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

MEMÓRIA E NÍVEIS DO FATOR NEUROTRÓFICO DERIVADO DO CÉREBRO (BDNF) EM UM MODELO ANIMAL DE ESTÁGIOS PRECOCE E TARDIO DE TRANSTORNO DE HUMOR BIPOLAR

GABRIEL RODRIGO FRIES

Orientadora
Profa. Dra. Maria Luiza Saraiva Pereira
Co-orientador
Prof. Dr. Flávio Kapczinski

Porto Alegre, fevereiro de 2011.

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GABRIEL RODRIGO FRIES

Dissertação 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 Mestre em Bioquímica.

Porto Alegre, fevereiro de 2011.

"*Zwar weiß ich viel, doch möcht ich alles wissen*."

(Fausto I, Verso 601 / Wagner - Johann Wolfgang von Goethe)

(Embora eu saiba muito, gostaria de saber tudo)

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PARTE I

RESUMO

O Transtorno do Humor Bipolar (THB) é um transtorno psiquiátrico grave e altamente incapacitante, sendo sua progressão associada a grandes prejuízos cognitivos aparentemente irreversíveis. Evidências sugerem que o fator neurotrófico derivado do cérebro (BDNF) tem um importante papel na neuroprogressão do THB, visto que seus níveis séricos encontram-se diminuídos em pacientes com múltiplos episódios em comparação com aqueles que tiveram apenas um episódio maníaco. Sendo assim, o objetivo do presente estudo foi avaliar a memória e os níveis de BDNF em um modelo animal de mania induzido por D-anfetamina (AMPH), em estágios precoce e tardio do THB. Para isso, ratos Wistar machos adultos foram divididos em grupos precoce (salina ou AMPH 2mg/kg i.p. por 7 dias) e tardio (salina ou AMPH 2mg/kg i.p. por 35 dias). Nos grupos tardios, as injeções ocorreram 5 vezes por 7 dias seguidos intercalados por 7 dias sem injeção. Uma semana após a última injeção, os ratos foram submetidos aos testes de habituação ao campo aberto ou esquiva inibitória passiva e, após, procedeu-se com a eutanásia e isolamento do hipocampo, córtex pré-frontal e região da amígdala. Os níveis de mRNA de BDNF foram dosados por PCR quantitativo em tempo real e os seus níveis protéicos foram dosados por ELISA sanduíche. A AMPH prejudicou a memória de habituação tanto no tratamento precoce quanto no tardio, sendo mais prejudicial no tratamento tardio ($p<0,05$). Este prejuízo foi acompanhado de uma redução nos níveis protéicos de BDNF no hipocampo e um aumento nos níveis de mRNA de BDNF no córtex pré-frontal. Na esquiva inibitória, todos os grupos (salina e AMPH, precoce e tardio) aprenderam o estímulo aversivo, porém a AMPH diminuiu significativamente o tempo de descida da plataforma dos ratos em comparação à salina. Não houve diferenças entre os grupos precoce e tardio, embora os ratos do grupo AMPH tardio apresentaram uma redução nos níveis protéicos de BDNF no córtex pré-frontal e um aumento nos níveis de mRNA de BDNF no hipocampo em comparação com o grupo AMPH precoce. Esses resultados sugerem que os prejuízos cognitivos observados com a progressão do THB podem estar associados a alterações nos níveis de BDNF no hipocampo e córtex pré-frontal.

ABSTRACT

Bipolar disorder (BD) is a severe and highly disabling psychiatric disorder whose progression has been associated with apparently irreversible cognitive impairments. Evidences suggest that brain-derived neurotrophic fator (BDNF) plays an importante role in BD neuroprogression, as its serum levels are reduced in patients who had multiple mood episodes in comparison to those with only episode. The objective of the present study was to evaluate memory and BDNF levels in an amphetamine (AMPH)-induced animal model of mania, in early and late stages of BD. In order to do that, adult male Wistar rats were divided into early groups (saline or AMPH 2mg/kg ip for 7 days) and late groups (saline or AMPH 2 mg/kg for 35 days). In the late groups, animals were injected for 7 days straight followed by 7 days without any injection, being this protocol repeated 5 times. One week after the last injection, rats were submitted to the open-field habituation test or passive inhibitory avoidance test. Right after that, the rats were killed and the hippocampus, prefrontal cortex, and amygdala region were isolated. BDNF mRNA levels were measured by quantitative Real Time PCR, and protein levels were measured by sandwich ELISA. AMPH impaired habituation memory both in early and late treatments, but memory was worse in the late group. This impairment was accompanied by a reduction of BDNF protein levels in hippocampus and an increase of BDNF mRNA levels in prefrontal cortex of late AMPH-treated rats. In the inhibitory avoidance, on the other hand, all groups (saline and AMPH, early and late) showed significant differences in latency to step-down between training and test trials, which show that even AMPH-treated rats have managed to learn the aversive stimulus. However, AMPH has significantly decreased the latency to step-down in the test when comparing to Sal-treated rats, which suggests an AMPH-induced impairment in this memory task. No difference in behavior was observed between late and early treatments for this test, although late AMPH-treated rats presented a reduction of BDNF protein levels in prefrontal cortex and an increase of BDNF mRNA levels in hippocampus in comparison to early AMPH-treated rats. These results suggest that the cognitive impairment in BD may be associated with alterations in BDNF levels in hippocampus and prefrontal cortex.

LISTA DE ABREVIATURAS

Akt	proteína cinase B
AMPA	alfa-amino-3-hidroxi-5-metil-4-isoxazol-propiônico
AMPH	D-anfetamina
ANOVA	análise de variância
BD	*Bipolar disorder*
BDNF	fator neurotrófico derivado do cérebro
BSA	albumina sérica bovina
CamKII	proteína cinase dependente de cálcio-calmodulina tipo II
cAMP	adenosina monofosfato cíclico
cDNA	DNA complementar
CREB	proteína de ligação ao elemento de resposta ao cAMP
DSM-IV	Manual para Diagnóstico e Estatística da Associação Psiquiátrica Americana, quarta revisão
eiF4E	fator de iniciação eucariótico 4E
ELISA	Ensaio imunossorvente ligado a enzima
Elk-1	proteína do tipo E-26 1
ERK	cinase regulada por sinal extracelular
GABA	ácido gama-aminobutírico
LTD	depressão de longa duração
LTM	memória de longa duração
LTP	potenciação de longa duração
MAPK	proteína cinase ativada por mitógeno
MAPKK	proteína cinase cinase ativada por mitógeno
mRNA	RNA mensageiro
mTOR	alvo da rapamicina em mamíferos
NGF	fator de crescimento neural
NMDA	N-metil-D-aspartato
PBS	salina tamponada com fosfato
PCR	reação em cadeia da polimerase
pCREB	proteína de ligação ao elemento de resposta ao cAMP fosforilada
PI3K	fosfatidil-inositol 3-cinase
PKA	proteína cinase A
PLC-γ	fosfolipase-C gama
PP1	proteína fosfatase 1
proBDNF	precursor do fator neurotrófico derivado do cérebro
qPCR	reação em cadeia da polimerase quantitativa
RE	retículo endoplasmático
SNC	sistema nervoso central
THB	Transtorno de Humor Bipolar
Trk	receptor tirosina cinase

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1. INTRODUÇÃO

1.1 Transtorno de Humor Bipolar

O Transtorno de Humor Bipolar (THB) é um transtorno mental complexo, multifatorial e potencialmente grave associado a episódios recorrentes e elevada morbidade clínica (Kilbourne *et al.,* 2004). Sua prevalência estimada é de aproximadamente 1% ao longo da vida (Merikangas, 2007), podendo chegar a 3% considerando as formas mais brandas do transtorno. Estudos populacionais sugerem que a prevalência do THB é semelhante entre homens e mulheres, sendo que o início dos sintomas ocorre geralmente entre o fim da adolescência e o início da vida adulta (Weissman, 1991).

A característica essencial do THB tipo I é um curso clínico caracterizado pela ocorrência de um ou mais episódios maníacos ou episódios mistos. Os indivíduos apresentam também com freqüência, um ou mais episódios depressivos. O THB tipo II caracteriza-se pela presença de episódios depressivos maiores e episódios hipomaníacos. Ambos apresentam sintomas que envolvem alterações no humor, cognição e comportamento. A intensidade dos sintomas é variável, acarretando prejuízos no desempenho das atividades diárias e sofrimento pessoal altamente incapacitante.

Os episódios de mania caracterizam-se por humor elevado ou euforia, hiperatividade com falta de necessidade de sono, fuga de idéias, aceleração do pensamento, aumento do impulso sexual, agressividade, pressão de fala e taquilalia, paranóia, impulsividade e um otimismo aumentado que geralmente se torna tão extremo a ponto de prejudicar o julgamento

dos pacientes (Belmaker, 2004). É a intensidade, o tipo e a cronicidade desses sintomas que determinam a subdivisão do diagnóstico entre mania ou hipomania. Na hipomania, as alterações são mais moderadas e podem ou não resultar em sérios problemas para o indivíduo. O episódio maníaco, segundo o Manual para Diagnóstico e Estatística da Associação Psiquiátrica Americana, quarta revisão (DSM-IV), define o diagnóstico do THB tipo I e, portanto, a diferença para o THB tipo II, onde apenas episódios hipomaníacos estão presentes.

Os episódios de depressão bipolar caracterizam-se por humor deprimido, perda de interesse, perda de apetite, perturbações no sono, retardo psicomotor, diminuição da velocidade de pensamento e fala, baixa autoestima e ideação suicida (Belmaker, 2004). Assim como nos episódios maníacos, a gravidade pode variar consideravelmente – de uma discreta lentificação física e mental, com quase nenhuma distorção cognitiva ou da percepção, até quadros de estupor depressivo, com delírios, alucinações e obnubilação da consciência (Goodwin & Jamison, 2007).

1.2 Neuroprogressão do THB

O THB apresenta um prognóstico a longo prazo muito pior do que anteriormente imaginado. Os pacientes frequentemente apresentam sintomas subsindrômicos associados a prejuízos cognitivos persistentes e declínio funcional decorrentes da progressão do transtorno (Kapczinski *et al*., 2008a). Há evidências mostrando que os pacientes em estágios iniciais da doença apresentam desfechos clínicos muito melhores do que aqueles com múltiplos episódios (Tohen *et al.*, 1990; Schuepbach *et al*., 2008). Por exemplo, pacientes com maior duração de doença ou aqueles que tiveram mais de três episódios são menos propensos a

responder ao tratamento farmacológico, particularmente ao lítio (Gelenberg *et al.*, 1989; Swann *et al*., 1999). Além disso, a duração dos períodos interepisódicos diminui à medida que o número de episódios aumenta (Kessing, 1998).

Essas observações clínicas são consistentes com estudos de imagem mostrando diferenças anatômicas em estruturas cerebrais específicas em decorrência da evolução da doença. Estudos morfométricos mostram que pacientes com THB apresentam alterações em várias estruturas em comparação a controles (Lopez-Larson *et al*., 2002; Lyoo *et al.*, 2004, Lyoo *et al.*, 2006). Alguns autores sugerem que essas mudanças neuroanatômicas tendem a ser mais pronunciadas após repetidos episódios e correlacionam-se positivamente com o tempo de duração da doença (Strakowski *et al*., 2002; Lyoo *et al*., 2006). Além disso, pacientes com apenas um episódio maníaco apresentam mínimas alterações em estruturas cerebrais (Yatham *et al*., 2007).

