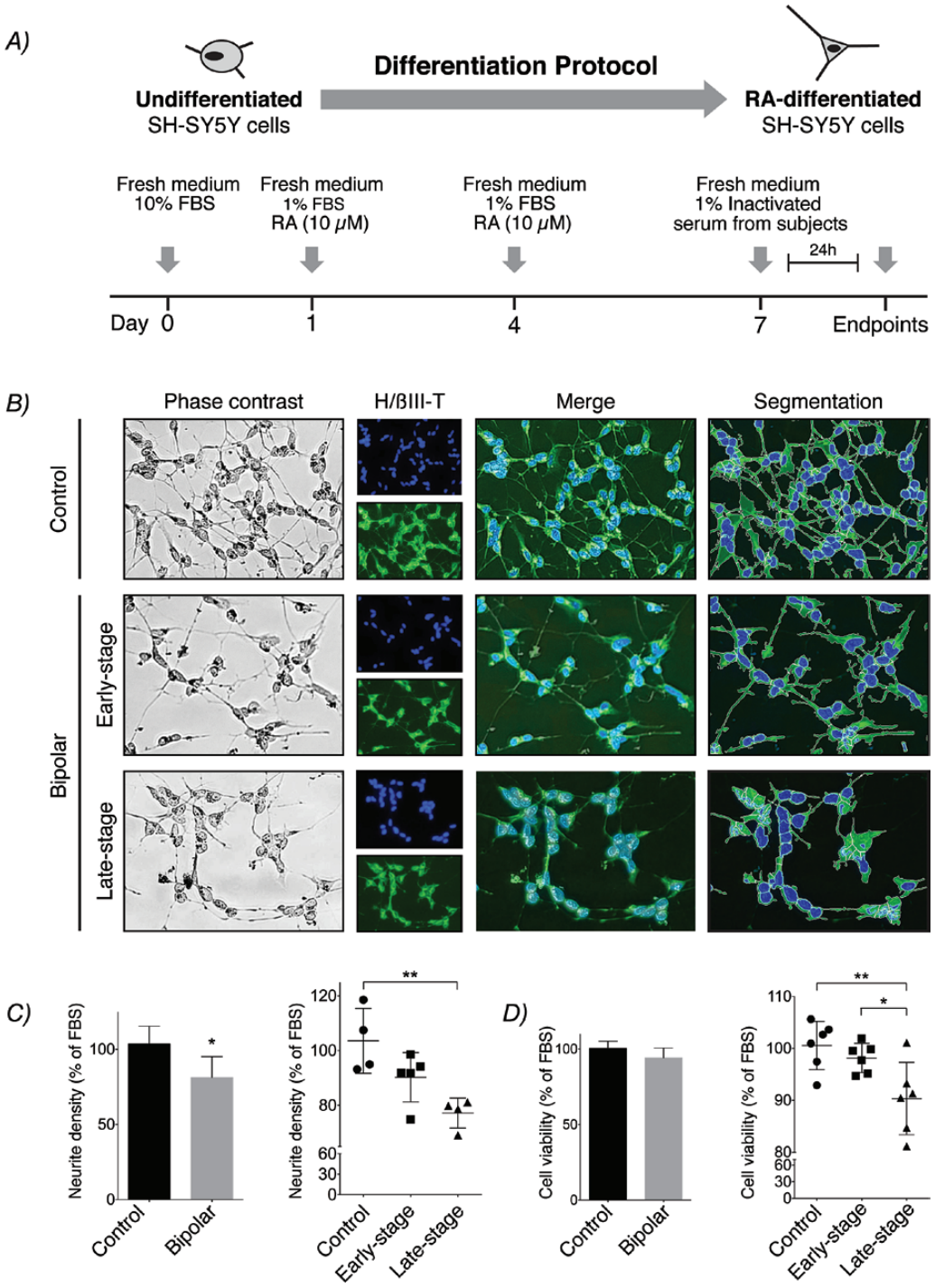


Figure 1. Protocol design, neurite density, and cell viability of retinoic acid (RA)-differentiated SH-SY5Y cells challenged with bipolar disorder (BD) serum. (A) RA differentiation protocol of human SH-SY5Y cells. At day 0, the exponentially-growing proliferative SH-SY5Y cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) / F12 (1:1) medium supplemented with 2 mM glutamine, 100 μg/μL gentamycin, and 0.25 mg/mL amphotericin B and containing 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere with 5% CO2 at 37°C. After 24 hours (day 1), the previous medium was removed and a fresh medium containing 1% of FBS and 10 μM of RA (differentiation medium) was added. Three days later (day 4), the differentiation medium was replaced by a fresh one. At day 7, SH-SY5Y cells were ready to perform the experiments of interest. The medium was replaced by fresh medium with 1% of serum of bipolar patients and controls, instead of FBS. The treatment lasted 24 h, when the endpoints were analyzed. (B) Representative phase contrast and fluorescent images of human RA-differentiated SH-SY5Y cells labeled with nuclear dye Hoechst 33342 (H) and anti-βIII tubulin (βIII-T) treated with the serums of bipolar patients and controls. Merge is the combination of phase contrast, H, and βIII-T images for analysis in the AutoQuant neurite software. Representative neurite segmentation shows the neurite density per cell body, identified by the AutoQuant neurite software. (C) Neurite density analysis. Comparison between bipolar patients and the control group ($p = 0.0153$) and between late-stage patients and controls ($p = 0.0089$) showed statistical differences. (D) Cell viability analysis. Comparison between bipolar patients and the control group did not show a statistical difference. However, when comparing the late-stage group of patients to the control group ($p = 0.0075$) and late- and early-stage patients ($p = 0.0290$) there was a statistical difference. Fetal bovine serum (FBS) was considered as 100% of cell viability. Data are presented as mean ± standard deviation. A *t*-test and one-way ANOVA were performed when appropriate. *Significant differences were considered when $p < 0.05$. **Significant differences were considered when $p < 0.01$.

In summary, we analyzed the effect of exposure to the serum of patients with BD in human cells differentiated into neuron-like cells. Our results showed neurotoxic activity in the serum of BD patients, particularly late-stage patients. In addition, we developed a new experimental model using neuronal RA-differentiated human cell cultures.

Acknowledgments

This work was supported by the Brazilian fund Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq/MS/SCTIE/DECIT – Pesquisas Sobre Doenças Neurodegenerativas (#466989/2014–8) and a fellowship from MCT/CNPq (#306439/2014-0) to Fábio Klamt and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) with a scholarship to Drs Wollenhaupt-Aguiar and Pfaffenseller.

Statement of Interest

Drs Wollenhaupt-Aguiar, Pfaffenseller, de Saraiva Chagas, Castro, Passos, and Klamt have no declaration of interest. Dr Kapczinski has received grants or research support from AstraZeneca, Eli Lilly, Janssen-Cilag, Servier, NARSAD, and the Stanley Medical Research Institute; has been a member of speakers' boards for AstraZeneca, Eli Lilly, Janssen, and Servier; and has served as a consultant for Servier. Dr Kauer-Sant'Anna is on speaker/advisory boards for, or has received research grants from, NARSAD, Stanley Medical Research Institute, CNPq-Universal, CNPq/INCT-TM, FIPE-HCPA, and Novartis.

References

Abe C, Ekman CJ, Sellgren C, Petrovic P, Ingvar M, Landen M (2015) Manic episodes are related to changes in frontal cortex: a longitudinal neuroimaging study of bipolar disorder 1. Brain 138:3440–3448.

Berk M, Kapczinski F, Andreazza AC, Dean OM, Giorlando F, Maes M, Yucel M, Gama CS, Dodd S, Dean B, Magalhaes PV, Amminger P, McGorry P, Malhi GS (2011) Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors. Neurosci Biobehav Rev 35:804–817.

Cao B, Passos IC, Mwangi B, Bauer IE, Zunta-Soares GB, Kapczinski F, Soares JC (2016) Hippocampal volume and verbal memory performance in late-stage bipolar disorder. J Psychiatr Res 73:102–107.

Fries GR, Vasconcelos-Moreno MP, Gubert C, Santos BT, da Rosa AL, Eisele B, Sartori J, Pfaffenseller B, Kapczinski F, Kauer-Sant'anna M (2013) Early apoptosis in peripheral blood mononuclear cells from patients with bipolar disorder. J Affect Disord 152–154:474–477.

Haarman BC, Riemersma-Van der Lek RF, de Groot JC, Ruhe HG, Klein HC, Zandstra TE, Burger H, Schoevers RA, de Vries EF, Drexhage HA, Nolen WA, Doorduin J (2014) Neuroinflammation in bipolar disorder A[(11)C]-(R)-PK11195 positron emission tomography study. Brain Behav Immun 40:219–225.

Hamilton M (1960) A rating scale for depression. J Neurol Neurosurg Psychiatry 23:56–62.

Herberth M, Koethe D, Levin Y, Schwarz E, Krzyszton ND, Schoeffmann S, Ruh H, Rahmoune H, Kranaster L, Schoenborn T, Leweke MF, Guest PC, Bahn S (2011) Peripheral profiling analysis for bipolar disorder reveals markers associated with reduced cell survival. Proteomics 11:94–105.

Kapczinski F, Dias VV, Kauer-Sant'Anna M, Frey BN, Grassi-Oliveira R, Colom F, Berk M (2009) Clinical implications of a staging model for bipolar disorders. Expert Rev Neurother 9:957–966.

Kapczinski F, Dal-Pizzol F, Teixeira AL, Magalhaes PV, Kauer-Sant'Anna M, Klamt F, Pasquali MA, Quevedo J, Gama CS, Post R (2010) A systemic toxicity index developed to assess peripheral changes in mood episodes. Mol Psychiatry 15:784–786.

Kapczinski F et al. (2014) Staging systems in bipolar disorder: an International Society for Bipolar Disorders task force report. Acta Psychiatr Scand 130:354–363.

Kauer-Sant'Anna M, Kapczinski F, Andreazza AC, Bond DJ, Lam RW, Young LT, Yatham LN (2009) Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. Int J Neuropsychop 12:447–458.

Lopes FM, Schroder R, da Frota ML, Jr., Zanotto-Filho A, Muller CB, Pires AS, Meurer RT, Colpo GD, Gelain DP, Kapczinski F, Moreira JC, Fernandes Mda C, Klamt F (2010) Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res 1337:85–94.

Merikangas KR, Akiskal HS, Angst J, Greenberg PE, Hirschfeld RM, Petukhova M, Kessler RC (2007) Lifetime and 12-month prevalence of bipolar spectrum disorder in the National Comorbidity Survey replication. Arch Gen Psychiatry 64:543–552.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Panizzutti B, Gubert C, Schuh AL, Ferrari P, Bristot G, Fries GR, Massuda R, Walz J, Rocha NP, Berk M, Teixeira AL, Gama CS (2015) Increased serum levels of eotaxin/CCL11 in late-stage patients with bipolar disorder: An accelerated aging biomarker? J Affect Disord 182:64–69.

Patel JP, Frey BN (2015) Disruption in the blood-brain barrier: the missing link between brain and body inflammation in bipolar disorder? Neural Plast 2015:708306.

Pfaffenseller B, Wollenhaupt-Aguiar B, Fries GR, Colpo GD, Burque RK, Bristot G, Ferrari P, Cereser KM, Rosa AR, Klamt F, Kapczinski F (2014) Impaired endoplasmic reticulum stress response in bipolar disorder: cellular evidence of illness progression. Int J Neuropsychop 17:1453–1463.

Politi P, Brondino N, Emanuele E (2008) Increased proapoptotic serum activity in patients with chronic mood disorders. Arch Med Res 39:242–245.

Rajkowska G (2002) Cell pathology in bipolar disorder. Bipolar Disord 4:105–116.

Rosa AR, Magalhaes PV, Czepielewski L, Sulzbach MV, Goi PD, Vieta E, Gama CS, Kapczinski F (2014) Clinical staging in bipolar disorder: focus on cognition and functioning. J Clin Psychiatry 75:e450–456.

Schonhofen P, de Medeiros LM, Bristot IJ, Lopes FM, De Bastiani MA, Kapczinski F, Crippa JA, Castro MA, Parsons RB, Klamt F (2015) Cannabidiol exposure during neuronal differentiation sensitizes cells against redox-active neurotoxins. Mol Neurobiol 52:26–37.

Strakowski SM, DelBello MP, Zimmerman ME, Getz GE, Mills NP, Ret J, Shear P, Adler CM (2002) Ventricular and periventricular structural volumes in first- versus multiple-episode bipolar disorder. Am J Psych 159:1841–1847.

Young RC, Biggs JT, Ziegler VE, Meyer DA (1978) A rating scale for mania: reliability, validity and sensitivity. Br J Psychiatry 133:429–435.

PARTE III

Discussão e conclusões

4. DISCUSSÃO

Como abordado nessa tese, o transtorno bipolar tem sido associado com alterações sistêmicas durante os episódios de humor e alterações estruturais no cérebro em pacientes que já passaram por múltiplos episódios agudos. Com o objetivo de entender melhor os mecanismos subjacentes a estas alterações, essa tese se propôs a estudar vias de regulação transcricional disfuncionais no córtex pré-frontal de pacientes bipolares para propor alvos moleculares que possam estar envolvidos na fisiopatologia da doença. Além disso, este trabalho caracterizou um modelo celular neuronal para avaliar estes alvos moleculares e o possível efeito da 'toxicidade sistêmica' sobre as células neuronais, propondo um modelo *in vitro* de estudo do transtorno bipolar.

Conforme revisado no capítulo 1, um conjunto crescente de evidências aponta para alterações em neuroplasticidade, rotas de inflamação e envolvendo estresse oxidativo no transtorno bipolar, principalmente durante os episódios agudos de humor. Com base nestas alterações periféricas e na disfunção celular potencialmente resultante, os episódios de humor têm sido associados a uma toxicidade sistêmica. Nossa revisão de achados da literatura demonstrou que biomarcadores periféricos relacionados às rotas acima diferenciam entre pacientes em episódios maníacos ou depressivos comparados a pacientes eutímicos ou indivíduos saudáveis. Muitas vezes, a alteração em apenas um destes biomarcadores apresenta pequeno tamanho de efeito, por isso a avaliação de múltiplos biomarcadores, como discutido no capítulo 1, poderia consistir em uma abordagem mais informativa quanto à fisiopatologia da doença e contribuir no desenvolvimento de estratégias de diagnóstico e tratamento. A avaliação da toxicidade sistêmica através de um conjunto de biomarcadores periféricos pode facilitar a compreensão sobre o prejuízo celular e o dano ao organismo associado a episódios de

humor recorrentes. Assim, esses biomarcadores periféricos teriam potencial de uso como indicadores da atividade da doença e/ou de sua progressão.

Nossa pesquisa sobre os possíveis mecanismos responsáveis pela toxicidade sistêmica associada aos episódios agudos sugeriu o envolvimento de organelas e rotas de sinalização tipicamente responsáveis pela manutenção da homeostase celular, como mitocôndria e retículo endoplasmático, que poderiam estar relacionados com a diminuição da resiliência celular frente a condições de estresse. O conhecimento sobre as possíveis rotas implicadas na atividade da doença pode propor novas abordagens de tratamento para um melhor manejo do transtorno bipolar. Como abordado no capítulo 1, alternativas interessantes podem envolver terapias adjuvantes que atuem sobre as vias comentadas anteriormente (inflamação, estresse oxidativo e neurotrofinas), como agentes com efeitos antioxidantes, anti-inflamatórios e neuroprotetores, que poderiam auxiliar no controle da toxicidade sistêmica.

Os achados revisados nesta tese, associando episódios agudos a uma significativa toxicidade sistêmica no transtorno bipolar, corroboram com a ideia de que esta doença pode ser considerada uma doença multissistêmica. No entanto, há um grande interesse em entender os mecanismos que contribuem para a disfunção resultante de múltiplos episódios de humor e a possível relação de alterações sistêmicas com alterações estruturais no cérebro, como aquelas exemplificadas no capítulo 2, no qual é ilustrado o caso de uma atrofia cortical e dilatação de ventrículos significativa em um paciente com histórico de múltiplos episódios de humor comparado com um paciente, do mesmo sexo e idade similar, que só vivenciou alguns episódios agudos. Alterações em estruturas cerebrais tem sido reportadas no transtorno bipolar, como a redução no volume do hipocampo esquerdo (Cao *et al*., 2016), do corpo caloso (Lavagnino *et al*.,

2015) e do córtex frontal (Abé *et al*., 2015), além de uma diminuição da densidade de substância branca no sistema límbico-frontal (Mwangi *et al*., 2016), sendo que estas alterações têm sido observadas em pacientes bipolares em estágios mais avançados da doença, ou seja, após múltiplos episódios de humor. Considerando os episódios como tóxicos, nosso grupo tem proposto que as vias associadas a esse processo de 'neuroprogressão' estão focadas também em inflamação, estresse oxidativo e falta de neuroproteção (Berk *et al*., 2011), como observado sistemicamente. Para estudar as possíveis vias disfuncionais em nível central, e que podem estar associadas com esse processo, é necessário avaliar tecido *postmortem* de pacientes bipolares.

Então, utilizando abordagens de bioinformática baseadas em redes, almejamos estudar mecanismos biológicos do transtorno bipolar através de uma assinatura regulatória transcricional do córtex pré-frontal. Para isso, como descrito no capítulo 3 desta tese, primeiramente analisamos um grande conjunto de dados de expressão gênica de córtex pré-frontal humano saudável ao longo da vida para construir uma rede de regulação de fatores de transcrição conhecidos e todos os seus alvos potenciais, para assim obter uma representação mais realista do funcionamento do córtex pré-frontal. Em seguida, identificamos os genes que são diferencialmente expressos no córtex pré-frontal de pacientes e indivíduos saudáveis a partir de dois conjuntos de dados de microarranjo de amostras de tecido *postmortem*, para avaliar se a assinatura gênica do transtorno bipolar é influenciada pela ativação ou repressão de fatores de transcrição específicos. O objetivo central do trabalho foi gerar hipóteses sobre a regulação transcricional do transtorno bipolar e identificar unidades regulatórias (regulons) disfuncionais no pré-frontal dos pacientes. Através desta abordagem, identificamos cinco regulons enriquecidos no fenótipo bipolar, ou seja, associados com a assinatura gênica da doença. Entre eles, e como nosso resultado principal, identificamos que o

regulon de *EGR3* estava consistentemente reprimido em ambas as redes transcricionais e assinaturas gênicas do transtorno bipolar avaliadas. Além disso, de forma inédita, descrevemos a rede de regulons associados com a estrutura pré-frontal.

EGR3 é um fator de transcrição classificado como *immediate early gene* (IEG), grupo de genes que desempenham um papel essencial nas respostas celulares que contribuem para a plasticidade neuronal em longo prazo e possivelmente em processos relacionados a modificações da função sináptica (Lanahan e Worley, 1998). *EGR3* pertence à família de proteínas de "resposta de crescimento precoce" (*early growth response* - EGR) composta por cinco membros: EGR1 (também conhecido como NGFI-A, Krox-24, Zif268), EGR2 (Krox-20), EGR3 (PILOT) e EGR4 (também conhecido como NGFI-C) (Beckmann e Wilce 1997; O'Donovan *et al*., 1999). Estes genes são expressos em níveis basais em todo o cérebro, incluindo o córtex, hipocampo e outras áreas límbicas, e os gânglios da base (O'Donovan *et al*., 1999).

EGR3 é essencial para a resposta normal ao estresse assim como para os mecanismos de neuroplasticidade induzidos por este tipo de resposta, incluindo a regulação da expressão gênica neuronal (Li *et al*., 2005; Gallitano *et al*., 2007; Maple *et al*., 2015). Estudos com animais sem *EGR3* funcional demonstram o papel deste fator de transcrição na resposta à novidade e ao estresse (Gallitano *et al*., 2007). Animais *EGR3*$^{-/-}$ apresentaram elevada reatividade diante de estresse moderado de manipulação e liberação acentuada de corticosterona em resposta a este estresse. Estes animais também mostraram reatividade alta em ambientes novos indicando que o comportamento é afetado consideravelmente pelo estresse associado à novidade. Essa hiper-reatividade ao estresse pode estar associada a alterações na memória dos animais já que estes apresentaram prejuízos no estabelecimento do processo de depressão de longa duração

(*LTD*) no hipocampo. Assim, observa-se que as alterações fisiológicas e comportamentais apresentadas por esses animais são consistentes com modelos de transtorno mental.

A expressão neuronal de *EGR3* é regulada pela atividade sináptica e pela sinalização via MAPK-ERK (O'Donovan *et al*., 1999; Li *et al*., 2007). *EGR3* é ativado por várias proteínas, incluindo neuroregulina 1 (*NRG1*), calcineurina (*CaN*) e receptores N-metil-D-aspartato (*NMDA*) (Hippenmeyer *et al*., 2002; Yamada *et al*., 2007; Huentelman *et al*., 2015). Além disso, BDNF induz a expressão de *EGR3* via PKC/MAPK (Roberts *et al*., 2006), processo que deve ser essencial ao desenvolvimento do sistema nervoso e ao desempenho cognitivo ao longo da vida.

EGR3 regula a expressão de diferentes genes e vias envolvidas em processos celulares importantes como plasticidade sináptica, extensão axonal, regulação de neurotrofinas e receptores. Entre estes genes, até o momento, é possível incluir aqueles que codificam *ARC* (*activity regulated cytoskeletal associated gene*) (Lyford *et al*., 1995, Li *et al*., 2005), receptor de GABA/subunidade 4 (*GABRA4*) (Roberts *et al*., 2006), receptor de NMDA/subunidades NR1 e NR2B (Gallitano *et al*., 2007; Kim *et al*., 2012) e receptor de neurotrofinas NGFR (*p75NTR*) (Dechant e Barde, 2002; Gao, Daugherty e Tourtellotte, 2007). Portanto, o envolvimento de *EGR3* em processos de memória, aprendizado e plasticidade sináptica é provavelmente mediado por estes, e outros ainda não identificados, genes alvos efetores.

Nossos dados sugerem que uma disfunção no regulon do *EGR3* pode estar envolvida no transtorno bipolar. Como *EGR3* é um regulon mestre (*i.e*., um fator de transcrição que exerce influências expressivas no fenótipo), alterações neste regulon podem refletir em alterações relevantes em toda a rede de genes que ele regula.

Considerando que a rede de genes e vias reguladas por *EGR3* está envolvida em processos neurobiológicos essenciais e que *EGR3* está associado com a translação de eventos ambientais em alterações duradouras na expressão gênica neural (O'Donovan *et al*., 1999; Gallitano *et al*., 2007), uma disfunção neste regulon sugere uma possível contribuição para a influência do ambiente e de vários genes na fisiopatologia do transtorno bipolar.

Os nossos resultados, sugerindo o envolvimento de *EGR3* na fisiopatologia do transtorno bipolar, possuem embasamento na literatura científica. Outros estudos têm indicado o *EGR3* como um alvo importante na área de psiquiatria. Como discutido anteriormente no capítulo 3, este fator de transcrição tem sido estudado principalmente na esquizofrenia, com trabalhos que mostram uma associação significativa de *EGR3* com a doença em populações asiáticas e de origem europeia (Yamada *et al*., 2007; Kim *et al*., 2010; Zhang *et al*., 2012; Huentelman *et al*., 2015). Além disso, um estudo envolvendo uma rede de fatores de transcrição e microRNAs relacionados com a esquizofrenia identificou o *EGR3* como gene central na rede de regulação (Guo *et al*., 2010). No transtorno bipolar, os achados relacionados ao *EGR3* são reduzidos e menos consistentes. Um estudo de associação baseada em famílias identificou uma associação nominal (que não atingiu o nível de significância estatística após correção para múltiplas comparações) de *EGR3* com o risco para o transtorno bipolar em crianças (Gallitano-Mendel *et al*., 2012). Outro trabalho identificou, entre genes envolvidos com o ritmo circadiano, somente o *EGR3* como estando associado com o transtorno bipolar (Mansour *et al*., 2009). É importante ressaltar que processos moleculares e genéticos possivelmente envolvidos no transtorno bipolar e na esquizofrenia têm sido demonstrados como altamente coincidentes (Shinozaki e Potash, 2014). Alguns genes de susceptibilidade conferem risco no desenvolvimento de várias doenças psiquiátricas,

e este parece ser o caso do *EGR3*. Nesse sentido, tem sido proposto que a via do *EGR3* quando disfuncional pode estar relacionada com o risco para doenças psicóticas, já que *EGR3* é ativado por várias proteínas que têm sido associadas com este risco, como a neuregulina 1 (*NRG1*), calcineurina (*CaN*) e receptores N-metil-D-aspartato (*NMDA*) (Hippenmeyer *et al*., 2002; Yamada *et al*., 2007; Huentelman *et al*., 2015) e por drogas que induzem psicose via receptores de serotonina (5-HT2ARs) (González-Maeso *et al*., 2003).

Ainda neste contexto, é possível propor algumas relações interessantes. Por exemplo, o sistema de monoaminas está envolvido na regulação da expressão de membros da família de EGRs (O'Donovan *et al*, 1999), sendo que deficiências em monoaminas têm sido há bastante tempo associadas com distúrbios afetivos. Estas doenças se caracterizam por prejuízos em funções cognitivas, que poderiam estar relacionados a uma possível alteração na expressão de EGRs. Além disso, *EGR3* é responsivo ao BDNF, conforme estudos que demonstram que esta neurotrofina induz a expressão de *EGR3* via PKC/MAPK (Roberts *et al*., 2006). A regulação da expressão gênica via BDNF é crítica para o desenvolvimento do sistema nervoso e pode estar relacionada com o desempenho cognitivo durante a vida adulta. Há muitos dados na literatura demonstrando que os níveis periféricos de BDNF estão diminuídos no transtorno bipolar, durante os episódios de humor (conforme discutido no capítulo 1) e também com a progressão da doença (Fernandes *et al*., 2011; Frey *et al*., 2013) de modo que o BDNF tem sido proposto como um fator relevante na resiliência celular diminuída associada ao transtorno (Manji *et al*., 2003; Berk *et al*., 2011). Embora os estudos nesta área apresentem limitações e resultados contraditórios, é interessante levantar a hipótese relacionando BDNF e *EGR3* no transtorno bipolar. Podemos especular que os níveis diminuídos de BDNF no transtorno (se houver correlação entre níveis periféricos e

centrais) poderiam influenciar na repressão do *EGR3* já que ele é regulado por BDNF. Por outro lado, *EGR3* pode induzir indiretamente a expressão de BDNF, conforme estudo que verificou que os níveis de receptores NMDA são controlados pelos fatores de transcrição CREB e *EGR3* e mediado por BDNF (Kim *et al*., 2012). Como a ativação de receptores NMDA estimula a síntese de BDNF, este estudo aponta para uma rede gênica regulatória de retroalimentação que pode aumentar a neurotransmissão para alterar o comportamento neuronal em determinadas condições. Considerando essa observação, podemos especular ainda que a repressão de *EGR3* poderia explicar os níveis baixos de BDNF no transtorno bipolar. Mais factível seria pensar numa rede de retroalimentação do que numa relação causal. De qualquer forma, essas observações são hipotéticas e obviamente mais pesquisas são requeridas para uma melhor compreensão da possível relação entre BDNF e *EGR3* compartilhando uma via biológica e o seu papel nesta doença.

É importante deixar claro que o nosso estudo não foi focado inicialmente no *EGR3* e sim na análise da regulação transcricional no transtorno bipolar, na qual o *EGR3* emergiu como a unidade regulatória mais robustamente enriquecida nas duas assinaturas gênicas da doença obtidas a partir de bancos de dados de microarranjo independentes. O fato do *EGR3* já ter sido o foco de estudos anteriores na psiquiatria, na nossa visão, contribui muito para a qualidade do nosso achado, tornando menos proeminente a chance de ele ter sido encontrado ao acaso. Ao contrário de identificarmos um gene ou região de significado desconhecido na nossa análise, identificamos um gene com papel interessante para a psiquiatria, visto que é altamente sensível aos estímulos ambientais que levam à plasticidade. Os resultados desta tese, em conjunto com achados recentes na área, sugerem o *EGR3* como um potencial alvo para estudos genéticos futuros com o objetivo de identificar mecanismos através dos quais o

ambiente e a predisposição genética interagem para influenciar em transtornos psiquiátricos. Além disso, os nossos resultados apontam para toda a rede centrada no *EGR3*. Assim, toda essa rede pode estar comprometida no transtorno, o que fortalece o interesse em estudar esta rede de genes para compreender como o ambiente e o genoma interagem para moldar o comportamento. Portanto, nosso achado forneceu alvos potenciais para avaliação experimental e desenvolvimento de novas terapias.

Os potenciais alvos para estudo no transtorno bipolar, identificados a partir dos nossos resultados, incluem tanto os genes pertencentes ao regulon do *EGR3* como também os genes pertencentes aos demais regulons enriquecidos no fenótipo bipolar: TSC22 domain family, member 4 (*TSC22D4*), interleukin enhancer-binding factor 2 (*ILF2*), Y-box binding protein 1 (*YBX1*) e MAP-kinase activating death domain (*MADD*). A lista completa dos potenciais genes-alvo de cada uma destas unidades (Anexo 5) foi obtida a partir do mapeamento das interações significativas entre os fatores de transcrição conhecidos e todos os alvos potenciais na matriz de expressão gênica, através de análise de coeficiente de variação e informação mútua para filtrar somente as interações consistentes. A tabela demonstra o modo de ação de cada fator de transcrição sobre os seus alvos (obtida pela análise de GSEA bicaudal, conforme explicado no capítulo 3). Valores positivos e negativos indicam predições de interação onde o fator de transcrição induz ou reprime o alvo, respectivamente. Entre estes alvos, destacam-se os alvos do *EGR3*, regulon enriquecido consistentemente em ambas as redes transcricionais avaliadas, e alguns destes alvos estão listados na Tabela 4 pela sua função relevante e por alguns serem também alvos de outros regulons enriquecidos no fenótipo bipolar.

Tabela 4. Lista de genes-alvo do regulon *EGR3* e seu modo de ação.

Gene Symbol	Gene Name	Mode of action
SLC6A17[#]	Solute Carrier Family 6 (Neutral Amino Acid Transporter), Member 17	0.8377
OMG[#]	Oligodendrocyte Myelin Glycoprotein	0.7885
GPR158[#]	G Protein-coupled Receptor 158	0.929
RAPGEF4[§]	Rap Guanine Nucleotide Exchange Factor 4	0.8804
GABRA1[§]	Gamma-aminobutyric Acid (GABA) A Receptor, Alpha 1	0.9127
USP2[§]	Ubiquitin Specific Peptidase 2	0.8276
PLCB1[§]	Phospholipase C, Beta 1	0.6316
COBL[§]	Cordon-Bleu WH2 Repeat Protein	0.8519
RASSF5[§]	Ras Association (RalGDS/AF-6) Domain Family, Member 5	0.7937
TRHDE[§]	Thyrotropin-Releasing Hormone Degrading Enzyme	0.9101
SORCS3[§]	Sortilin-Related VPS10 Domain Containing Receptor 3	0.4143
PACSIN1[§]	Protein Kinase C and Casein Kinase Substrate in Neurons 1	0.8678
HTR2A[§]	5-Hydroxytryptamine (Serotonin) Receptor 2A	0.7472
RGS4[§]	Regulator of G-Protein Signaling 4	0.887
DGKB	Diacylglycerol Kinase, Beta	0.7825
NETO1	Neuropilin (NRP) and Tolloid (TLL)-Like 1	0.7537
CRHBP	Corticotropin Releasing Hormone Binding Protein	0.6857
ILF2	Interleukin Enhancer Binding Factor 2	-0.7685
CRY2	Cryptochrome Circadian Clock 2	0.5579
EGR1	Early Growth Response 1	0.5441

Valores positivos e negativos indicam predições de interação onde o fator de transcrição induz ou inibe o alvo, respectivamente.
alvos incomuns para *EGR3* e outros dois dos regulons enriquecidos no transtorno bipolar.
§ alvos incomuns para *EGR3* e mais um dos regulons enriquecidos no transtorno bipolar.

Os alvos do *EGR3* estão envolvidos em diversos processos celulares, como vias de transdução de sinal, sobrevivência e diferenciação, neurotransmissão, processamento proteico, ritmo circadiano, inflamação, sinalização pelo cálcio, entre outros. Alguns destes genes são destacados aqui pelas suas funções, outros por ser o foco já em estudos com doenças psiquiátricas, o que reforça a importância deles em mecanismos inerentes a estas doenças e o possível envolvimento do *EGR3* (como regulon mestre) influenciando estas alterações. Entre os genes com papel de destaque está o gene *COBL*

(Cordon-Bleu WH2 Repeat Protein) que atua na reorganização do citoesqueleto de actina, regulando a morfogênese neuronal e a ramificação dos axônios e dendritos (Ahuja *et al*., 2007; Kessels *et al*., 2011); o gene *OMG* (Oligodendrocyte Myelin Glycoprotein) que codifica uma molécula de adesão celular envolvida no processo de mielinização no SNC (Vourc'h e Andres, 2004); *SLC6A17* (Solute Carrier Family 6 - Neutral Amino Acid Transporter, Member 17), transportador específico para neurotransmissores, aminoácidos e osmólitos (Reimer, 2013); e o gene *EGR1* (Early Growth Response 1) que é outro fator de transcrição da mesma família do *EGR3* e que está envolvido na plasticidade neuronal e formação da memória (Veyrac *et al*., 2014).

Relacionado também com a memória, o gene *NETO1* (Neuropilin and Tolloid-Like 1) está envolvido no desenvolvimento e manutenção de circuitos neuronais, regula plasticidade sináptica e cognição dependente do receptor N-metil-D-aspartato neuronal (NMDAR), pelo menos no contexto da aprendizagem espacial e memória (Ng *et al*, 2009). Entre outros genes, foi associado com o distúrbio de hiperatividade e déficit de atenção (Alemany *et al*., 2015). Já o gene *RAPGEF4* (Rap Guanine Nucleotide Exchange Factor 4, também conhecido como EPAC - exchange protein directly activated by cAMP) desempenha um papel específico e tempo-dependente na recuperação da memória (Ostroveanu *et al*., 2010) e, considerando isso, um estudo sugeriu EPAC como um novo alvo terapêutico para o tratamento de sintomas associados com a esquizofrenia (Kelly *et al*., 2009).

É importante observar o papel na neurotransmissão de genes-alvo de *EGR3*, como os receptores de GABA e serotonina. O receptor *GABRA1* (Gamma-aminobutyric Acid (GABA) A Receptor, Alpha 1) tem sido reportado entre genes candidatos no envolvimento da fisiopatologia do transtorno bipolar (Kato, Kuratomi e Kato, 2005) e

uma possível associação entre o haplótipo de *GABRA1* e transtornos de humor tem sido proposta (Horiuchi *et al*., 2004). Em relação à via da serotonina, níveis diminuídos de mRNA de *HTR2A* (5-Hydroxytryptamine (Serotonin) Receptor 2A) foram observados no córtex pré-frontal e no hipocampo de pacientes bipolares (López-Figueroa *et al*., 2004). Além disso, um haplótipo específico neste gene mostrou uma associação significativa com o transtorno bipolar (Xiang *et al*., 2014) e uma revisão recente demonstrou correlação de alterações genéticas nesta via com doenças psicóticas e com a resposta a antipsicóticos atípicos (Baou *et al*., 2016). A via de sinalização envolvendo o gene-alvo de *EGR3 CRHBP* (Corticotropin Releasing Hormone Binding Protein), associada com transmissão sináptica, transdução de sinal e comportamento, também tem sido associada com o transtorno bipolar (Nurnberger *et al*., 2014), o que vem de encontro com achados que mostram uma disfunção no eixo HPA (Hipotálamo-pituitária-adrenal) nesta doença (Fries *et al*., 2014).

Além disso, estudos bioquímicos e genéticos tem relacionado o transtorno bipolar e a esquizofrenia com uma falta de inibição da via do fosfatidilinositol. Os genes *RGS4* (Regulator of G-Protein Signaling 4) e *DGKB* (Diacylglycerol Kinase, Beta), importantes por regular a inibição dessa via, são alvos de *EGR3* e têm sido estudados em doenças psiquiátricas. O gene *RGS4* tem sido o foco de estudos com transtorno bipolar e esquizofrenia, com resultados significativos no último caso (Cordeiro *et al*., 2005; Ding e Hegde, 2009; Ding *et al*., 2016). Já o gene *DGKB* foi identificado como um gene de risco para o transtorno bipolar em dois estudos de associação do genoma. O primeiro estudo encontrou vários polimorfismos de nucleotídeo único (SNPs) em *DGKB* associados com o transtorno bipolar (Wellcome, 2007). No segundo trabalho, os 37 SNPs mais significativos dentro de uma gama de 550.000 SNPs foram genotipados

individualmente e, destes, a associação mais forte foi para um polimorfismo no gene *DGKB* (Baum *et al*., 2008).

Portanto, é notável que vários genes-alvo de *EGR3* possuem funções relevantes para o funcionamento do sistema nervoso e alguns deles já possuem associações com doenças psiquiátricas. Muitos estudos anteriores focando nestes genes ou em genes de papel similar não encontraram associações com o fenótipo bipolar. Nesse sentido, é importante destacar que genes únicos podem não apresentar poder estatístico suficiente para representar uma alteração considerável associada a alguma doença. Entretanto, quando analisamos uma rede que inclui vários genes importantes (possivelmente alterados na doença), a análise ganha poder estatístico porque considera a interação entre vários genes, como foi analisado no nosso estudo referente ao capítulo 3 desta tese.

Os resultados da literatura discutidos acima e as vias que os genes do regulon do *EGR3* estão envolvidos reforçam a importância do nosso achado e o seu significado biológico. A possível perda da função adequada desses genes poderia influenciar na resposta ao estresse no córtex pré-frontal nos pacientes bipolares, potencialmente contribuindo para alterações estruturais e, consequentemente, prejuízos cognitivos. Alterações em regulons mestres, principalmente em fatores de transcrição que fazem a translação de efeitos ambientais para mudanças biológicas, poderiam estar associadas a maior vulnerabilidade ao estresse de pacientes psiquiátricos e poderiam auxiliar a compreender porque eventos estressantes muitas vezes desencadeiam episódios da doença, aumentam a gravidade dos sintomas e levam a um pior prognóstico.

Ainda em relação aos resultados obtidos no estudo referente ao capítulo 3 desta tese, é importante discutir os outros regulons, além do *EGR3*, enriquecidos no

transtorno bipolar. A associação deles com o fenótipo bipolar nas duas redes transcricionais mostrou menor poder estatístico (como é o caso do *TSC22D4*) ou o enriquecimento não foi consistente nas duas redes transcricionais avaliadas (como é o caso de *ILF2*), onde não houve associação do regulon com a assinatura bipolar do estudo com todo o córtex pré-frontal (Sig 2) na rede transcricional de córtex pré-frontal adulto (TN2) (vide Figura 3 do capítulo 3). Isso indica que o enriquecimento destes regulons pode estar associado com algum fator relacionado à assinatura mais específica (só de neurônios, Sig 1) e/ou algum fator relacionado ao pré-frontal em outras fases do desenvolvimento além da fase adulta (encontrado só na rede TN1). Mais estudos são necessários para esclarecer essa questão.