1.2.1 Déficit cognitivo no THB

Pacientes com THB apresentam prejuízos cognitivos permanentes, e a extensão desses prejuízos é maior naqueles com múltiplos episódios em comparação com os pacientes que tiveram apenas um episódio de mania (Torres *et al*., 2007). O déficit cognitivo observado no THB está presente em todos os estados da doença, inclusive na eutimia, porém são acentuados durante os episódios de humor (Martínez-Arán *et al*., 2004; Vieta *et al*., 2010).

A importância da neurocognição reside na sua correlação com os desfechos funcionais, mesmo na ausência de sintomas residuais (Martínez-Arán *et al*., 2007). Melhores performances em atividades explorando a função executiva do córtex pré-frontal são

correlacionadas positivamente com o *status* ocupacional. Pacientes com maior duração da doença, maior número de hospitalizações, tentativas de suicídio e número de episódios prévios de mania tendem a apresentar piores desempenhos em atividades avaliando funções executivas e memória verbal (Vieta *et al*., 2010). Piores desfechos funcionais no THB também estão associados a um pior funcionamento pré-mórbido, morbidades depressivas, menores níveis educacionais, abuso de drogas, suporte social fraco e pobreza (Huxley e Baldessarini, 2007; Wingo *et al*., 2009).

Durante a eutimia, déficits cognitivos persistentes afetam a função executiva, memória verbal e atenção (Martinez-Arán *et al*., 2004; Robinson *et al*., 2006a; Mur *et al*., 2007). Essas disfunções cognitivas podem refletir padrões anormais de ativação cerebral (Benabarre *et al*, 2005; Strakowski *et al*., 2005), envolvendo o córtex pré-frontal na etiopatogênese do THB e sugerindo disfunções corticais-subcorticais-límbicas no transtorno (Malhi *et al*., 2004). Além disso, múltiplos episódios, particularmente maníacos e psicóticos, podem levar a prejuízos cognitivos mais graves (Martinez-Arán *et al*., 2004; Robinson *et al*., 2006b). Esses achados são corroborados por dados de neuroimagem, onde pacientes com múltiplos episódios apresentam ventrículos mais alargados e atrofia de substância cinzenta em comparação com pacientes em primeiro episódio (Strakowski *et al*., 2002).

1.3 Neurobiologia do THB

A neurobiologia do THB ainda não está totalmente esclarecida. Por ser um transtorno multifatorial, o THB pode ser resultante da interação entre fatores genéticos que causam susceptibilidade e fatores ambientais, como estresse e eventos traumáticos (Caspi e Moffitt,

2006; Barnett e Smoller, 2009). Um dos achados mais bem estabelecidos é o papel de fatores genéticos no THB, como evidenciado por estudos com gêmeos. Todos os estudos publicados a respeito mostraram uma maior concordância para o THB em gêmeos monozigóticos em comparação com gêmeos dizigóticos (revisado em Barnett e Smoller, 2009). Nas últimas duas décadas uma série de estudos de ligação e de associação foram realizados com o objetivo de buscar a base genética do transtorno, porém genes causais ou fatores de risco genéticos ainda não foram identificados. O fracasso na detecção de fatores genéticos robustos pode ser resultado de uma falta de homogeneidade clínica, baixo risco relativo de cada gene e/ou uma contribuição de múltiplas mutações raras (Barnett e Smoller, 2009).

Estudos de neuroquímica evidenciam a importância do neurotransmissor dopamina nos episódios de mania (Berk *et al*., 2007). Isso é evidenciado pelo fato que antipsicóticos com eficácia anti-maníaca são capazes de bloquear a neurotransmissão dopaminérgica (Yatham *et al*., 2002), além de que psicoestimulantes que aumentam os níveis de dopamina, como a D-anfetamina (AMPH), podem causar efeitos psicológicos semelhantes aos sintomas maníacos em indivíduos saudáveis (Mamelak, 1978). O episódio maníaco está associado a um aumento nos níveis de dopamina (Berk *et al*., 2007), e a administração de levodopa em pacientes com THB pode levar a episódios hipomaníacos (Peet e Peters, 1995).

Estudos neuroanatômicos indicam a presença de alterações no volume de regiões cerebrais específicas acompanhadas de atrofia ou perda celular no THB (Hajek *et al*., 2005). Estudos de imagem estrutural demonstraram volumes reduzidos de substância cinzenta em áreas do córtex orbital e pré-frontal medial, estriado ventral e hipocampo, assim como um alargamento significativo do terceiro ventrículo em comparação com controles saudáveis (Beyer e Krishnan, 2002). Estudos neuropatológicos *post-mortem* complementares mostraram

reduções anormais no volume cortical, redução na contagem de células gliais e/ou no tamanho dos neurônios do córtex pré-frontal subgenual, córtex orbital, córtex pré-frontal ântero-lateral e amígdala (Deep-Soboslay *et al*., 2008). Ainda não está bem estabelecido se essas anormalidades constituem déficits no desenvolvimento que conferem susceptibilidade a episódios de humor, mudanças compensatórias a processos patogênicos ou sequelas de episódios afetivos recorrentes *per se*. Pelo menos algumas dessas mudanças parecem ser reversíveis com o tratamento com estabilizadores de humor (Sassi *et al*., 2002), sugerindo os fatores neurotróficos como possíveis alvos no mecanismo de ação destes fármacos e um envolvimento destes na patofisiologia do THB.

1.3.1 Neurotrofinas

A família das neurotrofinas é composta por fatores regulatórios que medeiam a diferenciação e a sobrevivência de neurônios e modulam a transmissão e plasticidade sináptica (Bibel e Barde, 2000). Fatores neurotróficos endógenos foram descritos tradicionalmente como sendo capazes de aumentar a sobrevivência celular por prover o suporte trófico necessário para os neurônios; no entanto, o seu conjunto de ações também inclui a inibição da morte celular programada (Shaltiel *et al*., 2007). Os membros da família incluem o fator de crescimento neural (NGF), o fator neurotrófico derivado do cérebro (BDNF) e as neurotrofinas 3, 4, 5 e 6. Esses fatores podem ser secretados de forma constitutiva ou transitória e, frequentemente, de forma atividade-dependente (revisado em Shaltiel *et al*., 2007). Eles se ligam e ativam receptores tirosina-cinase (Trk) específicos e, consequentemente, vias de sinalização intracelular distintas (Barbacid, 1994).

Entre as neurotrofinas, o BDNF é a mais abundante no sistema nervoso central (SNC) e parece induzir efeitos neurotróficos e neuroprotetores de longo prazo (Murer *et al*., 2001). O BDNF também tem um papel na plasticidade sináptica e na liberação de neurotransmissores, facilitando a liberação de glutamato, ácido gama-aminobutírico (GABA), dopamina e serotonina (Tyler *et al*., 2002; Yoshii e Constantine-Paton, 2010). Já foi demonstrado que o estresse crônico diminui os níveis de BDNF no SNC de ratos, e a sua expressão é aumentada em diferentes regiões cerebrais após tratamento crônico com fármacos antidepressivos e estabilizadores de humor (Frey *et al*., 2006; Tankova *et al*., 2006). Tarefas de aprendizado estão associadas com um aumento dos níveis de mRNA de BDNF em ratos (Yamada e Nabeshima, 2003) e há indícios que o BDNF tem um papel importante na potenciação de longa duração (LTP) (Minichiello, 2009). Além disso, associação positiva entre os níveis séricos de BDNF e um teste de fluência verbal em humanos foi demonstrada, sugerindo, mais uma vez, a importância do BDNF em processos neurocognitivos (Dias *et al*., 2009).

Os efeitos biológicos do BDNF são exercidos pela ativação de pelo menos três vias de sinalização intracelular diferentes: fosfatidil-inositol 3-cinase/proteína cinase B (PI3K/Akt), fosfolipase C (PLC) e ERK-cinase ativada por mitógeno (ERK/MAPK) (Figura 1).

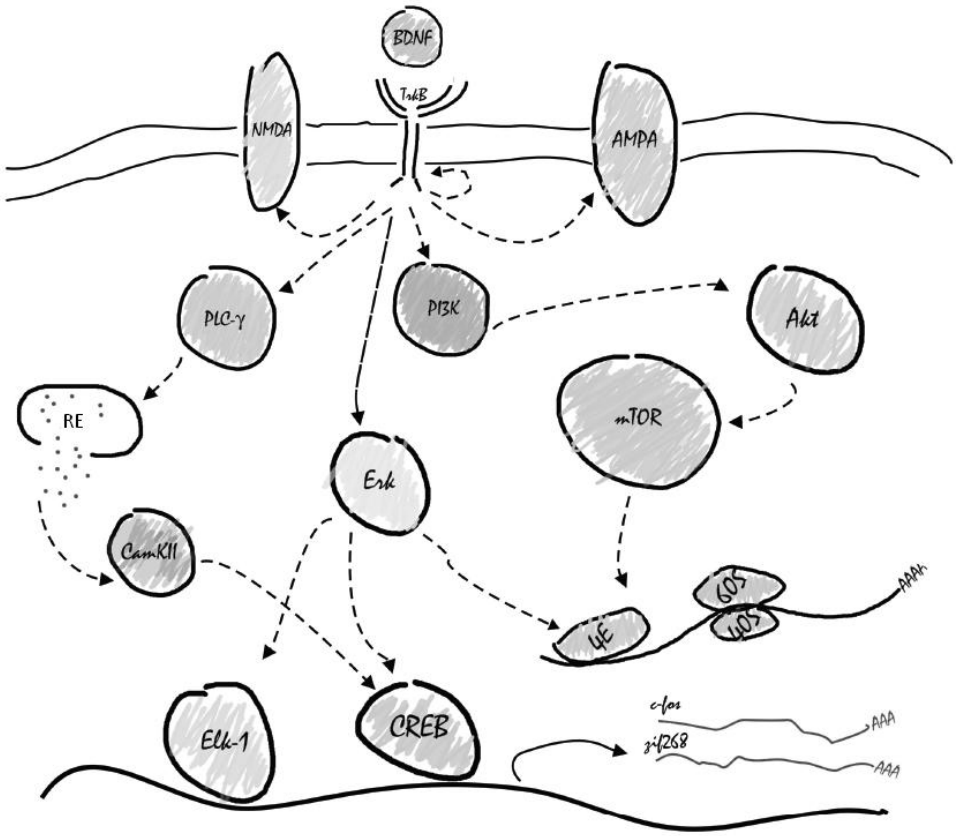

Figura 1. Vias de sinalização intracelular ativadas pela ligação do BDNF ao seu receptor TrkB. A ligação do BDNF ao TrkB pode rapidamente modular a atividade dos receptores glutamatérgicos NMDA e AMPA ou pode regular a transcrição e tradução através de três principais vias de sinalização intracelular que incluem a fosfatidil-inositol 3-cinase/proteína cinase B (PI3K/Akt), fosfolipase C-γ (PLC-γ) e a ERK/cinase ativada por mitógeno (ERK/MAPK). A ativação da PLC-γ leva à liberação de Ca^{2+} do retículo endoplasmático (RE) e ativação da cinase dependente de cálcio-calmodulina II (CamKII), resultando na fosforilação da proteína de ligação ao elemento de resposta ao cAMP (CREB) e ativação da transcrição. O BDNF pode também ativar a PI3K que, por sua vez, fosforila a proteína cinase B (Akt) e o alvo da rapamicina em mamíferos (mTOR) para regular o início da tradução através da modulação da atividade do fator de iniciação eucariótico 4E (eiF4E, ou 4E), entre outros substratos. A ativação da via da ERK/MAPK pode tanto regular a transcrição, pela fosforilação de CREB e da proteína do tipo E-26 1 (Elk-1), quanto a transcrição, por fosforilar eiF4E. A transcrição dependente de CREB pode envolver a síntese de mRNA de genes de expressão imediata como o c-fos ou o zif268 (adaptado de Bekinschtein *et al*., 2008).