De qualquer forma, estes regulons foram enriquecidos no transtorno bipolar e assim representam redes gênicas possivelmente relevantes para a fisiopatologia da doença além de sugerir alvos de estudo. Isso é reforçado pelo fato de alguns alvos deles serem incomuns com o regulon do *EGR3* (Tabela 4) e por possuírem funções importantes para o sistema nervoso como discutido anteriormente. O possível papel biológico destes regulons está abordado na Tabela 5. Há poucos estudos focando estes genes na área da psiquiatria. Por exemplo, o fator de transcrição *YBX1* é capaz de regular a expressão do polimorfismo no receptor da serotonina associado com a susceptibilidade para transtornos afetivos (Klenova *et al*., 2004). Além disso, a regulação do transportador de serotonina pelo lítio pode ser modulada por *YBX1* através de uma alteração genética polimórfica potencialmente associada ao transtorno bipolar (Roberts *et al*., 2007).

Tabela 5. Demais regulons enriquecidos no transtorno bipolar.

Regulon	Nome	Função descrita
TSC22D4	TSC22 (TGFß1-stimulated clone 22) Domain Family, Member 4	Controle de vários processos biológicos, como proliferação celular, diferenciação, senescência e apoptose. No SNC, parece estar associado com a proliferação, migração e maturação de sinapses (Canterini *et al*., 2012).
ILF2	Interleukin enhancer binding factor 2 (também conhecido por NF45)	Necessário para a expressão de IL-2 pelas células T. Envolvido na redistribuição de mRNA nuclear para o citoplasma, reparo de DNA e regulação do processamento de microRNA (Zhao *et al*., 2005).
YBX1	Y-box binding protein 1	Contribui para a regulação da tradução, modulando a interação entre o mRNA e fatores de iniciação eucarióticos.
MADD	MAP-kinase activating death domain (também conhecido por DENN)	Proteína adaptadora que interage com o domínio de morte do receptor de TNF-α para ativar MAPK e propagar o sinal apoptótico (Zhang, Zhou e Miller, 1998).

Nosso trabalho identificou o regulon *MADD* reprimido no fenótipo bipolar, assim como o *EGR3*, e os demais regulons (*TSC22D4*, *ILF2*, *YBX1*) se mostraram induzidos na assinatura gênica da doença. Nesse sentido, é interessante observar que *EGR3* atua sobre *ILF2* e vice-versa, em uma interação inibitória. Assim, faz sentido o regulon *ILF2* estar induzido na assinatura da doença já que ele é inibido por *EGR3* e este foi encontrado reprimido. O raciocínio inverso também é coerente, fechando um ciclo: como *ILF2* inibe *EGR3*, estando aumentado, este fator de transcrição contribui para a inibição de *EGR3* e sua repressão na assinatura da doença. Considerando que o

regulon do *ILF2* está induzido na assinatura gênica do transtorno bipolar, e que está envolvido na expressão de IL-2, é relevante mencionar que estudos têm demonstrado um aumento de IL-2 e do seu receptor perifericamente na mania e que estes níveis foram correlacionados com os sintomas da doença (Brietzke *et al*., 2009; Tsai *et al*., 2001; Munkholm, Vinberg e Kessing, 2013). Estudos futuros avaliando a possível relação entre achados da literatura no transtorno bipolar e alterações nos regulons abordados no nosso trabalho serão fundamentais para entender se uma disfunção na regulação transcricional nos pacientes é responsável pelas alterações bioquímicas e moleculares que vem sendo associadas ao transtorno.

Os nossos resultados são bastante inovadores, mas requerem confirmação por outros métodos para obtermos conclusões mais definitivas sobre o papel do *EGR3* e dos demais regulons no transtorno bipolar. Um passo importante para a validação dos nossos dados será avaliar os níveis de mRNA, por PCR em tempo real, dos fatores de transcrição e de alguns de seus alvos em amostras biológicas independentes, por exemplo, em pré-frontal *postmortem* de pacientes bipolares. Contudo, como é difícil obter amostras deste tipo, outras abordagens experimentais serão fundamentais para realizar a validação, como estudos em animais e em modelos celulares. Neste contexto, é relevante destacar que o nosso trabalho utilizou métodos de bioinformática inovadores que têm sido desenvolvidos para lidar com as limitações já conhecidas dos dados de genômica funcional, como dados de microarranjo (Castro *et al*., 2016). Além disso, como discutido anteriormente, estudos que demonstram que animais sem *EGR3* funcional apresentam alterações comportamentais e fisiológicas (resposta aumentada ao estresse, hiperatividade e dificuldade em se habituar a estímulos ambientais e sociais) coerentes com modelos de doença mental reforçam os nossos achados. Além disso, a hiperatividade nestes animais pode ser revertida com medicações antipsicóticas

utilizadas no tratamento do transtorno bipolar (Gallitano-Mendel *et al*., 2008; Williams *et al*., 2012).

As limitações do nosso estudo estão bem discutidas no capítulo 3 desta tese. Elas incluem o fato de termos utilizado somente dois bancos de dados de microarranjo para obter a assinatura da doença, o que foi em decorrência da falta de outros bancos disponíveis para a região de interesse no nosso trabalho: área BA9 do pré-frontal de pacientes bipolares. Escolhemos essa área já que o banco de dados de microarranjo foco da nossa análise foi aquele contendo expressão gênica de neurônios isolados desta região do córtex pré-frontal e selecionamos então outro banco com dados da mesma área para realizar a comparação, considerando que o perfil de expressão gênica difere de acordo com a região cerebral e lâminas no próprio tecido. Nesse sentido, estudos futuros são importantes para avaliar a regulação transcricional também em outras áreas do pré-frontal e em outras estruturas cerebrais, como hipocampo, de pacientes bipolares e verificar a especificidade dos nossos dados.

Outro fator limitante da nossa análise refere-se aos dados clínicos da amostra dos estudos de microarranjo, como a medicação utilizada pelos pacientes. Como esses dados são originalmente de outros grupos de pesquisa, algumas destas informações não se encontram disponíveis. Entretanto, com base em achados da literatura (principalmente em estudos com animais), como discutido no capítulo 3, é possível inferir que estabilizadores de humor, antipsicóticos e uso de substâncias de abuso provavelmente não interferiram nos nossos resultados, uma vez que estes fatores costumam estar associados com a indução de *EGR3* e de outros genes da mesma família e nossos dados apontam para a repressão deste regulon no transtorno bipolar.

A abordagem de bioinformática utilizada neste trabalho é bastante inovadora e se mostra proveitosa tanto como um primeiro passo na identificação de disfunções na regulação transcricional no córtex pré-frontal no transtorno bipolar (tópico relativamente negligenciado e mal compreendido), como é também um exemplo de aplicação da análise de reguladores mestres no estudo de transtornos neuropsiquiátricos. Este tipo de abordagem é especialmente inovador na área da psiquiatria e há um crescente interesse nessa metodologia, uma vez que a análise de dados publicamente disponíveis com ferramentas computacionais possibilita integrar dados obtidos a partir de técnicas de larga escala (como o microarranjo) para identificar potenciais vias e alvos relevantes para estudo e posterior validação experimental. Considerando a atual dificuldade em obter apropriados recursos financeiros e humanos na pesquisa, a abordagem por bioinformática oferece um maior embasamento na escolha do que realmente avaliar experimentalmente em estudos com células, animais e humanos, os quais costumam despender muito tempo, dinheiro e trabalho. Assim, esse método surge como alternativa robusta para identificar e focar em alvos interessantes para estudo de mecanismos envolvidos em doenças e ação de fármacos.

Nesse sentido, então, nos questionamos sobre qual a melhor forma de estudar o regulon do *EGR3* e seus alvos. Como nosso grupo de pesquisa tem utilizado o modelo de células SH-SY5Y diferenciadas, confirmamos a caracterização neuronal deste modelo por análise de expressão gênica utilizando microarranjo e, então, avaliamos o enriquecimento do regulon do *EGR3* nas células. Tal regulon se mostrou enriquecido nas células diferenciadas, indicando que os genes dessa rede transcricional são importantes para a diferenciação e que este modelo *in vitro* é apropriado para o estudo destes alvos.

Para avaliar o uso deste modelo celular no estudo da fisiopatologia do transtorno bipolar, como estratégia, nós decidimos avaliar a hipótese da toxicidade sistêmica associada à doença (como discutido no capítulo 1) no modelo de células diferenciadas. Para isso, utilizamos o soro de pacientes (representando a toxicidade sistêmica) para tratar as células, ao invés do usual soro fetal bovino. Verificamos que o soro dos pacientes (principalmente no caso dos soros de pacientes em estágios tardios de progressão da doença) diminuiu a densidade de neuritos das células diferenciadas e também diminuiu a viabilidade celular (somente no caso dos soros de pacientes em estágios tardios), indicando que os soros induziram neurotoxicidade nas células. Outros estudos anteriores também relataram o efeito tóxico do soro de pacientes bipolares, mas em células endoteliais humanas (Politi *et al*., 2008) e em células mononucleares de sangue periférico (Herberth *et al*., 2011).

É importante lembrar que alterações periféricas em marcadores inflamatórios, de neurotrofinas e estresse oxidativo têm sido relatadas em pacientes bipolares, especialmente durante os episódios da doença, sugerindo um padrão de 'toxicidade sistêmica' no transtorno como discutido anteriormente. Assim, acreditamos que o soro de pacientes bipolares, principalmente em estágios mais tardios de progressão da doença (apresentando dano cumulativo após vários episódios de humor), pode conter substâncias tóxicas que podem afetar as células neurais caso passem a barreira-hematoencefálica ou se houver alguma ruptura na integridade desta barreira permitindo passagem de substâncias do sangue periférico para o cérebro (Patel e Frey, 2015).

Nosso grupo tem proposto que prejuízos na neuroplasticidade podem levar a alterações estruturais no cérebro pela redução de neuritos e conexões na rede neuronal e costuma se referir a esse processo como neuroprogressão do transtorno bipolar. Tais

alterações têm sido observadas em pacientes que já passaram por vários episódios de humor (possivelmente maior exposição ao estresse, maior toxicidade sistêmica) conforme exemplificado e discutido no capítulo 2. Entretanto, o possível efeito da toxicidade sistêmica no cérebro ainda é desconhecido. Assim, neste estudo preliminar com as células SH-SY5Y, investigamos os efeitos da possível toxicidade dos soros de pacientes em células neuronais e propomos esse modelo experimental como uma maneira de desafiar as células e estudar alvos, vias e fármacos relacionados com o transtorno bipolar. Por exemplo, estudar as moléculas identificadas pela análise de mapa de conectividade conforme discutido no capítulo 4.

De forma geral, podemos propor que a disfunção no regulon do *EGR3* observada no nosso estudo esteja envolvida com aspectos da neuroprogressão no transtorno bipolar, considerando o papel do *EGR3* na neuroplasticidade. E, como esse fator de transcrição é ativado em resposta a estímulos de estresse, é possível supor que estressores cumulativos (por exemplo, episódios de humor recorrentes) podem agir como estímulos ambientais que induzem alterações específicas na expressão gênica, consequentemente levando a uma resposta mal adaptada ao estresse nos pacientes, o que envolveria também mecanismos sistêmicos incluindo aqueles vinculados à toxicidade periférica. Em última análise, essa disfunção poderia estar associada a uma resiliência neurobiológica reduzida, através da interação entre fatores ambientais e genéticos, influenciando na doença e possivelmente contribuindo com sintomas de prejuízo cognitivo e funcional observados no transtorno bipolar.

5. CONCLUSÕES

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

- Dados da literatura sugerem que biomarcadores periféricos relacionados às vias de inflamação, estresse oxidativo e neurotrofinas encontram-se alterados no transtorno bipolar, especialmente durante os episódios agudos de humor. Em conjunto, estas alterações têm sido associadas a uma toxicidade sistêmica da doença e aos prejuízos decorrentes de múltiplos episódios;

- É possível observar visualmente, através de imagens de ressonância magnética, pronunciada atrofia cortical e alargamento dos ventrículos em paciente bipolar após a experiência de múltiplos episódios, sugerindo que tais alterações estruturais estejam associadas com a toxicidade dos episódios e com a progressão do transtorno bipolar;

- Entre possíveis unidades regulatórias transcricionais disfuncionais no córtex pré-frontal de pacientes bipolares, o regulon do *EGR3* se mostrou reprimido consistentemente em duas assinaturas gênicas da doença nas duas redes transcricionais avaliadas;

- Análise de mapas de conectividade identificaram várias moléculas com potencial de modular as assinaturas gênicas do transtorno bipolar referente aos cinco regulons enriquecidos nas assinaturas da doença. Muitas destas associações são coerentes com dados relacionados ao transtorno bipolar, propondo estudos para validar os efeitos destas moléculas.

- O perfil de expressão gênica do modelo de células SH-SY5Y diferenciadas, através de análises de microarranjo, reforça a caracterização neuronal destas células e

sua aplicação em neurociências. Além disso, este modelo *in vitro* demonstrou ser adequado para estudar os alvos identificados no estudo de regulação transcricional, uma vez que o regulon do *EGR3* foi enriquecido pela diferenciação das células, sugerindo que tenha um papel relevante nesse processo;

- O soro de pacientes bipolares, especialmente aqueles em estágio mais tardio da doença, apresentou toxicidade às células SH-SY5Y diferenciadas, reduzindo os neuritos e a viabilidade destas células. Isto sugere, em última instância, que a toxicidade sistêmica poderia afetar células neuronais.

Em conclusão, nós propomos que a toxicidade sistêmica relacionada aos episódios recorrentes de humor possa influenciar nas alterações anatômicas cerebrais associadas com a progressão do transtorno bipolar, e a disfunção no regulon do *EGR3* poderia estar envolvida com aspectos desta neuroprogressão considerando o papel do *EGR3* na resposta ao estresse e na neuroplasticidade. Obviamente, estas são hipóteses que devem ser apropriadamente validadas em modelos experimentais, e sugerem alvos interessantes para o desenvolvimento de novos tratamentos.

6. PERSPECTIVAS

O trabalho relacionado a essa tese segue em andamento, com as análises de validação da técnica do microarranjo para confirmar os resultados de expressão diferencial de alguns genes identificados. Essas análises incluem:

- avaliar a expressão gênica, por PCR quantitativo em tempo real, de genes diferencialmente expressos nas células diferenciadas por sete dias com ácido retinóico e nas células proliferativas;

- avaliar a expressão gênica, por PCR quantitativo em tempo real, de genes pertencentes ao regulon do *EGR3* que foram diferencialmente expressos nas células diferenciadas.

Outro experimento que segue em andamento refere-se ao estudo do regulon do *EGR3* nas células SH-SY5Y diferenciadas, relacionando a toxicidade sistêmica associada ao transtorno bipolar e este regulon. Assim, como perspectivas desta tese:

- avaliar se o *EGR3* diminui nas células SH-SY5Y diferenciadas sob ação do soro de pacientes (análise por imunofluorescência, western blot e PCR em tempo real);

- avaliar o perfil de expressão gênica, através de microarranjo, destas células e comparar com a assinatura da doença abordada no capítulo 3, para validarmos os dados de bioinformática;

Como perspectiva também está um experimento envolvendo o silenciamento de *EGR3* por siRNA nas células SH-SY5Y diferenciadas para avaliar os efeitos na ausência deste gene, em relação a parâmetros como viabilidade celular, densidade de neuritos e análise de expressão gênica.

Além disso, será importante avaliar nas células SH-SY5Y diferenciadas algumas das moléculas selecionadas pela análise de CMAP. Nesse sentido:

- avaliar como as drogas selecionadas pela análise de CMAP e os fármacos tradicionalmente utilizados no tratamento do transtorno bipolar (lítio e valproato) modulam o regulon do *EGR3*.

- testar se estas drogas possuem efeito neuroprotetor nas células diferenciadas contra a toxicidade induzida pelos soros dos pacientes.

Desta forma, essas análises posteriores serão importantes para validar nossos resultados de bioinformática e para estabelecer o uso das células SH-SY5Y diferenciadas como modelo *in vitro* para estudo da fisiopatologia do transtorno bipolar, do efeito de drogas clássicas e de novas terapias. Em paralelo, será relevante avaliar os níveis de mRNA de alguns alvos de *EGR3* em amostras periféricas de pacientes bipolares e indivíduos saudáveis para entender como o regulon do *EGR3* atua perifericamente nestes pacientes.

REFERÊNCIAS

Abe, C., C. J. Ekman, C. Sellgren, P. Petrovic, M. Ingvar, and M. Landen. 'Manic Episodes Are Related to Changes in Frontal Cortex: A Longitudinal Neuroimaging Study of Bipolar Disorder 1', *Brain* **Vol. 138, No. Pt 11**, 3440-8, 2015.

Ahuja, R., R. Pinyol, N. Reichenbach, L. Custer, J. Klingensmith, M. M. Kessels, and B. Qualmann. 'Cordon-Bleu Is an Actin Nucleation Factor and Controls Neuronal Morphology', *Cell* **Vol. 131, No. 2**, 337-50, 2007.

Alemany, S., M. Ribases, N. Vilor-Tejedor, M. Bustamante, C. Sanchez-Mora, R. Bosch, V. Richarte, B. Cormand, M. Casas, J. A. Ramos-Quiroga, and J. Sunyer. 'New Suggestive Genetic Loci and Biological Pathways for Attention Function in Adult Attention-Deficit/Hyperactivity Disorder', *Am J Med Genet B Neuropsychiatr Genet*, 2015.

Altar, C. A., M. P. Vawter, and S. D. Ginsberg. 'Target Identification for Cns Diseases by Transcriptional Profiling', *Neuropsychopharmacology* **Vol. 34, No. 1**, 18-54, 2009.

Andreazza, A. C., F. Kapczinski, M. Kauer-Sant'Anna, J. C. Walz, D. J. Bond, C. A. Gonçalves, L. T. Young, and L. N. Yatham. '3-Nitrotyrosine and Glutathione Antioxidant System in Patients in the Early and Late Stages of Bipolar Disorder.', *J Psychiatry Neurosci* **Vol. 34, No. 4**, 263-71, 2009.

Aslam, S. P., K. A. Carroll, B. Naz, and A. O. Alao. 'Valacyclovir-Induced Psychosis and Manic Symptoms in an Adolescent Young Woman with Genital Herpes Simplex', *Psychosomatics* **Vol. 50, No. 3**, 293-6, 2009.

Bal-Price, A. K., C. Sunol, D. G. Weiss, E. van Vliet, R. H. Westerink, and L. G. Costa. 'Application of in Vitro Neurotoxicity Testing for Regulatory Purposes: Symposium Iii Summary and Research Needs', *Neurotoxicology*, 2008.

Baou, M., V. A. Boumba, P. Petrikis, G. Rallis, T. Vougiouklakis, and V. Mavreas. 'A Review of Genetic Alterations in the Serotonin Pathway and Their Correlation with Psychotic Diseases and Response to Atypical Antipsychotics', *Schizophr Res* **Vol. 170, No. 1**, 18-29, 2016.

Barbosa, I. G., N. P. Rocha, M. E. Bauer, A. S. de Miranda, R. B. Huguet, H. J. Reis, P. A. Zunszain, M. A. Horowitz, C. M. Pariante, and A. L. Teixeira. 'Chemokines in Bipolar Disorder: Trait or State?', *Eur Arch Psychiatry Clin Neurosci* **Vol. 263, No. 2**, 159-65, 2013.

Basso, K., A. A. Margolin, G. Stolovitzky, U. Klein, R. Dalla-Favera, and A. Califano. 'Reverse Engineering of Regulatory Networks in Human B Cells', *Nat Genet* **Vol. 37, No. 4**, 382-90, 2005.

Baum, A. E., N. Akula, M. Cabanero, I. Cardona, W. Corona, B. Klemens, T. G. Schulze, S. Cichon, M. Rietschel, M. M. Nothen, A. Georgi, J. Schumacher, M. Schwarz, R. Abou Jamra, S. Hofels, P. Propping, J. Satagopan, S. D. Detera-Wadleigh, J. Hardy, and F. J. McMahon. 'A Genome-Wide Association Study Implicates Diacylglycerol Kinase Eta (Dgkh) and Several Other Genes in the Etiology of Bipolar Disorder', *Mol Psychiatry* **Vol. 13, No. 2**, 197-207, 2008.

Beckmann, A. M., and P. A. Wilce. 'Egr Transcription Factors in the Nervous System', *Neurochem Int* **Vol. 31, No. 4**, 477-510; discussion 517-6, 1997.

Belmaker, R. H. 'Bipolar Disorder', *N Engl J Med* **Vol. 351, No. 5**, 476-86, 2004.

Berk, M., F. Kapczinski, A. C. Andreazza, O. M. Dean, F. Giorlando, M. Maes, M. Yucel, C. S. Gama, S. Dodd, B. Dean, P. V. Magalhaes, P. Amminger, P. McGorry, and G. S. Malhi. 'Pathways Underlying Neuroprogression in Bipolar Disorder: Focus on Inflammation, Oxidative Stress and Neurotrophic Factors', *Neurosci Biobehav Rev* **Vol. 35, No. 3**, 804-17, 2011.

Brietzke, E., L. Stertz, B. S. Fernandes, M. Kauer-Sant'anna, M. Mascarenhas, A. Escosteguy Vargas, J. A. Chies, and F. Kapczinski. 'Comparison of Cytokine Levels in Depressed, Manic and Euthymic Patients with Bipolar Disorder', *J Affect Disord* **Vol. 116, No. 3**, 214-7, 2009.

Brown, N. C., A. C. Andreazza, and L. T. Young. 'An Updated Meta-Analysis of Oxidative Stress Markers in Bipolar Disorder', *Psychiatry Res* **Vol. 218, No. 1-2**, 61-8, 2014.

Cao, B., I. C. Passos, B. Mwangi, I. E. Bauer, G. B. Zunta-Soares, F. Kapczinski, and J. C. Soares. 'Hippocampal Volume and Verbal Memory Performance in Late-Stage Bipolar Disorder', *J Psychiatr Res* **Vol. 73**, 102-7, 2016.

Carro, M. S., W. K. Lim, M. J. Alvarez, R. J. Bollo, X. Zhao, E. Y. Snyder, E. P. Sulman, S. L. Anne, F. Doetsch, H. Colman, A. Lasorella, K. Aldape, A. Califano, and A. Iavarone. 'The Transcriptional Network for Mesenchymal Transformation of Brain Tumours', *Nature* **Vol. 463, No. 7279**, 318-25, 2010.

Castro, M. A., I. de Santiago, T. M. Campbell, C. Vaughn, T. E. Hickey, E. Ross, W. D. Tilley, F. Markowetz, B. A. Ponder, and K. B. Meyer. 'Regulators of Genetic Risk of

Breast Cancer Identified by Integrative Network Analysis', *Nat Genet* **Vol. 48, No. 1**, 12-21, 2016.

Cheval, H., C. Chagneau, G. Levasseur, A. Veyrac, N. Faucon-Biguet, S. Laroche, and S. Davis. 'Distinctive Features of Egr Transcription Factor Regulation and Dna Binding Activity in Ca1 of the Hippocampus in Synaptic Plasticity and Consolidation and Reconsolidation of Fear Memory', *Hippocampus* **Vol. 22, No. 3**, 631-42, 2012.

Christy, B., and D. Nathans. 'Dna Binding Site of the Growth Factor-Inducible Protein Zif268', *Proc Natl Acad Sci U S A* **Vol. 86, No. 22**, 8737-41, 1989.

Cordeiro, Q., M. E. Talkowski, K. V. Chowdari, J. Wood, V. Nimgaonkar, and H. Vallada. 'Association and Linkage Analysis of Rgs4 Polymorphisms with Schizophrenia and Bipolar Disorder in Brazil', *Genes Brain Behav* **Vol. 4, No. 1**, 45-50, 2005.

Craddock, N., and I. Jones. 'Genetics of Bipolar Disorder', *J Med Genet* **Vol. 36, No. 8**, 585-94, 1999.

Craddock, N., V. Khodel, P. Van Eerdewegh, and T. Reich. 'Mathematical Limits of Multilocus Models: The Genetic Transmission of Bipolar Disorder', *Am J Hum Genet* **Vol. 57, No. 3**, 690-702, 1995.

Craddock, N., and P. Sklar. 'Genetics of Bipolar Disorder', *Lancet* **Vol. 381, No. 9878**, 1654-62, 2013.

Cunha, A. B., B. N. Frey, A. C. Andreazza, J. D. Goi, A. R. Rosa, C. A. Goncalves, A. Santin, and F. Kapczinski. 'Serum Brain-Derived Neurotrophic Factor Is Decreased in Bipolar Disorder During Depressive and Manic Episodes', *Neurosci Lett* **Vol. 398, No. 3**, 215-9, 2006.

Dechant, G., and Y. A. Barde. 'The Neurotrophin Receptor P75(Ntr): Novel Functions and Implications for Diseases of the Nervous System', *Nat Neurosci* **Vol. 5, No. 11**, 1131-6, 2002.

Ding, L., and A. N. Hegde. 'Expression of Rgs4 Splice Variants in Dorsolateral Prefrontal Cortex of Schizophrenic and Bipolar Disorder Patients', *Biol Psychiatry* **Vol. 65, No. 6**, 541-5, 2009.

Ding, L., M. Styblo, Z. Drobna, and A. N. Hegde. 'Expression of the Longest Rgs4 Splice Variant in the Prefrontal Cortex Is Associated with Single Nucleotide Polymorphisms in Schizophrenia Patients', *Front Psychiatry* **Vol. 7**, 26, 2016.

Drevets, W. C., and M. L. Furey. 'Replication of Scopolamine's Antidepressant Efficacy in Major Depressive Disorder: A Randomized, Placebo-Controlled Clinical Trial', *Biol Psychiatry* **Vol. 67, No. 5**, 432-8, 2010.

Edsjo, A., E. Lavenius, H. Nilsson, J. C. Hoehner, P. Simonsson, L. A. Culp, T. Martinsson, C. Larsson, and S. Pahlman. 'Expression of Trkb in Human Neuroblastoma in Relation to Mycn Expression and Retinoic Acid Treatment', *Lab Invest* **Vol. 83, No. 6**, 813-23, 2003.

Faganello, F. R., and R. Mattioli. 'Anxiolytic-Like Effect of Chlorpheniramine in Inhibitory Avoidance in Goldfish Submitted to Telencephalic Ablation', *Prog Neuropsychopharmacol Biol Psychiatry* **Vol. 31, No. 1**, 269-74, 2007.

Fernandes, B. S., M. Berk, C. W. Turck, J. Steiner, and C. A. Goncalves. 'Decreased Peripheral Brain-Derived Neurotrophic Factor Levels Are a Biomarker of Disease

Activity in Major Psychiatric Disorders: A Comparative Meta-Analysis', *Mol Psychiatry*, 2014.

Fernandes, B. S., C. S. Gama, K. M. Cereser, L. N. Yatham, G. R. Fries, G. Colpo, D. de Lucena, M. Kunz, F. A. Gomes, and F. Kapczinski. 'Brain-Derived Neurotrophic Factor as a State-Marker of Mood Episodes in Bipolar Disorders: A Systematic Review and Meta-Regression Analysis', *J Psychiatr Res* **Vol. 45, No. 8**, 995-1004, 2011.

Frey, B. N., A. C. Andreazza, J. Houenou, S. Jamain, B. I. Goldstein, M. A. Frye, M. Leboyer, M. Berk, G. S. Malhi, C. Lopez-Jaramillo, V. H. Taylor, S. Dodd, S. Frangou, G. B. Hall, B. S. Fernandes, M. Kauer-Sant'Anna, L. N. Yatham, F. Kapczinski, and L. T. Young. 'Biomarkers in Bipolar Disorder: A Positional Paper from the International Society for Bipolar Disorders Biomarkers Task Force', *Aust N Z J Psychiatry* **Vol. 47, No. 4**, 321-32, 2013.

Fries, G. R., M. P. Vasconcelos-Moreno, C. Gubert, B. T. Santos, A. L. da Rosa, B. Eisele, J. Sartori, B. Pfaffenseller, F. Kapczinski, and M. Kauer-Sant'anna. 'Early Apoptosis in Peripheral Blood Mononuclear Cells from Patients with Bipolar Disorder', *J Affect Disord* **Vol. 152-154**, 474-7, 2014.

Furey, M. L., and W. C. Drevets. 'Antidepressant Efficacy of the Antimuscarinic Drug Scopolamine: A Randomized, Placebo-Controlled Clinical Trial', *Arch Gen Psychiatry* **Vol. 63, No. 10**, 1121-9, 2006.

Gallitano, A. L., R. Tillman, V. Dinu, and B. Geller. 'Family-Based Association Study of Early Growth Response Gene 3 with Child Bipolar I Disorder', *J Affect Disord* **Vol. 138, No. 3**, 387-96, 2012.

Gallitano-Mendel, A., Y. Izumi, K. Tokuda, C. F. Zorumski, M. P. Howell, L. J. Muglia, D. F. Wozniak, and J. Milbrandt. 'The Immediate Early Gene Early Growth Response Gene 3 Mediates Adaptation to Stress and Novelty', *Neuroscience* **Vol. 148, No. 3**, 633-43, 2007.

Gallitano-Mendel, A., D. F. Wozniak, E. A. Pehek, and J. Milbrandt. 'Mice Lacking the Immediate Early Gene *EGR3* Respond to the Anti-Aggressive Effects of Clozapine yet Are Relatively Resistant to Its Sedating Effects', *Neuropsychopharmacology* **Vol. 33, No. 6**, 1266-75, 2008.

Gao, X., R. L. Daugherty, and W. G. Tourtellotte. 'Regulation of Low Affinity Neurotrophin Receptor (P75(Ntr)) by Early Growth Response (Egr) Transcriptional Regulators', *Mol Cell Neurosci* **Vol. 36, No. 4**, 501-14, 2007.

Geddes, J. R., and D. J. Miklowitz. 'Treatment of Bipolar Disorder', *Lancet* **Vol. 381, No. 9878**, 1672-82, 2013.

'Genome-Wide Association Study of 14,000 Cases of Seven Common Diseases and 3,000 Shared Controls'. *Nature* **Vol. 447, No. 7145**, 661-78, 2007.

Gershon, E. S., N. Alliey-Rodriguez, and C. Liu. 'After Gwas: Searching for Genetic Risk for Schizophrenia and Bipolar Disorder', *Am J Psychiatry* **Vol. 168, No. 3**, 253-6, 2011.

Gigante, A. D., L. T. Young, L. N. Yatham, A. C. Andreazza, F. G. Nery, L. T. Grinberg, H. Heinsen, and B. Lafer. 'Morphometric Post-Mortem Studies in Bipolar Disorder: Possible Association with Oxidative Stress and Apoptosis', *Int J Neuropsychopharmacol* **Vol. 14, No. 8**, 1075-89, 2011.

Gonzalez-Maeso, J., T. Yuen, B. J. Ebersole, E. Wurmbach, A. Lira, M. Zhou, N. Weisstaub, R. Hen, J. A. Gingrich, and S. C. Sealfon. 'Transcriptome Fingerprints Distinguish Hallucinogenic and Nonhallucinogenic 5-Hydroxytryptamine 2a Receptor Agonist Effects in Mouse Somatosensory Cortex', *J Neurosci* **Vol. 23, No. 26**, 8836-43, 2003.

Grande, I., P. V. Magalhães, M. Kunz, E. Vieta, and F. Kapczinski. 'Mediators of Allostasis and Systemic Toxicity in Bipolar Disorder', *Physiol Behav* **Vol. 106, No. 1**, 46-50, 2012.

Guo, A. Y., J. Sun, P. Jia, and Z. Zhao. 'A Novel Microrna and Transcription Factor Mediated Regulatory Network in Schizophrenia', *BMC Syst Biol* **Vol. 4**, 10, 2010.

Hamdani, N., R. Tamouza, and M. Leboyer. 'Immuno- Inflammatory Markers of Bipolar Disorder: A Review of Evidence', *Front Biosci (Elite Ed)* **Vol. 4**, 2170-82, 2012.

Hawton, K., L. Sutton, C. Haw, J. Sinclair, and L. Harriss. 'Suicide and Attempted Suicide in Bipolar Disorder: A Systematic Review of Risk Factors.', *J Clin Psychiatry* **Vol. 66, No. 6**, 693-704, 2005.

Helenius, D., P. M. Jorgensen, and H. C. Steinhausen. 'A Three Generations Nation-Wide Population Study of Family Load Estimates in Bipolar Disorder with Different Age at Onset', *J Affect Disord* **Vol. 150, No. 1**, 146-51, 2013.

Hellbom, E. 'Chlorpheniramine, Selective Serotonin-Reuptake Inhibitors (Ssris) and over-the-Counter (Otc) Treatment', *Med Hypotheses* **Vol. 66, No. 4**, 689-90, 2006.

Herberth, M., D. Koethe, Y. Levin, E. Schwarz, N. D. Krzyszton, S. Schoeffmann, H. Ruh, H. Rahmoune, L. Kranaster, T. Schoenborn, M. F. Leweke, P. C. Guest, and S. Bahn. 'Peripheral Profiling Analysis for Bipolar Disorder Reveals Markers Associated with Reduced Cell Survival', *Proteomics* **Vol. 11, No. 1**, 94-105, 2011.

Herdegen, T., and J. D. Leah. 'Inducible and Constitutive Transcription Factors in the Mammalian Nervous System: Control of Gene Expression by Jun, Fos and Krox, and Creb/Atf Proteins', *Brain Res Brain Res Rev* **Vol. 28, No. 3**, 370-490, 1998.

Hippenmeyer, S., N. A. Shneider, C. Birchmeier, S. J. Burden, T. M. Jessell, and S. Arber. 'A Role for Neuregulin1 Signaling in Muscle Spindle Differentiation', *Neuron* **Vol. 36, No. 6**, 1035-49, 2002.

Horiuchi, Y., J. Nakayama, H. Ishiguro, T. Ohtsuki, S. D. Detera-Wadleigh, T. Toyota, K. Yamada, M. Nankai, H. Shibuya, T. Yoshikawa, and T. Arinami. 'Possible Association between a Haplotype of the Gaba-a Receptor Alpha 1 Subunit Gene (Gabra1) and Mood Disorders', *Biol Psychiatry* **Vol. 55, No. 1**, 40-5, 2004.

Huang, E. J., and L. F. Reichardt. 'Neurotrophins: Roles in Neuronal Development and Function', *Annu Rev Neurosci* **Vol. 24**, 677-736, 2001.

Huentelman, M. J., L. Muppana, J. J. Corneveaux, V. Dinu, J. J. Pruzin, R. Reiman, C. N. Borish, M. De Both, A. Ahmed, A. Todorov, C. R. Cloninger, R. Zhang, J. Ma, and A. L. Gallitano. 'Association of Snps in *EGR3* and Arc with Schizophrenia Supports a Biological Pathway for Schizophrenia Risk', *PLoS One* **Vol. 10, No. 10**, e0135076, 2015.

Hughes, P., and M. Dragunow. 'Induction of Immediate-Early Genes and the Control of Neurotransmitter-Regulated Gene Expression within the Nervous System', *Pharmacol Rev* **Vol. 47, No. 1**, 133-78, 1995.

Jaffe, R. J., V. Novakovic, and E. D. Peselow. 'Scopolamine as an Antidepressant: A Systematic Review', *Clin Neuropharmacol* **Vol. 36, No. 1**, 24-6, 2013.

Jayaram, N., N. P. Rao, G. Venkatasubramanian, S. Sanmuganathan, R. V. Behere, S. Varambally, and B. N. Gangadhar. 'Acyclovir in Bipolar Disorder: A Mismatched Combination?', *Indian J Psychol Med* **Vol. 33, No. 2**, 208-9, 2011.

Jeon, W. J., B. Dean, E. Scarr, and A. Gibbons. 'The Role of Muscarinic Receptors in the Pathophysiology of Mood Disorders: A Potential Novel Treatment?', *Curr Neuropharmacol* **Vol. 13, No. 6**, 739-49, 2015.

Jones, M. W., M. L. Errington, P. J. French, A. Fine, T. V. Bliss, S. Garel, P. Charnay, B. Bozon, S. Laroche, and S. Davis. 'A Requirement for the Immediate Early Gene Zif268 in the Expression of Late Ltp and Long-Term Memories', *Nat Neurosci* **Vol. 4, No. 3**, 289-96, 2001.

Kaiser, T., G. Feng. 'Modeling psychiatric disorders for developing effective treatments', *Nat Med*. **Vol. 21, No.9**, 979-88, 2015.

Kapczinski, F., F. Dal-Pizzol, A. L. Teixeira, P. V. Magalhaes, M. Kauer-Sant'Anna, F. Klamt, J. C. Moreira, M. A. de Bittencourt Pasquali, G. R. Fries, J. Quevedo, C. S. Gama, and R. Post. 'Peripheral Biomarkers and Illness Activity in Bipolar Disorder', *J Psychiatr Res* **Vol. 45, No. 2**, 156-61, 2011.

Kapczinski, F., F. Dal-Pizzol, A. L. Teixeira, P. V. Magalhaes, M. Kauer-Sant'Anna, F. Klamt, M. A. Pasquali, J. Quevedo, C. S. Gama, and R. Post. 'A Systemic Toxicity Index Developed to Assess Peripheral Changes in Mood Episodes', *Mol Psychiatry* **Vol. 15, No. 8**, 784-6, 2010.

Kapczinski, F., V. V. Dias, M. Kauer-Sant'Anna, E. Brietzke, G. H. Vazquez, E. Vieta, and M. Berk. 'The Potential Use of Biomarkers as an Adjunctive Tool for Staging Bipolar Disorder', *Prog Neuropsychopharmacol Biol Psychiatry* **Vol. 33, No. 8**, 1366-71, 2009.

Kapczinski, F., V. V. Dias, M. Kauer-Sant'Anna, B. N. Frey, R. Grassi-Oliveira, F. Colom, and M. Berk. 'Clinical Implications of a Staging Model for Bipolar Disorders', *Expert Rev Neurother* **Vol. 9, No. 7**, 957-66, 2009.

Kapczinski, F., E. Vieta, A. C. Andreazza, B. N. Frey, F. A. Gomes, J. Tramontina, M. Kauer-Sant'anna, R. Grassi-Oliveira, and R. M. Post. 'Allostatic Load in Bipolar Disorder: Implications for Pathophysiology and Treatment', *Neurosci Biobehav Rev* **Vol. 32, No. 4**, 675-92, 2008.

Kaplan, D. R., K. Matsumoto, E. Lucarelli, and C. J. Thiele. 'Induction of Trkb by Retinoic Acid Mediates Biologic Responsiveness to Bdnf and Differentiation of Human Neuroblastoma Cells. Eukaryotic Signal Transduction Group', *Neuron* **Vol. 11, No. 2**, 321-31, 1993.

Kato, T., G. Kuratomi, and N. Kato. 'Genetics of Bipolar Disorder', *Drugs Today (Barc)* **Vol. 41, No. 5**, 335-44, 2005.

Kauer-Sant'Anna, M., F. Kapczinski, A. C. Andreazza, D. J. Bond, R. W. Lam, L. T. Young, and L. N. Yatham. 'Brain-Derived Neurotrophic Factor and Inflammatory Markers in Patients with Early- Vs. Late-Stage Bipolar Disorder.', *Int J Neuropsychopharmacol* **Vol. 12, No. 4**, 447-58, 2009.

Keck, P. E., Jr., S. L. McElroy, and S. M. Strakowski. 'Anticonvulsants and Antipsychotics in the Treatment of Bipolar Disorder', *J Clin Psychiatry* **Vol. 59 Suppl 6**, 74-81; discussion 82, 1998.

Kelly, M. P., J. M. Stein, C. G. Vecsey, C. Favilla, X. Yang, S. F. Bizily, M. F. Esposito, G. Wand, S. J. Kanes, and T. Abel. 'Developmental Etiology for Neuroanatomical and Cognitive Deficits in Mice Overexpressing Galphas, a G-Protein Subunit Genetically Linked to Schizophrenia', *Mol Psychiatry* **Vol. 14, No. 4**, 398-415, 347, 2009.