A transcrição do gene *Bdnf* é regulada principalmente pela proteína de ligação ao elemento de resposta ao cAMP (CREB) (Chalovich *et al*., 2006). Este fator de transcrição precisa ser fosforilado a pCREB para ser translocado ao núcleo e ativar a transcrição dos genes regulados por ele, entre os quais o *Bdnf (*Finkbeiner, 2000). Uma vez sintetizado e

processado, o mRNA é traduzido em uma forma precursora da proteína, chamada proBDNF. O proBDNF pode ser clivado proteoliticamente no meio intracelular por enzimas e secretado como BDNF maduro, ou pode ser secretado como proBDNF e ser clivado por proteases extracelulares (revisado em Lessmann *et al*., 2003).

Níveis séricos de BDNF estão diminuídos em pacientes com THB em episódios maníacos e depressivos (Cunha *et al*., 2006; de Olivera GS *et al*., 2009). Além disso, Kauer-Sant'Anna e colaboradores (2009) compararam pacientes com THB que haviam tido apenas um episódio de humor e pacientes com múltiplos episódios, e mostraram que os níveis de BDNF diminuem com o número de episódios. Esses dados levaram à hipótese de que as mudanças relacionadas ao aumento no número de episódios devem explicar, pelo menos em parte, algumas das mudanças estruturais observadas em pacientes com THB. Os níveis de BDNF também foram correlacionados negativamente com a duração da doença (Kauer-Sant'Anna *et al*., 2009), sugerindo um importante papel desta neurotrofina na progressão do THB (Kapczinski *et al.*, 2008b).

Um polimorfismo de base única (do inglês *single nucleotide polymorphism* – SNP), caracterizado pela troca de uma guanina por uma adenina no nucleotídeo 196 do gene *BDNF*, leva a substituição de uma valina por uma metionina no códon 66 do BDNF em humanos (Val66Met). Este polimorfismo está associado a prejuízos em memória episódica em voluntários saudáveis (Egan *et al.*, 2003) e piora significativa na performance no Teste Wisconsin de Classificação de Cartas (WCST) em pacientes com THB Val/Met em comparação com pacientes com o genótipo Val/Val (Rybakowski *et al*., 2006). Além disso, um estudo prospectivo demonstrou que pacientes portadores do alelo Met apresentam uma maior redução do lobo temporal em comparação com pacientes Val/Val em um período de 4

anos de seguimento (McIntosh *et al.*, 2007). De uma forma geral, esses resultados sugerem fortemente um envolvimento significativo do BDNF na patofisiologia do THB e no declínio cognitivo associado à duração e à progressão da doença.

1.4 Modelo animal de mania

O conhecimento limitado acerca da neurobiologia do THB dificulta o desenvolvimento de modelos animais para o transtorno, uma vez que este apresenta-se de forma muito heterogênea na população e não há alvos bioquímicos ou genéticos específicos consolidados que possam ser manipulados para o desenvolvimento dos modelos. No entanto, a tarefa é necessária – praticamente todas as medicações existentes foram descobertas ao acaso ou resultaram de testes de medicações aprovadas para outros usos em modelos animais existentes (no caso, antipsicóticos e anticonvulsivantes).

No caso do THB, o desenvolvimento de modelos animais adequados é particularmente difícil pelo fato do transtorno ser cíclico e apresentar uma grande heterogeneidade clínica. Os conhecimentos acerca de sua patofisiologia são ainda limitados e existem poucos endofenótipos clínicos bem validados. Os mecanismos de ação das medicações estabilizadoras de humor e os dados a respeito de genes que conferem susceptibilidade também são insuficientes. A maioria dos modelos animais utilizados é, portanto, específica para o episódio de mania ou para o episódio de depressão e poucos se aventuram em mimetizar a ciclicidade e a recorrência típica do THB em animais (Post, 2007).

Existem diferentes abordagens para o desenvolvimento de modelos animais: eles podem basear-se em sintomas, endofenótipos/patofisiologia ou na resposta a medicamentos. Um dos modelos animais de mania mais bem consolidados na literatura é o modelo induzido pela administração de AMPH em ratos Wistar (Frey *et al*., 2006), baseando-se no fato que há um aumento nos níveis de dopamina em pacientes durante o episódio de mania. Este experimento é tipicamente dividido em reversão e prevenção da mania. No modelo de reversão, os ratos recebem administrações diárias de 2mg/kg de AMPH ou salina (SAL) por 14 dias seguidos e passam a receber também injeções de lítio 47,5mg/kg, valproato de sódio 200mg/kg ou SAL nos últimos 7 dias. Neste caso, avalia-se a reversão dos efeitos da administração de AMPH pelo uso das medicações, mimetizando o tratamento agudo de um episódio maníaco. No modelo de prevenção, os ratos recebem administrações diárias dos estabilizadores de humor (nas mesmas doses utilizadas no modelo de reversão) por 14 dias e, nos últimos 7 dias, passam a receber também injeções de AMPH ou SAL. Desta forma, avalia-se a prevenção dos efeitos da AMPH pelo uso prévio das medicações, mimetizando um tratamento de manutenção no THB com estabilizadores de humor. O teste comportamental comumente utilizado é o campo aberto, possibilitando a avaliação de parâmetros exploratórios e locomotores. As validades de constructo e preditiva do modelo tem sido comprovadas (Frey *et al*., 2006; Andreazza *et al.*, 2008; Walz *et al*., 2008; Valvassori *et al*., 2010), correlacionando achados nos animais com dados clínicos e bioquímicos observados em pacientes.

1.5 Memória

Memória pode ser definida como a aquisição, o armazenamento e a evocação (aprendizado) de informações. As memórias podem ser classificadas de acordo com diferentes critérios: função (trabalho *versus* referência), conteúdo (declarativa/explícita *versus* procedural/implícita), duração (imediata ou de curta duração *versus* remota ou de longa duração), natureza (associativa *versus* não-associativa) ou motivação (apetitiva/recompensa *versus* aversiva) (Quillfedt, 2010). Do ponto de vista eletrofisiológico, a modificação dependente do uso de sinapses é um dos mecanismos celulares primários responsáveis pela formação de memória e pelo aprendizado.

Um dos mecanismos eletrofisiológicos mais estudados na formação de memórias é a chamada potenciação de longa duração (LTP) (Bliss e Collingridge, 1993). Este fenômeno corrobora a hipótese que o aprendizado envolve plasticidade sináptica e é definido como um aumento prolongado da magnitude da resposta sináptica de um neurônio pós-sináptico após uma estimulação curta e de alta freqüência de um neurônio pré-sináptico (Lent, 2008). A LTP já foi observada em diferentes regiões do SNC (hipocampo, córtex cerebral, amígdala e cerebelo) e é um fenômeno típico de sinapses excitatórias glutamatérgicas (Squire e Kandel, 2009). Envolvimento da sinalização induzida pelo receptor TrkB na LTP *in vitro* e *in vivo* foi demonstrado em modelos de aprendizado (Minichiello, 2009). A atividade neuronal que leva à LTP aumenta fortemente a expressão de BDNF em neurônios hipocampais em fatias cerebrais, sugerindo que sinais sinápticos fisiológicos podem induzir a liberação pós-sináptica de BDNF endógeno (Pang e Lu, 2004). Da mesma maneira, a deleção do gene *Bdnf* em camundongos *knockout* prejudica a indução normal da LTP precoce (E-LTP) na região CA1 em fatias hipocampais (Korte *et al.*, 1995). A importância do BDNF na consolidação da LTP

tardia (L-LTP) foi sugerida por estudos que mostram que esta neurotrofina estimula a síntese e o rápido transporte para os dendritos de moléculas de mRNA do gene *Arc* (proteína associada ao citoesqueleto regulada pela atividade de genes de expressão imediata), cuja função na L-LTP já foi anteriormente mostrada (revisado em Bramham, 2008). Achados recentes indicam também que a síntese de BDNF durante a L-LTP é crucial para a persistência da memória (Bekinschtein *et al*., 2007), o que comprova ainda mais a hipótese de que a estimulação do TrkB é fundamental para os mecanismos de plasticidade sináptica.

1.5.1 Testes comportamentais para o estudo da memória

A teoria da consolidação da memória, proposta por Müller e Pilzecker em 1900, é um dos paradigmas fundamentais da psicobiologia e neurofarmacologia da memória (McGaugh, 1966; Dudai, 2000, em Quillfeldt 2010). Uma consequência desta teoria é a possibilidade do planejamento das intervenções experimentais em dois ou três momentos na formação e evocação da memória. A formação da memória não é um processo instantâneo, sendo dividida em aquisição (melhor conhecida como aprendizado) e consolidação (a fase lábil durante a qual o traço de memória torna-se fisicamente armazenado). A evocação da memória, por sua vez, ocorre durante a re-exposição ao contexto de aprendizado, com ou sem o estímulo anteriormente recebido (e é a única forma de se provar que a memória foi realmente formada e armazenada) (Quillfeldt, 2010).

A aquisição da memória pode ocorrer após um ou vários *trials* de aprendizado (treino), quando os animais são expostos ao contexto controlado para diferentes variáveis. Imediatamente após o treino, e ainda durante várias horas, o traço de memória passará por

uma fase lábil (consolidação), estando suscetível a distúrbios que podem modificar o dado originalmente adquirido. Um teste de evocação de memória de longa duração (LTM) pode ser realizado em diferentes intervalos de tempo pós-treino, mas há um consenso na literatura de que este deve ocorrer no mínimo 6 horas após (geralmente após 24 horas) (Quillfeldt, 2010).

Diferentes testes comportamentais possibilitam o estudo de diferentes tipos de memória. O teste de habituação ao campo aberto, por exemplo, mede a memória do tipo não aversiva clássica não associativa. Este consiste em expor o animal a uma área aberta (*open field*), um novo ambiente sem qualquer estímulo aversivo ou apetitivo, e deixá-lo explorar a área livremente por um período pré-estabelecido de tempo (treino). Vinte e quatro horas após, o rato é re-exposto ao mesmo ambiente e o seu comportamento exploratório é observado. Uma diminuição significativa no comportamento exploratório no teste em comparação ao treino é um indício de habituação ao ambiente e, portanto, de aprendizado.