Kessels, M. M., L. Schwintzer, D. Schlobinski, and B. Qualmann. 'Controlling Actin Cytoskeletal Organization and Dynamics During Neuronal Morphogenesis', *Eur J Cell Biol* **Vol. 90, No. 11**, 926-33, 2011.

Kessing, L. V., and P. K. Andersen. 'Does the Risk of Developing Dementia Increase with the Number of Episodes in Patients with Depressive Disorder and in Patients with Bipolar Disorder?', *J Neurol Neurosurg Psychiatry* **Vol. 75, No. 12**, 1662-6, 2004.

Ketter, T. A., J. P. Houston, D. H. Adams, R. C. Risser, A. L. Meyers, D. J. Williamson, and M. Tohen. 'Differential Efficacy of Olanzapine and Lithium in Preventing Manic or Mixed Recurrence in Patients with Bipolar I Disorder Based on Number of Previous Manic or Mixed Episodes.', *J Clin Psychiatry* **Vol. 67, No. 1**, 95-101, 2006.

Kieseppa, T., T. Partonen, J. Haukka, J. Kaprio, and J. Lonnqvist. 'High Concordance of Bipolar I Disorder in a Nationwide Sample of Twins', *Am J Psychiatry* **Vol. 161, No. 10**, 1814-21, 2004.

Kim, J. H., D. S. Roberts, Y. Hu, G. C. Lau, A. R. Brooks-Kayal, D. H. Farb, and S. J. Russek. 'Brain-Derived Neurotrophic Factor Uses Creb and *EGR3* to Regulate Nmda Receptor Levels in Cortical Neurons', *J Neurochem* **Vol. 120, No. 2**, 210-9, 2012.

Kim, S. H., J. Y. Song, E. J. Joo, K. Y. Lee, Y. M. Ahn, and Y. S. Kim. '*EGR3* as a Potential Susceptibility Gene for Schizophrenia in Korea', *Am J Med Genet B Neuropsychiatr Genet* **Vol. 153b, No. 7**, 1355-60, 2010.

Klenova, E., A. C. Scott, J. Roberts, S. Shamsuddin, E. A. Lovejoy, S. Bergmann, V. J. Bubb, H. D. Royer, and J. P. Quinn. 'Yb-1 and Ctcf Differentially Regulate the 5-Htt Polymorphic Intron 2 Enhancer Which Predisposes to a Variety of Neurological Disorders', *J Neurosci* **Vol. 24, No. 26**, 5966-73, 2004.

Ko, S. W., K. I. Vadakkan, H. Ao, A. Gallitano-Mendel, F. Wei, J. Milbrandt, and M. Zhuo. 'Selective Contribution of Egr1 (Zif/268) to Persistent Inflammatory Pain', *J Pain* **Vol. 6, No. 1**, 12-20, 2005.

Kupfer, D. J. 'The Increasing Medical Burden in Bipolar Disorder', *JAMA* **Vol. 293, No. 20**, 2528-30, 2005.

Lamb, J., E. D. Crawford, D. Peck, J. W. Modell, I. C. Blat, M. J. Wrobel, J. Lerner, J. P. Brunet, A. Subramanian, K. N. Ross, M. Reich, H. Hieronymus, G. Wei, S. A. Armstrong, S. J. Haggarty, P. A. Clemons, R. Wei, S. A. Carr, E. S. Lander, and T. R.

Golub. 'The Connectivity Map: Using Gene-Expression Signatures to Connect Small Molecules, Genes, and Disease', *Science* **Vol. 313, No. 5795**, 1929-35, 2006.

Lanahan, A., and P. Worley. 'Immediate-Early Genes and Synaptic Function', *Neurobiol Learn Mem* **Vol. 70, No. 1-2**, 37-43, 1998.

Lapa, G. B., T. A. Mathews, J. Harp, E. A. Budygin, and S. R. Jones. 'Diphenylpyraline, a Histamine H1 Receptor Antagonist, Has Psychostimulant Properties', *Eur J Pharmacol* **Vol. 506, No. 3**, 237-40, 2005.

Lavagnino, L., B. Cao, B. Mwangi, M. J. Wu, M. Sanches, G. B. Zunta-Soares, F. Kapczinski, and J. Soares. 'Changes in the Corpus Callosum in Women with Late-Stage Bipolar Disorder', *Acta Psychiatr Scand* **Vol. 131, No. 6**, 458-64, 2015.

Leboyer, M., I. Soreca, J. Scott, M. Frye, C. Henry, R. Tamouza, and D. J. Kupfer. 'Can Bipolar Disorder Be Viewed as a Multi-System Inflammatory Disease?', *J Affect Disord* **Vol. 141, No. 1**, 1-10, 2012.

Lee, S. H., N. R. Wray, M. E. Goddard, and P. M. Visscher. 'Estimating Missing Heritability for Disease from Genome-Wide Association Studies', *Am J Hum Genet* **Vol. 88, No. 3**, 294-305, 2011.

Lee, W. P., and W. S. Tzou. 'Computational Methods for Discovering Gene Networks from Expression Data', *Brief Bioinform* **Vol. 10, No. 4**, 408-23, 2009.

Li, L., J. Carter, X. Gao, J. Whitehead, and W. G. Tourtellotte. 'The Neuroplasticity-Associated Arc Gene Is a Direct Transcriptional Target of Early Growth Response (Egr) Transcription Factors', *Mol Cell Biol* **Vol. 25, No. 23**, 10286-300, 2005.

Li, L., S. H. Yun, J. Keblesh, B. L. Trommer, H. Xiong, J. Radulovic, and W. G. Tourtellotte. '*EGR3*, a Synaptic Activity Regulated Transcription Factor That Is Essential for Learning and Memory', *Mol Cell Neurosci* **Vol. 35, No. 1**, 76-88, 2007.

Loebrich, S., and E. Nedivi. 'The Function of Activity-Regulated Genes in the Nervous System', *Physiol Rev* **Vol. 89, No. 4**, 1079-103, 2009.

Lopes, F. M., G. F. Londero, L. M. de Medeiros, L. L. da Motta, G. A. Behr, V. A. de Oliveira, M. Ibrahim, J. C. Moreira, L. O. Porciuncula, J. B. da Rocha, and F. Klamt. 'Evaluation of the Neurotoxic/Neuroprotective Role of Organoselenides Using Differentiated Human Neuroblastoma Sh-Sy5y Cell Line Challenged with 6-Hydroxydopamine', *Neurotox Res* **Vol. 22, No. 2**, 138-49, 2012.

Lopes, F. M., R. Schroder, M. L. da Frota, Jr., A. Zanotto-Filho, C. B. Muller, A. S. Pires, R. T. Meurer, G. D. Colpo, D. P. Gelain, F. Kapczinski, J. C. Moreira, C. Fernandes Mda, and F. Klamt. 'Comparison between Proliferative and Neuron-Like Sh-Sy5y Cells as an in Vitro Model for Parkinson Disease Studies', *Brain Res* **Vol. 1337**, 85-94, 2010.

Lopez-Figueroa, A. L., C. S. Norton, M. O. Lopez-Figueroa, D. Armellini-Dodel, S. Burke, H. Akil, J. F. Lopez, and S. J. Watson. 'Serotonin 5-Ht1a, 5-Ht1b, and 5-Ht2a Receptor Mrna Expression in Subjects with Major Depression, Bipolar Disorder, and Schizophrenia', *Biol Psychiatry* **Vol. 55, No. 3**, 225-33, 2004.

Louveau, A., I. Smirnov, T. J. Keyes, J. D. Eccles, S. J. Rouhani, J. D. Peske, N. C. Derecki, D. Castle, J. W. Mandell, K. S. Lee, T. H. Harris, and J. Kipnis. 'Structural and Functional Features of Central Nervous System Lymphatic Vessels', *Nature* **Vol. 523, No. 7560**, 337-41, 2015.

Lyford, G. L., K. Yamagata, W. E. Kaufmann, C. A. Barnes, L. K. Sanders, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, A. A. Lanahan, and P. F. Worley. 'Arc, a Growth Factor and Activity-Regulated Gene, Encodes a Novel Cytoskeleton-Associated Protein That Is Enriched in Neuronal Dendrites', *Neuron* **Vol. 14, No. 2**, 433-45, 1995.

Lyoo, I. K., Y. H. Sung, S. R. Dager, S. D. Friedman, J. Y. Lee, S. J. Kim, N. Kim, D. L. Dunner, and P. F. Renshaw. 'Regional Cerebral Cortical Thinning in Bipolar Disorder.', *Bipolar Disord* **Vol. 8, No. 1**, 65-74, 2006.

Magalhães, P. V., K. Jansen, R. T. Pinheiro, F. Klamt, A. L. Teixeira, R. A. da Silva, and F. Kapczinski. 'Systemic Toxicity in Early-Stage Mood Disorders.', *J Psychiatr Res* **Vol. 45, No. 10**, 1407-9, 2011.

Maher, B. 'Personal Genomes: The Case of the Missing Heritability', *Nature*, 2008.

Manji, H. K., J. A. Quiroz, J. L. Payne, J. Singh, B. P. Lopes, J. S. Viegas, and C. A. Zarate. 'The Underlying Neurobiology of Bipolar Disorder', *World Psychiatry* **Vol. 2, No. 3**, 136-46, 2003.

Manolio, T. A., F. S. Collins, N. J. Cox, D. B. Goldstein, L. A. Hindorff, D. J. Hunter, M. I. McCarthy, E. M. Ramos, L. R. Cardon, A. Chakravarti, J. H. Cho, A. E. Guttmacher, A. Kong, L. Kruglyak, E. Mardis, C. N. Rotimi, M. Slatkin, D. Valle, A. S. Whittemore, M. Boehnke, A. G. Clark, E. E. Eichler, G. Gibson, J. L. Haines, T. F. Mackay, S. A. McCarroll, and P. M. Visscher. 'Finding the Missing Heritability of Complex Diseases', *Nature* **Vol. 461, No. 7265**, 747-53, 2009.

Mansour, H. A., M. E. Talkowski, J. Wood, K. V. Chowdari, L. McClain, K. Prasad, D. Montrose, A. Fagiolini, E. S. Friedman, M. H. Allen, C. L. Bowden, J. Calabrese, R. S.

El-Mallakh, M. Escamilla, S. V. Faraone, M. D. Fossey, L. Gyulai, J. M. Loftis, P. Hauser, T. A. Ketter, L. B. Marangell, D. J. Miklowitz, A. A. Nierenberg, J. Patel, G. S. Sachs, P. Sklar, J. W. Smoller, N. Laird, M. Keshavan, M. E. Thase, D. Axelson, B. Birmaher, D. Lewis, T. Monk, E. Frank, D. J. Kupfer, B. Devlin, and V. L. Nimgaonkar. 'Association Study of 21 Circadian Genes with Bipolar I Disorder, Schizoaffective Disorder, and Schizophrenia', *Bipolar Disord* **Vol. 11, No. 7**, 701-10, 2009.

Maple, A. M., X. Zhao, D. I. Elizalde, A. K. McBride, and A. L. Gallitano. 'Htr2a Expression Responds Rapidly to Environmental Stimuli in an *EGR3*-Dependent Manner', *ACS Chem Neurosci* **Vol. 6, No. 7**, 1137-42, 2015.

Margolin, A. A., I. Nemenman, K. Basso, C. Wiggins, G. Stolovitzky, R. Dalla Favera, and A. Califano. 'Aracne: An Algorithm for the Reconstruction of Gene Regulatory Networks in a Mammalian Cellular Context', *BMC Bioinformatics* **Vol. 7 Suppl 1**, S7, 2006.

Margolin, A. A., K. Wang, W. K. Lim, M. Kustagi, I. Nemenman, and A. Califano. 'Reverse Engineering Cellular Networks', *Nat Protoc* **Vol. 1, No. 2**, 662-71, 2006.

Matza, L. S., K. S. Rajagopalan, C. L. Thompson, and G. de Lissovoy. 'Misdiagnosed Patients with Bipolar Disorder: Comorbidities, Treatment Patterns, and Direct Treatment Costs.', *J Clin Psychiatry* **Vol. 66, No. 11**, 1432-40, 2005.

McClellan, J., and M. C. King. 'Genetic Heterogeneity in Human Disease', *Cell* **Vol. 141, No. 2**, 210-7, 2010.

McCurdy, R. D., F. Féron, C. Perry, D. C. Chant, D. McLean, N. Matigian, N. K. Hayward, J. J. McGrath, and A. Mackay-Sim. 'Cell Cycle Alterations in Biopsied Olfactory Neuroepithelium in Schizophrenia and Bipolar I Disorder Using Cell Culture and Gene Expression Analyses', *Schizophr Res* **Vol. 82, No. 2-3**, 163-73, 2006.

McEwen, B. S., and P. J. Gianaros. 'Stress- and Allostasis-Induced Brain Plasticity', *Annu Rev Med* **Vol. 62**, 431-45, 2011.

McEwen, B. S., and J. C. Wingfield. 'The Concept of Allostasis in Biology and Biomedicine.', *Horm Behav* **Vol. 43, No. 1**, 2-15, 2003.

McIntyre, R. S., M. Danilewitz, S. S. Liauw, D. E. Kemp, H. T. Nguyen, L. S. Kahn, A. Kucyi, J. K. Soczynska, H. O. Woldeyohannes, A. Lachowski, B. Kim, J. Nathanson, M. Alsuwaidan, and V. H. Taylor. 'Bipolar Disorder and Metabolic Syndrome: An International Perspective', *J Affect Disord* **Vol. 126, No. 3**, 366-87, 2010.

Medeiros, Liana Marengo de, and Fabio Klamt. 'Diferenciação Colinérgica Da Linhagem De Neuroblastoma Humano Sh-Sy5y E Seu Uso Como Modelo in Vitro Da Doença De Alzheimer', (Ed.)^(Eds.), 2015.

Modabbernia, A., S. Taslimi, E. Brietzke, and M. Ashrafi. 'Cytokine Alterations in Bipolar Disorder: A Meta-Analysis of 30 Studies', *Biol Psychiatry* **Vol. 74, No. 1**, 15-25, 2013.

Mortensen, P. B., C. B. Pedersen, M. Melbye, O. Mors, and H. Ewald. 'Individual and Familial Risk Factors for Bipolar Affective Disorders in Denmark', *Arch Gen Psychiatry* **Vol. 60, No. 12**, 1209-15, 2003.

Munkholm, K., M. Vinberg, and L. Vedel Kessing. 'Cytokines in Bipolar Disorder: A Systematic Review and Meta-Analysis', *J Affect Disord* **Vol. 144, No. 1-2**, 16-27, 2013.

Mwangi, B., M. J. Wu, B. Cao, I. C. Passos, L. Lavagnino, Z. Keser, G. B. Zunta-Soares, K. M. Hasan, F. Kapczinski, and J. C. Soares. 'Individualized Prediction and Clinical Staging of Bipolar Disorders Using Neuroanatomical Biomarkers', *Biol Psychiatry Cogn Neurosci Neuroimaging* **Vol. 1, No. 2**, 186-194, 2016.

Ng, D., G. M. Pitcher, R. K. Szilard, A. Sertie, M. Kanisek, S. J. Clapcote, T. Lipina, L. V. Kalia, D. Joo, C. McKerlie, M. Cortez, J. C. Roder, M. W. Salter, and R. R. McInnes. 'Neto1 Is a Novel Cub-Domain Nmda Receptor-Interacting Protein Required for Synaptic Plasticity and Learning', *PLoS Biol* **Vol. 7, No. 2**, e41, 2009.

Nurnberger, J. I., Jr., D. L. Koller, J. Jung, H. J. Edenberg, T. Foroud, I. Guella, M. P. Vawter, and J. R. Kelsoe. 'Identification of Pathways for Bipolar Disorder: A Meta-Analysis', *JAMA Psychiatry* **Vol. 71, No. 6**, 657-64, 2014.

O'Donovan, K. J., W. G. Tourtellotte, J. Millbrandt, and J. M. Baraban. 'The Egr Family of Transcription-Regulatory Factors: Progress at the Interface of Molecular and Systems Neuroscience', *Trends Neurosci* **Vol. 22, No. 4**, 167-73, 1999.

O'Donovan, K. J., E. P. Wilkens, and J. M. Baraban. 'Sequential Expression of Egr-1 and Egr-3 in Hippocampal Granule Cells Following Electroconvulsive Stimulation', *J Neurochem* **Vol. 70, No. 3**, 1241-8, 1998.

Ostroveanu, A., E. A. van der Zee, U. L. Eisel, M. Schmidt, and I. M. Nijholt. 'Exchange Protein Activated by Cyclic Amp 2 (Epac2) Plays a Specific and Time-Limited Role in Memory Retrieval', *Hippocampus* **Vol. 20, No. 9**, 1018-26, 2010.

Patel, J. P., and B. N. Frey. 'Disruption in the Blood-Brain Barrier: The Missing Link between Brain and Body Inflammation in Bipolar Disorder?', *Neural Plast* **Vol. 2015**, 708306, 2015.

Pavletich, N. P., and C. O. Pabo. 'Zinc Finger-Dna Recognition: Crystal Structure of a Zif268-Dna Complex at 2.1 A', *Science* **Vol. 252, No. 5007**, 809-17, 1991.

Perez-Cadahia, B., B. Drobic, and J. R. Davie. 'Activation and Function of Immediate-Early Genes in the Nervous System', *Biochem Cell Biol* **Vol. 89, No. 1**, 61-73, 2011.

Pfaffenseller, B., G. R. Fries, B. Wollenhaupt-Aguiar, G. D. Colpo, L. Stertz, B. Panizzutti, P. V. Magalhaes, and F. Kapczinski. 'Neurotrophins, Inflammation and Oxidative Stress as Illness Activity Biomarkers in Bipolar Disorder', *Expert Rev Neurother* **Vol. 13, No. 7**, 827-42, 2013.

Pfaffenseller, B., B. Wollenhaupt-Aguiar, G. R. Fries, G. D. Colpo, R. K. Burque, G. Bristot, P. Ferrari, K. M. Cereser, A. R. Rosa, F. Klamt, and F. Kapczinski. 'Impaired Endoplasmic Reticulum Stress Response in Bipolar Disorder: Cellular Evidence of Illness Progression', *Int J Neuropsychopharmacol* **Vol. 17, No. 9**, 1453-63, 2014.

Piao, G., S. Saito, Y. Sun, Z. P. Liu, Y. Wang, X. Han, J. Wu, H. Zhou, L. Chen, and K. Horimoto. 'A Computational Procedure for Identifying Master Regulator Candidates: A Case Study on Diabetes Progression in Goto-Kakizaki Rats', *BMC Syst Biol* **Vol. 6 Suppl 1**, S2, 2012.

Politi, P., N. Brondino, and E. Emanuele. 'Increased Proapoptotic Serum Activity in Patients with Chronic Mood Disorders', *Arch Med Res* **Vol. 39, No. 2**, 242-5, 2008.

Post, R. M. 'Transduction of Psychosocial Stress into the Neurobiology of Recurrent Affective Disorder.', *Am J Psychiatry* **Vol. 149, No. 8**, 999-1010, 1992.

Post, R. M., J. Fleming, and F. Kapczinski. 'Neurobiological Correlates of Illness Progression in the Recurrent Affective Disorders', *J Psychiatr Res* **Vol. 46, No. 5**, 561-73, 2012.

Purcell, S. M., N. R. Wray, J. L. Stone, P. M. Visscher, M. C. O'Donovan, P. F. Sullivan, and P. Sklar. 'Common Polygenic Variation Contributes to Risk of Schizophrenia and Bipolar Disorder', *Nature* **Vol. 460, No. 7256**, 748-52, 2009.

Radio, N. M., and W. R. Mundy. 'Developmental Neurotoxicity Testing in Vitro: Models for Assessing Chemical Effects on Neurite Outgrowth', *Neurotoxicology* **Vol. 29, No. 3**, 361-76, 2008.

Reimer, R. J. 'Slc17: A Functionally Diverse Family of Organic Anion Transporters', *Mol Aspects Med* **Vol. 34, No. 2-3**, 350-9, 2013.

Roberts, D. S., Y. Hu, I. V. Lund, A. R. Brooks-Kayal, and S. J. Russek. 'Brain-Derived Neurotrophic Factor (Bdnf)-Induced Synthesis of Early Growth Response Factor 3 (*EGR3*) Controls the Levels of Type a Gaba Receptor Alpha 4 Subunits in Hippocampal Neurons', *J Biol Chem* **Vol. 281, No. 40**, 29431-5, 2006.

Roberts, J., A. C. Scott, M. R. Howard, G. Breen, V. J. Bubb, E. Klenova, and J. P. Quinn. 'Differential Regulation of the Serotonin Transporter Gene by Lithium Is Mediated by Transcription Factors, Ccctc Binding Protein and Y-Box Binding Protein 1, through the Polymorphic Intron 2 Variable Number Tandem Repeat', *J Neurosci* **Vol. 27, No. 11**, 2793-801, 2007.

Roedding, A. S., A. F. Gao, W. Au-Yeung, T. Scarcelli, P. P. Li, and J. J. Warsh. 'Effect of Oxidative Stress on Trpm2 and Trpc3 Channels in B Lymphoblast Cells in Bipolar Disorder', *Bipolar Disord* **Vol. 14, No. 2**, 151-61, 2012.

Rybakowski, J. K., A. Remlinger-Molenda, A. Czech-Kucharska, M. Wojcicka, M. Michalak, and J. Losy. 'Increased Serum Matrix Metalloproteinase-9 (Mmp-9) Levels in Young Patients During Bipolar Depression', *J Affect Disord* **Vol. 146, No. 2**, 286-9, 2013.

Sassi, R. B., J. A. Stanley, D. Axelson, P. Brambilla, M. A. Nicoletti, M. S. Keshavan, R. T. Ramos, N. Ryan, B. Birmaher, and J. C. Soares. 'Reduced Naa Levels in the Dorsolateral Prefrontal Cortex of Young Bipolar Patients', *Am J Psychiatry* **Vol. 162, No. 11**, 2109-15, 2005.

Schonhofen, P., L. M. de Medeiros, I. J. Bristot, F. M. Lopes, M. A. De Bastiani, F. Kapczinski, J. A. Crippa, M. A. Castro, R. B. Parsons, and F. Klamt. 'Cannabidiol Exposure During Neuronal Differentiation Sensitizes Cells against Redox-Active Neurotoxins', *Mol Neurobiol* **Vol. 52, No. 1**, 26-37, 2015.

Scola, G., and A. C. Andreazza. 'The Role of Neurotrophins in Bipolar Disorder', *Prog Neuropsychopharmacol Biol Psychiatry* **Vol. 56**, 122-8, 2015.

Scott, J., E. Paykel, R. Morriss, R. Bentall, P. Kinderman, T. Johnson, R. Abbott, and H. Hayhurst. 'Cognitive-Behavioural Therapy for Severe and Recurrent Bipolar Disorders: Randomised Controlled Trial.', *Br J Psychiatry* **Vol. 188**, 313-20, 2006.

Senba, E., and T. Ueyama. 'Stress-Induced Expression of Immediate Early Genes in the Brain and Peripheral Organs of the Rat', *Neurosci Res* **Vol. 29, No. 3**, 183-207, 1997.

Shinozaki, G., and J. B. Potash. 'New Developments in the Genetics of Bipolar Disorder', *Curr Psychiatry Rep* **Vol. 16, No. 11**, 493, 2014.

Smyth, G. K. 'Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments', *Stat Appl Genet Mol Biol* **Vol. 3**, Article3, 2004.

Soreca, I., E. Frank, and D. J. Kupfer. 'The Phenomenology of Bipolar Disorder: What Drives the High Rate of Medical Burden and Determines Long-Term Prognosis?', *Depress Anxiety* **Vol. 26, No. 1**, 73-82, 2009.

Staiger, J. F., S. Bisler, A. Schleicher, P. Gass, J. H. Stehle, and K. Zilles. 'Exploration of a Novel Environment Leads to the Expression of Inducible Transcription Factors in Barrel-Related Columns', *Neuroscience* **Vol. 99, No. 1**, 7-16, 2000.

Steckert, A. V., S. S. Valvassori, M. Moretti, F. Dal-Pizzol, and J. Quevedo. 'Role of Oxidative Stress in the Pathophysiology of Bipolar Disorder', *Neurochem Res* **Vol. 35, No. 9**, 1295-301, 2010.

Stertz, L., P. V. Magalhaes, and F. Kapczinski. 'Is Bipolar Disorder an Inflammatory Condition? The Relevance of Microglial Activation', *Curr Opin Psychiatry* **Vol. 26, No. 1**, 19-26, 2013.

Strakowski, S. M., M. P. DelBello, M. E. Zimmerman, G. E. Getz, N. P. Mills, J. Ret, P. Shear, and C. M. Adler. 'Ventricular and Periventricular Structural Volumes in First- Versus Multiple-Episode Bipolar Disorder.', *Am J Psychiatry* **Vol. 159, No. 11**, 1841-7, 2002.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 'Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles', *Proc Natl Acad Sci U S A* **Vol. 102, No. 43**, 15545-50, 2005.

Swann, A. C., C. L. Bowden, J. R. Calabrese, S. C. Dilsaver, and D. D. Morris. 'Differential Effect of Number of Previous Episodes of Affective Disorder on Response to Lithium or Divalproex in Acute Mania.', *Am J Psychiatry* **Vol. 156, No. 8**, 1264-6, 1999.

Torres, I. J., V. G. Boudreau, and L. N. Yatham. 'Neuropsychological Functioning in Euthymic Bipolar Disorder: A Meta-Analysis.', *Acta Psychiatr Scand Suppl***, No. 434**, 17-26, 2007.

Tsai, S. Y., Y. Y. Yang, C. J. Kuo, C. C. Chen, and S. J. Leu. 'Effects of Symptomatic Severity on Elevation of Plasma Soluble Interleukin-2 Receptor in Bipolar Mania', *J Affect Disord* **Vol. 64, No. 2-3**, 185-93, 2001.

Tsuang, Ming T., and Stephen V. Faraone. *The Genetics of Mood Disorders.*, Johns Hopkins University Press, 1990.

van Someren, E. P., L. F. Wessels, E. Backer, and M. J. Reinders. 'Genetic Network Modeling', *Pharmacogenomics* **Vol. 3, No. 4**, 507-25, 2002.

Veyrac, A., A. Besnard, J. Caboche, S. Davis, and S. Laroche. 'The Transcription Factor Zif268/Egr1, Brain Plasticity, and Memory', *Prog Mol Biol Transl Sci* **Vol. 122**, 89-129, 2014.

Vieta, E., D. Popovic, A. R. Rosa, B. Solé, I. Grande, B. N. Frey, A. Martinez-Aran, J. Sanchez-Moreno, V. Balanzá-Martínez, R. Tabarés-Seisdedos, and F. Kapczinski. 'The Clinical Implications of Cognitive Impairment and Allostatic Load in Bipolar Disorder.', *Eur Psychiatry*, 2012.

Vieta, E., M. Reinares, and A. R. Rosa. 'Staging Bipolar Disorder', *Neurotox Res* **Vol. 19, No. 2**, 279-85, 2011.

Viswanath, B., S. P. Jose, A. Squassina, J. Thirthalli, M. Purushottam, O. Mukherjee, V. Vladimirov, G. P. Patrinos, M. Del Zompo, and S. Jain. 'Cellular Models to Study Bipolar Disorder: A Systematic Review', *J Affect Disord* **Vol. 184**, 36-50, 2015.

Vourc'h, P., and C. Andres. 'Oligodendrocyte Myelin Glycoprotein (Omgp): Evolution, Structure and Function', *Brain Res Brain Res Rev* **Vol. 45, No. 2**, 115-24, 2004.

Weiner, M., L. Warren, and J. G. Fiedorowicz. 'Cardiovascular Morbidity and Mortality in Bipolar Disorder', *Ann Clin Psychiatry* **Vol. 23, No. 1**, 40-7, 2011.

'Who | the Global Burden of Disease: 2004 Update'. *WHO*, 2014.

Williams, A. A., W. M. Ingram, S. Levine, J. Resnik, C. M. Kamel, J. R. Lish, D. I. Elizalde, S. A. Janowski, J. Shoker, A. Kozlenkov, J. Gonzalez-Maeso, and A. L. Gallitano. 'Reduced Levels of Serotonin 2a Receptors Underlie Resistance of *EGR3*-Deficient Mice to Locomotor Suppression by Clozapine', *Neuropsychopharmacology* **Vol. 37, No. 10**, 2285-98, 2012.

Xiang, B., Z. Yang, Y. Lin, L. Guan, X. Li, W. Deng, Z. Jiang, G. Lao, Q. Wang, X. Hao, X. Liu, Y. Wang, L. Zhao, X. Ma, T. Li, L. Cao, and X. Hu. 'Genes in the

Serotonin Pathway Are Associated with Bipolar Affective Disorder in a Han Chinese Population', *Neurosci Bull* **Vol. 30, No. 1**, 33-42, 2014.

Yamada, K., D. J. Gerber, Y. Iwayama, T. Ohnishi, H. Ohba, T. Toyota, J. Aruga, Y. Minabe, S. Tonegawa, and T. Yoshikawa. 'Genetic Analysis of the Calcineurin Pathway Identifies Members of the Egr Gene Family, Specifically *EGR3*, as Potential Susceptibility Candidates in Schizophrenia', *Proc Natl Acad Sci U S A* **Vol. 104, No. 8**, 2815-20, 2007.

Yamagata, K., W. E. Kaufmann, A. Lanahan, M. Papapavlou, C. A. Barnes, K. I. Andreasson, and P. F. Worley. '*EGR3*/Pilot, a Zinc Finger Transcription Factor, Is Rapidly Regulated by Activity in Brain Neurons and Colocalizes with Egr1/Zif268', *Learn Mem* **Vol. 1, No. 2**, 140-52, 1994.

Zhang, R., S. Lu, L. Meng, Z. Min, J. Tian, R. K. Valenzuela, T. Guo, L. Tian, W. Zhao, and J. Ma. 'Genetic Evidence for the Association between the Early Growth Response 3 (*EGR3*) Gene and Schizophrenia', *PLoS One* **Vol. 7, No. 1**, e30237, 2012.

ANEXOS

ANEXO 1

Curr Psychiatry Rep (2012) 14:667–675
DOI 10.1007/s11920-012-0319-2

BIPOLAR DISORDERS (MA FRYE, SECTION EDITOR)

Staging and Neuroprogression in Bipolar Disorder

Gabriel Rodrigo Fries · Bianca Pfaffenseller · Laura Stertz · André Vinicius Contri Paz · Aroldo Ayub Dargél · Maurício Kunz · Flávio Kapczinski

Published online: 23 October 2012

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

G. R. Fries · B. Pfaffenseller · L. Stertz · A. V. C. Paz · A. A. Dargél · M. Kunz · F. Kapczinski (✉)
Laboratory of Molecular Psychiatry, Centro de Pesquisas Experimentais, Hospital de Clínicas de Porto Alegre, and INCT for Translational Medicine,
Rua Ramiro Barcelos,
2350, Porto Alegre, RS, Brazil
e-mail: flavio.kapczinski@gmail.com

G. R. Fries
e-mail: gabrielrfries@gmail.com

B. Pfaffenseller
e-mail: bianca.pfaffenseller@gmail.com

L. Stertz
e-mail: laurastertz@gmail.com

A. V. C. Paz
e-mail: andrecontri@hotmail.com

A. A. Dargél
e-mail: aroldodargel@gmail.com

M. Kunz
e-mail: maukunz@gmail.com

G. R. Fries · B. Pfaffenseller · L. Stertz · F. Kapczinski
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, UFRGS,
Rua Ramiro Barcelos,
2350, Porto Alegre, RS, Brazil

A. A. Dargél · M. Kunz · F. Kapczinski
Programa de Pós-Graduação em Medicina: Psiquiatria, Universidade Federal do Rio Grande do Sul, UFRGS,
Rua Ramiro Barcelos,
2350, Porto Alegre, RS, Brazil

[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.

Disclosure G. R. Fries: none; B. Pfaffenseller: none; L. Stertz: none; A. V. C. Paz: none; A. Ayub Dargél: none; M. Kunz: travel/accommodations/meeting expenses reimbursed by Eli Lilly and Company; F. Kapczinski: grants from NARSAD, Stanley Medical Research Institute, CNPq (National Council for Scientific and Technological Development), and CAPES, and payment for lectures including service on speakers bureaus from Eli Lilly and Company, Lundbeck, AstraZeneca, Servier, and Janssen.

References

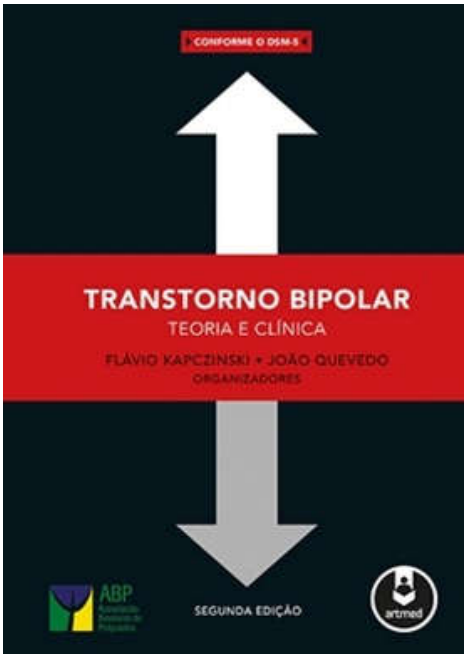
Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

1. • Post RM, Fleming J, Kapczinski F. Neurobiological correlates of illness progression in the recurrent affective disorders. J Psychiatr Res. 2012;46(5):561–73. This is a full review regarding progression of bipolar disorder, including data on clinical aspects, effects of medications and neurobiological findings.
2. •• Berk M, Kapczinski F, Andreazza AC, et al. Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors. Neurosci Biobehav Rev. 2011;35(3):804–17. *This review discuss the several biological mechanisms responsible for bipolar disorder progression, including oxidative stress, neurotrophic factors, among others.*
3. Kapczinski F, Vieta E, Andreazza AC, et al. Allostatic load in bipolar disorder: implications for pathophysiology and treatment. Neurosci Biobehav Rev. 2008;32(4):675–92.
4. • Berk M, Brnabic A, Dodd S, et al. Does stage of illness impact treatment response in bipolar disorder? Empirical treatment data and their implication for the staging model and early intervention. Bipolar Disord. 2011;13(1):87–98. *This study employs a staging model to classify the patients and shows that individuals at the earliest stages of illness have a more favourable response to treatment when compared to later stages*.
5. Kessing LV, Andersen PK, Mortensen PB. Predictors of recurrence in affective disorder. A case register study. J Affect Disord. 1998;49(2):101–8.
6. Roy-Byrne P, Post RM, Uhde TW, Porcu T, Davis D. The longitudinal course of recurrent affective illness: life chart data from research patients at the NIMH. Acta Psychiatr Scand Suppl. 1985;317:1–34.
7. Ketter TA, Houston JP, Adams DH, et al. Differential efficacy of olanzapine and lithium in preventing manic or mixed recurrence in patients with bipolar I disorder based on number of previous manic or mixed episodes. J Clin Psychiatry. 2006;67(1):95–101.
8. Swann AC, Bowden CL, Calabrese JR, Dilsaver SC, Morris DD. Differential effect of number of previous episodes of affective disorder on response to lithium or divalproex in acute mania. Am J Psychiatry. 1999;156(8):1264–6.
9. Scott J, Paykel E, Morriss R, et al. Cognitive-behavioural therapy for severe and recurrent bipolar disorders: randomised controlled trial. Br J Psychiatry. 2006;188:313–20.
10. Reinares M, Colom F, Rosa AR, et al. The impact of staging bipolar disorder on treatment outcome of family psychoeducation. J Affect Disord. 2010;123(1–3):81–6.
11. Matza LS, Rajagopalan KS, Thompson CL, de Lissovoy G. Misdiagnosed patients with bipolar disorder: comorbidities, treatment patterns, and direct treatment costs. J Clin Psychiatry. 2005;66(11):1432–40.
12. Rosa AR, González-Ortega I, González-Pinto A, et al. One-year psychosocial functioning in patients in the early vs. late stage of bipolar disorder. Acta Psychiatr Scand. 2012;125(4):335–41.
13. Torres IJ, Boudreau VG, Yatham LN. Neuropsychological functioning in euthymic bipolar disorder: a meta-analysis. Acta Psychiatr Scand Suppl. 2007;434:17–26.
14. Kessing LV, Andersen PK. Does the risk of developing dementia increase with the number of episodes in patients with depressive disorder and in patients with bipolar disorder? J Neurol Neurosurg Psychiatry. 2004;75(12):1662–6.
15. Hawton K, Sutton L, Haw C, Sinclair J, Harriss L. Suicide and attempted suicide in bipolar disorder: a systematic review of risk factors. J Clin Psychiatry. 2005;66(6):693–704.
16. Goldberg JF, Ernst CL. Features associated with the delayed initiation of mood stabilizers at illness onset in bipolar disorder. J Clin Psychiatry. 2002;63(11):985–91.
17. Post RM. Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. Am J Psychiatry. 1992;149(8):999–1010.
18. Vieta E, Reinares M, Rosa AR. Staging bipolar disorder. Neurotox Res. 2011;19(2):279–85.
19. Vieta E, Popovic D, Rosa AR, et al. The clinical implications of cognitive impairment and allostatic load in bipolar disorder. Eur Psychiatry 2012. doi:10.1016/j.eurpsy.2011.11.007.
20. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. Horm Behav. 2003;43(1):2–15.
21. Strakowski SM, Delbello MP, Adler CM. The functional neuroanatomy of bipolar disorder: a review of neuroimaging findings. Mol Psychiatry. 2005;10(1):105–16.