Outro teste comportamental muito utilizado é o teste de esquiva inibitória. Este teste envolve o aprendizado de inibição de uma resposta para evitar um estímulo aversivo. Na prática, este consiste em posicionar um rato sobre uma plataforma localizada ao lado de uma área metálica ligada a um circuito elétrico, de forma que, quando o animal explora o ambiente e desce da plataforma, ele recebe um choque curto de intensidade moderada. A memória é, portanto, do tipo aversiva. Vinte e quatro horas após o treino, o animal é reposicionado na plataforma e o tempo que ele demora para descer da mesma é contado. Um aumento significativo na latência para descer da plataforma é um indicativo de aprendizado. Este tipo de memória é de difícil classificação, pois envolve tanto um componente explícito e associativo (ao contexto) quando um condicionamento do tipo operante (ao choque), sendo, este último, considerado um tipo de memória implícita (Quillfeldt, 2010).

1.6 Justificativa

O THB é um transtorno psiquiátrico grave e altamente incapacitante e a sua progressão está associada a prejuízos cognitivos e alterações neuroanatômicas aparentemente irreversíveis. Até o presente momento, poucos estudos foram realizados para explorar as bases biológicas da neuroprogressão do THB, porém há fortes evidências de que as neurotrofinas tem um importante papel nestes mecanismos. Os níveis séricos de BDNF encontram-se diminuídos em pacientes com longo tempo de doença em comparação com pacientes com apenas um episódio, o que pode explicar, pelo menos em parte, o declínio cognitivo observado. Além disso, estudos recentes sugerem um papel fundamental da sinalização induzida por BDNF/TrkB nos processos de memória em diferentes regiões cerebrais, o que sugere que mecanismos dependentes de neurotrofinas ocorrendo no SNC podem estar associados com a progressão do THB. Um maior conhecimento acerca da neurobiologia desta progressão pode fornecer evidências fundamentais para o estudo de tratamentos profiláticos ou de manutenção da eutimia para pacientes com THB. Além disso, o estudo de regiões cerebrais específicas possibilitará um maior entendimento do papel de cada uma delas nos processos de neuroprogressão e, portanto, da patofisiologia do transtorno.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a memória e os níveis cerebrais de BDNF em um modelo animal de transtorno bipolar em estágio precoce e tardio.

2.2 Objetivos específicos

- Mimetizar os estágios precoce e tardio do transtorno bipolar em ratos com diferentes tempos de tratamento com D-anfetamina;
- Avaliar a memória de longa duração dos ratos no teste de esquiva inibitória;
- Avaliar a memória de habituação de longa duração dos ratos no teste de habituação ao campo aberto;
- Quantificar os níveis de mRNA do gene *Bdnf* e os níveis protéicos de BDNF no córtex pré-frontal, região da amigdala e hipocampo dos ratos.

PARTE II

3. RESULTADOS

Os resultados deste trabalho serão apresentados sob a forma de um artigo científico submetido para publicação na revista *The International Journal of Neuropsychopharmacology*.

3.1 Capítulo I

Assessment of memory and BDNF levels in an animal model of early and late stages of bipolar disorder

Title: Assessment of memory and BDNF levels in an animal model of early and late stages of bipolar disorder

Short title: Memory and BDNF in an animal model of bipolar disorder

Category: *Regular research article*

Authors: Gabriel Rodrigo Fries[1,3,4,5], Samira S Valvassori[2,3], Hugo Bock[4,5], Laura Stertz[1,3], Edemilson Mariot[2,3], Roger B Varela[2,3], Márcia Kauer-Sant'Anna[1,3], João Quevedo[2,3], Flávio Kapczinski[1,3], Maria Luiza Saraiva-Pereira[4,5]

Authors' affiliation:

[1]Bipolar Disorder Program and Laboratory of Molecular Psychiatry, Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil

[2]Laboratory of Neurosciences, Universidade do Extremo Sul Catarinense, Criciúma, Santa Catarina, Brazil

[3]INCT of Translational Medicine, Brazil

[4]Laboratory of Genetic Identification and Medical Genetic Service, Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil

[5]Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Brazil

Corresponding author:

Maria Luiza Saraiva-Pereira

Laboratório de Identificação Genética – Centro de Pesquisa Experimental

Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2350

CEP 90035-903 – Porto Alegre – RS – Brazil

Phone: + 55 51 3359-7689

Fax: + 55 51 3359-8010

Email: mlpereira@hcpa.ufrgs.br

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Abstract

Bipolar disorder (BD) is a severe psychiatric disorder whose progression has been associated with cognitive impairments. Evidences suggest that brain-derived neurotrophic fator (BDNF) plays an important role in BD neuroprogression. We aimed to evaluate memory and BDNF levels in an amphetamine (AMPH)-induced animal model of mania, in early and late stages of BD. Adult male Wistar rats were divided into early (saline or AMPH 2mg/kg ip for 7 days) and late groups (saline or AMPH 2 mg/kg for 35 days). After the open-field habituation or inhibitory avoidance test, the rats were killed and the hippocampus, prefrontal cortex and amygdala region were isolated. BDNF mRNA and protein levels were measured by quantitative Real Time PCR and ELISA, respectively. AMPH impaired habituation memory both in early and late treatments, but worsened in the late group. This was accompanied by reduced BDNF protein in hippocampus and increased BDNF mRNA in prefrontal cortex of late AMPH-treated rats. In the inhibitory avoidance, all groups learned the aversive stimulus, although AMPH has significantly decreased latency to step-down in the test compared to saline. No difference was observed between late and early treatments, although late AMPH-treated rats presented reduced BDNF protein in prefrontal cortex and increased mRNA in hippocampus compared to early AMPH-treated rats. These results suggest that the cognitive impairment in BD may be associated with alterations in BDNF levels in hippocampus and prefrontal cortex.

Key words: bipolar disorder, memory, BDNF

Introduction

Bipolar disorder (BD) is a severe chronic psychiatric disorder with an estimated prevalence of approximately 1% (Merikangas et al., 2007). The manic phase, one of its main features, is characterized by elated mood, pressure to keep talking, grandiosity, hyperactivity, flight of ideas and diminished need for sleep, and is typically intercalated with depressive and euthymic phases (Belmaker, 2004). Even though the acute mood episode itself has been associated with cognitive and functional impairments (Martinez-Aran et al., 2004), evidences suggest that they tend to get worse with disease progression, especially with the increase of number of acute episodes (Elshahawi et al., 2010; Torres et al., 2007). Longer duration of BD has been associated with many types of impaired neuropsychological performances in patients (Denicoff et al., 1999), and risk of dementia seems to increase with the number of episodes (Kessing and Andersen, 2004). In addition, longer duration of BD has been associated with reduced cerebral cortical thinning, which could partially begin to explain the progressive decline observed in cognitive function (Lyoo et al., 2006).

Among other changes related to illness progression, BD patients at a late stage of the illness present lower serum levels of brain-derived neurotrophic factor (BDNF) when compared to BD patients at an early stage and controls (Kauer-Sant'Anna et al., 2009), which suggests a reduction in neuroplasticity along with BD progression. BDNF is a member of the neurotrophin family that mediates differentiation and survival of neurons, and modulates synaptic transmission and plasticity (Shaltiel et al., 2007). In adult nervous system, BDNF displays a widespread distribution pattern, with high levels of mRNA and protein in hippocampus, amygdala, cerebral cortex, and hypothalamus

(Pruunsild et al., 2007). Reduced BDNF levels were observed in the serum of BD patients in manic and depressive episodes when compared to euthymic and controls (Cunha et al., 2006), and it is believed to be one of the key molecules involved in the neurobiology and treatment of BD (Kapczinski et al., 2008). BDNF plays also key roles in memory processes, promoting long-term potentiation (LTP) and inhibiting long-term depression (LTD) (reviewed by Cunha et al., 2010), and can modulate protein synthesis by transcriptional and translational mechanisms, which corroborates the idea of being required in the consolidation of long-term memory (LTM) (reviewed by Bekinschtein et al., 2008).

Manic episode has been consistently associated with an increase in dopamine levels (reviewed by Berk et al., 2007), and it is well recognized that administration of levodopa to patients with BD can lead to hypomanic episodes (Peet and Peters, 1995). In addition, direct acting dopamine agonists may induce mania in some BD patients, and it seems that selective dopamine antagonists are effective in controlling symptoms of mania (Yatham et al., 2002). Given the involvement of dopamine in cognitive processes and its relevance to the BD pathophysiology, dopamine-enhancing drugs appear to be interesting tools in the development of BD animal models. Among them, the psychostimulant-induced rodent animal model of mania has been shown to present predictive, construct and face validity, and seems to be reliable and useful in preclinical studies regarding BD (Andreazza et al., 2008; Machado-Vieira et al., 2004; Walz et al., 2008). In the model first described by Frey et al. (2006), the amphetamine (AMPH)-induced hyperactivity in rats was reversed and prevented by chronic injections of mood stabilizers. As a limitation, its face validity has usually focused primarily on

hyperactivity as a symptom resembling mania, and no further attention has been given to other behavioral features, including the cognitive decline seen in patients.

Up to date, no study has been conducted in order to understand the cognitive impairment induced by BD progression and its relationship with BDNF levels in different brain areas. Therefore, we sought to investigate two types of memory, habituation and inhibitory avoidance, in a psychostimulant-induced animal model of mania and the following BDNF changes induced in hippocampus, amygdala region and prefrontal cortex, all of which have distinct key roles in memory processes and have been implicated in BD pathophysiology.

Materials and Methods

Animals

Adult male Wistar rats (weight 250-350 g) were obtained from our local breeding and kept at the animal experimentation area of the Universidade do Extremo Sul Catarinense (UNESC). The animals were housed 5 per cage, with food and water ad libitum under a 12 hour light/dark cycle. All experimental procedures were performed with the approval of local ethics committee and according to recommendations of NIH Guide for the Care and Use of Laboratory Animals and Sociedade Brasileira de Neurociências e Comportamento (SBNeC).

Treatment

Treatment protocols were performed as follows: rats were randomly assigned in 4 groups: 1) early saline (7 days of saline i.p.); 2) early amphetamine (7 days of 2 mg/kg AMPH i.p.); 3) late saline (35 days of saline i.p.) and 4) late amphetamine (35 days of 2 mg/kg AMPH i.p.). In the late groups, injections occurred once a day for 7 days followed by 7 days of washout (no injection), and this protocol was repeated 5 times. One week after the last injection, all animals were subjected to behavioral tests (step-down inhibitory avoidance or habituation to open-field).

Behavioral tests

Habituation to open-field

Habituation to an open-field was carried out in a 40 × 60 cm open-field surrounded by 50-cm high walls made of brown plywood with a frontal glass wall. The floor of the open-field was divided into 12 equal rectangles by black lines. The animals

were gently placed on the left rear quadrant and left to explore the arena for 5 min (training session). Immediately afterwards, the animals were taken back to their home cages, and 24 h later submitted again to a similar open-field session (test session). Crossings of the black lines and rearings performed in both sessions were counted. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of habituation retention.

S**tep-down inhibitory avoidance test**

The inhibitory avoidance apparatus consisted of an acrylic box (50 × 25 × 25 cm) whose floor consisted of parallel-caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart, and a platform that was 7 cm wide and 2.5 cm high. Animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Training sessions were performed 7 days after the last injection. Immediately after stepping down on the grid, animals received a foot shock of 0.3 mA per 2.0 seconds. In test sessions carried out 24 hours after training, no foot shock was given and the step-down latency (maximum of 180 seconds) was used as a measure of retention. Behavioral tests were performed by the same person which was blind to experimental groups.

Isolation of brain structures

Right after the behavioral tests, animals were killed by decapitation and different brain structures were isolated: prefrontal cortex, hippocampus, and amygdala region. One hemisphere was immediately submerged in RNA stabilizing reagent (RNA*later*®, Ambion) and kept overnight at 4ºC, followed by long-term storage at -20ºC. The other hemisphere was stored at -80ºC until further analyses.

RNA extraction and cDNA synthesis

Total RNA was isolated from brain structures submerged in RNA*later*® (Sigma) using TRI Reagent® (Sigma), according to manufacture's instructions. RNA concentrations were evaluated using the fluorimetric method Quant-IT RNA Assay (Invitrogen) in the Qubit (Invitrogen) equipment. RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to manufacter's instructions. The amount of isolated RNA used for reverse-transcription reactions was 200 ng in a total reaction volume of 10 μL containing 2 μl of 10x RT buffer, 0.8 μl of 25x dNMT Mix (100 mM), 2 μl of 10x RT Random Primers and 1 μl of MultiScribe Reverse Transcriptase (50 units/ml). Reactions were performed for 10 min at 25ºC, 2 h at 37ºC and 5 s at 85ºC. Subsequently cDNA was kept at -20ºC until used for PCR amplification. A 1:5 dilution of cDNA was performed in water before qPCR.

Quantitative real-time PCR (qPCR)

The expression of *Bdnf* was measured by qPCR using an inventoried TaqMan FAM/MGB assay (Applied Biosystems, ID assay Rn01484928_m1). Expression values were normalized by beta-actin endogenous control expression using a TaqMan VIC/MGB endogenous control inventoried assay (Applied Biosystems, 4352340E). Reactions were performed in ABI Prism 7500 Fast equipment. Reactions were carried out in a total volume of 12 μl containing 6 μl of 2x TaqMan Gene Expression Master Mix (containing ROX, Amplitaq Gold DNA polimerase, AmpErase UNG, dATP, dCTP, dGTP, dUTP, and MgCl2), 0.6 μl of 20x TaqMan Gene Expression Assay, 0.6 μl of 20x TaqMan Endegenous Control, 3.8 μl of water and 1 μl of cDNA solution.

Cycling program consisted of 2 min at 50ºC and 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. All reactions were performed in triplicates. Relative expression levels were determined by the ddCt method, according to Livak and Schmittgen (2001).

Quantification of BDNF protein levels

Samples were homogenized in phosphate buffer solution (PBS) containing protease inhibitor cocktail (Sigma). BDNF levels in hippocampus, amygdala region, striatum, prefrontal cortex and serum were determined by sandwich-ELISA using monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, Minnesota). Briefly, microtiter plates (96-well flat-bottom) were coated overnight at room temperature with the monoclonal anti-BDNF antibody at 4 μg/ml in PBS. Then, plates were washed three times with wash buffer (PBS, pH 7.4, with 0.05% Tween 20) and were blocked for 1 hour at room temperature with PBS containing 5% nonfat milk powder. After washing, plates were coated for 2 hours at room temperature with the samples diluted 1:2 in sample diluent (PBS with 1% BSA) and standard curve ranged from 7.8 to 500 pg/mL of BDNF. Plates were washed again and a biotinylated anti-BDNF antibody was added at 0.2 μg/mL in PBS, followed by incubation for 2 hours at room temperature. After washing, incubation with streptavidin-peroxidase conjugate (diluted 1:200 in sample diluent) was performed for 20 min at room temperature. Afterwards, plates were washed and incubated with substrate for 20 minutes at room temperature. Finally, stop solution (H_2SO_4 1M) was added and the amount of BDNF was determined by reading the absorbance at 450 nm. The standard curve demonstrated a direct relationship between optical density (OD) and BDNF concentration. Total protein was measured by Bradford's method using bovine serum albumin as a standard.

Statistical analyses

The statistical analyses were performed in the SPSS 16.0 software for Windows (SPSS, Inc.). Data are presented as mean ± standard deviation (SD), and were all fitted to normal distribution, except for the inhibitory avoidance data (these are presented as median and interquartile range). For the open-field habituation data, paired t-test was used to compare Training *vs.* Test values, and independent t-test was used to compare AMPH *vs.* saline groups. To compare early *vs.* late groups, independent t-test was performed using the variation between training and test values. For the inhibitory avoidance test, we performed Wilcoxon signed-rank tests to compare Training *vs.* Test values of latency to step-down, and Mann-Whitney test to compare AMPH *vs.* saline groups. One-way ANOVA was performed for comparing means between groups of the biochemical data, followed by post-test of Tukey. In all cases, $p<0.05$ was used as index of significance.

Results

Open-field habituation

Saline-treated rats showed significantly lower crossings and rearings in the Test when comparing with Training, both in Early (paired t-test; crossings – $t(13)=2.47$, $p=0.28$ and rearings – $t(13)=2.707$, $p=0.018$) and Late treatments (paired t-test; crossings – $t(14)=8.4$, $p=0.00$ and rearings – $t(14)$-6.036, $p=0.00$) (Figure 1). No such difference was found in AMPH-treated rats, although crossings on the Test were significantly higher than in the Late AMPH group when compared to Late saline (independent t-test; $t(23)=-2.763$, $p=0.01$). Memory was significantly worse in AMPH Late group when compared to AMPH Early group, as seen by crossings values (independent t-test; $t(25)=2.636$, $p=0.014$).

Inhibitory avoidance

Both saline- and AMPH-treated rats presented significantly higher latency to step-down when comparing Test and Training values in Early (Wilcoxon signed-ranks test; saline – $Z=-2.803$, $p=0.005$; AMPH – $Z=-2.347$, $p=0.019$) and Late (Wilcoxon signed-ranks test; saline – $Z=-3.408$, $p=0.001$; AMPH – $Z=-2.927$, $p=0.003$) treatments (Figure 2). AMPH-treated rats showed significantly lower latency times in Test when compared to saline-treated rats in Early (Mann-Whitney test; $U=3.5$, $Z=-3.519$, $p=0.000$) and Late (Mann-Whitney test; $U=13$, $Z=-4.137$, $p=0.000$) treatments. No difference was found between Early and Late treatments.

BDNF mRNA levels

BDNF mRNA level results are presented in Figure 3. In rats who underwent open-field habituation, prefrontal cortex mRNA were significantly higher in Late AMPH group when compared to Early AMPH group (one-way ANOVA; $F(3,51)=7.158$, $p=0.000$). No other differences were observed.

In rats who underwent inhibitory avoidance test, hippocampal BDNF mRNA levels were significantly higher in Late AMPH group when compared to Late saline group (one-way ANOVA; $F(3,52)=8.437$, $p=0.004$) and Early AMPH group (one-way ANOVA; $F(3,52)=8.437$, $p=0.000$). No other differences were observed.

BDNF protein levels

BDNF protein level results are presented in Figure 4. Hippocampal BDNF protein levels were significantly lower in Late AMPH group when compared to Early AMPH for those rats who underwent open-field habituation (one-way ANOVA; $F(3,54)=3.931$, $p=0.008$). In the inhibitory avoidance test, prefrontal cortex BDNF protein levels were reduced in Late AMPH rats when compared to Early AMPH rats (one-way ANOVA; $F(3,53)=3.941$, $p=0.016$). No further differences were found to be significant among groups.

Discussion

Our results showed that AMPH impairs open-field habituation memory both in early and late treatments, which suggests that this kind of hippocampus-dependent memory is very sensitive to AMPH. We have also found that impairment by late treatment was significantly more than that by early one as seen by variation difference between training and test trials comparing early and late AMPH groups. These impairments in behavioral test were accompanied by BDNF protein levels reduction in hippocampus and BDNF mRNA levels increase in prefrontal cortex in the late stage group. In the inhibitory avoidance learning, on the other hand, all groups showed significant differences in latency to step-down between training and test trials. This indicates that even AMPH-treated rats have managed to learn aversive stimulus. However, AMPH has significantly decreased the latency to step-down in the test when compared to saline-treated rats, which suggests an AMPH-induced impairment in this memory task. No difference in behavior was observed between late and early treatments for this test, although late AMPH-treated rats presented a reduction of BDNF protein levels in prefrontal cortex and an increase of BDNF mRNA levels in hippocampus when comparing to early AMPH-treated rats. As far as we are aware, this is the first study ever to evaluate habituation and inhibitory avoidance memory in an animal model of mania and the BDNF levels associated with these behaviors.

Stimulants such as AMPH have been shown to improve as well as impair different memory tasks, and there are evidences that suggest dose-dependent effect on memory (Arnsten, 2006). AMPH acts by increasing dopamine levels in the synaptic cleft that have been previously shown to regulate the expression of essential proteins for

the establishment of lasting neuronal plasticity (Küppers and Beyer, 2001). Different doses and treatment schemes of AMPH have been used to act on memory acquisition, consolidation and recall in different ways. Object recognition was impaired in AMPH-treated male and female rats (Bisagno et al., 2003), and impairment has also been found in short-term memory of a spatial delayed matching-to-sample task after AMPH treatment (Kesner et al., 1981). Although AMPH enhances LTP at low doses, it impairs LTP at high doses, which suggests a biphasic action of this drug on memory (Xu et al., 2010). This effect appears to be due to, at least partially, activation and inactivation of the intracellular cAMP-PKA signaling cascade coupled mainly to dopamine receptors (Xu et al., 2010). Moreover, chronic AMPH treatment can be neurotoxic (Rodríguez-Casado et al., 2007; Yamamoto et al., 2010) in selected brain areas, which might contribute to the treatment-induced memory impairment.

The two memory behavioral tasks used in our study seem to be differentially affected by AMPH treatment. The open-field habituation, one of the most elementary forms of learning, was shown to be more sensitive to AMPH, being impaired significantly by both early and late treatments. There are some evidences suggesting that this kind of memory depends basically on hippocampus, and does not require as many signaling pathways activation as required by inhibitory avoidance task (Vianna et al., 2001). This task, on the other hand, seems to depend mainly on entorhinal cortex, parietal cortex, amygdala, and hippocampus (Izquierdo et al., 1997; Zanatta et al., 1997), and all of them seem to be essential for memory retrieval one day after training (Izquierdo et al., 1997). The differential neurotoxic action of AMPH in specific brain areas might explain the difference in modulation of these different types of memory.

Although consistently reported, the mechanisms of AMPH-induced dopaminergic neurotoxicity are still unclear. Angelucci et al. (2007) found that chronic AMPH treatment can reduce levels of nerve growth factor (NGF) and BDNF in selected brain regions, and a reduction in hippocampus has also been reported (Frey et al., 2006). BDNF has been involved in consolidation of many types of memories in different brain areas, being critical for persistence of LTM storage in the hippocampus (Bekinschtein et al., 2008). The time point when BDNF is increased during memory formation has not been completely elucidated. There are evidences suggesting that BDNF achieves a peak of concentration few hours after the training session (in the consolidation phase) (Rossato et al., 2009), but little is known concerning its levels right after memory recall (as assessed by our study).