22. Lyoo IK, Sung YH, Dager SR, et al. Regional cerebral cortical thinning in bipolar disorder. Bipolar Disord. 2006;8(1):65–74.
23. Rajkowska G, Halaris A, Selemon LD. Reductions in neuronal and glial density characterize the dorsolateral prefrontal cortex in bipolar disorder. Biol Psychiatry. 2001;49(9):741–52.
24. López-Larson MP, DelBello MP, Zimmerman ME, Schwiers ML, Strakowski SM. Regional prefrontal gray and white matter abnormalities in bipolar disorder. Biol Psychiatry. 2002;52(2):93–100.
25. Ongür D, Drevets WC, Price JL. Glial reduction in the subgenual prefrontal cortex in mood disorders. Proc Natl Acad Sci USA. 1998;95(22):13290–5.
26. Sassi RB, Brambilla P, Hatch JP, et al. Reduced left anterior cingulate volumes in untreated bipolar patients. Biol Psychiatry. 2004;56(7):467–75.
27. Doris A, Belton E, Ebmeier KP, Glabus MF, Marshall I. Reduction of cingulate gray matter density in poor outcome bipolar illness. Psychiatry Res. 2004;130(2):153–9.
28. Drevets WC, Price JL, Simpson JR, et al. Subgenual prefrontal cortex abnormalities in mood disorders. Nature. 1997;386 (6627):824–7.
29. Bora E, Fornito A, Yücel M, Pantelis C. Voxelwise meta-analysis of gray matter abnormalities in bipolar disorder. Biol Psychiatry. 2010;67(11):1097–105.
30. Rosso IM, Killgore WD, Cintron CM, et al. Reduced amygdala volumes in first-episode bipolar disorder and correlation with cerebral white matter. Biol Psychiatry. 2007;61(6):743–9.
31. Javadapour A, Malhi GS, Ivanovski B, et al. Hippocampal volumes in adults with bipolar disorder. J Neuropsychiatry Clin Neurosci. 2010;22(1):55–62.
32. Berk M, Hallam K, Malhi GS, et al. Evidence and implications for early intervention in bipolar disorder. J Ment Health. 2010;19 (2):113–26.
33. Mills NP, Delbello MP, Adler CM, Strakowski SM. MRI analysis of cerebellar vermal abnormalities in bipolar disorder. Am J Psychiatry. 2005;162(8):1530–2.
34. Hwang J, Lyoo IK, Dager SR, et al. Basal ganglia shape alterations in bipolar disorder. Am J Psychiatry. 2006;163(2):276–85.
35. Strakowski SM, DelBello MP, Sax KW, et al. Brain magnetic resonance imaging of structural abnormalities in bipolar disorder. Arch Gen Psychiatry. 1999;56(3):254–60.
36. Atmaca M, Ozdemir H, Yildirim H. Corpus callosum areas in first-episode patients with bipolar disorder. Psychol Med. 2007;37 (5):699–704.
37. Barnea-Goraly N, Chang KD, Karchemskiy A, Howe ME, Reiss AL. Limbic and corpus callosum aberrations in adolescents with bipolar disorder: a tract-based spatial statistics analysis. Biol Psychiatry. 2009;66(3):238–44.
38. Brambilla P, Nicoletti M, Sassi RB, et al. Corpus callosum signal intensity in patients with bipolar and unipolar disorder. J Neurol Neurosurg Psychiatry. 2004;75(2):221–5.
39. Moorhead TW, McKirdy J, Sussmann JE, et al. Progressive gray matter loss in patients with bipolar disorder. Biol Psychiatry. 2007;62(8):894–900.
40. Frey BN, Zunta-Soares GB, Caetano SC, et al. Illness duration and total brain gray matter in bipolar disorder: evidence for neurodegeneration? Eur Neuropsychopharmacol. 2008;18(10):717–22.
41. Strakowski SM, DelBello MP, Zimmerman ME, et al. Ventricular and periventricular structural volumes in first- versus multiple-episode bipolar disorder. Am J Psychiatry. 2002;159 (11):1841–7.
42. Rajkowska G. Cell pathology in bipolar disorder. Bipolar Disord. 2002;4(2):105–16.
43. Hunsberger JG, Austin DR, Chen G, Manji HK. Cellular mechanisms underlying affective resiliency: the role of glucocorticoid receptor- and mitochondrially-mediated plasticity. Brain Res. 2009;1293:76–84.
44. Schloesser RJ, Huang J, Klein PS, Manji HK. Cellular plasticity cascades in the pathophysiology and treatment of bipolar disorder. Neuropsychopharmacology. 2008;33(1):110–33.
45. Kato T. Molecular neurobiology of bipolar disorder: a disease of 'mood-stabilizing neurons'? Trends Neurosci. 2008;31(10):495–503.
46. Perova T, Wasserman MJ, Li PP, Warsh JJ. Hyperactive intracellular calcium dynamics in B lymphoblasts from patients with bipolar I disorder. Int J Neuropsychopharmacol. 2008;11(2):185–96.
47. McCurdy RD, Féron F, Perry C, et al. Cell cycle alterations in biopsied olfactory neuroepithelium in schizophrenia and bipolar I disorder using cell culture and gene expression analyses. Schizophr Res. 2006;82(2–3):163–73.
48. Stork C, Renshaw PF. Mitochondrial dysfunction in bipolar disorder: evidence from magnetic resonance spectroscopy research. Mol Psychiatry. 2005;10(10):900–19.
49. So J, Warsh JJ, Li PP. Impaired endoplasmic reticulum stress response in B-lymphoblasts from patients with bipolar-I disorder. Biol Psychiatry. 2007;62(2):141–7.
50. Hayashi A, Kasahara T, Kametani M, et al. Aberrant endoplasmic reticulum stress response in lymphoblastoid cells from patients with bipolar disorder. Int J Neuropsychopharmacol. 2009;12 (1):33–43.
51. •• Manji H, Kato T, Di Prospero NA, et al. Impaired mitochondrial function in psychiatric disorders. Nat Rev Neurosci. 2012;13 (5):293–307. *This study reviews data on mitochondrial function in psychiatric disorders, including clinical and preclinical data*.
52. • Du J, Wang Y, Hunter R, et al. Dynamic regulation of mitochondrial function by glucocorticoids. Proc Natl Acad Sci USA. 2009;106(9):3543–8. *This study shows that glucocorticoids have a biphasic effect on mitochondrial functions, relating chronic stress to reduced mitochondrial membrane potential, calcium holding capacity and oxygen consumption*.
53. Gong Y, Chai Y, Ding JH, Sun XL, Hu G. Chronic mild stress damages mitochondrial ultrastructure and function in mouse brain. Neurosci Lett. 2011;488(1):76–80.
54. Daban C, Vieta E, Mackin P, Young AH. Hypothalamic-pituitary-adrenal axis and bipolar disorder. Psychiatr Clin North Am. 2005;28(2):469–80.
55. Benes FM, Matzilevich D, Burke RE, Walsh J. The expression of proapoptosis genes is increased in bipolar disorder, but not in schizophrenia. Mol Psychiatry. 2006;11(3):241–51.
56. Eastwood SL, Harrison PJ. Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a Western blot study of synaptophysin, GAP-43 and the complexins. Brain Res Bull. 2001;55(5):569–78.
57. Pfaffenseller B. Disfunção na Resposta ao Estresse do Retículo Endoplasmático em linfócitos de pacientes com Transtorno de Humor Bipolar. Dissertation. Porto Alegre: Universidade Federal do Rio Grande do Sul; 2012.
58. Bachmann RF, Schloesser RJ, Gould TD, Manji HK. Mood stabilizers target cellular plasticity and resilience cascades: implications for the development of novel therapeutics. Mol Neurobiol. 2005;32(2):173–202.
59. Kim HW, Rapoport SI, Rao JS. Altered expression of apoptotic factors and synaptic markers in postmortem brain from bipolar disorder patients. Neurobiol Dis. 2010;37(3):596–603.
60. Fernandes BS, Gama CS, Ceresér KM, et al. Brain-derived neurotrophic factor as a state-marker of mood episodes in bipolar disorders: a systematic review and meta-regression analysis. J Psychiatr Res. 2011;45(8):995–1004.
61. Walz JC, Andreazza AC, Frey BN, et al. Serum neurotrophin-3 is increased during manic and depressive episodes in bipolar disorder. Neurosci Lett. 2007;415(1):87–9.
62. Walz JC, Magalhães PV, Giglio LM, et al. Increased serum neurotrophin-4/5 levels in bipolar disorder. J Psychiatr Res. 2009;43(7):721–3.

63. Rosa AR, Frey BN, Andreazza AC, et al. Increased serum glial cell line-derived neurotrophic factor immunocontent during manic and depressive episodes in individuals with bipolar disorder. Neurosci Lett. 2006;407(2):146–50.
64. Kapczinski F, Dal-Pizzol F, Teixeira AL, et al. Peripheral biomarkers and illness activity in bipolar disorder. J Psychiatr Res. 2011;45(2):156–61.
65. Andreazza AC, Kapczinski F, Kauer-Sant'Anna M, et al. 3-Nitrotyrosine and glutathione antioxidant system in patients in the early and late stages of bipolar disorder. J Psychiatry Neurosci. 2009;34(4):263–71.
66. Kauer-Sant'Anna M, Kapczinski F, Andreazza AC, et al. Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. Int J Neuropsychopharmacol. 2009;12(4):447–58.
67. Sapolsky RM. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry. 2000;57(10):925–35.
68. Grande I, Fries GR, Kunz M, Kapczinski F. The role of BDNF as a mediator of neuroplasticity in bipolar disorder. Psychiatry Investig. 2010;7(4):243–50.
69. Young LT. Neuroprotective effects of antidepressant and mood stabilizing drugs. J Psychiatry Neurosci. 2002;27(1):8–9.
70. Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. Nat Rev Cancer. 2005;5(11):845–56.
71. Barbosa IG, Huguet RB, Mendonça VA, et al. Increased plasma levels of brain-derived neurotrophic factor in patients with long-term bipolar disorder. Neurosci Lett. 2010;475(2):95–8.
72. Barbosa IG, Rocha NP, Huguet RB, et al. Executive dysfunction in euthymic bipolar disorder patients and its association with plasma biomarkers. J Affect Disord. 2012;137(1–3):151–5.
73. Lin PY. State-dependent decrease in levels of brain-derived neurotrophic factor in bipolar disorder: a meta-analytic study. Neurosci Lett. 2009;466(3):139–43.
74. Magalhães PV, Jansen K, Pinheiro RT, et al. A nested population-based case-control study on peripheral inflammation markers and brainderived neurotrophic factor in early-stage mood disorders. Istambul: Presented at the 5th Biennial Conference of the International Society for Bipolar Disorders; 2012.
75. Hope S, Dieset I, Agartz I, et al. Affective symptoms are associated with markers of inflammation and immune activation in bipolar disorders but not in schizophrenia. J Psychiatr Res. 2011;45 (12):1608–16.
76. Tsai SY, Chung KH, Wu JY, et al. Inflammatory markers and their relationships with leptin and insulin from acute mania to full remission in bipolar disorder. J Affect Disord. 2012;136(1–2):110–6.
77. Barbosa IG, Huguet RB, Mendonça VA, et al. Increased plasma levels of soluble TNF receptor I in patients with bipolar disorder. Eur Arch Psychiatry Clin Neurosci. 2011;261(2):139–43.
78. Kunz M, Ceresér KM, Goi PD, et al. Serum levels of IL-6, IL-10 and TNF-α in patients with bipolar disorder and schizophrenia: differences in pro- and anti-inflammatory balance. Rev Bras Psiquiatr. 2011;33(3):268–74.
79. Söderlund J, Olsson SK, Samuelsson M, et al. Elevation of cerebrospinal fluid interleukin-1ß in bipolar disorder. J Psychiatry Neurosci. 2011;36(2):114–8.
80. Barbosa IG, Rocha NP, Bauer ME, et al. Chemokines in bipolar disorder: Trait or state? Eur Arch Psychiatry Clin Neurosci. 2012. doi:10.1007/s00406-012-0327-6.
81. Herberth M, Koethe D, Levin Y, et al. Peripheral profiling analysis for bipolar disorder reveals markers associated with reduced cell survival. Proteomics. 2011;11(1):94–105.
82. Kato T. Mitochondrial dysfunction as the molecular basis of bipolar disorder: therapeutic implications. CNS Drugs. 2007;21(1):1–11.
83. Regenold WT, Phatak P, Marano CM, et al. Elevated cerebrospinal fluid lactate concentrations in patients with bipolar disorder and schizophrenia: implications for the mitochondrial dysfunction hypothesis. Biol Psychiatry. 2009;65(6):489–94.
84. Halliwell B. Free radicals and antioxidants: updating a personal view. Nutr Rev. 2012;70(5):257–65.
85. Magalhães PV, Jansen K, Pinheiro RT, et al. Peripheral oxidative damage in early-stage mood disorders: a nested population-based case-control study. Int J Neuropsychopharmacol. 2011;19:1–8.
86. Andreazza AC, Kauer-Sant'anna M, Frey BN, et al. Oxidative stress markers in bipolar disorder: a meta-analysis. J Affect Disord. 2008;111(2–3):135–44.
87. Kunz M, Gama CS, Andreazza AC, et al. Elevated serum superoxide dismutase and thiobarbituric acid reactive substances in different phases of bipolar disorder and in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry. 2008;32(7): 1677–81.
88. Yumru M, Savas HA, Kalenderoglu A, et al. Oxidative imbalance in bipolar disorder subtypes: a comparative study. Prog Neuropsychopharmacol Biol Psychiatry. 2009;33(6):1070–4.
89. Simon NM, Smoller JW, McNamara KL, et al. Telomere shortening and mood disorders: preliminary support for a chronic stress model of accelerated aging. Biol Psychiatry. 2006;60(5):432–5.
90. Elvsåshagen T, Vera E, Bøen E, et al. The load of short telomeres is increased and associated with lifetime number of depressive episodes in bipolar II disorder. J Affect Disord. 2011;135(1–3):43–50.
91. Berk M, Hallam KT, McGorry PD. The potential utility of a staging model as a course specifier: a bipolar disorder perspective. J Affect Disord. 2007;100(1–3):279–81.
92. Kapczinski F, Dias VV, Kauer-Sant'Anna M, et al. Clinical implications of a staging model for bipolar disorders. Expert Rev Neurother. 2009;9(7):957–66.
93. McGorry PD, Purcell R, Hickie IB, et al. Clinical staging: a heuristic model for psychiatry and youth mental health. Med J Aust. 2007;187(7 Suppl):S40–2.
94. Magalhães PV, Dodd S, Nierenberg AA, Berk M. Cumulative morbidity and prognostic staging of illness in the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD). Australian & New Zealand Journal of Psychiatry. Aust N Z J Psychiatry. 2012. doi:10.1177/0004867412460593.
95. Magalhães PV, Kapczinski F, Nierenberg AA, Deckersbach T, Weisinger D, Dodd S, Berk M. Illness burden and medical comorbidity in the Systematic Treatment Enhancement Program for Bipolar Disorder. Acta Psychiatr Scand. 2012;125(4):303–308.
96. Reinares M, Papachristou E, Harvey P, Mar Bonnín C, Sánchez-Moreno J, Torrent C, Ayuso-Mateos JL, Ploubidis GB, Vieta E, Frangou S. Towards a clinical staging for bipolar disorder: Defining patient subtypes based on functional outcome. J Affect Disord. doi:10.1016/j.jad.2012.06.005.
97. McGorry PD. Staging in neuropsychiatry: a heuristic model for understanding, prevention and treatment. Neurotox Res. 2010;18 (3–4):244–55.

ANEXO 2



2

Fisiopatologia do transtorno bipolar: novas tendências

Gabriel Rodrigo Fries
Bianca Pfaffenseller
Flávio Kapczinski

INTRODUÇÃO

As bases biológicas do transtorno bipolar (TB) são, via de regra, complexas e multifatoriais. Diversos estudos têm sido realizados com o objetivo de identificar genes, proteínas, modificações pós-traducionais, metabólitos e endofenótipos capazes de diferenciar pacientes e indivíduos saudáveis (também chamados de "controles"). Conforme esperado, muitas alterações já foram identificadas em pacientes, a maioria das quais ainda tem sido explorada em estudos clínicos e pré-clínicos buscando desenvolver tratamentos mais eficazes e inovadores. No entanto, a grande quantidade de dados produzida nos últimos anos ainda não foi

Capítulo: Fisiopatologia do Transtorno Bipolar

Autores: Gabriel Rodrigo Fries, Bianca Pfaffenseller, Flávio Kapczinski

Introdução

As bases biológicas do transtorno bipolar são, por via de regra, complexas e multifatoriais. Diferentes estudos têm sido realizados com o objetivo de se identificar genes, proteínas, modificações pós-traducionais, metabólitos e endofenótipos capazes de diferenciar pacientes e controles. Conforme esperado, muitas alterações já foram identificadas em pacientes, a maioria das quais ainda tem sido explorada em estudos clínicos e pré-clínicos com vista ao desenvolvimento de tratamentos mais eficazes e inovadores (Figura 1). No entanto, a grande quantidade de dados produzida nos últimos anos ainda não foi suficiente para, de fato, identificar as causas específicas do transtorno, muito menos dos mecanismos pontuais associados à resposta ao tratamento ou à melhora dos sintomas. Como consequência, o transtorno bipolar ainda não apresenta um prognóstico de sucesso, e muitos pacientes acabam fazendo uso da abordagem "tentativa e erro" até encontrar a medicação ideal para o seu tratamento. Limitações à parte, não há dúvidas de que um entendimento detalhado das bases biológicas do transtorno é o principal requisito para o seu tratamento e manejo adequado.

Neste capítulo, discutiremos brevemente alguns dos principais mecanismos biológicos descritos para o transtorno bipolar (Figura 2). Em muitos casos, como veremos a seguir, ainda se desconhece se as alterações são associadas à causa do transtorno ou às suas consequências. Além disso, a maior parte dos achados refere-se a alterações identificadas na periferia (em células do sangue, por exemplo), o que é justificado pelo fato de uma coleta de sangue ser muito menos invasiva do que a coleta de tecido cerebral. Neste contexto, estudos *post-mortem* ou de neuroimagem tornaram-se os grandes responsáveis pelos achados no sistema nervoso, muitos dos quais inclusive se correlacionam com os achados periféricos. Vamos iniciar a discussão das bases biológicas do transtorno bipolar analisando achados neuroquímicos em diferentes neurotransmissores, seguido de achados relacionados à resiliência celular e, por fim, alterações sistêmicas. Em seguida, discutiremos a perspectiva da identificação e uso de possíveis 'biomarcadores' da doença e de como o entendimento das bases biológicas do transtorno podem melhorar o seu tratamento e, como consequência, a qualidade de vida dos pacientes. A herdabilidade e o caráter genético do transtorno, os quais também desempenham um papel fundamental na sua patofisiologia, serão discutidos posteriormente em um capítulo específico.

1. Alterações neuroquímicas no transtorno bipolar

Vamos iniciar nossa discussão acerca da fisiopatologia do transtorno bipolar explorando algumas alterações relatadas nos sistemas de neurotransmissores, com foco no sistema dopaminérgico e glutamatérgico.

Muitos estudos têm sugerido um papel fundamental da dopamina no transtorno bipolar, a qual se mostrou aumentada em pacientes durante os episódios de mania. A administração de d-anfetamina, por exemplo, uma droga estimulante que induz um aumento dos níveis de dopamina na fenda sináptica, pode induzir sintomas semelhantes à mania em indivíduos saudáveis, podendo também induzir episódios maníacos em pacientes bipolares. De fato, muitos modelos animais de mania empregados no estudo pré-clínico do transtorno bipolar consistem na administração de drogas que aumentam a neurotransmissão dopaminérgica nos animais. Neste contexto, a resolução dos sintomas já foi também associada a uma diminuição na neurotransmissão dopaminérgica, corroborando achados anteriores que sugerem um estudo hiperdopaminérgico nos episódios agudos de humor e a necessidade de revertê-lo com o tratamento.

Além da dopamina, níveis aumentados de glutamato+glutamina foram descritos em regiões do córtex pré-frontal de pacientes bipolares *in vivo*, sugerindo um aumento da neurotransmissão glutamatérgica no transtorno. Esse aumento pode estar associado à disfunção mitocondrial no transtorno bipolar, como discutido nas próximas seções deste capítulo. Além disso, marcadores de excitotoxicidade já foram descritos em regiões cerebrais de pacientes, e o lítio parece ser capaz de proteger células da excitotoxicidade glutamatérgica *in vitro*. De fato, medicamentos de ação antiglutamatérgica, como a lamotrigina, podem ser eficazes no tratamento do transtorno. De forma conjunta, os sistemas glutamatérgico e dopaminérgico podem ainda interagir em vários níveis, regulando aspectos-chave da neurotransmissão e neuroplasticidade (1).

Além disso, estudos *post-mortem* também sugerem um aumento do *turnover* de noradrenalina em áreas corticais e talâmicas de pacientes bipolares, enquanto estudos *in vivo* relatam níveis aumentados de noradrenalina e seu principal metabólito, 3-metoxi-4-hidroxifenilglicol, em pacientes maníacos em comparação àqueles em depressão. Além disso, níveis reduzidos do ácido 5-hidroxiindolacético (5-HIAA), principal metabólito da serotonina, foram descritos em um subgrupo de pacientes, especialmente aqueles apresentando impulsividade, agressão e tentativas de suicídio. Um estudo também já mostrou uma redução na ligação de radioisótopos ao

transportador de serotonina (o qual capta serotonina da fenda sináptica) em plaquetas e no mesencéfalo de pacientes deprimidos. Em conjunto, esses achados sugerem um envolvimento da neurotransmissão dopaminérgica, glutamatérgica, noredrenérgica e serotonérgica no transtorno bipolar.

Vale ressaltar que esses achados não excluem a possibilidade de outros neurotransmissores também estarem envolvidos na fisiopatologia do transtorno. De fato, uma hipótese recente sugere que a depressão bipolar esteja associada a um aumento da função colinérgica e a mania a um aumento da função das catecolaminas (dopamina e noradrenalina). Neste sentido, o aumento de acetilcolina na depressão poderia afetar receptores de acetilcolina muscarínicos e nicotínicos de forma compensatória (2).

2. Resiliência celular e plasticidade

2.1 Resiliência e morte celular

Várias evidências têm demonstrado a presença de alterações neuroanatômicas em pacientes com transtorno bipolar, como a redução da massa cinzenta no córtex pré-frontal dorsolateral e o alargamento dos ventrículos laterais, e uma possível relação destas com prejuízos cognitivos e funcionais. Particularmente, essas alterações parecem ser amplificadas com a progressão do transtorno. Ainda não está claro se estas alterações estão mais relacionadas com a perda de neurônios em si (o que caracterizaria uma *neurodegeneração*) ou se refletem uma diminuição na conectividade entre estas células (prejuízos na *neuroplasticidade*), embora esta última possibilidade seja a mais provável.

Na tentativa de se explicar algumas destas alterações estruturais, estudos recentes têm sugerido que a fisiopatologia do transtorno bipolar envolve prejuízos na resiliência celular, a qual pode ser definida como a habilidade das células de se adaptarem e sobreviverem frente a diferentes estímulos de dano. Alguns tipos celulares, por exemplo, podem modular a comunicação com outras células de acordo com estes estímulos, o que chamamos de plasticidade celular, ou neuroplasticidade, quando se refere aos neurônios. É através deste processo que os neurônios restabelecem ou formam conexões sinápticas diante de condições específicas, permitindo ao sistema nervoso central mudar e adaptar-se tanto durante o desenvolvimento neural quanto em situações novas ao longo da vida adulta (como na

adaptação a lesões e eventos estressores). Evidências têm indicado que alterações em diversas vias de sinalização intracelular podem afetar a resiliência celular e a neuroplasticidade no transtorno bipolar através de mecanismos envolvendo neurotransmissores, glicocorticóides, fatores neurotróficos e sinalização pelo cálcio.

Entre estes achados, já se demonstrou que pacientes com transtorno bipolar apresentam níveis diminuídos de N-acetil-aspartato no córtex pré-frontal e no hipocampo (3). Isso pode indicar um prejuízo na arborização dendrítica e nas sinapses de pacientes, já que esta molécula é abundante em neurônios maduros e representa integridade neuronal, reflexo da viabilidade e funcionamento adequado das células neurais. Nesse contexto de integridade celular, níveis aumentados de fatores apoptóticos em tecidos periféricos e centrais no transtorno bipolar também tem sido relatados, assim como uma diminuição de fatores antiapoptóticos, como Bcl-2 e BDNF. Além disso, um estudo recente reportou um aumento de apoptose em células periféricas de pacientes bipolares (4). Em conjunto, estes trabalhos têm mostrado que as células de pacientes com transtorno bipolar apresentam uma menor resiliência, possivelmente ativando vias de morte celular. De fato, um estudo mostrou que células do neuroepitélio olfatório de pacientes apresentam um aumento da vulnerabilidade à morte celular em comparação a controles saudáveis (5).

Assim, sugere-se que prejuízos em neuroplasticidade podem levar ao encolhimento de estruturas do cérebro de pacientes devido a uma redução no número ou na complexidade das conexões neurais. Além disso, o prejuízo em vias de sinalização associadas com a sobrevivência de diferentes tipos celulares pode ser um dos fatores responsáveis pelo prejuízo clinicamente observado na resiliência de pacientes e na maior vulnerabilidade destes indivíduos a condições ambientais estressoras. Os mecanismos responsáveis por esta resiliência celular diminuída no transtorno bipolar ainda são desconhecidos, mas possivelmente envolvem vias de sinalização e organelas com papel importante na homeostase celular, como a mitocôndria e o retículo endoplasmático (RE). Neste sentido, estímulos em decorrência de uma disfunção mitocondrial ou estresse do retículo endoplasmático costumam ativar mecanismos de resiliência na tentativa de restabelecer o equilíbrio na célula.

Alterações em algumas destas vias tem sido descritas no transtorno bipolar, e há indícios de que elas tenham uma função importante na modulação da sobrevivência/morte celular de pacientes. Uma destas vias é a sinalização pelo cálcio, a qual possui uma regulação fina para prevenir o aumento de cálcio citosólico e, consequentemente, perturbações nas mais variadas enzimas dependentes de cálcio e morte celular. Alterações nos níveis intracelulares de cálcio frequentemente são

encontradas no transtorno bipolar, incluindo níveis elevados de cálcio em células do sangue periférico de pacientes e distúrbios na sua homeostase (6). Esta elevação pode ser causada por disfunções em organelas que tipicamente desempenham papeis centrais no sequestro, tamponamento e armazenamento do cálcio intracelular, como o RE e as mitocôndrias.

2.2 Estresse do RE

Estudos têm sugerido o envolvimento de disfunções no RE na fisiopatologia do transtorno bipolar com base em achados que mostram que os pacientes apresentam alterações na chamada 'resposta a proteínas mal enoveladas' (UPR, do inglês *unfolded protein response*), na qual as células ativam mecanismos de proteção para restaurar a homeostase após o acúmulo de proteínas no lúmen do RE (Figura 2). Esse processo é fisiologicamente ativado pelas células em resposta ao estresse do RE através da indução da expressão de chaperonas, as quais atuam para controlar o aumento das proteínas mal enoveladas, e de uma diminuição temporária da síntese global de proteínas até o equilíbrio ser restabelecido. Entretanto, se a UPR não for suficiente para restaurar o balanço, o estresse prolongado do RE pode levar à morte celular.

Nesse contexto, tem sido demonstrado que células de pacientes com transtorno bipolar apresentam prejuízos na indução da UPR frente ao estresse do RE, o que poderia culminar com a ativação de vias apoptóticas. Em um estudo recente, por exemplo, linfócitos de pacientes com transtorno bipolar, em contraste com células de indivíduos saudáveis, falharam na indução de proteínas da via de sinalização da UPR e apresentaram também níveis aumentados de morte celular em resposta ao estresse do RE induzido *in vitro* (7). Esse prejuízo foi ainda mais pronunciado em pacientes em estágio mais avançado de progressão da doença.

Em conjunto, o estresse do RE tem sido considerado um dos mecanismos responsáveis pela menor resiliência celular de pacientes com transtorno bipolar. É interessante observar que esta resposta debilitada ao estresse do RE pode estar relacionada a várias disfunções neuronais relatadas nestes pacientes, considerando que componentes da UPR também estão envolvidos no desenvolvimento e plasticidade neural, maturação e transporte de vários receptores, bem como na sinalização por cálcio (8-10). Além disso, os mecanismos de ação de estabilizadores do humor parecem envolver esta via celular, uma vez que o tratamento crônico com lítio e valproato pode aumentar a expressão de chaperonas do RE *in vivo* (11, 12).

O RE está proximamente associado com as mitocôndrias em aspectos morfológicos e funcionais, principalmente através da comunicação pelo cálcio (13). Assim, alterações nas interações RE-mitocôndrias, culminando com as alterações nos níveis intracelulares de cálcio descritas no transtorno bipolar, podem influenciar na regulação do metabolismo celular, levar à disfunção mitocondrial e, em alguns casos, morte celular (14).

2.3 Disfunção mitocondrial e estresse crônico

Um conjunto abrangente de evidências, as quais compreendem estudos genéticos, de imagem e *post-mortem,* tem sugerido um papel fundamental da disfunção mitocondrial no transtorno bipolar (15, 16). Entre os achados de alterações mitocondriais em pacientes destacam-se o metabolismo energético debilitado, níveis alterados de cálcio intracelular, alterações nos complexos da cadeia respiratória, regulação diferencial de genes associados com a mitocôndria, e alterações no formato e na distribuição destas organelas. O papel da disfunção mitocondrial no transtorno bipolar é ainda reforçado por trabalhos relatando que estabilizadores de humor e antidepressivos podem melhorar a função mitocondrial (17, 18).

Estas alterações podem estar associadas aos efeitos da exposição crônica ao estresse, o qual é frequentemente associado com a fisiopatologia do transtorno bipolar. O eixo do estresse, comumente conhecido como eixo hipotálamo-hipófise-adrenal (HPA, do inglês *hypothalamic-pituitary-adrenal*), está comprometido em pacientes com transtornos do humor, os quais suprimem de forma ineficiente a liberação de cortisol em resposta ao teste de supressão com dexametasona (19) e apresentam níveis séricos de cortisol aumentados independente da fase da doença (20). Esta deficiência no eixo HPA em regular os níveis de cortisol circulantes, levando a um aumento exagerado dos níveis de cortisol durante o estresse, pode ter importantes consequências em longo prazo aos pacientes, uma vez que os glicocorticóides possuem papéis fundamentais na interação entre mediadores alostáticos e sistemas de neurotransmissores/peptídeos cerebrais.

Nessa linha, estudos *in vitro* e com modelos animais têm demonstrado que o estresse crônico e a exposição crônica aos glicocorticóides podem causar disfunção mitocondrial, levando à redução no consumo de oxigênio, no potencial de membrana mitocondrial e na capacidade de armazenamento de cálcio com consequente ativação de apoptose (21, 22). Adicionalmente, glicocorticóides podem agravar a inflamação (23), contribuir com mecanismos de excitotoxicidade no sistema nervoso central, e ainda induzir diminuição de dendritos por inibir a neurogênese (24). Desta forma,

sugere-se que a disfunção mitocondrial e o estresse crônico podem prejudicar a resiliência celular.

3. Alterações sistêmicas

Conforme mencionado anteriormente, a maioria das informações disponíveis acerca da biologia do transtorno bipolar foi inicialmente descrita no sangue e em suas células. Neste sentido, as alterações sistêmicas encontradas têm possibilitado a descoberta de vários mecanismos associados ao transtorno, embora estas não necessariamente reflitam alterações no sistema nervoso central. De forma geral, a grande parte dos achados se concentra em alterações em fatores neurotróficos, em marcadores de estresse oxidativo, e em inflamação. Vamos discuti-los brevemente nas próximas seções.

3.1 Neurotrofinas

As neurotrofinas compreendem uma classe de proteínas altamente abundantes no sistema nervoso com funções fundamentais na sobrevivência, crescimento, e plasticidade das células neuronais. O fator neurotrófico derivado do cérebro (BDNF, do inglês *brain-derived neurotrophic factor*) é a neurotrofina mais abundante no cérebro de mamíferos adultos, e é fundamental para a neuroplasticidade e para os mecanismos de ação das medicações utilizadas no tratamento do transtorno bipolar (25).

Níveis séricos de BDNF foram inicialmente descritos como estando reduzidos em pacientes em episódios agudos de mania e depressão, não havendo diferenças em comparação a controles ou pacientes em eutimia. Esses resultados foram posteriormente confirmados por estudos de meta-análise (26, 27), sugerindo um importante papel do BDNF na eutimia e na remissão dos sintomas. Ainda, os níveis de BDNF sérico já foram associados a prejuízos cognitivos e também à progressão do transtorno. Especificamente, pacientes em estágios tardios da doença (com maior tempo de doença e um maior número de episódios de humor) apresentaram menores níveis séricos de BDNF em comparação a pacientes em início de doença (28). Neste sentido, sugere-se que a progressão do transtorno bipolar esteja associada a uma redução em mecanismos de neuroplasticidade, o que pode, pelo menos em parte, explicar a redução na resposta ao tratamento e o maior prejuízo cognitivo observado em pacientes com maior número de episódios (29).

Além do BDNF, diversos estudos também sugerem alterações significativas nos níveis de outras neurotrofinas em pacientes, incluindo a neurotrofina-4/5, neurotrofina-3, fator neurotrófico derivado da glia (GDNF) e o fator de crescimento neural (NGF). De forma conjunta, estes achados dão suporte à hipótese de que um prejuízo significativo em mecanismos de neuroplasticidade esteja envolvido na fisiopatologia do transtorno bipolar.

3.2 Estresse oxidativo

O termo “estresse oxidativo” se refere a um desbalanço entre a produção de espécies reativas e as defesas antioxidantes (30), ou ainda a um distúrbio no balanço entre pró-oxidantes e antioxidantes na direção dos primeiros, levando a um dano oxidativo. O dano oxidativo a biomoléculas, como os lipídeos, proteínas e DNA, pode comprometer a função celular de diferentes maneiras, o que justifica a relevância de tais mecanismos em diversas patologias.

No caso do transtorno bipolar, uma série de evidências sugere um importante papel do estresse oxidativo na sua patofisiologia (31). Uma meta-análise recente mostrou que um marcador de peroxidação lipídica e o dano ao DNA/RNA estão significativamente elevados em pacientes quando comparados a controles (32). A atividade de uma das principais enzimas antioxidantes, a superóxido dismutase (SOD), também já foi descrita como estando aumentada em pacientes, porém especificamente naqueles em episódio agudo de mania ou depressão. A atividade de outra enzima, a catalase, se mostrou diminuída em pacientes eutímicos medicados e aumentada em pacientes maníacos não medicados.

Mais do que refletirem a atividade da doença, as alterações sistêmicas relacionadas ao estresse oxidativo também já foram sugeridas como sendo fundamentais na progressão do transtorno bipolar (33). As atividades das enzimas glutationa redutase e glutationa S-transferase, por exemplo, foram descritas como estando aumentadas em pacientes em estágios tardios do transtorno em comparação àqueles em estágios iniciais e controles. Por outro lado, outras alterações já estão presentes desde o início do transtorno, como o aumento dos níveis de 3-nitrotirosina e de carbonil, ambos marcadores de dano oxidativo a proteínas.

Marcadores de estresse oxidativo também já foram encontrados em estudos *post-mortem* em pacientes com transtorno bipolar, como o aumento na oxidação proteica e dos níveis de 3-nitrotirosina, e uma diminuição nos níveis de glutationa no córtex pré-frontal de pacientes. Os mecanismos responsáveis por essas alterações diversas ainda não são conhecidos, porém os estudos existentes sugerem um papel

da disfunção mitocondrial como um dos principais responsáveis pelo aumento das espécies reativas e, portanto, do estresse oxidativo, em pacientes com transtorno bipolar (34, 35).

3.3 Inflamação

O número de estudos envolvendo o papel da inflamação no transtorno bipolar tem aumentado substancialmente nos últimos anos. Entre os marcadores inflamatórios avaliados, destacam-se as citocinas, proteínas responsáveis pela comunicação intercelular no sistema imune, por atuarem também em processos neurais como plasticidade sináptica e, muitas vezes, com a capacidade de causar toxicidade e apoptose em neurônios e células da glia (36).

Os episódios de humor têm sido caracterizados como estados pró-inflamatórios, baseando-se em achados que mostram aumento nos níveis periféricos de citocinas pró-inflamatórias no transtorno bipolar. Os dados mais consistentes sugerem um aumento nos níveis de interleucina (IL)-6 e do fator de necrose tumoral alfa (TNF-α) durante a mania e a depressão (37-39). Entre pacientes eutímicos, já verificou-se que o TNF-α e a IL-6 encontravam-se elevados independentemente do estágio da doença, mas de forma mais expressiva em pacientes em estágios mais avançados de progressão (28). Já a IL-10, uma citocina anti-inflamatória, foi encontrada aumentada somente no estágio precoce. Mais recentemente, observou-se um aumento dos níveis plasmáticos das quimiocinas CCL11, CCL24 e CXCL10, e uma diminuição de CXCL8 em pacientes bipolares crônicos comparados a indivíduos saudáveis (40).

Outro estudo observou um perfil de expressão gênica inflamatória com características aberrantes em monócitos de pacientes com transtorno bipolar, sendo algumas delas relacionadas às citocinas comumente correlacionadas com o transtorno, principalmente TNF-α e IL-6 (41). Neste contexto, o TNF-α se tornou um dos principais alvos na pesquisa do transtorno bipolar por ser um dos principais mediadores pró-inflamatórios. Seus efeitos podem ser influenciados por outras citocinas (pró- e anti-inflamatórias), agindo em vias de neuroplasticidade, resiliência e sobrevivência celular (42). De fato, o tratamento de células periféricas sanguíneas de controles saudáveis com o soro de pacientes em eutimia (cuja expressão sérica apresentava alterações na expressão de proteínas pro-/anti-inflamatórias) levou a uma diminuição na viabilidade celular das mesmas (43), indicando um aumento na resposta

inflamatória e, provavelmente, a morte de células do sistema imune de pacientes com transtorno bipolar.

A origem do desbalanço imunológico observado no transtorno bipolar é ainda desconhecida. Estudos futuros são necessários com enfoque na influência de fatores ambientais no perfil inflamatório de pacientes, como o estresse, sono, alterações no ritmo circadiano, má qualidade de vida e longos períodos de exposição a drogas. Estes apresentam um alto risco para desenvolver comorbidades clínicas, como doenças cardiovasculares, síndrome metabólica e diabetes (44, 45), sendo que a principal relação entre essas comorbidades e o transtorno bipolar parece ser a presença de inflamação sistêmica crônica e de altos níveis de marcadores inflamatórios. Justamente devido ao crescente conjunto de evidências que sugerem uma inflamação crônica leve na periferia e no cérebro de pacientes (46), alguns autores tem se referido ao transtorno bipolar como uma doença inflamatória multisistêmica (47, 48).

Conclusões

Conforme discutimos ao longo das seções anteriores, a fisiopatologia do transtorno bipolar é complexa, multifatorial, e envolve a participação de mecanismos centrais e periféricos (Figura 1). Além disso, os achados sugerem a existência de alterações especificas durante os episódios agudos de humor (conhecidos, portanto, como marcadores de atividade da doença) e outras que se alteram em função da progressão do transtorno. Vale ressaltar ainda que, além dos mecanismos discutidos neste capítulo, as bases biológicas do transtorno envolvem ainda uma série de alterações em vias de sinalização celular (como aquelas associados à ativação da proteína G, monofosfato cíclico de adenosina, fosfatidilinositol e Wnt), neuroinflamação, disfunção glial, entre outros (Figura 3).

Além disso, muitas das alterações relatadas, principalmente os achados de estresse oxidativo e inflamação sistêmica, podem, em parte, colaborar para a maior incidência de comorbidades clínicas em pacientes (como, por exemplo, a obesidade, a síndrome metabólica e as doenças cardiovasculares) (49). De fato, o conjunto de alterações periféricas constitui um quadro de 'toxicidade sistêmica' que se encontra elevado durante os episódios agudos, porém se estabiliza durante a eutimia (50) (Figura 4). Neste contexto, tais achados periféricos poderão desempenhar uma importante ferramenta diagnóstica a partir do momento em que forem, de fato, validados como biomarcadores. Especificamente, muitos deles poderão auxiliar no manejo de sintomas, na predição de resposta a possíveis medicações, e na

identificação de estágios específicos do transtorno. Em última instância, poderão identificar populações mais homogêneas de pacientes com transtorno bipolar e, assim, contribuir para um tratamento mais adequado dos mesmos.

Figuras

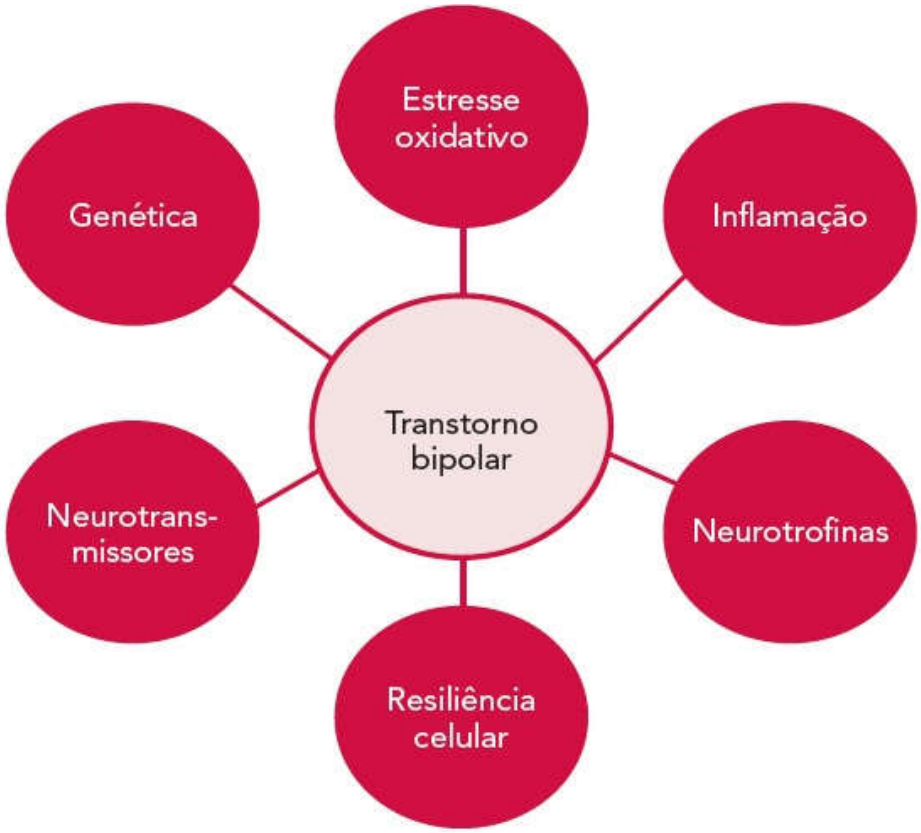


Figura 1. Fisiopatologia do transtorno bipolar.

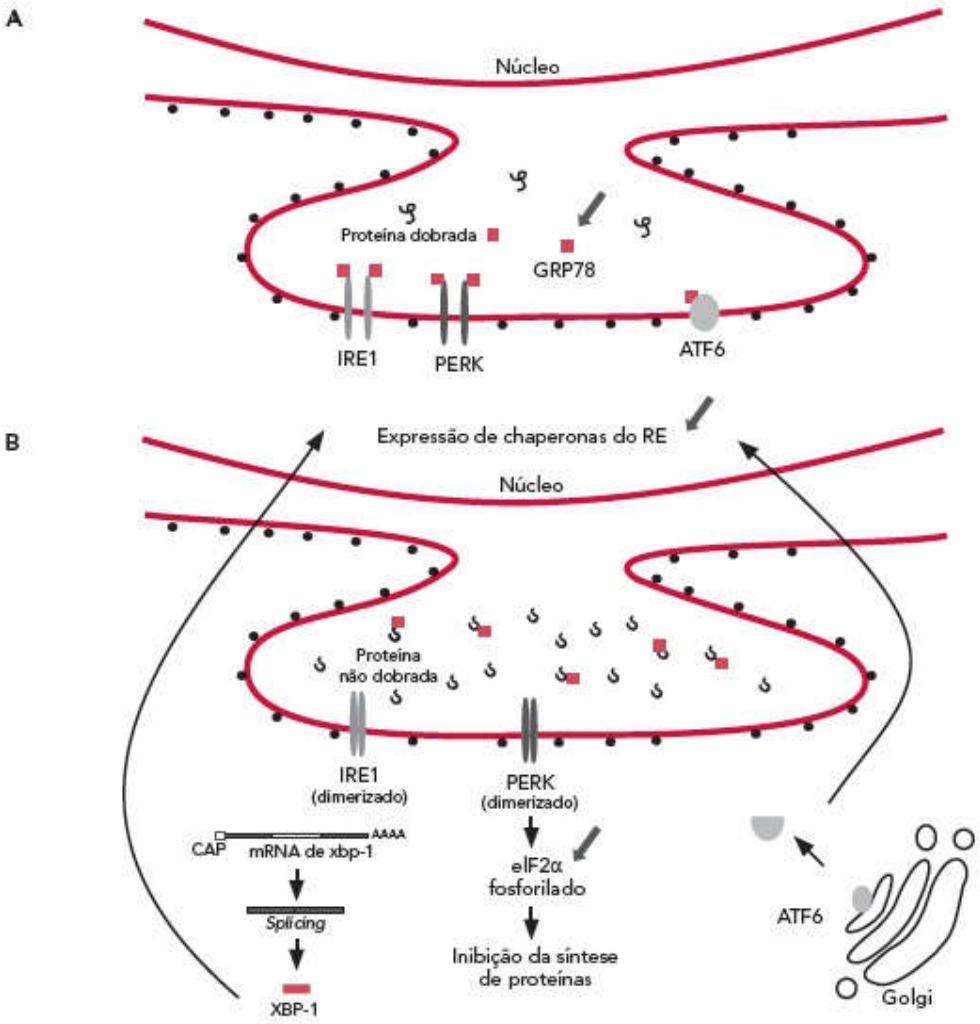


Figura 2. Vias de sinalização ativadas pelo estresse do retículo endoplasmático.

Neurotransmissão	Neurotrofinas	Estresse oxidativo	Inflamação	Resiliência celular
↑ Dopamina ↑ Glutamato ↑ Noradrenalina ↓ 5-HIAA	↓ BDNF ↑ NT-3 ↑ NT-4/5 ↑ GDNF ↓ NGF	↑ NO ↑ Dano ao DNA/RNA ↑ Atividade da SOD ↓ Atividade da CAT ↑ 3-NT ↑ PCC ↓ GSH	↑ TNF-α ↑ IL-6 ↑ IL-10	↑ Disfunção mitocondrial ↓ Resposta ao estresse do RE ↑ Apoptose ↑ Cálcio

Figura 3. Alterações centrais e periféricas descritas no transtorno bipolar.

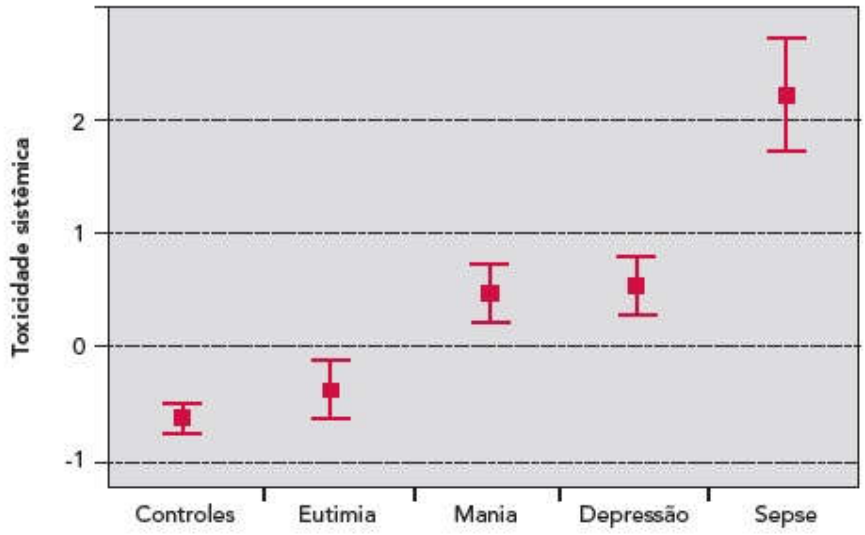


Figura 4. Toxicidade sistêmica no transtorno bipolar.

Referências

1. de Bartolomeis A, Buonaguro EF, Iasevoli F, Tomasetti C. The emerging role of dopamine-glutamate interaction and of the postsynaptic density in bipolar disorder pathophysiology: Implications for treatment. J Psychopharmacol. 2014;28(6):505-26.
2. van Enkhuizen J, Janowsky DS, Olivier B, Minassian A, Perry W, Young JW, et al. The catecholaminergic-cholinergic balance hypothesis of bipolar disorder revisited. Eur J Pharmacol. 2014.
3. Sassi RB, Stanley JA, Axelson D, Brambilla P, Nicoletti MA, Keshavan MS, et al. Reduced NAA levels in the dorsolateral prefrontal cortex of young bipolar patients. Am J Psychiatry. 2005;162(11):2109-15.
4. Fries GR, Vasconcelos-Moreno MP, Gubert C, Santos BT, da Rosa AL, Eisele B, et al. Early apoptosis in peripheral blood mononuclear cells from patients with bipolar disorder. J Affect Disord. 2014;152-154:474-7.
5. McCurdy RD, Féron F, Perry C, Chant DC, McLean D, Matigian N, et al. Cell cycle alterations in biopsied olfactory neuroepithelium in schizophrenia and bipolar I disorder using cell culture and gene expression analyses. Schizophr Res. 2006;82(2-3):163-73.
6. Perova T, Wasserman MJ, Li PP, Warsh JJ. Hyperactive intracellular calcium dynamics in B lymphoblasts from patients with bipolar I disorder. Int J Neuropsychopharmacol. 2008;11(2):185-96.
7. Pfaffenseller B, Wollenhaupt-Aguiar B, Fries GR, Colpo GD, Burque RK, Bristot G, et al. Impaired endoplasmic reticulum stress response in bipolar disorder: cellular evidence of illness progression. Int J Neuropsychopharmacol. 2014;17(9):1453-63.
8. Vandenberghe W, Nicoll RA, Bredt DS. Interaction with the unfolded protein response reveals a role for stargazin in biosynthetic AMPA receptor transport. J Neurosci. 2005;25(5):1095-102.
9. Verkhratsky A. The endoplasmic reticulum and neuronal calcium signalling. Cell Calcium. 2002;32(5-6):393-404.
10. Weng WC, Lee WT, Hsu WM, Chang BE, Lee H. Role of glucose-regulated Protein 78 in embryonic development and neurological disorders. J Formos Med Assoc. 2011;110(7):428-37.
11. Chen B, Wang JF, Young LT. Chronic valproate treatment increases expression of endoplasmic reticulum stress proteins in the rat cerebral cortex and hippocampus. Biol Psychiatry. 2000;48(7):658-64.
12. Shao L, Sun X, Xu L, Young LT, Wang JF. Mood stabilizing drug lithium increases expression of endoplasmic reticulum stress proteins in primary cultured rat cerebral cortical cells. Life Sci. 2006;78(12):1317-23.
13. Giorgi C, De Stefani D, Bononi A, Rizzuto R, Pinton P. Structural and functional link between the mitochondrial network and the endoplasmic reticulum. Int J Biochem Cell Biol. 2009;41(10):1817-27.
14. Wang X, Eno CO, Altman BJ, Zhu Y, Zhao G, Olberding KE, et al. ER stress modulates cellular metabolism. Biochem J. 2011;435(1):285-96.
15. Manji H, Kato T, Di Prospero NA, Ness S, Beal MF, Krams M, et al. Impaired mitochondrial function in psychiatric disorders. Nat Rev Neurosci. 2012;13(5):293-307.
16. Cataldo AM, McPhie DL, Lange NT, Punzell S, Elmiligy S, Ye NZ, et al. Abnormalities in mitochondrial structure in cells from patients with bipolar disorder. Am J Pathol. 2010;177(2):575-85.
17. Valvassori SS, Rezin GT, Ferreira CL, Moretti M, Gonçalves CL, Cardoso MR, et al. Effects of mood stabilizers on mitochondrial respiratory chain activity in brain of rats treated with d-amphetamine. J Psychiatr Res. 2010;44(14):903-9.
18. Bachmann RF, Wang Y, Yuan P, Zhou R, Li X, Alesci S, et al. Common effects of lithium and valproate on mitochondrial functions: protection against methamphetamine-induced mitochondrial damage. Int J Neuropsychopharmacol. 2009;12(6):805-22.
19. Daban C, Vieta E, Mackin P, Young AH. Hypothalamic-pituitary-adrenal axis and bipolar disorder. Psychiatr Clin North Am. 2005;28(2):469-80.
20. Cervantes P, Gelber S, Kin FN, Nair VN, Schwartz G. Circadian secretion of cortisol in bipolar disorder. J Psychiatry Neurosci. 2001;26(5):411-6.
21. Du J, Wang Y, Hunter R, Wei Y, Blumenthal R, Falke C, et al. Dynamic regulation of mitochondrial function by glucocorticoids. Proc Natl Acad Sci U S A. 2009;106(9):3543-8.
22. Gong Y, Chai Y, Ding JH, Sun XL, Hu G. Chronic mild stress damages mitochondrial ultrastructure and function in mouse brain. Neurosci Lett. 2011;488(1):76-80.
23. Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM. The stressed CNS: when glucocorticoids aggravate inflammation. Neuron. 2009;64(1):33-9.
24. Sapolsky RM. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry. 2000;57(10):925-35.
25. Grande I, Fries GR, Kunz M, Kapczinski F. The role of BDNF as a mediator of neuroplasticity in bipolar disorder. Psychiatry Investig. 2010;7(4):243-50.
26. Fernandes BS, Gama CS, Ceresér KM, Yatham LN, Fries GR, Colpo G, et al. Brain-derived neurotrophic factor as a state-marker of mood episodes in bipolar disorders: a systematic review and meta-regression analysis. J Psychiatr Res. 2011;45(8):995-1004.
27. Fernandes BS, Berk M, Turck CW, Steiner J, Gonçalves CA. Decreased peripheral brain-derived neurotrophic factor levels are a biomarker of disease activity in major psychiatric disorders: a comparative meta-analysis. Mol Psychiatry. 2014;19(7):750-1.
28. Kauer-Sant'Anna M, Kapczinski F, Andreazza AC, Bond DJ, Lam RW, Young LT, et al. Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. Int J Neuropsychopharmacol. 2009;12(4):447-58.
29. Fries GR, Pfaffenseller B, Stertz L, Paz AV, Dargél AA, Kunz M, et al. Staging and neuroprogression in bipolar disorder. Curr Psychiatry Rep. 2012;14(6):667-75.
30. Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans. 2007;35(Pt 5):1147-50.
31. Steckert AV, Valvassori SS, Moretti M, Dal-Pizzol F, Quevedo J. Role of oxidative stress in the pathophysiology of bipolar disorder. Neurochem Res. 2010;35(9):1295-301.

32. Brown NC, Andreazza AC, Young LT. An updated meta-analysis of oxidative stress markers in bipolar disorder. Psychiatry Res. 2014;218(1-2):61-8.
33. Berk M, Kapczinski F, Andreazza AC, Dean OM, Giorlando F, Maes M, et al. Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors. Neurosci Biobehav Rev. 2011;35(3):804-17.
34. Andreazza AC, Shao L, Wang JF, Young LT. Mitochondrial complex I activity and oxidative damage to mitochondrial proteins in the prefrontal cortex of patients with bipolar disorder. Arch Gen Psychiatry. 2010;67(4):360-8.
35. Clay HB, Sillivan S, Konradi C. Mitochondrial dysfunction and pathology in bipolar disorder and schizophrenia. Int J Dev Neurosci. 2011;29(3):311-24.
36. Kraft AD, McPherson CA, Harry GJ. Heterogeneity of microglia and TNF signaling as determinants for neuronal death or survival. Neurotoxicology. 2009;30(5):785-93.
37. Brietzke E, Stertz L, Fernandes BS, Kauer-Sant'anna M, Mascarenhas M, Escosteguy Vargas A, et al. Comparison of cytokine levels in depressed, manic and euthymic patients with bipolar disorder. J Affect Disord. 2009;116(3):214-7.
38. Munkholm K, Vinberg M, Berk M, Kessing LV. State-related alterations of gene expression in bipolar disorder: a systematic review. Bipolar Disord. 2012;14(7):684-96.
39. Modabbernia A, Taslimi S, Brietzke E, Ashrafi M. Cytokine alterations in bipolar disorder: a meta-analysis of 30 studies. Biol Psychiatry. 2013;74(1):15-25.
40. Barbosa IG, Rocha NP, Bauer ME, de Miranda AS, Huguet RB, Reis HJ, et al. Chemokines in bipolar disorder: Trait or state? Eur Arch Psychiatry Clin Neurosci. 2012.
41. Padmos RC, Hillegers MH, Knijff EM, Vonk R, Bouvy A, Staal FJ, et al. A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. Arch Gen Psychiatry. 2008;65(4):395-407.
42. Brietzke E, Kapczinski F. TNF-alpha as a molecular target in bipolar disorder. Prog Neuropsychopharmacol Biol Psychiatry. 2008;32(6):1355-61.
43. Herberth M, Koethe D, Levin Y, Schwarz E, Krzyszton ND, Schoeffmann S, et al. Peripheral profiling analysis for bipolar disorder reveals markers associated with reduced cell survival. Proteomics. 2011;11(1):94-105.
44. McIntyre RS, Danilewitz M, Liauw SS, Kemp DE, Nguyen HT, Kahn LS, et al. Bipolar disorder and metabolic syndrome: an international perspective. J Affect Disord. 2010;126(3):366-87.
45. Weiner M, Warren L, Fiedorowicz JG. Cardiovascular morbidity and mortality in bipolar disorder. Ann Clin Psychiatry. 2011;23(1):40-7.
46. Hamdani N, Tamouza R, Leboyer M. Immuno- inflammatory markers of bipolar disorder: a review of evidence. Front Biosci (Elite Ed). 2012;4:2170-82.
47. Leboyer M, Soreca I, Scott J, Frye M, Henry C, Tamouza R, et al. Can bipolar disorder be viewed as a multi-system inflammatory disease? J Affect Disord. 2012;141(1):1-10.
48. Stertz L, Magalhães PV, Kapczinski F. Is bipolar disorder an inflammatory condition? The relevance of microglial activation. Curr Opin Psychiatry. 2013;26(1):19-26.
49. Sylvia LG, Shelton RC, Kemp DE, Bernstein EE, Friedman ES, Brody BD, et al. Medical burden in bipolar disorder: findings from the Clinical and Health Outcomes Initiative in Comparative Effectiveness for Bipolar Disorder study (Bipolar CHOICE). Bipolar Disord. 2014.
50. Kapczinski F, Dal-Pizzol F, Teixeira AL, Magalhaes PV, Kauer-Sant'Anna M, Klamt F, et al. A systemic toxicity index developed to assess peripheral changes in mood episodes. Mol Psychiatry. 2010;15(8):784-6.

ANEXO 3

Journal of Affective Disorders 152-154 (2014) 474–477

Contents lists available at ScienceDirect

Journal of Affective Disorders

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

Preliminary communication

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

Gabriel Rodrigo Fries [a,b,*], Mirela Paiva Vasconcelos-Moreno [a,c], Carolina Gubert [a,b], Barbara Tietböhl Martins Quadros dos Santos [a], André Luiz Schuh Teixeira da Rosa [a], Bárbara Eisele [a], Juliana Sartori [a], Bianca Pfaffenseller [a,b], Flávio Kapczinski [a,b,c], Márcia Kauer-Sant'Anna [a,b,c]

[a] Laboratory of Molecular Psychiatry, Centro de Pesquisas Experimentais, Hospital de Clínicas de Porto Alegre, and INCT for Translational Medicine, Brazil
[b] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, UFRGS, Brazil
[c] Programa de Pós-Graduação em Medicina: Psiquiatria, Universidade Federal do Rio Grande do Sul, UFRGS, Brazil

ARTICLE INFO

Article history:
Received 15 May 2013
Accepted 31 July 2013
Available online 16 August 2013

Keywords:
Bipolar disorder
PBMCs
Apoptosis
Cell viability

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

* Corresponding author at: Laboratory of Molecular Psychiatry, Centro de Pesquisas Experimentais, Hospital de Clínicas de Porto Alegre, and INCT for Translational Medicine, 2350, CEP 90035903, Porto Alegre-RS, Brazil. Tel.: +55 51 335 98845; fax: +55 51 33598 846.
E-mail address: gabrielrfries@gmail.com (G.R. Fries).

0165-0327/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jad.2013.07.027

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).

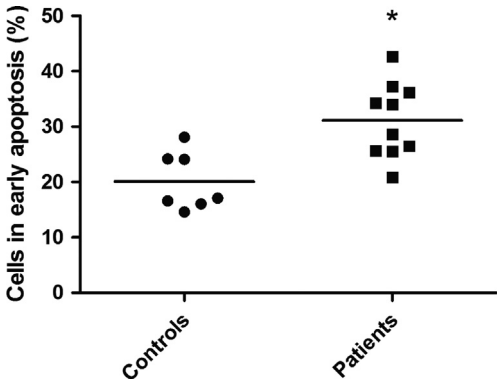


Fig. 1. Early apoptosis in BD patients and controls. *$P = 0.002$, independent *t*-test.

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.

Role of funding source

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundo de Incentivo a Pesquisas e Eventos (FIPE–HCPA).

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.

Acknowledgment

None.

References

Andreazza, A.C., Kauer-Sant'anna, M., Frey, B.N., Bond, D.J., Kapczinski, F., Young, L. T., Yatham, L.N., 2008. Oxidative stress markers in bipolar disorder: a meta-analysis. Journal of Affective Disorders 111 (2–3), 135–144.

Andreazza, A.C., Frey, B.N., Erdtmann, B., Salvador, M., Rombaldi, F., Santin, A., Gonçalves, C.A., Kapczinski, F., 2007. DNA damage in bipolar disorder. Psychiatry Research 153 (1), 27–32.

Bachmann, R.F., Schloesser, R.J., Gould, T.D., Manji, H.K., 2005. Mood stabilizers target cellular plasticity and resilience cascades: implications for the development of novel therapeutics. Molecular Neurobiology 32 (2), 173–202.

Bei, E., Salpeas, V., Pappa, D., Anagnostara, C., Alevizos, V., Moutsatsou, P., 2009. Phosphorylation status of glucocorticoid receptor, heat shock protein 70, cytochrome c and Bax in lymphocytes of euthymic, depressed and manic bipolar patients. Psychoneuroendocrinology 34, 1162–1175.

Brietzke, E., Kapczinski, F., 2008. TNF-alpha as a molecular target in bipolar disorder. Progress in Neuro-Psychopharmacology and Biological Psychiatry 32 (6), 1355–1361.

Clay, H.B., Sillivan, S., Konradi, C., 2011. Mitochondrial dysfunction and pathology in bipolar disorder and schizophrenia. International Journal of Developmental Neuroscience 29 (3), 311–324.

Fernandes, B.S., Gama, C.S., Ceresér, K.M., Yatham, L.N., Fries, G.R., Colpo, G., de Lucena, D., Kunz, M., Gomes, F.A., Kapczinski, F., 2011. Brain-derived neurotrophic factor as a state-marker of mood episodes in bipolar disorders: a systematic review and meta-regression analysis. Journal of Psychiatric Research 45 (8), 995–1004.

Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S., Gottlieb, E., Green, D.R., Hengartner, M.O., Kepp, O., Knight, R.A., Kumar, S., Lipton, S.A., Lu, X., Madeo, F., Malorni, W., Mehlen, P., Nuñez, G., Peter, M.E., Piacentini, M., Rubinsztein, D.C., Shi, Y., Simon, H.U., Vandenabeele, P., White, E., Yuan, J., Zhivotovsky, B., Melino, G., Kroemer, G., 2012. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death and Differentiation 19 (1), 107–120.

Gigante, A.D., Young, L.T., Yatham, L.N., Andreazza, A.C., Nery, F.G., Grinberg, L.T., Heinsen, H., Lafer, B., 2011. Morphometric post-mortem studies in bipolar disorder: possible association with oxidative stress and apoptosis. International Journal of Neuropsychopharmacology 14 (8), 1075–1089.

Hayashi, A., Kasahara, T., Kametani, M., Toyota, T., Yoshikawa, T., Kato, T., 2009. Aberrant endoplasmic reticulum stress response in lymphoblastoid cells from patients with bipolar disorder. International Journal of Neuropsychopharmacology 12 (1), 33–43.

Herberth, M., Koethe, D., Levin, Y., Schwarz, E., Krzyszton, N.D., Schoeffmann, S., Ruh, H., Rahmoune, H., Kranaster, L., Schoenborn, T., Leweke, M.F., Guest, P.C.,

Bahn, G., 2011. Peripheral profiling analysis for bipolar disorder reveals markers associated with reduced cell survival. Proteomics 11 (1), 94–105.
Kapczinski, F., Dal-Pizzol, F., Teixeira, A.L., Magalhaes, P.V., Kauer-Sant'Anna, M., Klamt, F., Pasquali, M.A., Quevedo, J., Gama, C.S., Post, R., 2010. A systemic toxicity index developed to assess peripheral changes in mood episodes. Molecular Psychiatry 15 (8), 784–786.
Kapczinski, F., Dal-Pizzol, F., Teixeira, A.L., Magalhaes, P.V., Kauer-Sant'Anna, M., Klamt, F., Moreira, J.C., de Bittencourt Pasquali, M.A., Fries, G.R., Quevedo, J., Gama, C.S., Post, R., 2011. Peripheral biomarkers and illness activity in bipolar disorder. Journal of Psychiatric Research 45 (2), 156–161.
Kauer-Sant'Anna, M., Kapczinski, F., Andreazza, A.C., Bond, D.J., Lam, R.W., Young, L.T., Yatham, L.N., 2009. Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. International Journal of Neuropsychopharmacology 12 (4), 447–458.
Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., El-Deiry, W.S., Golstein, P., Green, D.R., Hengartner, M., Knight, R.A., Kumar, S., Lipton, S.A., Malorni, W., Nuñez, G., Peter, M.E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino, G., 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death and Differentiation 16 (1), 3–11.
Krysko, D.V., Agostinis, P., Krysko, O., Garg, A.D., Bachert, C., Lambrecht, B.N., Vandenabeele, P., 2011. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. Trends in Immunology 32 (4), 157–164.
Kupfer, D.J., 2005. The increasing medical burden in bipolar disorder. JAMA 293 (20), 2528–2530.
Nandra, K.S., Agius, M., 2012. The differences between typical and atypical antipsychotics: the effects on neurogenesis. Psychiatria Danubina 24 (Suppl 1), S95–99.
O'Brien, S.M., Scully, P., Scott, L.V., Dinan, T.G., 2006. Cytokine profiles in bipolar affective disorder: focus on acutely ill patients. Journal of Affective Disorders 90 (2-3), 263–267.
Politi, P., Brondino, N., Emanuele, E., 2008. Increased proapoptotic serum activity in patients with chronic mood disorders. Archives of Medical Research 39 (2), 242–245.
Roshanaei-Moghaddam, B., Katon, W., 2009. Premature mortality from general medical illnesses among persons with bipolar disorder: a review. Psychiatric Services 60 (2), 147–156.
Shao, L., Martin, M.V., Watson, S.J., Schatzberg, A., Akil, H., Myers, R.M., Jones, E.G., Bunney, W.E., Vawter, M.P., 2008. Mitochondrial involvement in psychiatric disorders. Annals of Medicine 40 (4), 281–295.
Song, N., Boku, S., Nakagawa, S., Kato, A., Toda, H., Takamura, N., Omiya, Y., Kitaichi, Y., Inoue, T., Koyama, T., 2012. Mood stabilizers commonly restore staurosporine-induced increase of p53 expression and following decrease of Bcl-2 expression in SH-SY5Y cells. Progres in Neuropsychopharmacology and Biological Psychiatry 38 (2), 183–189.
Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., Kroemer, G., 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature Reviews Molecular Cell Biology 11, 700–714.

ANEXO 4

Title Page

Title: Dissimilar Mechanism of Action and Dopamine Transporter Dependency of 6-Hydroxydopamine-Induced Toxicity in Undifferentiated and RA- Differentiated SH-SY5Y Human Neuroblastoma Cells

Authors: Fernanda M. Lopes[1]*, Leonardo L. da Motta[1], Marco A. De Bastiani[1], Bianca Pfaffenseller[1], Bianca W. Aguiar[1], Luiz F. de Souza[2], Geancarlo Zanatta[1], Daiani M. Vargas[1]; Patrícia Schönhofen[1], Giovana F. Londero[1], Liana M. de Medeiros[1], Valder N. Freire[3], Richard B. Parsons[4], Alcir L. Dafre[2], Mauro A. A. Castro[5], Fabio Klamt[1]*.

Filiation: [1] Laboratory of Cellular Biochemistry, Department of Biochemistry, ICBS/UFRGS, 90035-003 Porto Alegre (RS), Brazil; [2] Cellular Defenses Laboratory, Department of Biochemistry, Biological Sciences Centre, Federal University of Santa Catarina (UFSC), 88040-900 Florianopolis (SC), Brazil; [3]Department of Physics at Federal University of Ceará (UFC), 60455-760 Fortaleza (CE), Brazil; [4] Institute of Pharmaceutical Science, King's College London, 150 Stamford Street, London SE1 9NH, UK; [5] Bioinformatics and Systems Biology Laboratory, Federal University of Paraná (UFPR), Polytechnic Center, 81520-260 Curitiba (PR), Brazil.

Correspondence to: Fernanda Martins Lopes, M.Sc and Prof. Fábio Klamt, PhD Laboratório de Bioquímica Celular (LBC), Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul

(UFRGS). 2600 Ramiro Barcelos St., Porto Alegre (RS), Brazil, 90035-003. Phone: +55 51 3308-5556; FAX: +55 51 3308-5535, e-mail:fe.m.lopes@gmail.com and fabio.klamt@ufrgs.br

Abstract

Background: Research on Parkinson's disease (PD) and drug development are hampered by the lack of suitable human *in vitro* models that simply and accurately recreate the conditions of disease. To counteract this, many attempts to differentiate cell lines, such as the human SH-SY5Y neuroblastoma cells, into more neuron-like cells have been performed, resulting in partial expression of neuronal markers. Here we aimed to characterize relevant neuronal features to discriminate undifferentiated and retinoic acid (RA)-differentiated SH-SYSY cells, and explored potential differences between both cell models in response to the neurotoxin 6-hydroxydopamine (6-OHDA).

Results: RA-differentiated SH-SY5Y cells have low proliferative rate, a pronounced neuronal morphology with high expression of genes related to synapse vesicle cycle and present more similarities to dopaminergic neurons, which are the target cells of PD. Since increased oxidative stress is an early event in PD, we also explored the differences in redox parameters of both models. Undifferentiated and RA-differentiated SH-SY5Y cells are extremely dissimilar regarding the overall capacity and the nature of their antioxidant defenses and, even though RA-differentiated SH-SY5Y cells present a higher basal antioxidant capacity with high resistance against H_2O_2 insult, they are two-fold more sensitive to 6-OHDA. Noteworthily, 6-OHDA's toxicity was significantly decreased by dithiothreitol (DTT), a cell-permeable thiol reducing agent, and by 3α-Bis-4-fluorophenyl-methoxytropane, a dopamine transporter (DAT) inhibitor, particularly in RA-differentiated SH-SY5Y cells, that also presented higher DAT immunocontent.

Conclusions: DAT inhibition can protect only RA-differentiated cells from the oxidative damage and neuronal cell death caused by 6-OHDA, mimicking a phenomenon described for *in vivo* experimental models, but still controversial for *in vitro* models. Moreover, these data demonstrates, for the first time, crucial differences in the mechanism of action of 6-OHDA

operating in undifferentiated and RA-differentiated SH-SY5Y cells. These differences could potentially impact our understanding of the pathological mechanisms of PD and the development of new therapies and drugs for the management of the disease.

Keywords (3-to-10 words): SH-SY5Y cells, retinoic acid, Parkinson disease, experimental model, 6-hydroxydopamine, Dopamine transporter

Background

Parkinson's disease (PD) pathological hallmark is the loss of dopaminergic neurons in *substantia nigra pars compacta* (SNpc) and decreased dopamine (DA) innervation in the striatum [1,2]. Although PD is mainly associated at the cellular level with oxidative stress [3] and mitochondrial dysfunction [4], the functional changes operating during the initial stage of PD remains unknown [5]. These limitations are widely attributed to the lack of reliable human *in vitro* cell model for studying the molecular mechanisms underlying the pathophysiology of PD [6,7]. The main cause of this is the difficulty to reproduce the complex physiological and biochemical features of a human dopaminergic neuron *in vitro* [8].

In this context, the human neuroblastoma cell line SH-SY5Y is the most used *in vitro* model for this disturb [9–11], mainly because it express catecholamine synthesis machinery [12]. Although SH-SY5Y cells are widely used as PD model, it does not present neuronal properties such as remain permanently in post-mitotic state and present important proteins responsible for chemical synapse [6]. Interestingly, the *in vitro* differentiation of the human SH-SY5Y neuroblastoma cell line into a neuronal phenotype was established more than 30 years ago. The differentiation protocols commonly use retinoic acid (RA) as a neurotrophin that causes changes in morphology and leads to cell cycle arrest [13]. However, there are only few studies addressing the differences between the undifferentiated and RA-differentiated SH-SY5Y cells [14–21].

Besides the use of human dopaminergic (neuron-*like*) cells, it is also necessary mimics the pathophysiology of PD to have a complete experimental *in vitro* model for this disturb [22]. For over 40 years, the catecholamine-derived neurotoxin 6-hydroxydopamine (6-OHDA) has been used to induce dopaminergic cell death in several preclinical experimental models of this disorder [23]. Once inside the neuron, 6-OHDA accumulates in the cytosol and its non-enzymatically auto-oxidation causes reactive oxygen species and *p*-quinones production, as well as a decrease of antioxidants levels [24,25]. Moreover, high levels of 6-OHDA can be found in human biopsies of caudade nucleus [26] and in urine [27] of PD patients.

The main characteristic of 6-OHDA is its similarity with dopamine, which enables the toxin to enter into the dopaminergic neuron via dopamine transporter (DAT) and, then, cause

cellular damage [28,29]. The role of DAT in 6-OHDA-induced neurotoxicity is well established *in vivo* [30], however it is still controversial in *in vitro* studies. Previous works showed that DAT inhibitors are not able to protect from the damage caused by 6-OHDA [31]. On the other hand, it was found a partial protection against this toxin in primary cultures of dopaminergic neurons [32]. These controversial findings may be related to the *in vitro* cellular model used since most of these experiments were performed in cells with low expression levels of DAT, which includes undifferentiated SH-SY5Y cells. Is spite of the large amount of data related to this subject, there are no studies showing the role of DAT in 6-OHDA toxicity in RA-differentiated SH-SY5Y cells.

In this present work, we aim to characterize the differences between undifferentiated and differentiated SH-SY5Y cells regarding gene expression of important networks related to dopaminergic neurons machinery, morphology and redox metabolism. After explored these features, we evaluated the response of both models to 6-OHDA-cytotoxicity. Here, for the first time, we showed the role of DAT dependency in cell death induced by 6-OHDA only in RA-differentiated cells.

Results and Discussion

Neuronal *in vitro* experimental models have always been an issue in neurosciences because of the difficulty in mimicking nervous system complexity [6–8]. Moreover, most cellular models do not present relevant neuronal morphology and neurochemical features. In this context, even though SH-SY5Y cells does not present mature neuronal cell features, it is the most used *in vitro* model to study PD. Neuronal characteristics of SH-SY5Y can be increased by RA-induced-differentiation, however just a few studies explored the differences between undifferentiated and RA-differentiated cells [17,18,33,34].

Neuronal characterization

Neurons are specialized cells that process and transmit information through electrical and chemical signals and rarely undergo cellular divisions and are commonly known as permanent post-mitotic cells [35] . Hence, it is important to evaluate whether any *in vitro* models present these relevant features. In order to analyze the neuronal features of undifferentiated and RA-differentiated cells (differentiation protocol described in Fig. 1), we first evaluate the

effect of RA-treated in the proliferation rates and cell cycle distribution. Cellular growth was accessed by cell counting during different culturing time. Our data showed a significant decrease of proliferation rates in RA-differentiated cells (Fig. 2a). Previous studies showed that lowering the serum levels in culture medium can cause cell cycle arrest in G1/G0 phase and a decrease in proliferation rates, and the combination with RA leads to terminal differentiation of neuroblast cells [36–38]. On the other hand, here we found that the decrease in cellular growth in RA-differentiated cells was associated with a decrease in S phase in combination with G2-M arrest (Fig. 2b). This feature is commonly found in cells treated with BDNF, another important neurotrophin [39,40].

To further investigate this, we analyzed gene expression of the cell cycle network by microarray analysis. No statically significant differences between the two phenotypes were found (Fig. 2c), which was expected since the gene set network used (curated by KEGG pathways) covers the cellular machinery related to both cell cycle arrest and proliferation processes. The components enriched in RA-differentiated SH-SY5Y cells are summarized (Fig. 2c). These genes are associated with cell cycle arrest, for instance, cyclin-dependent protein kinases (CDK) inhibitors (*e.g.*: p18, p19, p21 and p27) and genes related to G2-M arrest, such as *GDD45G* and *SMAD3* [39,41,42].

We then evaluated the changes in cell morphology by the quantification of neurite density in both cellular models (Fig. 2d,e). A significant increase in neurite density was found in RA-differentiated cells ($P<0.0001$), suggesting a change from epithelial (as defined by ATCC for SH-SY5Y cells) [43] to a stellate morphology after the differentiation process. The term neurite refers to axons and dendrites extended by neuronal cell lines. The *in vitro* quantification of neurite density is an important morphological parameters for neuroscience [6,8]. Hence, the increase of neurite density in RA-differentiated cells represents a great advantage to this cellular model, since these structures are part of chemical synapse and can be used as endpoint in neurotoxicological evaluations [44,45].

After the morphological characterization, we aimed to analyze which cellular model possesses a better machinery to support chemical synapses. One important step of this process is attributed to the set of genes involved in synaptic vesicle cycle. Using landscape

analysis and Gene Set Enrichment Analysis (GSEA), we found a significant increase in gene expression level of synaptic vesicle cycle components (gene set according to KEGG pathways) in RA-differentiated cells compared to undifferentiated ones (P<0.05) (Fig. 2f,g). In table 1 we present the list of genes that contribute individually to global changes in expression levels. The synaptic vesicle cycle consists of exocytosis followed by endocytosis and recycle [46,47]. At first, vesicles are loaded with neurotransmiters, which requires active transporter and proton pump that provides the pH and electrochemical gradients. At this part it is fundamental the role of H^+-ATPase transporters in combination with solute carrier, such as *SLC18A1*, *SLC18A3* and *SLC17A8* (Table 1) [48]. Once the vesicles are loaded, they must tether near to the release sites. After that, the vesicle must be primed before be competent to the fusion. *UNC13*, *RIMS1* and syntaxin are proteins strongly related to this process [49]. The primed vesicles can undergo fusion processes that are regulated by SNARE proteins, such as SNAP-25, NSF and complexins [50,51]. At the end, the synaptic vesicles incorporated to the plasma membrane are retrieved by endocytosis, which many proteins have been described that participated in this process (dynamins and clathrins, for instance) [52]. In our study, all genes abovementioned were found to be up-regulated in RA-differentiated cells (Fig. 2f,g; Table 1).

Taken together, our results pointed to the high diverse phenotypes presented by undifferentiated and RA-differentiated SH-SY5Y cell models. The undifferentiated cells exhibit an epithelial morphology, low expression of genes related to chemical synapse and has high proliferation rates (tumoral phenotype). On the other hand, RA-differentiated SH-SY5Y cells present low proliferation rates, a pronounced neuronal morphology and an enrichment of the machinery responsible for perform basic neuronal functions (neuronal phenotype) (Fig. 2).

Dopaminergic characterization

Both cellular models (undifferentiated and RA-differentiated SH-SY5Y cells) are widely used to study the molecular and cellular mechanisms of PD, which has the dopaminergic neurons as the major target cells. Thus, after studying the differences in general neuronal properties, we investigate several dopaminergic features in the tumoral and neuronal phenotypes of SH-SY5Y cells. At first, we evaluated global differences in gene expression of the dopaminergic synapse network, using gene set enrichment analysis (GSEA). Even though we did not found a global significant difference between phenotypes (Figure 3a) – probably

because this gene set includes genes involved in both pre- and post-synaptic processes –, PKA, MAPK, CAMKII and PP2A genes, which are major regulators of tyrosine hydroxylase (TH) activity [53,54] , were all significantly enriched in the neuronal phenotype. The complete list of genes significantly enriched in RA-differentiated cells is listed in table 3.