Some considerations have to be taken into account when analyzing our data. As mentioned above, AMPH treatment itself might be able to induce modifications on BDNF levels. Such modification could not be assessed by our work considering that all animals were assigned to a behavioral test after the treatment, and BDNF levels were not assessed before these tests. There has been shown that memory behavioral tests, including those used in our work, might be able to modulate BDNF levels as well. We cannot rule out that levels measured in our samples are a consequence of such tests, and not specifically of the pharmacological treatment previously performed. This surely restricts our conclusions on AMPH-induced modulation of BDNF levels, but also leads to an interesting observation that AMPH has somehow an effect on test-induced changes in BDNF. Up to date, the mechanisms by which those tests modulate BDNF levels remain unclear. A sequence of biochemical events in the hippocampus and other brain areas has been described during memory behavior tests, leading to

phosphorylation and activation of transcription factor CREB (cAMP response element-binding) (reviewed by Cunha et al., 2010). CREB has been shown to activate BDNF gene expression *in vitro* and *in vivo*, and may be responsible for modulation of BDNF levels observed in behavior tests (Ou and Gean, 2007; Tao et al., 1998). Evidences suggest that BDNF is required in the consolidation phase of those memories (reviewed by Bekinschtein et al., 2008), but little is known about its permanent changes or its function on memory retrieval. Further studies are essential to clarify this issue.

It is interesting to note that the two different memory tests modulated BDNF in different ways, suggesting a specific control of such levels according to type of memory being tested. Results presented here suggest that the impairment in open-field habituation is associated to reduction of BDNF protein levels in hippocampus and increase of mRNA levels in prefrontal cortex. It is possible that both areas somehow cross-talk in the modulation of BDNF levels, which is also supported by our data on inhibitory avoidance learning. In that case, the impairment was accompanied by reduction of BDNF protein levels in prefrontal cortex and an increase of mRNA levels in hippocampus. This increase in levels of mRNA with no increase in protein levels may be explained by the fact that the BDNF mRNAs are translated firstly into a precursor form of the protein, which is then later processed to its mature form (Chen et al., 2005). In our study, only the mature BDNF was assessed, thus we were unable to detect whether the increased mRNAs had already been translated. The amygdala region showed no significant difference in BDNF levels, which might suggest that this neurotrophin does not take part in the memory recall mechanism for these two behavioral tests in amygdala or that AMPH lacks neurotoxic/neurotrophic actions in this specific area of the brain.

Viana et al. (2001) suggested that biochemistry of the early period of consolidation of habituation is different from that of avoidance task or others, not involving LTP-like mechanisms sensitive to inhibitors of protein kinase A (PKA), mitogen-activated protein kinase kinase (MAPKK), D1 receptors, or N-methyl-D-aspartate (NMDA) receptors. The only resemblance between these two memories was found to be their sensitivity to a calcium-calmodulin dependent protein kinase II (CaMKII). There are no evidences of differences or similarities between them regarding memory recall and BDNF modulation. In this task further studies are required to clarify these mechanisms and to bring further insight into our results.

In conclusion, our results suggest that the cognitive impairment observed in BD patients may be related to alterations in BDNF levels in hippocampus and prefrontal cortex, and that these two brain areas may cross-talk in order to modulate such changes. Mechanisms by which those alterations occur were not yet fully described, but it seems to be dependent of type of memory being activated and of the number of previous mood episodes (here mimicked by AMPH injections). These data support the hypothesis of specific brain alterations occurring with the BD progression and strongly suggests that BDNF-related neuroplasticity mechanisms may play an important role in the episode-related cognitive decline showed in BD.

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References

Martinez-Aran A, Vieta E, Colom F, Reinares M, *et al.* (2004). Cognitive function across manic or hypomanic, depressed, and euthymic states in bipolar disorder. *American Journal of Psychiatry* **161**, 262–270.

Andreazza AC, Kauer-Sant'Anna M, Frey BN, Stertz L, *et al.* (2008). Effects of mood stabilizers on DNA damage in an animal model of mania. *Journal of Psychiatry and Neuroscience* **33(6)**, 516-24.

Angelucci F, Gruber SH, El Khoury A, Tonali PA, *et al.* (2007). Chronic amphetamine treatment reduces NGF and BDNF in the rat brain. *European Neuropsychopharmacology* **17**(12), 756-62.

Arnsten AF (2006). Stimulants: Therapeutic actions in ADHD. *Neuropsychopharmacology* **31**(11), 2376-83.

Bekinschtein P, Cammarota M, Izquierdo I, Medina JH (2008). BDNF and memory formation and storage. *Neuroscientist* **14(**2), 147-56.

Belmaker RH (2004). Medical Progress: Bipolar Disorder. *New England Journal of Medicine* **351**, 476-86.

Berk M, Dodd S, Kauer-Sant'anna M, Malhi GS, *et al.* (2007). Dopamine dysregulation syndrome: implications for a dopamine hypothesis of bipolar disorder. *Acta Psychiatrica Scandinavica Supplementum* **434**, 41-9.

Bisagno V, Ferguson D, Luine VN (2003). Chronic D-amphetamine induces sexually dimorphic effects on locomotion, recognition memory, and brain monoamines. *Pharmacology, Biochemistry, and Behavior* **74**(4), 859-67.

Chen Z, Ieraci A, Teng H, Dall H, *et al.* (2005). Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *Journal of Neuroscience* **25**(26). 6156-6166.

Cunha AB, Frey BN, Andreazza AC, Goi JD, *et al.* (2006). Serum brain-derived neurotrophic factor is decreased in bipolar disorder during depressive and manic episodes. *Neuroscience Letters* **398**(3), 215-9.

Cunha C, Brambilla R, Thomas KL (2010). A simple role for BDNF in learning and memory? *Frontiers in Molecular Neuroscience* **3**, 1-14.

Denicoff KD, Ali SO, Mirsky AF, Smith-Jackson EE, *et al.* (1999). Relationship between prior course of illness and neuropsychological functioning in patients with bipolar disorder. *Journal of Affective Disorders* **56**(1), 67-73.

Elshahawi HH, Essawi H, Rabie MA, Mansour M, *et al.* (In Press). Cognitive functions among euthymic bipolar I patients after a single manic episode versus recurrent episodes. *Journal of Affective Disorders*.

Frey BN, Andreazza AC, Ceresér KM, Martins MR, *et al.* (2006). Effects of mood stabilizers on hippocampus BDNF levels in an animal model of mania. *Life Sciences* **79**(3), 281-6.

Izquierdo I, Quillfeldt JA, Zanatta MS, Quevedo J, *et al.* (1997). Sequential role of hippocampus and amygdala, entorhinal cortex and parietal cortex in formation and retrieval of memory for inhibitory avoidance in rats. *European Journal of Neuroscience* **9**, 786-793.

Kapczinski F, Frey BN, Kauer-Sant'Anna M, Grassi-Oliveira R (2008). Brain-derived neurotrophic factor and neuroplasticity in bipolar disorder. *Expert Reviews of Neurotherapeutics* **8**(7), 1101-13.

Kauer-Sant'Anna M, Kapczinski F, Andreazza AC, Bond DJ, *et al.* (2009). Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. *International Journal of Neuropsychopharmacology* **12**(4), 447-58.

Kesner RP, Bierley RA, Pebbles P (1981). Short-term memory: the role of d-amphetamine. *Pharmacology, Biochemistry, and Behavior* **15**(5), 673-6.

Kessing LV, Andersen PK (2004). Does the risk of developing dementia increase with the number of episodes in patients with depressive disorder and in patients with bipolar disorder? *Journal of Neurology, Neurosurgery and Psychiatry* **75**(12), 1662-6.

Küppers E, Beyer C (2001). Dopamine regulates brain-derived neurotrophic factor (BDNF) expression in cultured embryonic mouse striatal cells. *Neuroreport* **12**(6), 1175-9.

Lyoo IK, Sung YH, Dager SR, Friedman SD, *et al.* (2006). Regional cerebral cortical thinning in bipolar disorder. *Bipolar Disorders* **8**(1), 65-74.

Machado-Vieira R, Kapczinski F, Soares JC (2004). Perspectives for the development of animal models of bipolar disorder. *Progress in Neuropsychopharmacology and Biological Psychiatry* **28**(2), 209-24.

Merikangas KR, Akiskal HS, Angst J, Greenberg PE, *et al.* (2007). Lifetime and 12-month prevalence of bipolar spectrum disorder in the National Comorbidity Survey replication. *Archives of General Psychiatry* **64**, 543–552.

Ou LC, Gean PW (2007). Transcriptional regulation of brain-derived neurotrophic factor in the amygdala during consolidation of fear memory. *Molecular Pharmacology* **72**(2), 350-8.

Peet M, Peters S (1995). Drug-induced mania. *Drug Saffety* **12**(2), 146-53.

Pruunsild P, Kazantseva A, Aid T, Palm K, *et al.* (2007). Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. *Genomics* **90**(3), 397-406.

Rodríguez-Casado A, Alvarez I, Toledano A, de Miguel E, *et al*. (2007). Amphetamine effects on brain protein structure and oxidative stress as revealed by FTIR microspectroscopy. *Biopolymers* **86**(5-6), 437-46.

Rossato JI, Bevilaqua LR, Izquierdo I, Medina JH, *et al.* (2009). Dopamine controls persistence of long-term memory storage. *Science* **325**(5943), 1017-20.

Shaltiel G, Chen G, Manji HK (2007). Neurotrophic signaling cascades in the pathophysiology and treatment of bipolar disorder. *Current Opinion in Pharmacology* **7**(1), 22-6.

Torres IJ, Boudreau VG, Yatham LN (2007). Neuropsychological functioning in euthymic bipolar disorder: a meta-analysis. *Acta Psychiatrica Scandinavica Supplementum* **434**, 17-26.

Vianna MR, Izquierdo LA, Barros DM, de Souza MM, *et al.* (2001). Pharmacological differences between memory consolidation of habituation to an open field and inhibitory avoidance learning. *Brazilian Journal of Medical and Biological Research* **34**(2), 233-40.

Walz JC, Frey BN, Andreazza AC, Ceresér KM, *et al*. (2008). Effects of lithium and valproate on serum and hippocampal neurotrophin-3 levels in an animal model of mania. *Journal of Psychiatric Research* **42**(5), 416-21.

X Tao, S Finkbeiner, DB Arnold, AJ Shaywitz*, et al*. (1998). Ca2+ influx regulates BDNF transcription by a CREB family transcription factor. *Neuron* **20**, 709-26.

Xu TX, Ma Q, Spealman RD, Yao WD (In Press). Amphetamine modulation of long-term potentiation in the prefrontal cortex: dose dependency, monoaminergic contributions, and paradoxical rescue in hyperdopaminergic mutant. *Journal of Neurochemistry*.

Yamamoto BK, Moszczynska A, Gudelsky GA (2010). Amphetamine toxicities: classical and emerging mechanisms. *Annals of the New York Academy of Sciences* **1187**, 101-21.

Yatham LN, Liddle PF, Shiah IS, Lam RW, *et al.* (2002). PET study of [(18)F]6-fluoro-L-dopa uptake in neuroleptic- and mood-stabilizer-naive first-episode nonpsychotic mania: effects of treatment with divalproex sodium. *American Journal of Psychiatry* **159**(5), 768-74.

Zanatta MS, Quillfeldt JH, Schaeffer E, Schmitz PK, *et al.* (1997). Involvement of the hippocampus, amygdala, entorhinal cortex and posterior parietal cortex in memory consolidation. *Brazilian Journal of Medical and Biological Research* **30**, 235-240.