Moreover, we used differential gene expression analysis, where we verified the expression levels of the most used dopaminergic markers derived from SNpc presynaptic neurons [55,56]. The genes evaluated in this study are from cathecolamine synthesis (*DDC*, *GCH1* and *TH*), degradation (MAOA, MAOB, COMT), and synapse (*SLC18A1*, *SLC18A2*, *SLC6A3*, *DRD2*). *DR2*, *GHC* and *SLC18A1* have their expression levels significantly increased in RA-differentiated cells as compared to undifferentiated cells (Figure 3b).

Hence, we found that both phenotypes of SH-SY5Y cells express some levels of the dopaminergic machinery necessary to produce and release DA. It is well known that neuroblastoma cancers (as the primary tumor that SH-SY5Y cells were isolated and immortalized) present, as tumoral markers, catecholamines [57,58]. Due to this feature, undifferentiated cells are commonly used as PD model. On the other hand, many lines of evidence showed that RA-differentiated cells increase the protein levels of dopaminergic markers, such as tyrosine hydroxylase [13,18,59,60]. In contrast, other works showed no difference in this parameter [14,17]. These discrepancy found in the literature might be attributed to the differentiation protocols used, since there are noticeable differences between them, such as incubation time, cell densities, serum concentration and differentiation agent (*e.g.*: retinoic acid, staurosporine, brain derived neurotrophic factor) [14,16–18,33,60]

At last, dopamine (DA) levels were investigated using an immunohistochemical approach in both SH-SY5Y phenotypes. In Fig. 3c, we verified that both models produce DA, as expected. Although we decided to not address directly the amount DA, as fluorescence signals are a qualitative parameter, we can verified that DA immunoreactivity was consistently more intense in all RA-differentiated cells preparations (Fig. 3c). In this evaluation, we can conclude that RA-differentiation cells increase the levels of pre-synaptic dopaminergic markers, which potentiate the dopaminergic phenotype. Due to this, after the differentiation process, RA-differentiated SH-SY5Y cells are more similar to PD- target-cell and would be more relevant to use it in studies regarding this disease.

Taking all these data into account, we can conclude that, during the RA-differentiation process, SH-SY5Y cells acquire several desired neuronal (morphological and neurochemical) features and, more relevant for the use as an *in vitro* cell model for PD, potentiate a dopaminergic phenotype.

Redox characterization

Oxidative stress is an ubiquitously observed clinical hallmark of neurodegenerative disorders [61,62]. Neuronal dysfunction and death due to oxidative stress might causally contribute to the pathogenesis of progressive degeneration found in Parkinson's disease [3,63–66]. In PD, dopaminergic neurons are exposed to a chronic oxidative damage, mostly attributed to the high levels of iron present in SNpc, the hydroxyl radical ($HO^{\bullet}$) produced by dopamine metabolism [67] and the decrease in mitochondrial Complex I (NADH:ubiquinone oxidoreductase) activity [4]. Furthermore, based on the pivotal importance played by oxidative stress in neurodegenerative diseases, the endogenous machinery responsible for the basal enzymatic (and non-enzymatic) antioxidant defenses and other relevant redox parameters should be consistently characterized when establishing any relevant *in vitro* cell model of PD.

To do so, we first evaluated the gene expression levels of the human antioxidant network (according to KEGG pathways) using GSEA approach. Although there are no global significant differences between undifferentiated and RA-differentiated cells in this parameter (Fig. 4a), a set of antioxidant genes was found to be enriched in RA-differentiated cells. The genes that contributed to this enrichment are mostly related to thiol metabolism, such as *GLRX* and *GLRX2* (glutaredoxins), *SRN1* (sulforedoxin), *PDIA6* (protein disulfide isomerase) and *TMX4* (thioredoxin associated protein) (Fig. 4a). We also verified the expression levels of the antioxidant using differential expression as a statistical analysis of microarray data. As a result, *CAT*, *SOD*, *GPX3* and *GPX7* genes presented an overexpression in RA-differentiated cell ($P<0.05$). In contrast, *GPX1*, *GPX2* and *GPX6* were overexpressed in undifferentiated SH-SY5Y cells ($P<0.05$) (data not shown).

To strengthen these differences, we validated *in vitro* the microarray data by evaluating the activity of several enzymes involved in first line antioxidant defenses (such as catalase - CAT, Superoxide Dismutase - SOD, Glutathione Peroxidase - GPx, Glutathione Reductase -

GR, Thioredoxin Reductase - TrxR and Glutathione-*S*-Transferase - GST), the level of non-enzymatic antioxidant defenses (glutathione and reduced thiol levels) and the *steady-state* H_2O_2 generated by both SH-SY5Y phenotypes (Table 3). Our *in vitro* validation revealed that: *i*) both SH-SY5Y phenotypes are extremely dissimilar regarding the overall capacity and the nature of their antioxidant defenses; *ii*) RA-differentiated cells present a higher basal antioxidant capacity, which explain their higher resistance against H_2O_2 insult; and *iii*) Undifferentiated cells generates higher rates of H_2O_2, a phenomenon described for transformed (tumoral) cells [68] . Previous data showed that a moderate increase of oxidative stress, especially the increase of H_2O_2 levels, can act as proliferative signal in cancer cells [69,70].

Regarding the antioxidant capacity of RA-differentiated SH-SY5Y cells, it is well known that neuronal cells have low levels of antioxidants, thus they are more prone to suffer from oxidative damage when compared to other types of cells [61,71]. Interestingly, our data showed that the neuronal phenotype has a higher overall antioxidant capacity when compared to the tumoral phenotype. Even though there is a significant increase in most of antioxidant activities in RA-differentiated cells, these values are still low when compared to the human brain (Table 3) [61]. The most intriguing data regarding these redox evaluations is that RA-differentiated SH-SY5Y cells were more resistant to H_2O_2, but they present a higher susceptibly to 6-OHDA cytotoxicity (Table 3), as previously described [18]. Even though 6-OHDA toxicity acts trough oxidative stress, the higher antioxidant capacity observed is not able to protect RA-differentiated cells from the cell death. This data suggest another mechanism operating in RA-differentiated SH-SY5Y cells.

6-OHDA is an analog of dopamine widely used in preclinical experimental models to mimic PD through the generation of a massive oxidative damage in cells [72]. Although the selectivity of 6-OHDA for dopaminergic neurons found in *in vivo* experiments may be due to fact that this toxin acts as a substrate for the dopamine transporter (DAT) many lines evidence obtained from *in vitro* experiments have shown that this neurotoxin acts extracellularly, where 6-OHDA is rapidly auto-oxidized by molecular oxygen to form anion superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the corresponding *p*-quinones [30,31,73] Using undifferentiated SH-SY5Y cells, previous studies showed that 6-OHDA's toxicity is inhibited by catalase. Moreover, glutathione (a cell-impermeable thiol) can protect undifferentiated SH-SY5Y cells from 6-OHDA,

and the same study also found that DAT inhibitor (DATi) failed to decrease 6-OHDA's cytotoxicity [74]. Nonetheless, despite the extensive use of 6-OHDA, the precise molecular mechanism by which this toxin destroys specifically dopaminergic neurons has not been fully delineated.

In order to investigate the inconsistencies between *in vivo* and *in vitro* studies, we first pre-incubate undifferentiated and RA-differentiated with two thiol reducing agents: *tris*(2-carboxyethyl)phosphine (TCEP), a cell-impermeable compound, or dithiothreitol (DTT), a cell-permeable small-molecule, before challenging cells with 6-OHDA (Fig. 4b,d) [75–77]. Interestingly, no differences were found between both cellular models when TCEP were used to protect cells against 6-OHDA-oxidant insult (Fig. 4c). On the other hand, DTT was able to prevent 60% of 6-OHDA-dependent cytotoxicity in RA-differentiated cells, in contrast to only 24% in undifferentiated cells ($P<0.0005$) (Fig. 4e). Hence, these data demonstrates, for the first time, significant differences in the mechanism of 6-OHDA's toxicity between undifferentiated and RA-differentiated SH-SY5Y cells, suggesting that, in RA-differentiated cells, part of the oxidative dysfunction caused by 6-OHDA involves the uptake of the neurotoxin (or some metabolite, such as *p*-quinones) and the intracellular auto-oxidation.

The role of DAT in 6-OHDA-induced cell death

In order to investigate this, we evaluated the role of DAT in the toxicity induced by 6-OHDA in both cellular models. *In vivo*, this toxin is taken-up by DAT, a classical neuronal dopaminergic marker. DAT is the major regulator of dopamine neurotransmition and is responsible for the re-uptake of dopamine from the synaptic cleft [78] . However, the role played by DAT in *in vitro* studies is still controversial [31,32,73]. Fig. 5a shows an increase in DAT immunocontent in RA-differentiated cells ($P<0.01$), corroborating with previous studies. We then investigated whether the inhibition of this transporter interferes in the extent of cell death caused by this neurotoxin. To do that, we first examined by modeling through molecular docking how 3α-Bis-4-fluorophenyl-methoxytropane, a DAT inhibitor (DATi), and 6-OHDA interacts with DAT (Fig. 5b), and compared the *K*i (inhibitory constant) and *E* (ligation energy) of this interaction with dopamine (DA) and *p*-quinone values (Table 4).

In order to evaluate the ability of our procedure to find reliable conformations for ligands in the DAT binding site (PDB ID 4M48) [79] the redocking of nortiptilyne was used as input to tune the docking machinery. Redocking procedure was performed using as input geometry either the crystallographic nortripilyne data or a 3D-generated structure using quantum geometry optimization methods. As depicted in Supplementary Fig. 1, the best result was obtained after the classical refinement of docking poses. The result obtained using crystallographic geometry as input was similar to the crystallographic coordinates, with RMSD values of 1.1260 Å. The classical energy minimization procedure improved this result to a RMSD value of 0.7049 Å. Similar results were obtained employing as input geometry the nortriptilyne molecule optimized through quantum methods *in vacuum*, showing a RMSD of 1.1830 Å, decreasing to 0.7050 Å after classical optimization. These results convinced us that the achieved parameterization of Autodock 4.0 followed by classical refinement was adequate to dock ligands molecules (such as dopamine, 6-hydroxydopamine and 3α-Bis-4-fluorophenyl-methoxytropane) which input geometries were obtained *in vacuum* through quantum optimization methods in the binding site of DAT.

When docked into the binding pocket of DAT, 3α-Bis-4-fluorophenyl-methoxytropane showed similar orientation as observed for nortriptilyne with superposition of aromatic rings and orientation of the amine group toward TM1 and TM6 (Fig. 5b). Indeed, the amine group of 3α-Bis-4-fluorophenyl-methoxytropane forms and hydrogen bond with the carbonyl of Phe319, at 2.246 Å of distance, and sterically prevents Phe319 and RM6a from closing the extracellular gate, in the same fashion as suggested for nortriptilyne [79]. Thus, 3α-Bis-4-fluorophenyl-methoxytropane inhibits DAT by preventing substrate binding and stabilizing the outward-open conformation.

Due to their structural similarities, dopamine and its metabolite 6-hydroxydopamine seem to bind in the same orientation into DAT (Fig. 5b), being, as far as we know, demonstrated for the first time that dopamine, the DAT inhibitor 3α-Bis-4-fluorophenyl-methoxytropane, *p*-quinone (one major metabolite of 6-OHDA auto-oxidation) and 6-OHDA, all compete sterically for the same binding site in DAT, especially through the spatial blockage of Asp46 residue (Asp79 in DAT from *Homo sapiens*), which is pivotal for the interaction of DAT with substrate through a saline bridge with dopamine amine group (Fig. 5b) [80–82].

Interestingly, the steric blockage in the same binding site showed in Fig. 5b suggest a competitive inhibition. Actually, since we found a lower ligation energy of DATi for DAT in comparison to *p*-quinone and 6-OHDA, but higher for dopamine, our docking data suggest that DATi blocks completely the interaction of dopamine with DAT, but only partially *p*-quinone and 6-OHDA interactions with DAT (Table 4).

After this, we performed a pharmacological inhibition of DAT in both cellular models, through the treatment with DATi before challenge cells with 6-OHDA. Our findings showed that DAT inhibition causes significant changes in H_2O_2 production and cellular viability only in RA-differentiated cells (Fig. 5c,e). In Fig. 5d,f, we verified a decrease of approximately 50% in H_2O_2 production and 41% in cellular death caused by 6-OHDA in RA-differentiated SH-SY5Y cells. On the other hand, DATi was not able to prevent H_2O_2 generation and cell death caused by the toxin in undifferentiated SH-SY5Y cells. So, DAT inhibition can protect RA-differentiated cells from the damage caused by 6-OHDA, mimicking a phenomenon described for *in vivo* experimental models, but still controversial for *in vitro* models. Previous studies have found that undifferentiated and RA-differentiated cells have distinct susceptibilities to another widely used PD-inducing neurotoxin, MPTP [21,83]. On the other hand, studies that investigate the role of DATi in preventing 6-OHDA's neurotoxicity failed to achieve cellular protection [31,73,84]. Most of them used cells with low levels of DAT (*e.g.* undifferentiated SH-SY5Y cells) and high doses of 6-OHDA (*e.g.* 100 μM). Our data showed that RA-differentiated cells were more sensitive to 6-OHDA, and DAT inhibition led to a reduction of the damage caused by this neurotoxin.

Conclusion

We found that undifferentiated and RA-differentiated SH-SY5Y cells are two distinguish phenotypes due to the differences found in cells morphology, cell growth process, dopaminergic markers and redox metabolism. These features may contribute with the two different mechanism of action for 6-OHDA-cytotoxicty found in both models. In the neuronal phenotype, we found DAT-dependency in 6-OHDA-induced cell death. This may be related to the fact that this phenotype present the necessary features to mimic a dopaminergic cell. Many previous works used undifferentiated cells as PD model to study molecular mechanisms, to test potential drugs for the treatment of this disease and also to evaluate 6-OHDA's mechanisms of action and cellular targets. However, our data showed that undifferentiated cells does not present

neuronal properties, which it can be a great bias for these studies, and could be associated, at least in part, to the limitations in our understanding of PD pathophysiology and, consequently, the lack of potential drugs to treat the disease. Hence, our data support the use of RA-differentiated cells as an *in vitro* model of PD.

Methods

Chemicals

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Retinoic acid is from Enzo® and protein contents were measured by the Bradford assay [85].

Cell Culture

Exponential growing human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, USA) were maintained in a mixture 1:1 of Ham's F12 and Dulbeco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cripion®), 2 mM of glutamine, 100 U/mL of penicillin, 100 µg/µL of garamicine and 250 µg of amphotericin B in a humidified atmosphere of 5% of CO_2 at 37ºC.

Cellular differentiation was performed by lowering the FBS in culture medium to 1% plus RA at 10 µM during 7 days. The design of the differentiation protocol is outlined in Fig 1. Cells were seeded in the following densities: 1.8 x 10^5 cells in a 96-well plate, 6 x 10^4 cells in a 24-well plate, 10^5 cells in 12-well plate and 2.5 x 10^6 cells in a 75 cm^2 flask (~75% of confluence) at the day 0 in a cell medium containing 10% of FBS. After 24 hours (day 1), this medium was removed and the differentiation protocol was performed, using a differentiation medium containing 1% FBS and 10 µM of RA. At the day 4, the medium was replaced, and at the day 7, cells were used for experiments or treated with neurotoxins/compounds of interest.

To conclude it is important to point that the achievement of the differentiation protocol depends on 3 factors: (*i*) the confluence of the cells in day 1 must be around 75% (higher confluence inhibits neurite outgrowth, and lower confluence leads SH-SY5Y cells to detach); (*ii*) the cell medium should be used at maximum in 2 weeks to avoid glutamine decomposition; and

(*iii*) the RA must be freshly prepared at the day of the medium replacements. RA stock solutions were prepared in absolute ethanol and the concentration determined using E^{M}(351 nm) = 45000 [18]

RNA isolation and microarray assay

The cells were harvested and the RNA samples were obtained trough the TRIzol Reagent (Life Technologies) following by purification (Qiagen RNeasy Mini Kit #74 104 and #79 254 - Free RNase DNase Set Qiagen). After that, the microarray was performed using the chip GeneChip® PrimeView™ Human Gene Expression Array (Affymetrix™). The samples were collected at the day 0 (undifferentiated cells), day 4 and day 7 (RA-differentiated cells) as shown in Fig 1.

Enrichment analysis and expression values

Four genes sets were analyzed in both undifferentiated and RA-differentiated SH-SY5Y cells: synapse vesicle cycle, cell cycle, dopaminergic synapse and antioxidant (extracted from KEGG platform - http://www.genome.jp/kegg/pathway.html).

Gene set enrichment analysis (GSEA) was used to identify genes that contribute individually to global changes in expression levels in a given microarray dataset. GSEA considers experiments with genome-wide expression profiles from two classes of samples (*e.g.* undifferentiated *vs.* 4-day-RA-differentiated cells or undifferentiated *vs.* 7-day-differentiated cells). Genes were ranked based on the correlation between their expression and the class distinction. Given a prior defined network (*e.g.* synaptic vesicle cycle), the GSEA determines if the members of these sets of genes are randomly distributed or primarily found at the top or bottom of the ranking [86].

To access the logarithm of gene expression, raw CEL files were analysed using the R/Bioconductor pipeline. The data was normalized by Robust Multi-array Average (RMA) in the AFFY package, log (base 2) transformed, and batch-corrected with ComBat in the SVA package.

Neurite Density and Dopamine immunoreactivity

Neurite density was analyzed by immunofluorescence by using anti-βIII tubulin antibody (Alexa 488-conjugated) and with Nuclear dye Hoechst 33342 (0.25 µg/µL). The dopamine

reactivity was evaluated using an anti-dopamine antibody (1:250) followed by incubation with secondary antibody (Alexa 488-conjugated). Randomly selected images were captured using an Olympus IX70 inverted microscope and analyzed with NIS-elements software. Neurite density was assessed using the AutoQuant Neurite software (implemented in *R*), and expressed as arbitrary units (A.U.) [87].

Cellular growth and cell cycle

DNA composition was evaluated by using propidium iodide (PI), ow cytometry (BD Accuri, USA). The results were express in percent of cells in each cell cycle phase (G0/G1, S, G2/M). The cellular growth was evaluated through cell counting in a Neubauer Chamber.

Antioxidant enzymes activities

We also evaluated the redox status in both undifferentiated and RA-differentiated SH-SY5Y cells. The medium was removed and cells were washed twice with PBS. Then the cells were frozen-thawed three times in 10 mM PBS. The cell extracts were centrifuged (1000*g*/ 5 minutes) and the supernatant collected [44].

It was evaluated the following antioxidant enzymes activities: Glutathione peroxidase (GPx) (E.C.: 1.11.1.9) activity by measuring the oxidation of NADPH at 340 nm in the presence of GSH, *tert*-butylhydroperoxide and glutathione reductase [88]. Catalase (CAT) (E.C.: 1.11.1.6) activity was measured by H_2O_2 consumption of at 240 nm [89]. Superoxide dismutase (SOD) (E.C.: 1.15.1.1) activity was measured by inhibition by superoxide-dependent adrenaline autoxidation at 480 nm [90]. Thioredoxin reductase activity (TrxR) (E.C.: 1.8.1.9) was evaluated through DTNB reduction in the presence of NADPH at 340 nm [91]. Glutathione reductase (GR) (E.C.: 1.8.1.7) activity was measured by NADPH consuption at 340 nm [92]. The activity of glutathione S-transferase (GST) (E.C.: 2.5.1.13) was determined by forming the conjugate of GSH and chloro-dinitrobenzene (CDNB) [93]. Reduced thiol levels were measured by Elman´s assay [94] and GSH content by Akerboom and Sies [95].

Cytotoxicity parameters

The cytotoxicity induced by 6-OHDA and H_2O_2 were analyzed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At the end of treatment, cells were washed with PBS and a new medium containing 0,5 mg/mL MTT was added. Then, cells were incubated for 1 hour at 37ºC. After that, DMSO was added to solubilize the formazan salt. Finally, the absorbance was determined in a microplate reader (SoftMax Pro, Molecular Devices, USA) at wavelengths of 560 nm and 630 nm.

Reducing-thiol- agents experiments

The role of reducing agents in 6-OHDA-cytotoxicity was verified through pre-treatment with DTT or TCEP in both cell models for 1 hour in 37 C. After this, cells were treated with the LD_{50} dose of 6-OHDA and cell viability was verified by MTT assay.

DAT immunocontent and pharmacological inhibition

Cells were washed with PBS and ressuspended in a Tris-buffer (pH 7.0) with protease inhibitor (Roche®) and lysed by sonication. To evaluate the changes in DAT immunocontent during the RA-differentiation process, western blot analysis was performed using anti-DAT antibody (1:1000) from Santa Cruz® Biotechnology (Dallas, Texas, USA). For the load control, membranes were then striped and reprobed with rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000) from Abcam® (Cambridge, MA, USA).

To investigate the DAT dependency in 6-OHDA-induced cell death in both models, cells were pre-incubated during 30 minutes with 20 µM of DAT pharmacologic inhibitor (DATi = α-bis (4-fluorophenyl) methoxytropane hydrochloride) (Sigma®). Following this, cells were exposure to DL_{50} 6-OHDA during 24 hours (35 µM for undifferentiated cells and 15 µM for RA-differentiated cells) (Lopes *et al.,* 2010). At the end of the treatment, cell viability was verified through MTT assay. H_2O_2 and reactive species generation were determined by AmplexRed® and DCF-DA oxidation, respectively.

Structural data

The calculations performed in this study have taken full advantage of the X-ray crystal structure of the *Drosophila melanogaster* dopamine transporter (PDB ID 4M48) at 3.0 Å of resolution [79]. The structure was modified in order to replace mutated residues for native ones

using Discovery Studio 3.1 package. The protonation state of the receptor was adjusted according to results obtained from the PROPKA 3.1 web server tool [96] and from the Protonation tool in Discovery Studio 3.1 package. The protonation state set up at physiological pH of nortriptilyne was accomplished using the Marvin Sketch code version 5.5.0.1 (Marvin Beans Suite – ChemAxon) and the molecular structure was obtained through the addition of a single hydrogen atom to the amine group with its charge adjusted to +1 (electron charge -1).

Molecular Docking

Molecular docking was performed using Autodock4. To validate the docking protocol adopted in this work we performed the redocking of nortriptilyne in the DAT binding site, as describe elsewhere [97,98] using two distinct input conformations: (*i*) nortriptilyne in its crystallographic conformation, (*ii*) nortriptilyne at the minimum energy configuration obtained after classical annealing followed by quantum DFT (GGA-TS functional) geometry optimization in vacuum using DMOL3 code. The same procedure described in (*ii*) was employed to obtain the molecular structures of dopamine, 6-hydroxydopamine and 3α-Bis-4-fluorophenyl-methoxytropane hydrochloride for docking input. Docking was performed 20 times using the Lamarckian genetic algorithm (GA), a GA with 25,000,000 energy evaluations per run, population size set to 150, and a maximum of 27,000 generations per run. At the end, a thousand poses were obtained (50 poses per output) and clustered within a RMSD tolerance of 1.0 Å using Autodock Tools [99,100]

Dose selection and construction of the ligand-DAT complexes

The best results were obtained based in our visual inspection and energy score. When more than one representative cluster was observed, the best pose of each cluster was classically energy minimized into the binding site of DAT and the score of the selected poses was revalidated, in order to ensure the accuracy of the method, through a classical binding energy calculation (Eb) as described below:

$$Eb = EDAT + L - (EDAT + EL) \quad (1)$$

At the right side of Eq. (1), the first term EDAT + L is the total energy of the system formed by ligand bond in DAT; EDAT is the total energy of the DAT alone, while the third term

EL is the total energy of the ligand molecule alone. Ligand-DAT complexes were prepared using the crystallographic DAT structure after the removal of nortriptilyne. Every complex was classically optimized in two consecutive steps: (*i*) only hydrogen atoms were free to move during optimization; (*ii*) all hydrogen atoms and the all atoms of the ligand molecule were free to move during optimization. The classical optimization procedure was performed using the Forcite code with the force field CVFF, the convergence tolerances set to 2×10^{-5} kcal/mol (total energy variation), 0.001 kcal/mol.Å (maximum force per atom), and 1×10^{-5} Å (maximum atomic displacement).

Molecule Drawing and Images Acquisition

Marvin Sketch code version 5.5.0.1- 2011, ChemAxon (http://www.chemaxon.com), was used to draw the 2D ligands structure and to predict its protonation state at physiological pH. The images were prepared using PyMol 1.3 [101].

Statistical Analysis

Data are expressed as means ± S.E.M. of at least 3 independent experiments carried out in triplicate and student's *t*-test and one-way ANOVA were used ($P < 0.05$) (GraphPad® Software 5.0).

Competing interests

The authors declare that they have no competing interests.

Author's contributions

F.M.L., L.L.B, L.F.S, D.M.V, P.S., G.F.L. and L.M performed experiments. B.P. and B.W.A performed the RNA extraction for the microarray analysis, G.Z. performed the molecular docking. F.M.L, L.L.B, M.A.D.B, M.A.A.C., R.B.P., A.L.D. and F.K. analyzed and interpreted the data. F.M.L, and F.K. conceived and designed the experiments. F.M.L and F.K. wrote the manuscript.

Acknowledgements

Brazilians funds CNPq/MS/SCTIE/DECIT - Pesquisas Sobre Doenças Neurodegenerativas (#466989/2014-8), MCT/CNPq INCT-TM (#573671/2008-7), Programa de Doutorado Sanduíche no Exterior - PDSE/CAPES (#14581/2013-2), and Rapid Response Innovation Award/MJFF (#1326-2014) provided the financial support without interference in the ongoing work. FK received a fellowship from MCT/CNPq (#306439/2014-0).

Figure Legends

Figure 1: Protocol design of the RA-induced differentiation. At the day 0, exponentially growing SH-SY5Y cells were seeded in cell medium containing 10 % of FBS. After 24 hours (day 1), the previous medium was removed and fresh medium containing 1 % of FBS and 10 μM of RA (differentiation medium) was added. 3 days later (day 4), the differentiation medium was replaced by a fresh one. At the day 7, SH-SY5Y cells are ready to perform the experiments of interest.

Figure 2: Neuronal characterization of undifferentiated and RA-differentiated SH-SY5Y cells was performed by evaluating: (*i*) proliferation rates and cell cycle distribution; (*ii*) morphometric analysis; and (*iii*) synaptic vesicle network. (a) Cellular growth was performed by cell counting during 7 days in undifferentiated and RA-differentiated cells. (b) Cell cycle analysis was evaluated by propidium iodide (PI) - ow cytometry. The representative image of the cell cycle analysis in undifferentiated cells and RA-differentiated cells, which the results were express in percent of cells in each cell cycle phase (G0/G1, S, G2/M). (c) Enrichment analysis of the cell cycle network in undifferentiated and RA-differentiated SH-SY5Y cells using GSEA, showing the genes upregulated in RA-differentiated cells. (d) Morphological parameter evaluated by tubulin immunofluorescene of undifferentiated and RA-differentiated SH-SY5Y cells. Representative fluorescence microscopy images of undifferentiated and RA-differentiated (e) and the quantification of the neurite density per cell body using AutoQuant Neurite software. (f) Expression of synaptic vesicle cycle network in undifferentiated and RA-differentiated SH-SY5Y cells. STRING representation of synaptic vesicle cycle network gene interactions and landscape

analysis demonstrating elevated expression of this network in 7-day-RA-differentiated SH-SY5Y cells compared to undifferentiated ones, generated with ViaComplex® V1.0. Color gradient (Z-axis). *P* value refers to bootstrap analysis comparing cell lines. (g) Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels found in RA-differentiated cells in the syntactic vesicle cycle network. Data are presented as mean±SD of four independent experiments carried out in triplicates (n=4). *P<0.05 (Student t test). Transcripts obtained as described in Material & Methods section. Nominal *P* value of enrichment analysis obtained from GSEA (*P* < 0.05).

Figure 3: Dopaminergic characterization of undifferentiated and RA-differentiated SH-SY5Y cells. (a) Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels found in RA-differentiated cells in the dopaminergic synapse network using GSEA. (b) Differential expression levels of pre-synaptic dopaminergic markers in undifferentiated and RA-differentiated cells. (c) Immunocytochemical dopamine detection. Representative fluorescence microscopy images of undifferentiated and RA-differentiated. Data are presented as mean±SD of four independent experiments carried out in triplicates (n= 4). *P<0.05 (Student t test)

Figure 4: Redox characterization of undifferentiated and RA-differentiated SH-SY5Y (a) Enrichment analysis used to identify the genes that contributed individually to the global changes in expression levels found in RA-differentiated cells in the antioxidant network using GSEA. The table present the genes upregulated in RA-differentiated cells. (b,c,d,e)The role of thiol-reducing agents in 6-OHDA-induced cell death in undifferentiated and RA-differentiated SH-SY5Y cells. Both cellular models were treated with and cell-impermeable (b) and cell-permeable (d) thiol-reducing-agents, followed by DL_{50} of 6-OHDA exposure during 24 hours. Cell viability was evaluated by MTT and the results were expressed as a percentage of the control. (c,e) After this, we analyzed the percentage of 6-OHDA-induced-cell-death-inhibition for each thiol-reducing agent in both cellular models. Data are presented as mean±SD of four independent experiments carried out in triplicates (n= 4). *P<0.05 (Student t test)

Figure 5: Evaluation of DAT role in 6-OHDA-induced-cell-death in undifferentiated and RA-differentiated SH-SY5Y **(**a) Changes in the DAT immunocontent (dopaminergic cell marker)

were evaluated by western blot. Representative densitometric analysis of bands and immunoblot of DAT, using GAPDH as loading control. (b) Superposition of DAT inhibitor and 6-OHDA into the binding site of DAT showing how 6-OHDA is spatially blockage from forming a salt bridge with Asp46. (c) Evaluation of DAT inhibition in cell viability, H_2O_2 (d) and reactive species production (e) in undifferentiated and RA-differentiated SH-SY5Y cells challenged with 6-OHDA. Cells were treated during 30 minutes with DATi before the exposure to DL_{50} 6-OHDA during 24 hours. Cell viability was evaluated by MTT and the results were expressed as a percentage of the control. (c,e) After this, we analyzed the role of DATi in 6-OHDA-induced-cell-death in both cellular models. Data are presented as mean±SD of four independent experiments carried out in triplicates (n=4). *P<0.05 (Student t test).

References

[1] W.R. Gibb, Neuropathology of the substantia nigra., Eur. Neurol. 31 Suppl 1 (1991) 48–59.

[2] W.R. Gibb, Neuropathology of Parkinson's disease and related syndromes., Neurol. Clin. 10 (1992) 361–76.

[3] R.G. Fariello, Experimental support for the implication of oxidative stress in the genesis of parkinsonian syndromes., Funct. Neurol. 3 407–12.

[4] A.H. Schapira, V.M. Mann, J.M. Cooper, D. Dexter, S.E. Daniel, P. Jenner, et al., Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease., J. Neurochem. 55 (1990) 2142–5.

[5] S. Mullin, A.H. V Schapira, Pathogenic mechanisms of neurodegeneration in Parkinson disease., Neurol. Clin. 33 (2015) 1–17.

[6] N.M. Radio, W.R. Mundy, Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth., Neurotoxicology. 29 (2008) 361–76.

[7] C.W. Olanow, K. Kieburtz, A.H. V Schapira, Why have we failed to achieve neuroprotection in Parkinson's disease?, Ann. Neurol. 64 Suppl 2 (2008) S101–10.

[8] A.K. Bal-Price, H.T. Hogberg, L. Buzanska, S. Coecke, Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered., Neurotoxicol. Teratol. 32 36–41.

[9] A. Krishna, M. Biryukov, C. Trefois, P.M.A. Antony, R. Hussong, J. Lin, et al., Systems

genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease., BMC Genomics. 15 (2014) 1154.

[10] C. Alquézar, E. Barrio, N. Esteras, A. de la Encarnación, F. Bartolomé, J.A. Molina, et al., Targeting cyclin D3/CDK6 activity for treatment of Parkinson's disease., J. Neurochem. 133 (2015) 886–97.

[11] X. Ye, Y. Han, L. Zhang, W. Liu, J. Zuo, MTERF4 regulates the mitochondrial dysfunction induced by MPP(+) in SH-SY5Y cells., Biochem. Biophys. Res. Commun. 464 (2015) 214–20.

[12] J.L. Biedler, S. Roffler-Tarlov, M. Schachner, L.S. Freedman, Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones., Cancer Res. 38 (1978) 3751–7.

[13] S. Påhlman, A.I. Ruusala, L. Abrahamsson, M.E. Mattsson, T. Esscher, Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation., Cell Differ. 14 (1984) 135–44.

[14] M. Encinas, M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Ceña, et al., Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells., J. Neurochem. 75 (2000) 991–1003.

[15] S.P. Presgraves, T. Ahmed, S. Borwege, J.N. Joyce, Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists., Neurotox. Res. 5 (2004) 579–98.

[16] R. Constantinescu, A.T. Constantinescu, H. Reichmann, B. Janetzky, Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y., J. Neural Transm. Suppl. (2007) 17–28.

[17] Y.-T. Cheung, W.K.-W. Lau, M.-S. Yu, C.S.-W. Lai, S.-C. Yeung, K.-F. So, et al., Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research., Neurotoxicology. 30 (2009) 127–35.

[18] F.M. Lopes, R. Schröder, M.L.C. da Frota, A. Zanotto-Filho, C.B. Müller, A.S. Pires, et al., Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies., Brain Res. 1337 (2010) 85–94.

[19] D.W. Luchtman, C. Song, Why SH-SY5Y cells should be differentiated., Neurotoxicology. 31 (2010) 164–5; author reply 165–6.

[20] M.L.C. da Frota Junior, A.S. Pires, F. Zeidán-Chuliá, I.J. Bristot, F.M. Lopes, M.A. de Bittencourt Pasquali, et al., In vitro optimization of retinoic acid-induced neuritogenesis and TH endogenous expression in human SH-SY5Y neuroblastoma cells by the antioxidant Trolox., Mol. Cell. Biochem. 358 (2011) 325–34.

[21] L. Schneider, S. Giordano, B.R. Zelickson, M. S Johnson, G. A Benavides, X. Ouyang, et al., Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress., Free Radic. Biol. Med. 51 (2011)

2007–17.

[22] P. Jenner, Functional models of Parkinson's disease: a valuable tool in the development of novel therapies., Ann. Neurol. 64 Suppl 2 (2008) S16–29.

[23] U. Ungerstedt, G.W. Arbuthnott, Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system., Brain Res. 24 (1970) 485–93.

[24] R. Soto-Otero, E. Méndez-Alvarez, A. Hermida-Ameijeiras, A.M. Muñoz-Patiño, J.L. Labandeira-Garcia, Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease., J. Neurochem. 74 (2000) 1605–12.

[25] Y. Izumi, H. Sawada, N. Sakka, N. Yamamoto, T. Kume, H. Katsuki, et al., p-Quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly., J. Neurosci. Res. 79 (2005) 849–60.

[26] H.C. Curtius, M. Wolfensberger, B. Steinmann, U. Redweik, J. Siegfried, Mass fragmentography of dopamine and 6-hydroxydopamine. Application to the determination of dopamine in human brain biopsies from the caudate nucleus., J. Chromatogr. 99 (1974) 529–40.

[27] R. Andrew, D.G. Watson, S.A. Best, J.M. Midgley, H. Wenlong, R.K. Petty, The determination of hydroxydopamines and other trace amines in the urine of parkinsonian patients and normal controls., Neurochem. Res. 18 (1993) 1175–7.

[28] A. Ljungdahl, T. Hökfelt, G. Jonsson, C. Sachs, Autoradiographic demonstration of uptake and accumulation of 3H-6-hydroxydopamine in adrenergic nerves., Experientia. 27 (1971) 297–9.

[29] P.T. Redman, B.S. Jefferson, C.B. Ziegler, O. V Mortensen, G.E. Torres, E.S. Levitan, et al., A vital role for voltage-dependent potassium channels in dopamine transporter-mediated 6-hydroxydopamine neurotoxicity., Neuroscience. 143 (2006) 1–6.

[30] Y. Glinka, M. Gassen, M.B. Youdim, Mechanism of 6-hydroxydopamine neurotoxicity., J. Neural Transm. Suppl. 50 (1997) 55–66.

[31] A. Storch, A. Kaftan, K. Burkhardt, J. Schwarz, 6-Hydroxydopamine toxicity towards human SH-SY5Y dopaminergic neuroblastoma cells: independent of mitochondrial energy metabolism., J. Neural Transm. 107 (2000) 281–93.

[32] C. Cerruti, D.M. Walther, M.J. Kuhar, G.R. Uhl, Dopamine transporter mRNA expression is intense in rat midbrain neurons and modest outside midbrain., Brain Res. Mol. Brain Res. 18 (1993) 181–6.

[33] L. Agholme, T. Lindström, K. Kågedal, J. Marcusson, M. Hallbeck, An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons., J. Alzheimers. Dis. 20 (2010) 1069–82.

[34] Z. Xun, D.-Y. Lee, J. Lim, C.A. Canaria, A. Barnebey, S.M. Yanonne, et al., Retinoic acid-induced differentiation increases the rate of oxygen consumption and enhances the spare respiratory capacity of mitochondria in SH-SY5Y cells., Mech. Ageing Dev. 133 (2012) 176–85.

[35] E. Kandel, Principles of Neural Science, Fifth Edition, 2013.

[36] H.J. Ross, The antiproliferative effect of trans-retinoic acid is associated with selective induction of interleukin-1 beta, a cytokine that directly inhibits growth of lung cancer cells., Oncol. Res. 8 (1996) 171–8.

[37] W. Poluha, D.K. Poluha, B. Chang, N.E. Crosbie, C.M. Schonhoff, D.L. Kilpatrick, et al., The cyclin-dependent kinase inhibitor p21 (WAF1) is required for survival of differentiating neuroblastoma cells., Mol. Cell. Biol. 16 (1996) 1335–41.

[38] B. Hämmerle, Y. Yañez, S. Palanca, A. Cañete, D.J. Burks, V. Castel, et al., Targeting neuroblastoma stem cells with retinoic acid and proteasome inhibitor., PLoS One. 8 (2013) e76761.

[39] K. Herrup, The involvement of cell cycle events in the pathogenesis of Alzheimer's disease., Alzheimers. Res. Ther. 2 (2010) 13.

[40] M.C. Ovejero-Benito, J.M. Frade, Brain-derived neurotrophic factor-dependent cdk1 inhibition prevents G2/M progression in differentiating tetraploid neurons., PLoS One. 8 (2013) e64890.

[41] A. Garcia-Garcia, L. Zavala-Flores, H. Rodriguez-Rocha, R. Franco, Thiol-redox signaling, dopaminergic cell death, and Parkinson's disease., Antioxid. Redox Signal. 17 (2012) 1764–84.

[42] L.T. Kaufmann, C. Niehrs, Gadd45a and Gadd45g regulate neural development and exit from pluripotency in Xenopus., Mech. Dev. 128 401–11.

[43] www.atcc.org/Products/All/CRL-2266.aspx, (n.d.).

[44] F.M. Lopes, G.F. Londero, L.M. de Medeiros, L.L. da Motta, G.A. Behr, V.A. de Oliveira, et al., Evaluation of the neurotoxic/neuroprotective role of organoselenides using differentiated human neuroblastoma SH-SY5Y cell line challenged with 6-hydroxydopamine., Neurotox. Res. 22 (2012) 138–49.

[45] J. Llorens, A.A. Li, S. Ceccatelli, C. Suñol, Strategies and tools for preventing neurotoxicity: to test, to predict and how to do it., Neurotoxicology. 33 (2012) 796–804.

[46] T.C. Sudhof, The synaptic vesicle cycle., Annu. Rev. Neurosci. 27 (2004) 509–47.

[47] J. Rizo, J. Xu, The Synaptic Vesicle Release Machinery., Annu. Rev. Biophys. 44 (2015) 339–67.

[48] K.W. Beyenbach, H. Wieczorek, The V-type H+ ATPase: molecular structure and

function, physiological roles and regulation., J. Exp. Biol. 209 (2006) 577–89.

[49] J.M. Madison, S. Nurrish, J.M. Kaplan, UNC-13 interaction with syntaxin is required for synaptic transmission., Curr. Biol. 15 (2005) 2236–42.

[50] S. Pabst, J.W. Hazzard, W. Antonin, T.C. Südhof, R. Jahn, J. Rizo, et al., Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions., J. Biol. Chem. 275 (2000) 19808–18.

[51] K. Hu, J. Carroll, C. Rickman, B. Davletov, Action of complexin on SNARE complex., J. Biol. Chem. 277 (2002) 41652–6.

[52] K. Takei, O. Mundigl, L. Daniell, P. De Camilli, The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin., J. Cell Biol. 133 (1996) 1237–50.

[53] P.R. Dunkley, L. Bobrovskaya, M.E. Graham, E.I. von Nagy-Felsobuki, P.W. Dickson, Tyrosine hydroxylase phosphorylation: regulation and consequences., J. Neurochem. 91 (2004) 1025–43.

[54] S.C. Daubner, T. Le, S. Wang, Tyrosine hydroxylase and regulation of dopamine synthesis., Arch. Biochem. Biophys. 508 (2011) 1–12.

[55] H. Xie, L. Hu, G. Li, SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease., Chin. Med. J. (Engl). 123 (2010) 1086–92.

[56] J.A. Korecka, R.E. van Kesteren, E. Blaas, S.O. Spitzer, J.H. Kamstra, A.B. Smit, et al., Phenotypic Characterization of Retinoic Acid Differentiated SH-SY5Y Cells by Transcriptional Profiling, PLoS One. 8 (2013) e63862.

[57] B. Hero, D.H. Hunneman, M. Gahr, F. Berthold, Evaluation of catecholamine metabolites, mIBG scan, and bone marrow cytology as response markers in stage 4 neuroblastoma., Med. Pediatr. Oncol. 36 (2001) 220–3.

[58] R. Howman-Giles, P.J. Shaw, R.F. Uren, D.K. V Chung, Neuroblastoma and other neuroendocrine tumors., Semin. Nucl. Med. 37 (2007) 286–302.

[59] M. Miloso, D. Villa, M. Crimi, S. Galbiati, E. Donzelli, G. Nicolini, et al., Retinoic acid-induced neuritogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent., J. Neurosci. Res. 75 (2004) 241–52.

[60] R. Filograna, L. Civiero, V. Ferrari, G. Codolo, E. Greggio, L. Bubacco, et al., Analysis of the Catecholaminergic Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation, PLoS One. 10 (2015) e0136769.

[61] B. Halliwell, Oxidative stress and neurodegeneration: where are we now?, J. Neurochem. 97 (2006) 1634–58.

[62] E. Radi, P. Formichi, C. Battisti, A. Federico, Apoptosis and oxidative stress in

neurodegenerative diseases., J. Alzheimers. Dis. 42 Suppl 3 (2014) S125–52.

[63] J. Blesa, I. Trigo-Damas, A. Quiroga-Varela, V.R. Jackson-Lewis, Oxidative stress and Parkinson's disease, Front. Neuroanat. 9 (2015) 91.

[64] P. del Hoyo, A. García-Redondo, F. de Bustos, J.A. Molina, Y. Sayed, H. Alonso-Navarro, et al., Oxidative stress in skin fibroblasts cultures from patients with Parkinson's disease., BMC Neurol. 10 (2010) 95.

[65] R.B. Mythri, C. Venkateshappa, G. Harish, A. Mahadevan, U.B. Muthane, T.C. Yasha, et al., Evaluation of markers of oxidative stress, antioxidant function and astrocytic proliferation in the striatum and frontal cortex of Parkinson's disease brains., Neurochem. Res. 36 (2011) 1452–63.

[66] C. Venkateshappa, G. Harish, R.B. Mythri, A. Mahadevan, M.M.S. Bharath, S.K. Shankar, Increased oxidative damage and decreased antioxidant function in aging human substantia nigra compared to striatum: implications for Parkinson's disease., Neurochem. Res. 37 (2012) 358–69.

[67] Z.D. Zhou, Y.H. Lan, E.K. Tan, T.M. Lim, Iron species-mediated dopamine oxidation, proteasome inhibition, and dopaminergic cell demise: implications for iron-related dopaminergic neuron degeneration., Free Radic. Biol. Med. 49 (2010) 1856–71.

[68] L. Policastro, B. Molinari, F. Larcher, P. Blanco, O.L. Podhajcer, C.S. Costa, et al., Imbalance of antioxidant enzymes in tumor cells and inhibition of proliferation and malignant features by scavenging hydrogen peroxide., Mol. Carcinog. 39 (2004) 103–13.

[69] L.L. da Motta, M.A. De Bastiani, F. Stapenhorst, F. Klamt, Oxidative stress associates with aggressiveness in lung large-cell carcinoma., Tumour Biol. (2015).

[70] H. Sies, Role of metabolic H2O2 generation: redox signaling and oxidative stress., J. Biol. Chem. 289 (2014) 8735–41.

[71] X. Wang, E.K. Michaelis, Selective neuronal vulnerability to oxidative stress in the brain., Front. Aging Neurosci. 2 (2010) 12.

[72] J. Bové, C. Perier, Neurotoxin-based models of Parkinson's disease., Neuroscience. 211 (2012) 51–76.

[73] V. Lehmensiek, E.-M. Tan, S. Liebau, T. Lenk, H. Zettlmeisl, J. Schwarz, et al., Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant alpha-synucleins related to Parkinson's disease., Neurochem. Int. 48 (2006) 329–40.

[74] J. Iglesias-González, S. Sánchez-Iglesias, E. Méndez-Álvarez, S. Rose, A. Hikima, P. Jenner, et al., Differential toxicity of 6-hydroxydopamine in SH-SY5Y human neuroblastoma cells and rat brain mitochondria: protective role of catalase and superoxide dismutase., Neurochem. Res. 37 (2012) 2150–60.

[75] M.-F. Hsu, S.-P. Sun, Y.-S. Chen, C.-R. Tsai, L.-J. Huang, L.-T. Tsao, et al., Distinct

effects of N-ethylmaleimide on formyl peptide- and cyclopiazonic acid-induced Ca2+ signals through thiol modification in neutrophils., Biochem. Pharmacol. 70 (2005) 1320–9.

[76] A.J. Schindler, R. Schekman, In vitro reconstitution of ER-stress induced ATF6 transport in COPII vesicles., Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 17775–80.

[77] J. van den Akker, E. VanBavel, R. van Geel, H.L. Matlung, B. Guvenc Tuna, G.M.C. Janssen, et al., The redox state of transglutaminase 2 controls arterial remodeling., PLoS One. 6 (2011) e23067.

[78] S. Ramamoorthy, T.S. Shippenberg, L.D. Jayanthi, Regulation of monoamine transporters: Role of transporter phosphorylation., Pharmacol. Ther. 129 (2011) 220–38.

[79] A. Penmatsa, K.H. Wang, E. Gouaux, X-ray structure of dopamine transporter elucidates antidepressant mechanism., Nature. 503 (2013) 85–90.

[80] S. Kitayama, S. Shimada, H. Xu, L. Markham, D.M. Donovan, G.R. Uhl, Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding., Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7782–5.

[81] M. Indarte, J.D. Madura, C.K. Surratt, Dopamine transporter comparative molecular modeling and binding site prediction using the LeuT(Aa) leucine transporter as a template., Proteins. 70 (2008) 1033–46.

[82] X. Huang, C.-G. Zhan, How dopamine transporter interacts with dopamine: insights from molecular modeling and simulation., Biophys. J. 93 (2007) 3627–39.

[83] Y.X. Yang, M.M.K. Muqit, D.S. Latchman, Induction of parkin expression in the presence of oxidative stress., Eur. J. Neurosci. 24 (2006) 1366–72.

[84] K. Hanrott, L. Gudmunsen, M.J. O'Neill, S. Wonnacott, 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta., J. Biol. Chem. 281 (2006) 5373–82.

[85] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding., Anal. Biochem. 72 (1976) 248–54.

[86] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, et al., Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles., Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15545–50.

[87] P. Schönhofen, L.M. de Medeiros, I.J. Bristot, F.M. Lopes, M.A. De Bastiani, F. Kapczinski, et al., Cannabidiol Exposure During Neuronal Differentiation Sensitizes Cells Against Redox-Active Neurotoxins., Mol. Neurobiol. 52 (2015) 26–37.

[88] A. Wendel, Glutathione peroxidase., Methods Enzymol. 77 (1981) 325–33.

[89] H. Aebi, Catalase in vitro., Methods Enzymol. 105 (1984) 121–6.

[90] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase., J. Biol. Chem. 247 (1972) 3170–5.

[91] E.S. Arnér, L. Zhong, A. Holmgren, Preparation and assay of mammalian thioredoxin and thioredoxin reductase., Methods Enzymol. 300 (1999) 226–39.

[92] I. Carlberg, B. Mannervik, Glutathione reductase., Methods Enzymol. 113 (1985) 484–90.

[93] M.J. Pabst, W.H. Habig, W.B. Jakoby, Glutathione S-transferase A. A novel kinetic mechanism in which the major reaction pathway depends on substrate concentration., J. Biol. Chem. 249 (1974) 7140–7.

[94] G.L. ELLMAN, Tissue sulfhydryl groups., Arch. Biochem. Biophys. 82 (1959) 70–7.

[95] T.P. Akerboom, H. Sies, Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples., Methods Enzymol. 77 (1981) 373–82.

[96] http://propka.ki.ku, (n.d.).

[97] I. Halperin, B. Ma, H. Wolfson, R. Nussinov, Principles of docking: An overview of search algorithms and a guide to scoring functions., Proteins. 47 (2002) 409–43.

[98] M.K. Mohammad, I.M. Al-Masri, M.O. Taha, M.A.S. Al-Ghussein, H.S. Alkhatib, S. Najjar, et al., Olanzapine inhibits glycogen synthase kinase-3beta: an investigation by docking simulation and experimental validation., Eur. J. Pharmacol. 584 (2008) 185–91.

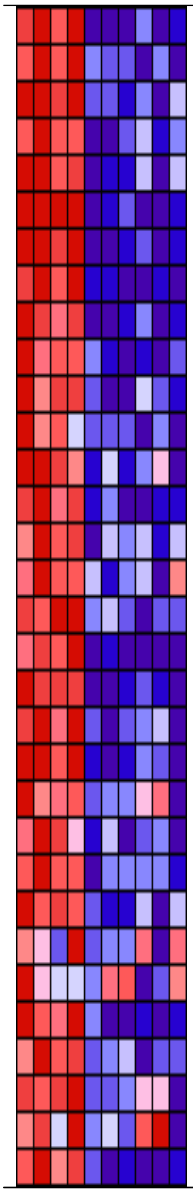
[99] M.F. Sanner, Python: a programming language for software integration and development., J. Mol. Graph. Model. 17 (1999) 57–61.

[100] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, et al., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility., J. Comput. Chem. 30 (2009) 2785–91.

[101] Schrodinger LLC (2010) The PyMOL Molecular Graphics System, version 1.3r1. , (n.d.).

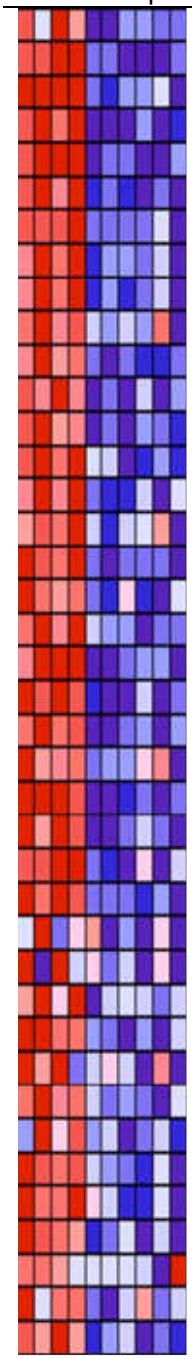
Tables

Table 1: Core enrichment from the synaptic vesicle network in 7-days RA- differentiated SH-SY5Y cells compared to undifferentiated cells.

Heat Map	Gene Symbol	Gene Name
	*SLC18A1*	solute carrier family 18 (vesicular), member 1
	ATP6V1G2	ATPase, H+ transporting, V1 subunit G2
	NSF	N-ethylmaleimide-sensitive factor
	ATP6V0D1	ATPase, H+ transporting, V0 subunit d1
	ATP6V0E2	ATPase, H+ transporting V0 subunit e2
	SNAP25	synaptosomal-associated protein, 25kDa
	ATP6V0E1	ATPase, H+ transporting, V0 subunit e1
	STXBP1	syntaxin binding protein 1
	DNM1	dynamin 1
	ATP6V1C1	ATPase, H+ transporting, V1 subunit C1
	DNM3	dynamin 3
	CPLX3	complexin 3
	CPLX1	complexin 1
	AP2A2	adaptor-related protein complex 2, alpha 2 sub.
	ATP6V0C	ATPase, H+ transporting, V0 subunit c
	RIMS1	regulating synaptic membrane exocytosis 1
	STX3	syntaxin 3
	ATP6V1H	ATPase, H+ transporting, V1 subunit H
	ATP6V1D	ATPase, H+ transporting, V1 subunit D
	AP2B1	adaptor-related protein complex 2, beta 1 sub.
	ATP6V1B2	ATPase, H+ transporting, V1 subunit B2
	CACNA1B	calcium channel, L type, alpha 1B subunit
	SLC18A3	solute carrier family 18 (vesicular), member 3
	AP2M1	adaptor-related protein complex 2, mu 1 subunit
	CLTC	clathrin, heavy chain (Hc)
	SLC17A8	solute carrier family 17, member 8
	ATP6V1G3	ATPase, H+ transporting, V1 subunit G3
	ATP6V1A	ATPase, H+ transporting, V1 subunit A
	CLTA	clathrin, light chain (Lca)
	STX2	syntaxin 2
	UNC13A	unc-13 homolog A (C. elegans)
	ATP6V1E1	ATPase, H+ transporting, V1 subunit E1

Data generated with Gene Set Enrichment Analysis (GSEA) comparing 7-days RA-differentiated cells (n=4) *vs*. undifferentiated SH-SY5Y cells (n=6) transcripts obtained as described in Methods section. Nominal *p*-value of enrichment analysis obtained from GSEA ($p<0.05$).

Table 2: Core enrichment from the dopaminergic synapse network in 7-days RA-differentiated SH-SY5Y cells compared to undifferentiated cells.

Heat Map	Gene Symbol	Gene Name
	*ITPR2*	inositol 1,4,5-triphosphate receptor, type 2
	CREB5	cAMP-responsive element binging protein 5
	MAOB	monoamine oxidase B
	SLC18A1	vesicular monoamine transporter (family 18), A1
	GNG8, 9, 2	G protein, gamma 8, 7, 2
	PPP2R2C	protein phosphatase 2A 55, reg. sub. B gamma
	PPP2R5B	protein phosphatase 2A 56, reg. sub. B, beta
	FOS	c-Fos transcription factor
	DRD2	dopamine receptor 2
	CLOCK	circadian locomotor output cycle kaput
	AKT1	RAC-alpha serine/threonine- protein kinase
	PPP2R5A	protein phosphatase 2A 56, reg. sub. B, alpha
	CREB3L2	cAMP-responsive element binding prot. 3-like 2
	MAPK10, 9, 8	mitogen-activating protein kinase 10, 9, 8
	ADCY5	adenylate cyclase 5
	PPP2R5C	protein phosphatase 2A 56, reg. sub. gamma
	CREB3	cAMP-responsive element binding protein 3
	GNAQ	G protein (q) subunit alpha
	PRKACA	protein kinase C, catalytic subunit alpha
	CACNA1B	calcium channel, voltage-depend., N, alpha 1B
	GNAS	GNAS complex locus
	KIF5C	kinesin heavy chain isoform 5C
	PLCB4	1-PIP-4,5 phosphodiesterase Beta 4
	CAMK2G	calcium/calmodulin-dependent PK II gamma
	GNGT1	G protein (T) subunit gamma-T1
	PLCB1	1-PIP-4,5 phosphdiesterase Beta 1
	ATF6B	activating transcription factor 6 beta
	PPP2CA	protein phosphatase 2A, cat. sub. alpha
	GNG3	G protein, subunit gamma-3
	PPP2R2D	protein phosphatase 2A 55, reg. sub. B delta
	GNB5	G protein, subunit beta-5
	PPP2CB	protein phosphatase 2A, cat. sub. beta
	GNB1	G protein, subunit beta-1
	KCNJ3	K inward-rectifying channel, subfamily J, mem. 3
	MAPK14	mitogen-activating protein kinase 14 (p38)
	GNG12	G protein, subunit gamma-12
	GNAI3	G protein, alpha inhibiting activity 3
	CREB1	cAMP-responsive element binding protein 1
	GSK3B	glycogen synthase kinase 3 beta
	PRKACB	cAMP-dependent protein kinase, cat. sub. beta

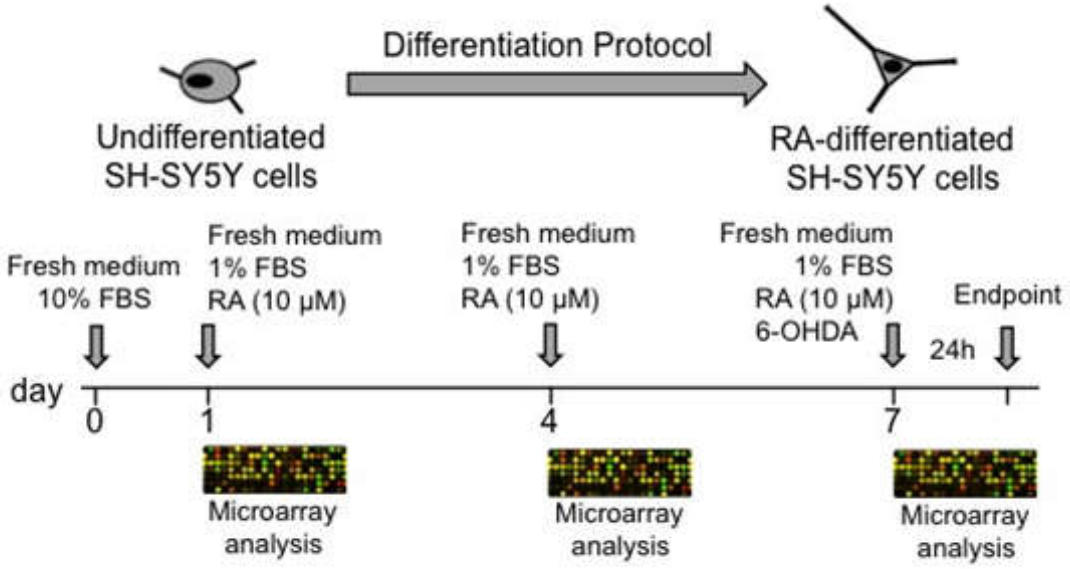
Data generated with Gene Set Enrichment Analysis (GSEA) comparing 7-days RA-differentiated cells (n=4) *vs*. undifferentiated SH-SY5Y cells (n=6) transcripts obtained as described in Methods section. Nominal *p*-value of enrichment analysis obtained from GSEA ($p<0.05$).

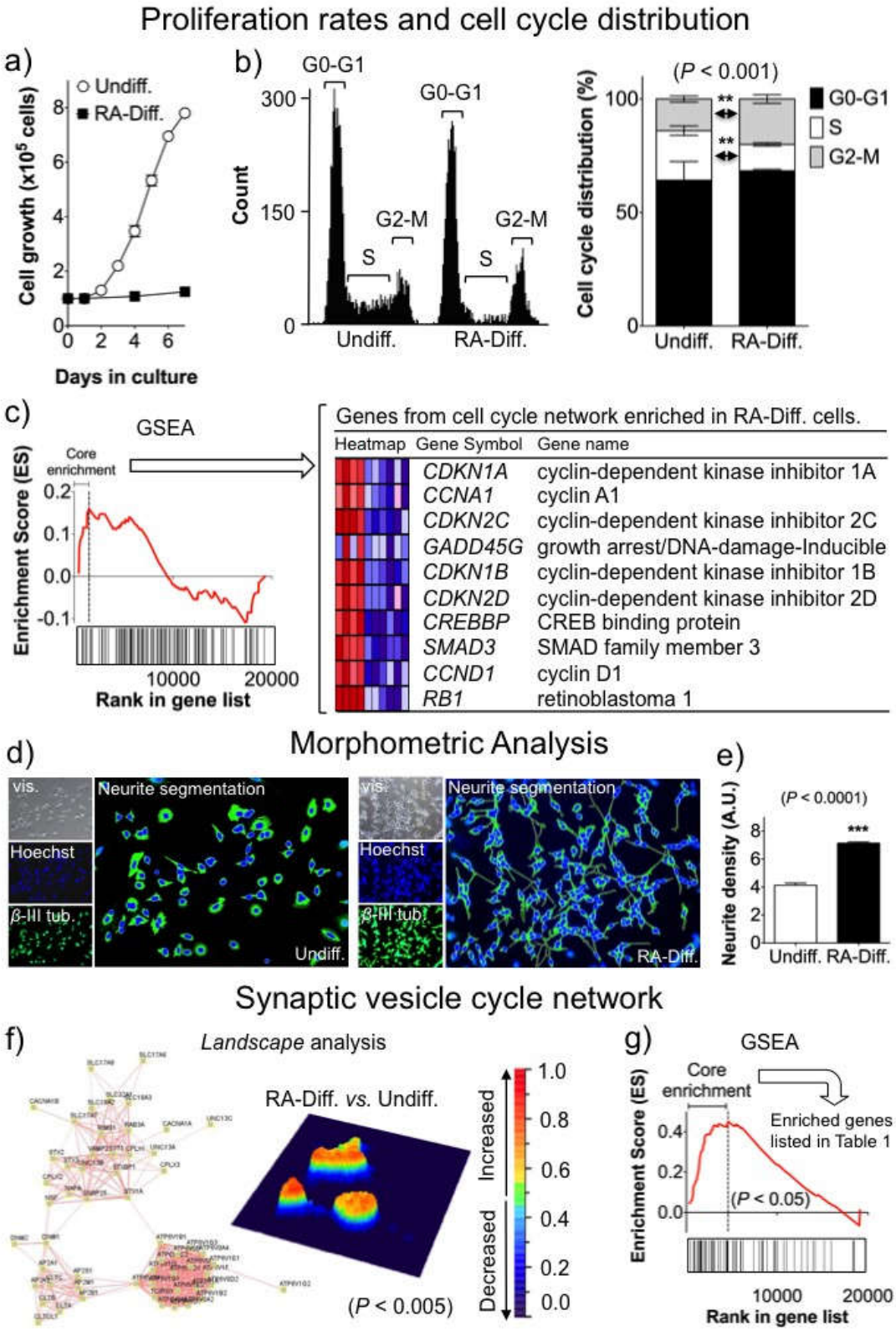
Table 3. *In Vitro* Evaluation of Redox Parameters in Undifferentiated and 7-days RA-Differentiated Human SH-SY5Y Neuroblastoma Cells.

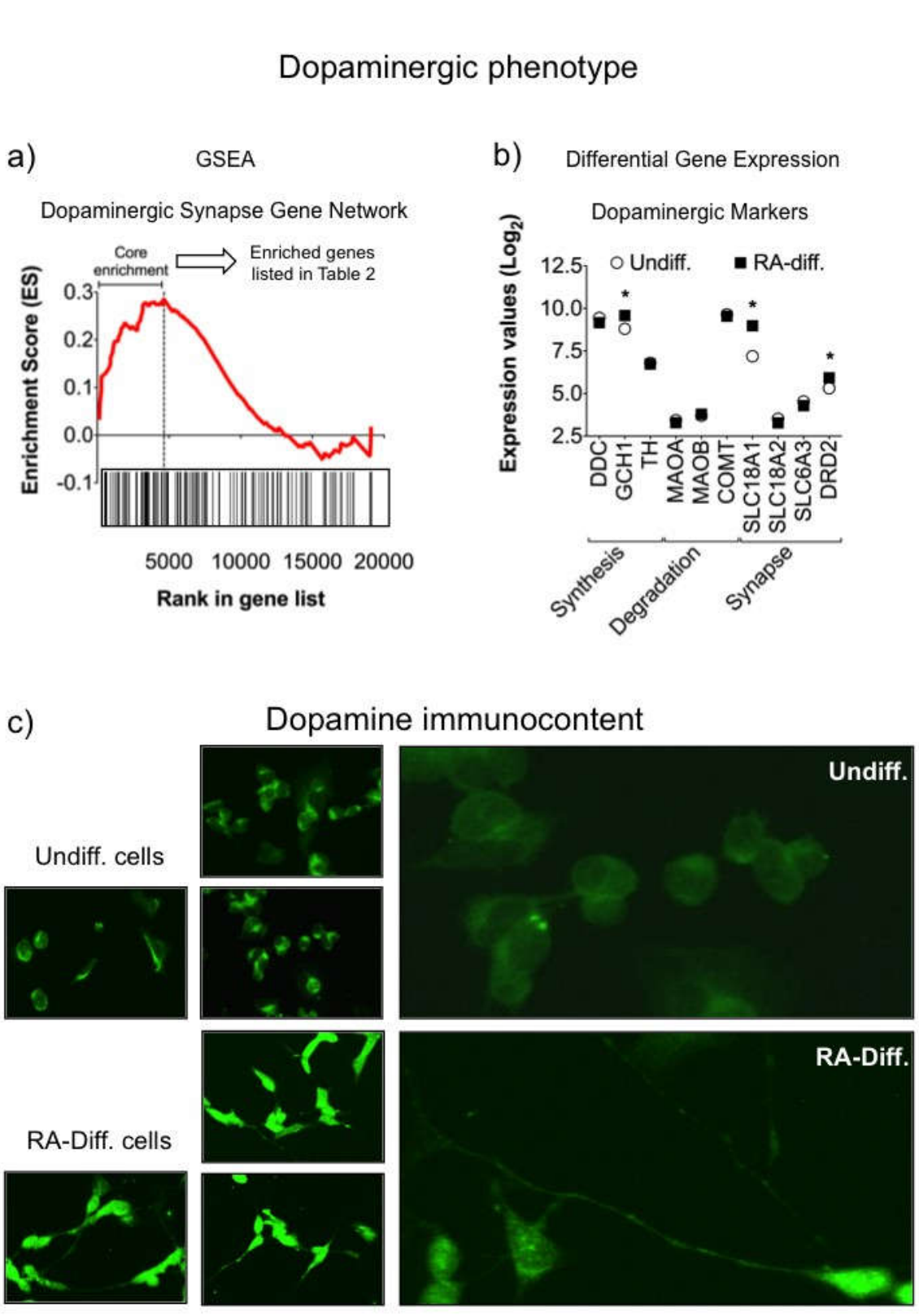
	Undifferentiated	RA-Differentiated	Fold change	*P*
Antioxidant enzymes defenses				
CAT (U/mg)	0.43 ± 0.07	**1.43 ± 0.16**	**3.32**	0.046
GPx (U/mg)	2.83 ± 0.50	3.85 ± 0.99	1.36	0.2336
SOD (U/mg)	10.18 ± 4.42	19.52 ± 3.09	1.92	0.0803
GR (nmol/mg)	16.47 ± 1.86	**25.46 ± 1.94**	**1.54**	0.0291
TrxR (nmol/mg)	**23.51 ± 1.59**	11.08 ± 0.54	**0.47**	0.0003
GST (U/mg)	9.96 ± 2.57	**25.31 ± 1.62**	**2.54**	0.0031
Non-enzymatic defenses				
Thiol Levels (nmol/mg)	17.57 ± 3.95	**39.16 ± 3.70**	**2.22**	0.0026
GSH levels (nmol/mg)	**16.39 ± 1.00**	6.96 ± 0.98	**0.42**	0.0008
LD_{50} (μM)				

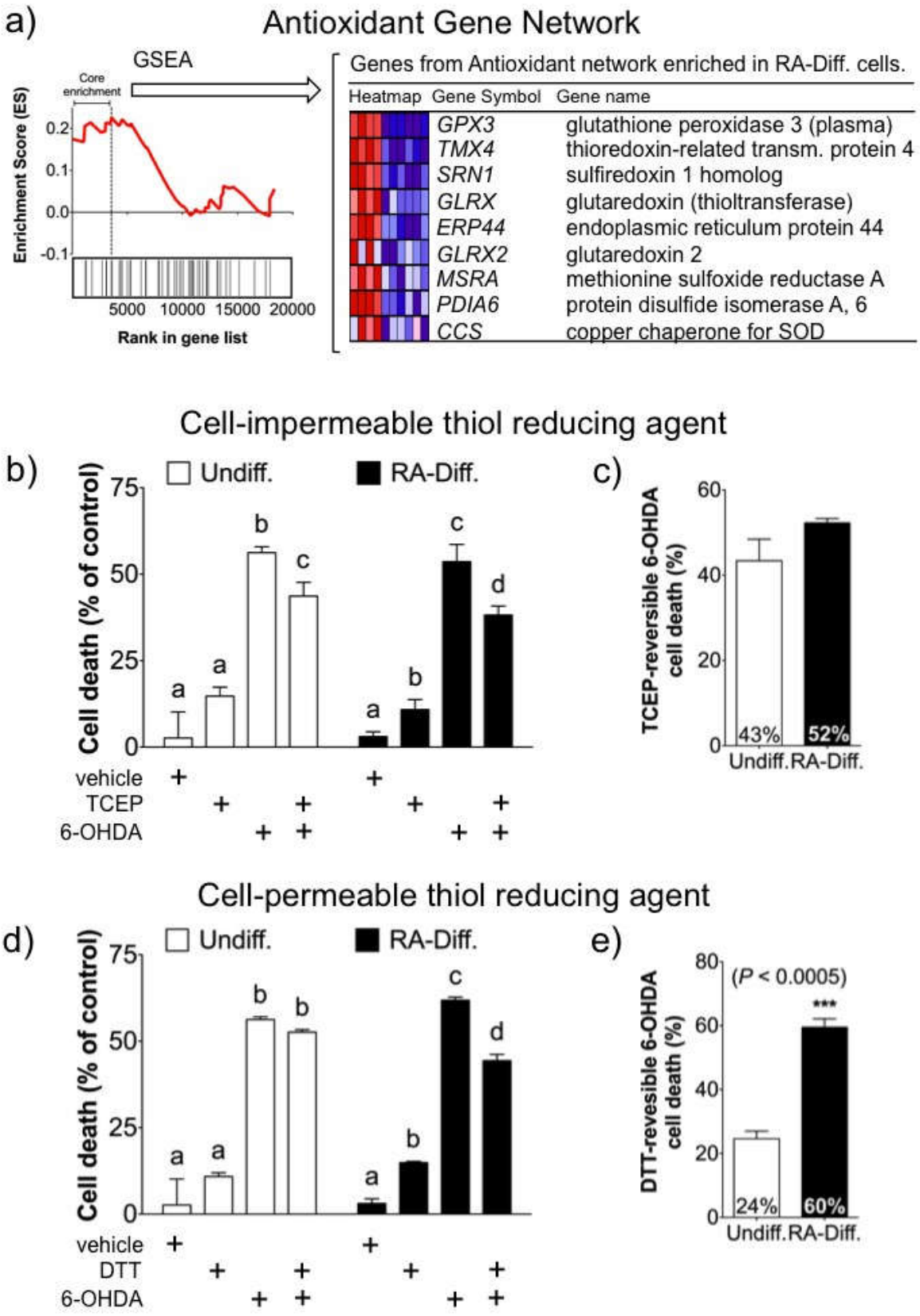
Data represent mean ± S.E.M. of at least four independent experiments (n = 4). P values indicate statistic differences between experimental groups (Student's t-test). Abbreviations: CAT, Catalase; GPx, Glutathione Peroxidase; SOD, Superoxide Dismutase; GR, Glutathione Reductase; TrxR, Thioredoxin Reductase, GST, Glutathione-S-Transferase; GSH, Glutathione; 6-OHDA, 6-hydroxydopamine.

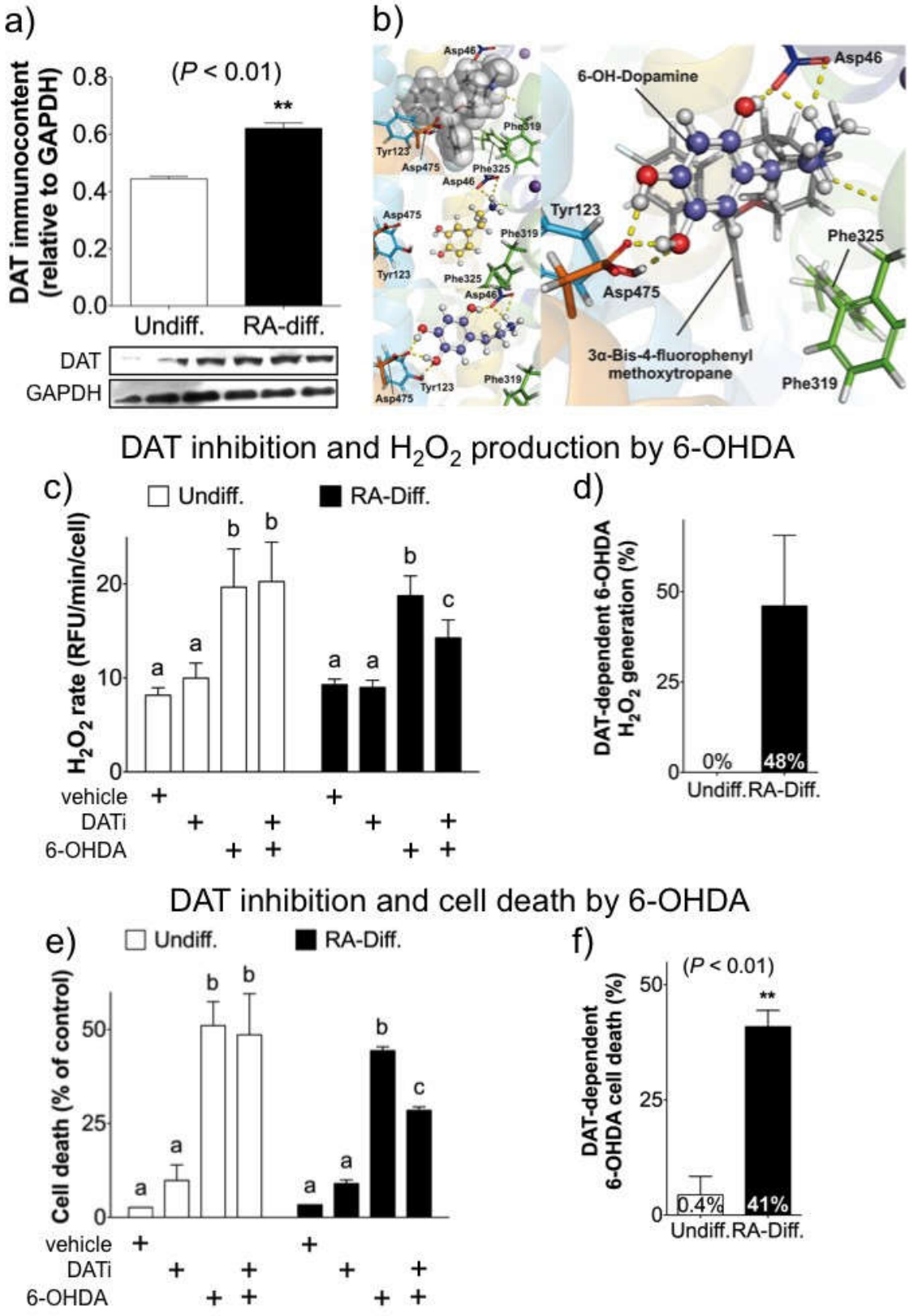
6-OHDA	**35.00 ± 2.03**	15.00 ± 0.866	**0.42**	0.0001




Proliferation rates and cell cycle distribution
a)
Undiff.
RA-Diff.
Cell growth (x10^5 cells)
Days in culture
b)
G0-G1
S
G2-M
Count
Undiff.
RA-Diff.
(P < 0.001)
Cell cycle distribution (%)
G0-G1
S
G2-M
c)
GSEA
Core enrichment
Enrichment Score (ES)
Rank in gene list
Genes from cell cycle network enriched in RA-Diff. cells.
Heatmap
Gene Symbol
Gene name
CDKN1A cyclin-dependent kinase inhibitor 1A
CCNA1 cyclin A1
CDKN2C cyclin-dependent kinase inhibitor 2C
GADD45G growth arrest/DNA-damage-Inducible
CDKN1B cyclin-dependent kinase inhibitor 1B
CDKN2D cyclin-dependent kinase inhibitor 2D
CREBBP CREB binding protein
SMAD3 SMAD family member 3
CCND1 cyclin D1
RB1 retinoblastoma 1
Morphometric Analysis
d)
vis.
Hoechst
β-III tub.
Neurite segmentation
Undiff.
RA-Diff.
e)
(P < 0.0001)
Neurite density (A.U.)
Undiff.
RA-Diff.
Synaptic vesicle cycle network
f)
Landscape analysis
RA-Diff. vs. Undiff.
Increased
Decreased
(P < 0.005)
g)
GSEA
Core enrichment
Enriched genes listed in Table 1
(P < 0.05)
Enrichment Score (ES)
Rank in gene list


Dopaminergic phenotype
a)
GSEA
Dopaminergic Synapse Gene Network
Core enrichment
Enriched genes listed in Table 2
Enrichment Score (ES)
0.3
0.2
0.1
0.0
-0.1
5000
10000
15000
20000
Rank in gene list
b)
Differential Gene Expression
Dopaminergic Markers
Expression values (Log2)
Undiff.
RA-diff.
12.5
10.0
7.5
5.0
2.5
DDC
GCH1
TH
MAOA
MAOB
COMT
SLC18A1
SLC18A2
SLC6A3
DRD2
Synthesis
Degradation
Synapse
c)
Dopamine immunocontent
Undiff. cells
RA-Diff. cells
Undiff.
RA-Diff.


a)
Antioxidant Gene Network
GSEA
Core enrichment
Enrichment Score (ES)
0.2
0.1
0.0
-0.1
5000
10000
15000
20000
Rank in gene list
Genes from Antioxidant network enriched in RA-Diff. cells.
Heatmap
Gene Symbol
Gene name
GPX3
glutathione peroxidase 3 (plasma)
TMX4
thioredoxin-related transm. protein 4
SRN1
sulfiredoxin 1 homolog
GLRX
glutaredoxin (thioltransferase)
ERP44
endoplasmic reticulum protein 44
GLRX2
glutaredoxin 2
MSRA
methionine sulfoxide reductase A
PDIA6
protein disulfide isomerase A, 6
CCS
copper chaperone for SOD
Cell-impermeable thiol reducing agent
b)
Undiff.
RA-Diff.
Cell death (% of control)
75
50
25
0
a
a
b
c
a
b
c
d
vehicle
TCEP
6-OHDA
c)
TCEP-reversible 6-OHDA cell death (%)
60
40
20
0
43%
52%
Undiff.
RA-Diff.
Cell-permeable thiol reducing agent
d)
Undiff.
RA-Diff.
Cell death (% of control)
75
50
25
0
a
a
b
b
a
b
c
d
vehicle
DTT
6-OHDA
e)
DTT-revesible 6-OHDA cell death (%)
80
60
40
20
0
(P < 0.0005)
***
24%
60%
Undiff.
RA-Diff.


a)
b)
DAT immunocontent (relative to GAPDH)
(P < 0.01)
**
Undiff.
RA-diff.
DAT
GAPDH
6-OH-Dopamine
Asp46
Tyr123
Asp475
Phe325
Phe319
3α-Bis-4-fluorophenyl methoxytropane
DAT inhibition and H2O2 production by 6-OHDA
c)
Undiff.
RA-Diff.
H2O2 rate (RFU/min/cell)
vehicle
DATi
6-OHDA
d)
DAT-dependent 6-OHDA H2O2 generation (%)
0%
48%
Undiff. RA-Diff.
DAT inhibition and cell death by 6-OHDA
e)
Cell death (% of control)
f)
DAT-dependent 6-OHDA cell death (%)
0.4%
41%

ANEXO 5

Lista de genes-alvo das unidades regulatórias enriquecidas no transtorno bipolar e seu modo de ação.