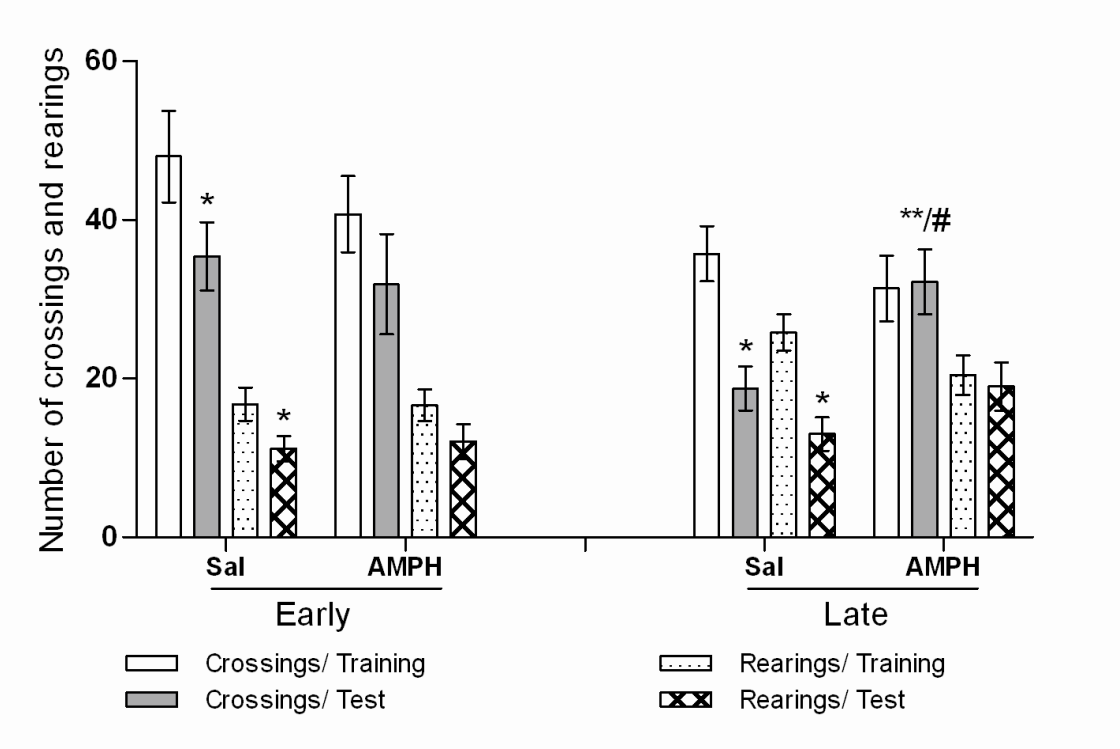

Figure 1. Open-field habituation test.

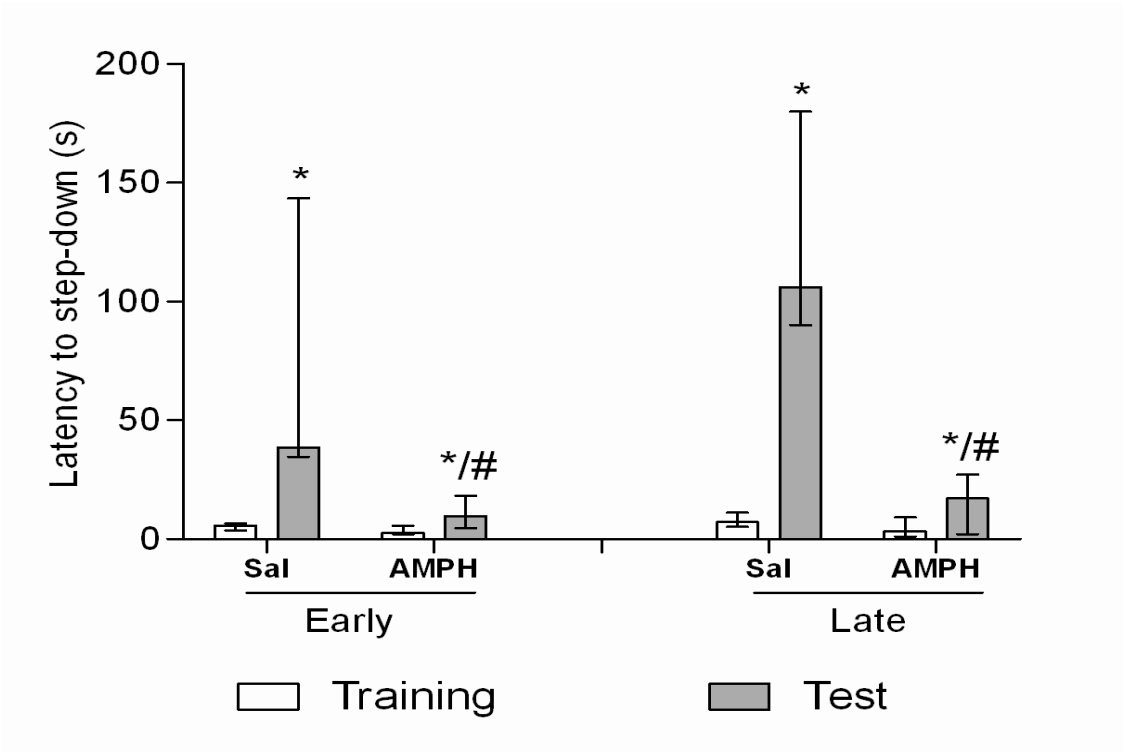

Figure 2. One-trial step-down inhibitory avoidance test.

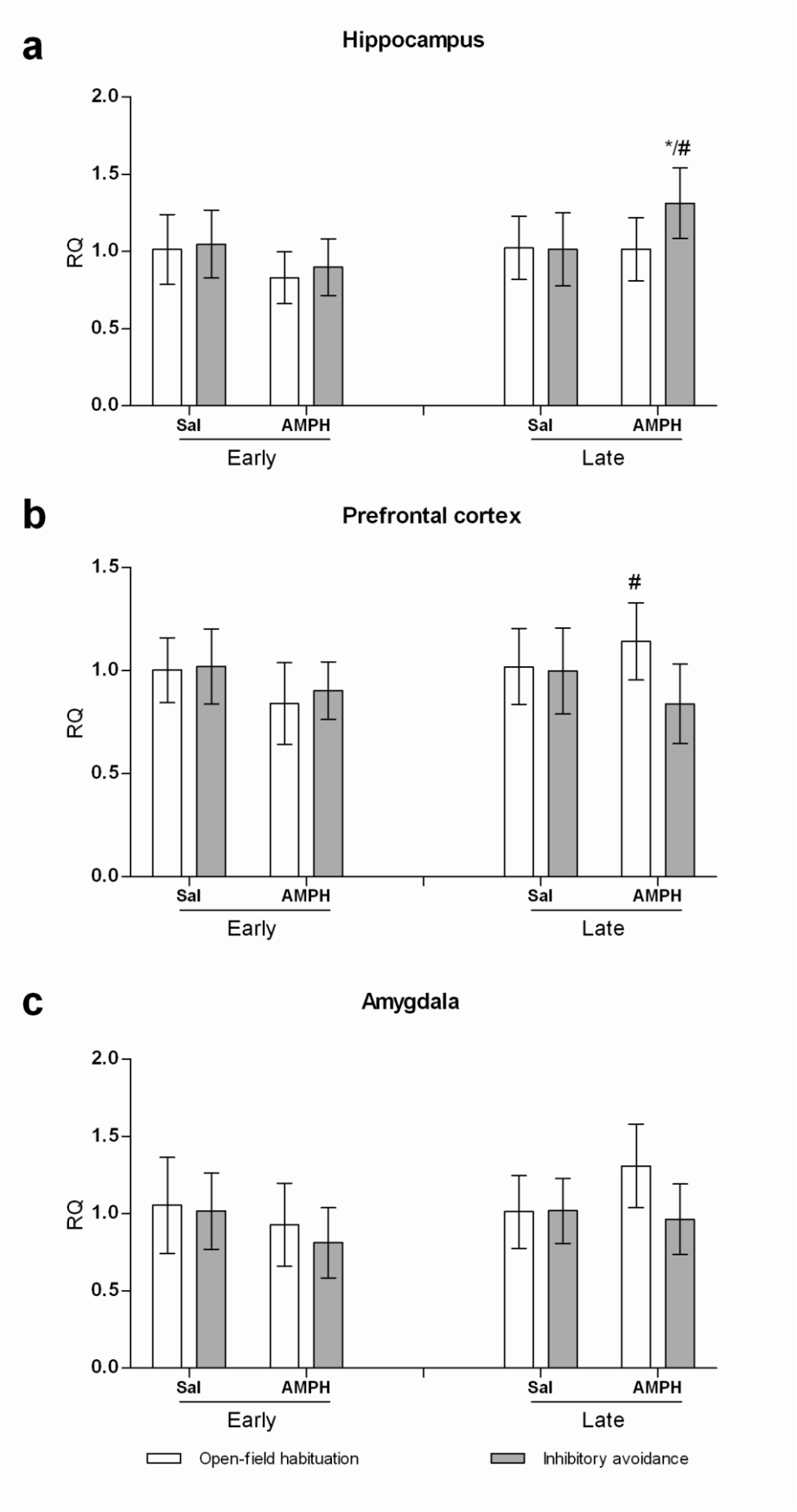

Figure 3. Relative expression of *Bdnf* gene in different brain structures.