Tabela 6. Lista de genes-alvo das unidades regulatórias enriquecidas no transtorno bipolar e seu modo de ação. Valores positivos e negativos indicam predições de interação onde o fator de transcrição induz ou inibe o alvo, respectivamente.

EGR3		***TSC22D4***		***ILF2***		***YBX1***		***MADD***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
ACVR1C	0.5923	*C7orf61*	0.4093	*CYFIP2*	-0.7035	*GRHL3*	-0.2079	*UBQLN2*	0.2692
NUDT9P1	0.3667	*PACSIN3*	0.5302	*CMAS*	-0.449	*BFAR*	0.5355	*HS6ST3*	0.3563
OLFM1	0.7138	*FGF1*	0.4927	*ZFAND6*	0.6702	*POU6F1*	-0.2942	*AACS*	0.3239
LOC100505576	0.6675	*GPR37L1*	1.067	*PRPSAP2*	0.9165	*TXLNB*	-0.4358	*FAM126B*	0.2742
ST8SIA5	0.5775	*GSN*	0.4411	*IQCB1*	0.4375	*NUAK1*	-0.5395	*ICA1*	0.4863
UHRF1BP1L	0.2764	*GALNTL2*	0.7489	*KCNC2*	-0.7202	*TMEM132C*	-0.581	*GPR158*	0.4476
RAPGEF4	0.8804	*FNBP1L*	-0.7849	*ATP6V0E2*	-0.5219	*HNRNPAB*	0.7754	*IDS*	0.4268
C2orf55	0.9681	*ZNF542*	-0.3963	*VBP1*	0.4134	*SPNS1*	0.3219	*PLCB1*	0.526
GABRA1	0.9127	*EML3*	0.4574	*HS6ST3*	-0.4171	*COX4NB*	0.4346	*PDP1*	0.4754
VIP	0.7359	*MOBP*	0.8879	*GEMIN2*	0.4088	*SOX11*	11.683	*TRIM9*	0.3269
GPR158	0.929	*GM2A*	0.9921	*UBE2D3*	0.4798	*FABP7*	0.6761	*PLEKHA6*	0.4024
USP2	0.8276	*KCNJ10*	0.9192	*MLX*	0.6575	*CAPS2*	-0.9088	*PTPRN2*	0.3514
EPHX4	0.8634	*CYP27A1*	0.4957	*FAM174A*	-0.6813	*SPINK2*	-0.2237	*CAND2*	0.3516
LINC00086	0.7963	*PADI2*	0.9337	*TPD52*	-0.7865	*STARD13*	-0.5805	*PIK3C3*	-0.2217
PCSK2	0.5643	*IRF9*	0.529	*TUBA3C*	0.7855	*HNRNPH1*	0.843	*SYNJ1*	0.5199
SERTM1	0.4217	*KCNJ16*	0.4166	*HNRNPA3*	0.7238	*LACTB2*	-0.4228	*SH2D3C*	0.2308
ANKRD55	0.4285	*FBXO2*	0.822	*SIAE*	-0.9348	*SCN1B*	-0.914	*ARHGAP32*	0.3782
PLCB1	0.6316	*CELF1*	-0.7566	*AGPAT1*	-0.5197	*SYNGR1*	-0.6044	*PPP2CA*	0.2818
PRPS2	0.4408	*EDNRB*	0.5092	*SLIT3*	-0.3144	*CCDC25*	-0.898	*PKNOX2*	0.4094
KCTD8	0.8149	*AGXT2L1*	0.9348	*HNRNPAB*	0.8498	*HHAT*	-0.5251	*DAGLA*	0.3734
SCG5	0.4063	*BBOX1*	0.7921	*ORMDL1*	0.481	*ZNF204P*	-0.6094	*CDS2*	0.4915
C19orf66	0.7981	*PLP1*	0.7968	*KIAA0368*	-0.7513	*CCDC92*	-0.581	*KCNJ3*	0.4686
CHRD	0.7891	*GLUL*	0.8405	*EFHA1*	0.4813	*RAB8A*	0.6817	*PANK2*	0.2797

EGR3		***TSC22D4***		***ILF2***		***YBX1***		***MADD***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
SELS	-0.567	*GPR62*	0.804	*RBM3*	0.6007	*PPP1CB*	0.4879	*SNORA56*	-0.173
SYNPR	0.6515	*DLGAP1*	-0.6015	*ANP32A*	0.5347	*ARNT*	-0.187	*EXOSC8*	-0.4684
SPRYD3	0.8005	*TUBA4A*	0.7746	*ICA1*	-0.618	*ERLEC1*	-0.8308	*GUCY1B3*	0.4656
COBL	0.8519	*MOG*	0.7487	*NGLY1*	0.7361	*PEX19*	-0.6282	*LOC338758*	0.4688
RASSF5	0.7937	*SLC48A1*	0.975	*SRSF3*	0.5462	*RPSA*	0.7726	*LMBR1*	0.3024
SSTR1	0.5789	*SLC9A9*	0.8054	*RTN3*	-0.2352	*PTH2*	0.4236	*C1orf74*	0.2021
PRKCB	0.8576	*GSTM5*	0.1847	*DAP3*	0.5437	*MKX*	-0.634	*AHSP*	-0.1798
MYBPC3	0.333	*AKT3*	-0.484	*ABCA5*	-0.5484	*RAB6A*	0.4329	*MITD1*	-0.3806
CAP2	0.6697	*NEU4*	0.5246	*CABIN1*	-0.6246	*PRPF19*	-0.6343	*SLC6A17*	0.4434
EGR1	0.5441	*ALDH1L1*	0.9893	*RNASEH2A*	0.9235	*PVALB*	-0.8004	*GNB5*	0.1802
PELI2	-0.7116	*COPZ2*	0.501	*NNAT*	0.9535	*KCNS3*	-0.7914	*MKL2*	0.4643
TRHDE	0.9101	*ALB*	0.1998	*RAPGEF4*	-0.8588	*ACTG1*	0.8283	*PDS5B*	0.1881
GRASP	0.8622	*CRYL1*	0.7921	*IGF2BP2*	0.8862	*LHFPL2*	0.2631	*MKL1*	0.2663
KCNIP3	0.7349	*PTGDS*	0.657	*LGI3*	-0.7976	*DYNLT1*	10.537	*UNC80*	0.4433
NETO1	0.7537	*AKR1C3*	0.731	*C2orf55*	-10.107	*PLXNB2*	0.4272	*C1orf21*	0.2974
DGKB	0.7825	*PAQR6*	0.9533	*MEX3A*	0.7969	*C17orf76-AS1*	0.9105	*DUSP8*	0.5331
OMG	0.7885	*TST*	0.6597	*MCM10*	0.8591	*TPT1*	0.3678	*SLC25A12*	0.2976
MFSD4	10.285	*TMEM229A*	0.9986	*GABRA1*	-0.9148	*N4BP2L2*	-0.4986	*RFPL1-AS1*	0.3585
SNCA	0.4934	*KLRC3*	0.5693	*CCNB2*	0.8324	*MAP7D2*	-0.954	*SNAP91*	0.4109
GDA	0.8561	*CLMN*	0.8009	*GPR158*	-0.9735	*EIF4EBP1*	0.9095	*SRP19*	-0.2231
CYP26B1	0.6151	*NA*	0.9404	*USP2*	-0.8863	*NCKIPSD*	-0.6071	*ATL1*	0.4538
FAM150B	0.3004	*TECR*	0.63	*SV2B*	-0.9203	*EFHD2*	-10.557	*HTT*	0.1868
PRKCA	0.5741	*GRAMD3*	0.9417	*EPHX4*	-0.909	*LZTFL1*	-0.4878	*GBF1*	0.2231
LOC338758	0.6364	*APLN*	0.4922	*SLC12A5*	-1.002	*THBS1*	0.4662	*KIAA0284*	0.3958
METTL17	-0.4574	*TRIM47*	0.4327	*LINC00086*	-0.8982	*THEMIS*	-0.853	*SYNRG*	-0.3004
KIAA1671	0.8168	*LARP6*	0.2925	*MCF2L2*	-0.7299	*PDE8B*	-0.7064	*BEX4*	0.2225

EGR3		***TSC22D4***		***ILF2***		***YBX1***		***MADD***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
SLC6A17	0.8377	*CSRP1*	0.9358	*GDI2*	0.8492	*SH3BP2*	0.8783	*SORCS3*	0.4308
NME5	0.5932	*CAPN14*	-0.3887	*CDK2*	0.6949	*RAB5B*	-0.5476	*9/15/2016*	-0.2717
KCTD16	0.5133	*ATP1B2*	0.6485	*CENPV*	0.8296	*HNRNPU*	0.8971	*CACNA2D3*	0.2704
TPBG	0.6447	*ENPP2*	0.8081	*TACC1*	-0.8132	*SYNJ1*	-0.6016	*RPTOR*	0.2746
SLC7A14	0.8106	*ACADVL*	0.5368	*NTRK2*	-0.3185	*RAB10*	0.7779	*SH2D2A*	-0.227
RASGRP1	0.5203	*BACH2*	-0.7607	*TUBA4A*	-0.8889	*TCEAL8*	-0.4881	*RAB3GAP1*	0.3124
XCL2	0.4545	*AQP4*	0.7324	*OLFM3*	-0.5333	*EIF3E*	0.7825	*GSK3A*	0.2056
CXorf1	0.5657	*MAL*	0.7074	*HNRNPH1*	0.8749	*EEF2*	0.5342	*PANX2*	0.455
FAM84A	0.4896	*ADD3*	0.7564	*AFAP1L2*	-0.7026	*DNAH6*	-0.233	*MAFG*	0.2964
DNAL4	0.4722	*DZIP1*	0.7332	*PCSK1*	-0.8267	*MAPRE1*	0.8126	*SNX6*	-0.3625
CMTM4	0.5891	*TMEM176B*	0.3234	*ELMO1*	-0.4663	*HEY1*	0.4146	*SYNGAP1*	0.2305
LSG1	-0.2958	*TNS3*	0.4122	*PTPN3*	-0.7161	*BCL2L2*	-10.222	*KIAA1467*	0.3554
SORCS3	0.4143	*NOTCH4*	0.5106	*PCNA*	0.8505	*CST11*	-0.2975	*SLC25A22*	0.5183
PTMA	-0.2634	*AQP1*	0.4514	*VIM*	0.5421	*COX11*	-0.4327	*RFK*	0.2219
BRMS1L	0.4124	*SPOCK3*	0.7677	*CEP57*	0.8226	*CA13*	-0.2759	*TMCO1*	-0.2131
LANCL2	0.3999	*TMPRSS13*	-0.227	*KCNAB1*	-0.7367	*SNORA67*	0.5515	*EPT1*	0.2638
ATP2B1	0.5583	*ARRDC2*	0.8237	*JAK1*	-0.2707	*NKIRAS1*	-0.7156	*NOC2L*	0.3173
ADAMTS19	0.3144	*ERMN*	0.6869	*CSE1L*	0.8875	*CBLN4*	-0.6011	*C19orf12*	0.2188
RBM45	-0.2329	*PLOD2*	0.5596	*WHAMMP3*	-0.6969	*EHD3*	-0.9684	*ASTN1*	0.2898
RPS14	-0.2998	*MT1F*	0.7681	*WHAMMP2*	-0.6231	*PANK2*	-0.2914	*MPST*	-0.2207
RGS7	0.3626	*C5orf32*	0.7611	*PDP1*	-0.5179	*PCOLCE2*	0.2701	*DDX47*	-0.2652
SLC25A30	-0.2641	*FLJ30838*	-0.8415	*RAE1*	10.589	*PHF15*	-0.9357	*RHOBTB2*	0.4432
SPOCK2	0.7888	*PRSS12*	-0.4993	*TMEM184B*	-0.7582	*GLRB*	-0.6435	*GALNT13*	0.341
TMEM22	0.5565	*HTRA1*	0.7372	*SMAP2*	-0.6492	*CBY1*	0.5955	*PSMB1*	-0.2362
FADD	-0.2871	*HPR*	0.322	*POLR2D*	0.6132	*TOMM34*	-0.7116	*C12orf53*	0.296
SLC25A1	-0.2224	*MYLK*	0.901	*ZNF207*	0.9449	*TAF4B*	-0.8322	*FRY*	0.2393

EGR3		***TSC22D4***		***ILF2***		***YBX1***		***MADD***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
SPSB3	-0.5901	*VPS53*	-0.3026	*TMSB15A*	0.8653	*GNB1*	0.4556	*TCEB1*	0.3709
RNF11	0.3386	*PMP2*	0.8479	*FLAD1*	0.5352	*CD93*	0.263	*EPHA4*	0.4399
TMEM60	-0.1748	*METTL7A*	0.7163	*CYLD*	-0.2686	*YPEL2*	-0.5367	*FP588*	0.306
TTC28-AS1	0.4293	*ADI1*	0.352	*TRPM3*	-0.2431	*GNB2L1*	0.8028	*TSPYL1*	0.3816
ZDHHC23	0.633	*GATSL3*	0.3332	*SMARCE1*	0.7671	*EIF4A1*	0.6679	*TCEAL1*	0.4559
PDE2A	0.5781	*OMG*	0.787	*HJURP*	0.7933	*ADCK3*	-0.4473	*KCND2*	0.3245
MET	0.654	*ROBO3*	0.4755	*TRPM2*	-0.8457	*ARAP2*	-0.5332	*BAG4*	0.4463
OXCT1	0.471	*TPP1*	0.8468	*HNRNPA1L2*	0.5869	*SMYD2*	-0.5974	*FLJ33996*	0.5958
MYRIP	0.714	*METRN*	0.5849	*DTYMK*	0.5825	*FIGF*	-0.5519	*CBX6*	0.464
PACSIN1	0.8678	*SNORD24*	-0.3395	*MORF4L2*	0.7746	*LMBR1*	-0.3581	*AP2A2*	0.3281
PCDH20	0.5585	*LAMP2*	0.7235	*APBB1*	-0.642	*ALG2*	-0.6061	*JPH3*	0.3766
CRY2	0.5579	*NWD1*	0.3	*DCTN5*	0.6691	*ISCA1*	-0.3291	*RAPGEFL1*	0.5404
ILF2	-0.7685	*GPR146*	0.8699	*PPP1R13B*	-0.602	*UQCRQ*	-0.3285	*RUNDC1*	0.3803
ZBBX	0.3775	*ALDH4A1*	0.7882	*C19orf66*	-0.8841	*C22orf46*	-0.6163	*KIAA0232*	0.2946
CRHBP	0.6857	*CAPZB*	10.624	*REEP5*	-0.5899	*SMEK2*	0.8753	*VPS13D*	0.2346
RXFP1	0.6422	*FAM189A2*	0.6368	*API5*	0.4785	*NAT8L*	-12.052	*HIST1H2AJ*	-0.3197
STYK1	0.5582	*C11orf95*	-0.8699	*CHRD*	-0.833	*NFE2L1*	-0.7809	*CDS1*	0.4695
GAD2	0.7233	*ACSF2*	0.5958	*SERPINI1*	-0.6483	*FLJ39639*	-0.309	*CYP4X1*	0.3738
ANKRD43	0.8815	*KANK1*	0.8258	*SPRYD3*	-0.9403	*LYSMD2*	-0.6658	*MEF2D*	0.4404
SPARCL1	0.8222	*SGCD*	0.5964	*COBL*	-0.9652	*VAMP1*	-0.7482	*NXPH1*	0.2368
LOC401442	0.6084	*MID1IP1*	0.7079	*SLC25A3*	0.4631	*MAT2B*	-0.6015	*IQSEC3*	0.4778
GPC2	-0.8069	*RARRES3*	0.7637	*USP39*	0.5036	*ATP6V1H*	-0.5231	*CCDC85A*	0.4723
CX3CL1	0.8175	*BTD*	0.5999	*ACTG1*	0.9651	*UNC80*	-0.4563	*LOC283683*	0.2984
RPL11	-0.2724	*NFATC1*	0.3922	*PPARGC1A*	-0.7311	*TPBG*	-0.6734	*DKC1*	-0.3106
WDYHV1	-0.2675	*AXL*	0.4886	*RASSF5*	-0.897	*KMO*	-0.7466	*C17orf51*	0.4552
CACNG3	0.8922	*BDH2*	0.6169	*HAUS1*	0.788	*NR3C2*	-0.8056	*ARHGAP44*	0.59

EGR3		***TSC22D4***		***ILF2***		***YBX1***		***MADD***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
COL24A1	0.6346	*CST2*	0.7021	*C17orf76-AS1*	0.787	*GLRX2*	-0.7449	*SLITRK4*	0.3547
MEF2D	0.7583	*LPAR2*	-0.7859	*NUSAP1*	0.9311	*FAM98A*	-0.4835	*AKAP6*	0.2806
CDH9	0.7381	*ASPA*	0.6791	*CUL4A*	0.381	*C6orf106*	-0.8719	*PLCXD3*	0.4821
C8orf46	0.9839	*EPHX1*	0.5549	*IFT81*	0.7969	*ANKRD46*	0.4844		
IQSEC3	0.5143	*PBXIP1*	0.3758	*MAP7D2*	-0.83	*CISD1*	-0.1799		
HTR2A	0.7472	*C20orf7*	-0.2929	*EIF4EBP1*	0.8645	*GRSF1*	-0.6512		
KLF9	0.7813	*ITGB4*	0.7026	*SNRNP40*	0.6297	*ARGLU1*	0.4261		
RGS4	0.887	*ARHGEF6*	0.5719	*CLK2*	0.4542	*FGFR1OP2*	-0.6383		
B3GNT1	0.6552	*HSDL2*	0.502	*GART*	0.7912	*PELI3*	-0.9286		
C13orf30	0.302	*PRR18*	0.9003	*MAPK14*	0.4236	*FAM20A*	0.2221		
WSCD2	0.7187	*C2orf88*	0.7538	*HACL1*	0.2414	*FAM125A*	0.2596		
RASGEF1A	0.8935	*SRSF12*	-0.6338	*NASP*	0.7073	*GLT8D1*	0.4705		
		PPAP2C	0.6847	*RBFOX3*	-0.662	*PPM1H*	-0.9952		
		C10orf54	0.5723	*RIOK1*	0.7062	*PIP4K2C*	-0.8401		
		ASXL3	-0.7645	*DCTD*	-0.5473	*NDUFV2*	-0.448		
		LCAT	0.4268	*ITPK1*	-0.863	*PABPC1*	0.35		
		SRPX	0.3405	*MKRN2*	-0.4578	*CPNE2*	0.2412		
		RGS3	0.4686	*SNRPD3*	0.4237	*CHCHD4*	-0.4888		
		ACY1	0.6476	*EIF4A3*	10.264	*RWDD2B*	-0.4713		
		AMPD3	0.8782	*EIF3L*	0.8581	*GFOD1*	-0.4872		
		LOC652276	-0.4938	*CENPH*	0.8171	*RPLP0*	0.7682		
		ACACB	0.657	*BMI1*	0.7612	*MTIF2*	-0.6913		
		ARHGAP31	0.5833	*HNRNPU*	0.9379	*TCTN1*	0.5634		
		RPL32	-0.69	*ATAT1*	0.7892	*PAWR*	0.6037		
		CTSH	0.6545	*TRHDE*	-0.9485	*DPP3*	0.4279		
		WIPI1	0.2208	*SLC17A7*	-0.8029	*GOLPH3L*	-0.5496		

TSC22D4		***ILF2***		***YBX1***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
IGSF3	-0.7802	*EIF3E*	0.9599	*RIOK3*	0.5679
CHDH	0.6978	*NRIP3*	-0.6552	*RPS3*	0.267
PPP1R36	0.4255	*CNOT2*	0.7412	*C20orf132*	-0.4413
IL17RB	0.7554	*IPCEF1*	-0.9101	*RPL13AP20*	0.6149
UBTD1	0.5311	*TPMT*	-0.5347	*HGD*	-0.5948
HOXC10	-0.4157	*PRKCE*	-0.4789	*ARPC5*	0.5333
C10orf90	0.7672	*DHPS*	0.711	*FBXW11*	-0.5277
SNORA28	-0.3733	*LINC00461*	0.7741	*TRAF4*	0.599
TLCD1	0.3861	*SYP*	-0.6124	*EEF1B2*	0.4081
RAPGEF3	0.6133	*GTF2B*	0.6973	*UBL4A*	-0.3215
LOC254559	-0.2941	*VASN*	-0.736	*SH3PXD2B*	0.3777
DAZAP2	0.5006	*BCL2L2*	-0.8058	*NDUFC2*	-0.7162
ABHD1	0.6073	*PNMAL2*	-0.7803	*C7orf25*	-0.3831
RRBP1	0.6271	*VMP1*	0.6088	*MKRN1*	0.6147
TUBGCP4	-0.2328	*HBG1*	0.8503	*C12orf75*	0.6448
LOC389033	0.2973	*OSGIN2*	-0.5193	*RPL18*	0.2033
UBQLN3	-0.3327	*ACTL6B*	0.5468	*PLA2G6*	-0.2891
C10orf25	-0.238	*ANKRD19P*	-0.6364	*DNAJC19*	-0.5011
TAPBPL	0.5369	*KCNJ3*	-0.5848	*EEF1A1*	0.4497
RASSF4	0.4536	*NOL11*	0.9085	*PPIB*	0.2463
PPIF	0.4184	*OMG*	-0.8782	*CXorf56*	-0.221
VAV3	0.2783	*NAE1*	0.8436	*HNRNPA1P10*	0.5484
TBC1D16	0.4031	*MFSD4*	-0.9367	*ETS2*	-0.9615
UBP1	-0.2479	*WTAP*	0.5624	*MAP2K5*	-0.4863
SLC25A20	0.4128	*UMPS*	0.6628	*RPL13A*	0.3084
C15orf24	0.3005	*IRF2*	-0.3338	*PIGK*	-0.4002

TSC22D4		***ILF2***		***YBX1***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
SLC39A11	0.4657	*RBM4*	0.8878	*FGF2*	-0.2083
RAC1	0.3194	*ITSN1*	0.4554	*RPL4*	0.522
TPCN1	0.6997	*PPP1R14B*	0.9181	*RPL39*	0.5207
ITM2C	0.7239	*YWHAZ*	0.3883	*STMN4*	0.388
ACADSB	0.287	*USP10*	0.7097	*ZNF576*	-0.6192
POMGNT1	0.4936	*CCNB1IP1*	0.7701	*C1orf31*	-0.2417
LIN7A	-0.2922	*RBM4B*	0.6854	*RPL14*	0.3998
LHFPL4	0.3013	*GPR162*	-0.5962	*MAD2L2*	0.6499
ABHD14B	0.7664	*PSMA4*	0.5231	*PRSS33*	-0.2241
NME3	0.2252	*PNPLA8*	0.2253	*COX6C*	-0.2071
PI16	0.5446	*SRSF5*	0.2078	*AQP11*	-0.1905
C1orf122	0.5597	*HPCAL4*	-0.7709	*LINC00515*	-0.7075
RUNX1T1	-0.2568	*HES6*	0.7461	*KLHL26*	-0.327
OPLAH	0.5127	*EXOSC8*	0.7875	*MBOAT2*	0.4562
UBR1	-0.3169	*FAM19A3*	-0.3819	*CHST11*	0.4295
PYGM	0.5178	*HNRNPH3*	0.6916	*RPS18*	0.3332
TTLL4	0.293	*GDA*	-0.8443	*ANKRD56*	-0.3469
MGC16275	0.4936	*EIF2B1*	0.6407	*C7orf73*	0.7518
KLHL29	-0.5775	*RAB3A*	-0.609	*MRPL3*	0.4067
GPC5	0.4534	*KNDC1*	-0.8863	*PARP6*	0.4925
PLEKHF1	0.6547	*RFC4*	0.7689	*CCDC47*	-0.3928
KIAA0391	0.3191	*TMEM53*	-0.274	*LOC390940*	0.3304
RENBP	0.6908	*PCDH9*	-0.8087	*RBP5*	-0.2875
LRP10	0.784	*ACADM*	0.2442	*DNAJB9*	-0.4634
C6orf174	-0.4484	*HMGN1*	0.8844	*DHRS1*	0.5115
ADHFE1	0.7008	*ATP2B2*	-0.7149	*NT5DC1*	-0.572

TSC22D4		***ILF2***		***YBX1***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
LBH	-0.1965	*SEC22A*	0.6359	*SYNDIG1*	-0.4344
SORT1	0.628	*SCFD1*	0.8145	*SEPHS2*	-0.4057
ATP13A4	0.344	*C2orf72*	-0.2113	*KAT7*	-0.613
SDS	0.6106	*C14orf109*	0.7128	*PGLS*	0.6234
CHML	-0.5571	*B3GALTL*	-0.401	*PGBD4*	-0.2265
LOC283481	0.3692	*LGI1*	-0.762	*CACYBP*	-0.4619
SELENBP1	0.7541	*MAPK1*	-0.6867	*EPM2A*	-0.4038
IZUMO4	0.6822	*TECPR2*	-0.6459	*RPSAP9*	0.6296
FANCG	-0.5069	*PAIP2*	0.2771	*IQCA1*	-0.4952
CAMKMT	0.6096	*RAB40B*	-0.7299	*CCDC167*	0.7754
SERHL2	0.2908	*C12orf35*	0.7813	*KDELR1*	0.5091
GRIN2C	0.6422	*ZDHHC5*	-0.4873	*LOC257396*	-0.7116
FSCN3	-0.2926	*KCNMA1*	-0.692	*SPAG8*	-0.4504
SCT	0.5532	*TTC7A*	-0.4505	*ARMC9*	-0.4039
TRMT1L	0.3023	*SLC6A17*	-0.7881	*GPCPD1*	0.2923
ZNF664-FAM101A	-0.6837	*TSEN34*	0.3772	*WASF3*	-0.4937
C10orf128	0.5382	*CXorf26*	0.7986	*RARS2*	0.8958
S100A16	0.3219	*MICAL2*	-0.854	*MDH1B*	-0.4115
SSPN	0.7959	*SCAMP5*	-0.6281	*TMEM184C*	-0.4258
EPB41L2	0.7777	*CUL2*	0.3849	*SLC4A10*	-0.3884
LINC00467	0.4968	*KIAA1107*	-0.8001	*KDELR3*	-0.2618
PHGDH	0.5978	*DKFZP434I0714*	0.8633	*PRUNE2*	-0.7496
APLP1	0.1649	*MKL2*	-0.8829	*DOK5*	0.8811
DIRC2	0.3347	*LPAR2*	0.7893	*ADCYAP1*	-0.6339
SUCLG2	0.5579	*NAPB*	-0.9327	*DHCR24*	-0.5222
WFS1	0.8032	*HIST1H2BG*	0.4807	*LOC100507507*	-0.3804

TSC22D4		***ILF2***		***YBX1***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
TFEB	0.7823	*NME6*	0.7568	*RPL18A*	0.2594
NKX2-2	0.8541	*ARL5A*	0.5199	*HDHD2*	-0.3278
ACSBG1	0.7597	*TMEM132E*	-0.6405	*MFSD6*	-0.4814
PLLP	0.8801	*C2CD2L*	-0.6397	*STEAP1*	-0.7724
CLDN10	0.5396	*PRKX*	0.7796	*TCFL5*	-0.4979
APCDD1	0.4794	*DUSP8*	-0.5266	*GPATCH4*	-0.3694
APOE	0.6921	*ZNF259*	0.773	*FAM122B*	-0.5884
C1QTNF5	0.3581	*ARMCX6*	0.4945	*INSM2*	-0.7852
TSPAN15	0.4772	*H2AFY2*	0.8532	*VPS35*	-0.4651
SNTA1	0.7773	*C6orf106*	-0.797	*RPS2P32*	0.9189
C16orf89	0.4631	*SLC7A14*	-0.9435	*TUBB2B*	0.5217
HDAC11	0.7017	*POU3F3*	0.6978	*MAGEE1*	-0.5552
CCDC152	0.526	*ALG9*	0.488	*C21orf128*	-0.6271
TTC18	0.615	*ZNF460*	0.3421	*MEMO1*	0.7629
ZNF524	0.4022	*CLPTM1*	-0.8131	*ZAN*	0.3081
HEBP1	0.2268	*C1orf9*	-0.2365	*PITPNB*	-0.1953
C13orf15	0.7569	*CASP6*	0.783	*TMOD3*	0.5693
C14orf159	0.807	*ARF4*	10.464	*STAMBPL1*	-0.9474
HSD3B7	0.2732	*ATL1*	-0.5877	*RCAN2*	-0.8397
TIMP4	0.4623	*KIAA1191*	0.7044	*MFN2*	-0.3776
S100A13	0.865	*TCP1*	10.253	*VPS33A*	-0.3172
ZCWPW1	0.4662	*UBE2G2*	0.3349	*MRPS6*	0.4066
C12orf39	0.543	*PPM1H*	-10.097	*PAH*	-0.8221
MT1X	0.7485	*CACNG2*	-0.4055	*FAM58A*	-0.4897
ACSS1	0.6267	*KARS*	0.6761	*CREBL2*	-0.68
KLF15	0.6309	*SEC14L1*	-0.2152	*CBX7*	-11.363

TSC22D4		***ILF2***		***YBX1***	
SYMBOL	Mode of action	SYMBOL	Mode of action	SYMBOL	Mode of action
SLC25A35	0.314	*ZNF410*	0.7654	*ATP2B3*	-0.861
RASL12	0.5514	*ESD*	0.4847	*RALBP1*	-0.5754
SLC25A48	0.552	*ATP8A2*	-0.4149	*WASL*	-0.6851
NTSR2	0.9659	*ATG4A*	0.4711	*LHFPL5*	-0.5748
NUDT10	-0.5517	*ZC3H12B*	-0.7718	*ZCRB1*	-0.7615
EPHX2	0.563	*ATP1B1*	-0.3842	*GNG8*	0.502
ALDH6A1	0.624	*BCL10*	0.8561	*ANGPTL5*	-0.624
TNFSF13	0.6603	*C12orf32*	0.9562	*COPS7A*	-0.597
KAT2B	0.6764	*NIP7*	0.4383	*TLL2*	-0.8015
PGCP	0.7594	*SNORD36A*	0.7595	*HNRNPA1*	0.8683
TPPP3	0.8874	*RPS6*	0.2903	*HTR2A*	-0.7934
SLC9A3R1	0.748	*FAM103A1*	0.3927	*LOC283683*	-0.3007
MT1M	0.8629	*SPRYD7*	-0.6708	*SENP2*	-0.7811
MERTK	0.481	*NUDT1*	0.6406	*STXBP5L*	-10.508
ALDOC	0.8877	*MED15*	-0.4745	*EEF1G*	0.3411
SLC25A18	0.8431	*TUSC2*	-0.5302		
PEX10	0.4396	*NDEL1*	-0.3605		
LHPP	0.8019	*C1orf173*	-0.5942		
CD9	0.7563	*LOC100288974*	0.5553		
PASK	-0.6045	*MEF2A*	-0.4437		
SLC15A2	0.3466	*ANKRD10-IT1*	0.7837		
PDHB	0.3729	*ARL1*	-0.9142		
PLTP	0.5482	*SV2A*	-0.4906		
MEX3B	-0.7951	*FBXL12*	0.4564		
LOC284408	-0.2362	*GRID1*	-0.2557		
RETSAT	0.418	*HNRNPD*	0.8807		

TSC22D4		***ILF2***	
SYMBOL	Mode of action	SYMBOL	Mode of action
CHADL	0.8797	*CTNNBL1*	0.6873
SCAMP2	0.6173	*VAPA*	0.222
MAP3K15	-0.2443	*C10orf107*	-0.3181
RYR1	0.7071	*MRS2*	0.3941
SLC12A4	0.6268	*SIPA1L3*	-0.3383
PLSCR4	0.6893	*TNFSF12*	-0.7942
ALDH1A1	0.9199	*EIF3D*	0.8537
HOGA1	0.7319	*KLHDC2*	0.4611
RAMP1	0.8749	*FDFT1*	0.2882
GPRC5B	0.7541	*ASB5*	-0.4025
NPC2	0.449	*TIAM1*	-0.7066
NKX6-2	0.7871	*PNISR*	0.8144
DBNDD2	0.7453	*SERPINA13*	-0.2518
KATNAL2	0.6745	*EIF3F*	0.7282
SEC14L5	0.8025	*EIF4E*	0.426
PIEZO1	0.5362	*PANX2*	-0.5616
AGT	0.7482	*HADH*	0.4638
PHYHD1	0.5624	*TEX2*	-0.5244
APOD	0.6629	*DUSP11*	0.5607
EFHD1	0.9501	*METTL13*	0.2382
LOC100131607	0.9275	*TNPO1*	0.3591
SPON1	0.2849	*RPS5*	0.4867
PAPLN	0.5835	*SLC25A22*	-0.5158
SCRG1	0.8502	*MPP2*	-0.7008
S100A1	0.7213	*MINA*	-0.4195
CYP2J2	0.9818	*XRCC6*	10.148

TSC22D4		***ILF2***	
SYMBOL	Mode of action	SYMBOL	Mode of action
RNASE1	0.7329	*PLEKHA3*	-0.5064
HSD17B6	0.5968	*STAU1*	0.5325
DAAM2	0.9107	*TRAPPC4*	0.4967
PLA2G16	0.9303	*RNF170*	0.4065
CPM	0.8994	*RPL7*	0.3046
VASH2	-0.7699	*TSSC1*	0.4693
MT1E	0.8676	*SECISBP2*	0.5303
MT1DP	0.8541	*ATP5L2*	0.2803
GFAP	0.7162	*NUP85*	0.6359
PAMR1	0.2202	*ZNF570*	0.433
HHLA3	0.3925	*KDM1A*	0.8454
CYP4F11	0.4896	*C11orf73*	0.5229
GPR17	0.5045	*NXT1*	0.7687
CBR1	0.7884	*RQCD1*	0.2262
BRP44L	0.7065	*SIRT6*	0.5449
PEX11G	0.4825	*NARS2*	0.4714
PAQR8	0.8588	*SLBP*	0.4577
		SPOCK2	-10.077
		SNRPD1	0.3217
		COPS7B	-0.2599
		LIG1	0.369
		PURA	-0.7854
		PPAPDC3	-0.7924
		SRY	0.2377
		TSPAN5	0.4587
		VPS29	0.3375

ILF2	
SYMBOL	Mode of action
BOD1	0.5462
C10orf2	0.4391
SMG7	-0.3826
DGUOK	0.533
MRPL1	0.3352
RNF34	0.5528
DTD1	0.5632
SCYL1	-0.6171
SNORA70	0.5627
UBE2V2	0.4559
NDRG3	-0.7373
DNAJC3	-0.3699
SLC35A1	0.6765
RFC2	0.7584
REPS2	-0.547
MGC72080	0.5193
APEX1	0.7589
RIC8B	0.3304
DGAT2	-0.4804
DBNL	-0.3025
SNORD80	0.5169
ARNTL	-0.56
SSBP3	-0.513
RBM34	0.6173
MRPS10	0.4601
ODC1	0.8481

ILF2	
SYMBOL	Mode of action
BSDC1	-0.2124
MRPS35	0.6153
EIF3H	0.7283
LAPTM4B	0.2371
KIAA0664	-0.5735
STS	-0.4991
CHN1	-0.8755
TTC7B	-0.2567
NME7	0.7599
RPL7L1	0.4663
CCR6	-0.2713
HRASLS5	-0.5087
POLB	0.9279
RPL10L	0.7875
ACAT2	0.7657
BAG4	-0.4703
KCNA4	-0.2397
PWP1	0.5581
CCT2	0.7704
PGBD5	-0.3434
INTS12	0.747
NCALD	-0.7736
FBXL16	-0.7085
FLJ33996	-0.4724
UNC5A	-0.6625
GXYLT1	0.3025

ILF2	
SYMBOL	Mode of action
FUS	0.4826
RBP1	0.3007
FSTL4	-0.8002
EPB41L2	-0.7623
HNRNPR	0.2779
RPL10A	0.4435
TLN2	-0.7111
PACSIN1	-0.9876
CALY	-0.8527
LOC100508196	-0.3922
SNORD6	0.8804
AKR1C1	0.6707
LARGE	-0.3538
SEMA3D	-0.3626
CCK	-0.7925
KLHL2	-0.4971
THY1	-0.8561
GPR83	-0.7715
NALCN	-0.8409
ME1	-0.8356
ENO2	-0.6548
NUP88	0.6831
CCT8	0.9995
EPHB4	0.3045
FKBP15	0.7014
SUMO1	0.5132

ILF2	
SYMBOL	Mode of action
TMEM38A	-0.7731
DUSP12	0.9001
NIM1	-0.7503
KCNB1	-0.844
PNMAL1	-0.3893
CHRM1	-0.6797
SCN4B	-0.8217
CSRP2	0.8986
PRNP	-0.6235
DACT3	-0.5914
WBP2	-0.8017
MYCN	0.7785
PSMD13	0.6117
CDC42BPB	-0.4787
TNFSF4	-0.2831
SSBP2	0.7922
PPIP5K1	-0.5932
C16orf5	-0.7857
EGR3	-0.7685
ANKRD43	-0.9078
TOM1L2	-0.8163
SPARCL1	-0.8784
PRRT3	-0.6173
NEUROG2	0.8672
CDS1	-0.7618
GPC2	0.9625

FLJ43663	-0.7824
KLRG1	0.5337
NAP1L2	-0.6827
FAM200A	0.6563
FAM134B	-0.4171
ASPHD1	-0.8172
ATP2B3	-0.7864
IMPDH2	0.9551
RAMP1	-0.7989
OGDHL	-0.7969
CIT	-0.8238
ATF1	0.7043
DIRAS1	-0.5841
UBE2E2	-0.791
C8orf46	-10.592
SNAPC3	0.9293
SYNGR3	-0.6227
KIAA1324L	-0.4572
BIRC5	0.7902
C1orf115	-0.6805
PINK1	-0.8892
GABBR2	-0.4918
DLGAP2	-0.5654
CCDC85A	-0.6618
HNRNPA1	0.9198
ZNF286A	0.8414
PHYHIP	-0.8831
CTXN3	-0.6856

RGS4	-0.8985
VASH2	0.7811
XK	-0.9339
VGF	-0.7082
C17orf51	-0.6175
RRAGC	0.4795
LRP11	-0.7558
RASGEF1A	-0.8443
ARHGAP44	-0.5814
METTL3	0.7757
C9orf69	-0.5034
ARF3	-0.6356
DNAH7	-0.4663
GJC1	0.5151