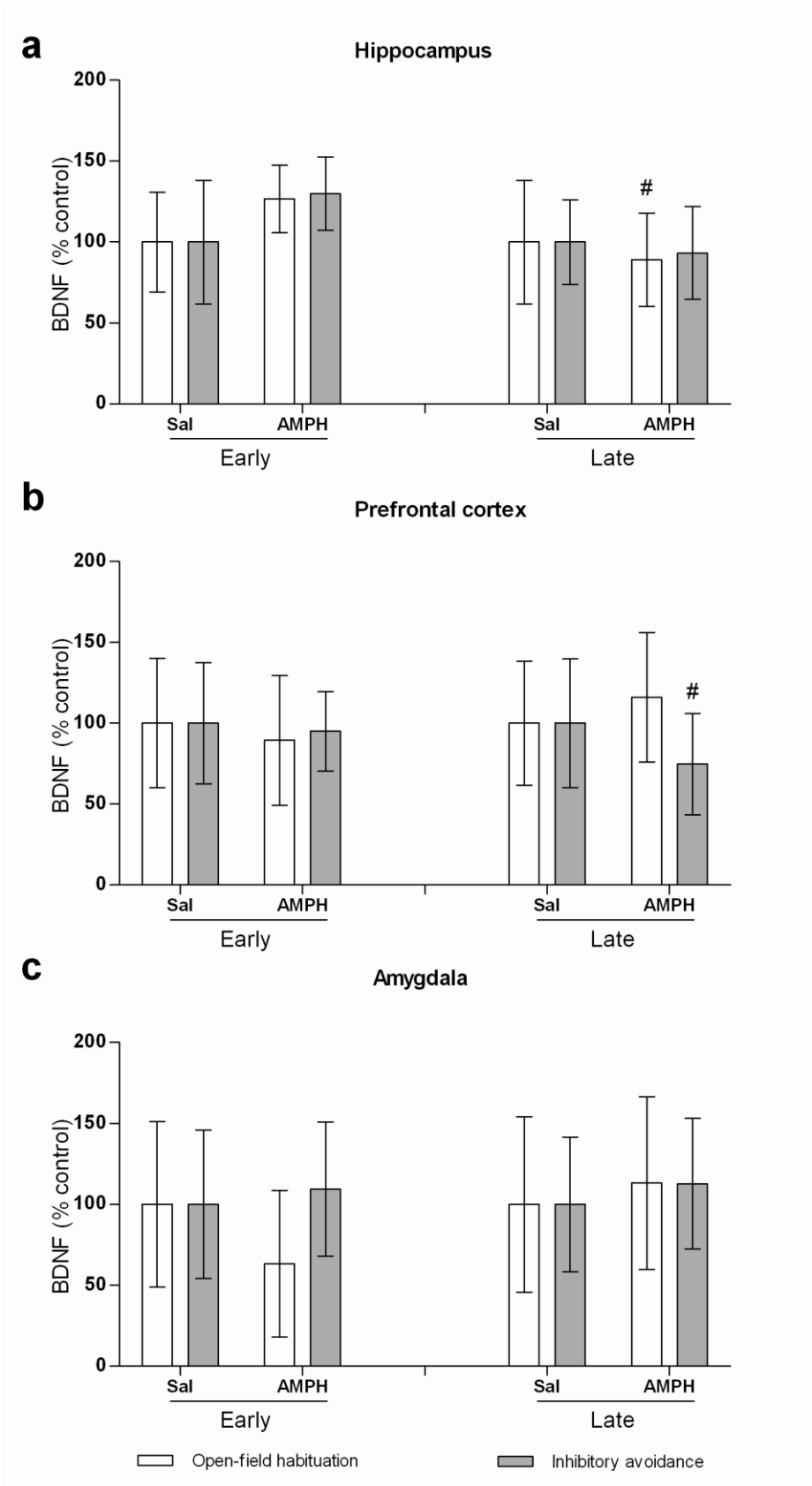

Figure 4. BDNF protein levels in different brain structures.

Figure Legends

Figure 1. Open-field habituation test. Crossings and rearings of training and test session performance (mean ± SD) in 5-min sessions in an open field with a 24-h interval between sessions. N=15 per group. *P<0.05 compared to training (paired t-test); **P<0.05 compared to saline (independent t-test); #P<0.05 for the comparison of variation between test and training sessions between early and late treatments (independent t-test).

Figure 2. One-trial step-down inhibitory avoidance test. Latency to step-down (s) in training and test session performance (median ± interquartile ranges), with a 24-h interval between sessions. N=15 per group. *P<0.05 compared to training (Wilcoxon signed-ranks test); #P<0.05 compared to saline (Mann-whitney test).

Figure 3. Relative expression of Bdnf gene in different brain structures. Results are presented as mean ± SD (N=15). A) Hippocampus; B) Prefrontal cortex, and C) Amygdala region. White bars represent data for open-field habituation and grey bars for inhibitory avoidance. Relative ratios of mRNA levels were calculated using the ddCT method normalized by ACTB as endogenous control and results of saline groups were used as calibrator. Results were compared with one-way ANOVA, followed by post-test of Tukey. *P<0.05 compared to saline; #P<0.05 compared to Early AMPH.

Figure 4. BDNF protein levels in different brain structures. Results are presented as percentage of control (saline groups) ± SD (N=15). A) Hippocampus; B) Prefrontal cortex and C) Amygdala region. White bars represent data for open-field habituation and grey bars

represent inhibitory avoidance. Results were compared with one-way ANOVA, followed by post-test of Tukey. #P<0.05 compared to Early AMPH.

PARTE III

4. DISCUSSÃO

A associação observada em pacientes entre a neuroprogressão do THB e declínio cognitivo sugere o envolvimento de mecanismos neurotóxicos associados ao aumento no número de episódios de humor (Torres *et al.*, 2007; Kauer-Sant'Anna *et al.*, 2009). A neurotrofina BDNF já foi associada à progressão do THB e é, por isso, considerada relevante nos processos envolvidos no declínio cognitivo observado (Kauer-Sant'Anna *et al.*, 2009). Nesta dissertação avaliamos a memória de habituação ao campo aberto e de esquiva inibitória passiva em um modelo animal de mania em estágios precoce e tardio do THB, além dos níveis de mRNA e dos níveis protéicos de BDNF em diferentes estruturas cerebrais. Observamos um prejuízo de memória induzido pelo tratamento com AMPH em ambos os testes comportamentais, havendo ainda uma diferença entre os grupos precoce e tardio no teste de habituação. A modulação dos níveis de BDNF foi específica para cada um dos testes, sendo ainda específica para cada área cerebral estudada.

Os resultados comportamentais obtidos confirmam que o nosso modelo animal é capaz de mimetizar características clínicas dos pacientes com THB. Este é o primeiro trabalho avaliando memória em um modelo animal de mania. Déficits em memória e outros parâmetros cognitivos já foram descritos em pacientes com THB (Torres *et al.*, 2007). Pacientes eutímicos avaliados após um único episódio de mania apresentaram prejuízos na atenção, nas funções executivas e no escore total de memória em comparação com sujeitos controles saudáveis. Além disso, eles obtiveram melhores resultados em comparação com pacientes eutímicos avaliados após múltiplos episódios recorrentes de humor com relação à atenção e função executiva (Elshahawi *et al.*, 2010). A duração da doença também já foi

negativamente correlacionada com escores em testes de função executiva (Thompson *et al.*, 2005), velocidade psicomotora (Martinez-Arán *et al.*, 2004) e memória verbal (Cavanagh *et al.*, 2002).

Neste contexto, um estudo de espectroscopia de prótons por ressonância magnética mostrou associação entre o tempo de duração da doença e perda neuronal hipocampal medida pelos níveis de N-acetil aspartato (Deicken *et al.*, 2003). Já foi proposto que episódios sucessivos causam danos sutis a áreas cerebrais e, assim, levam a prejuízos neurológicos e cognitivos em pacientes. No entanto, evidências associando os déficits cognitivos com indicadores de gravidade ou progressão da doença ainda não são consistentes. O BDNF pode ser um dos responsáveis pelos mecanismos associados à progressão do THB e os resultados apresentados nesse trabalho indicam que os níveis desta neurotrofina podem ser regulados no hipocampo e no córtex pré-frontal nestas circunstâncias.

As consequências observadas em função do número de episódios de humor sugerem uma aparente toxicidade associada aos episódios agudos. De fato, a hipercortisolemia induzida pelo estresse durante um episódio de humor pode resultar em toxicidade de células neuronais, diminuição nos receptores de glicocorticóides e eventual morte celular e tecidual (Altshuler, 2004). Kauer-Sant'Anna e colaboradores (2009) mostraram que os níveis séricos de BDNF estão diminuídos em pacientes com THB com múltiplos episódios em comparação com pacientes em início de doença, o que sugere que uma importante defesa neuroprotetora está também diminuída. O aumento de toxicidade associada a uma diminuição de mecanismos protetores pode ser responsável pelas mudanças neuroanatômicas associadas à progressão do THB, o que, por sua vez, justificaria o declínio cognitivo observado. É interessante observar que hipocampo e córtex pré-frontal possuem receptores para glicocorticóides, além de fortes

conexões recíprocas (Rajkowska, 2000), o que corrobora com os nossos achados de um envolvimento de ambas as estruturas no déficit de memória observado no nosso modelo animal.

A descoberta de mecanismos moleculares associados a transtornos mentais como o THB depende significativamente de modelos pré-clínicos bem estabelecidos e confiáveis. Atualmente, modelos animais ideais para doenças mentais não existem. No caso do THB, mais especificamente, modelos animais de mania e depressão são distintos, além de que modelos do transtorno em si não estão bem validados. Mesmo assim, seu uso permite estudar regiões cerebrais específicas em resposta a estímulos variados, tanto morfo- quanto bioquimicamente. Em humanos, tal abordagem se limita a estudos *post-mortem*. O modelo utilizado neste trabalho baseia-se na hiperativação dopaminérgica observada em episódios maníacos (Berk *et al.*, 2007) e é considerado um dos mais bem validados na literatura, principalmente em se tratando da validade de constructo (Machado-Vieira *et al*., 2004; Andreazza *et al*., 2008; Walz *et al*., 2008). No entanto, a validade aparente foi pouco explorada até o momento, tendo sido limitada durante anos à avaliação da hiperlocomoção dos animais. Os resultados encontrados neste trabalho mostraram um declínio cognitivo associado ao tratamento com AMPH, o que mimetiza observações clínicas do episódio maníaco e, portanto, aumenta a validade aparente do modelo animal em questão.

Nossos resultados sugerem diferenças farmacológicas entre os dois testes comportamentais de memória utilizados, incluindo a modulação destes pela AMPH e as alterações induzidas nos níveis cerebrais de BDNF. Diferentes estruturas cerebrais são ativadas em tipos de memória distintos, havendo inclusive um controle temporal do envolvimento de diferentes áreas durante o período de consolidação das mesmas (Izquierdo *et al*., 1997). Baseando-se nas já conhecidas diferenças farmacológicas existentes entre a

esquiva inibitória e o teste de habituação ao campo aberto (Vianna *et al*., 2001), as diferenças observadas no efeito da AMPH sobre cada um dos testes tornam-se justificáveis e sugerem novos estudos para o esclarecimento destes mecanismos. A AMPH, tanto por 7 (grupo precoce) quanto por 35 dias (grupo tardio), foi capaz de prejudicar a memória dos ratos nos testes empregados. Por outro lado, o uso de psicoestimulantes pode melhorar a memória em determinadas situações, principalmente em função da dose utilizada. Xu e colaboradores (2010) mostraram que baixas doses de AMPH (0,1 mg/kg) aumentam a LTP, porém doses elevadas prejudicam-na significativamente. Este prejuízo parece ser mediado por uma ativação excessiva de receptores D1 e D2 de dopamina e envolve a sinalização pós-sináptica dependente da proteína fosfatase I (PP1) (Xu *et al.*, 2010). Além disso, altas doses de AMPH diminuem a atividade de receptores N-metil-D-aspartato (NMDA), provavelmente como consequência da redução nos níveis de suas subunidades NR1/NR2A na membrana sináptica (Xu *et al.*, 2009). Estudos em diversos modelos mostram que o NMDAR é fundamental para a formação de memórias e para a formação da LTP (Li e Tsien, 2009). Considerando anfetaminas, a meta-anfetamina (METH) parece ser significativamente mais tóxica para o SNC do que a AMPH e mostrou efeito prejudicial em uma série de testes cognitivos em animais (Bisagno *et al.*, 2001; Schröder *et al.*, 2003; Marshall *et al*., 2007).

A AMPH é muito ativa nas vias de recompensa mesolímbica e mesocortical, sendo o estriado, o núcleo *accumbens* e o estriado ventral seus sítios primários de ação (Del Arco *et al*., 1999; Drevets *et al*., 2001). A AMPH aumenta os níveis de dopamina na fenda sináptica, possuindo ações vesiculares (aumentando a concentração do neurotransmissor no citosol do neurônio pré-sináptico) e interagindo com o transportador de dopamina DAT (induzindo, assim, um transporte reverso de dopamina do neurônio pré-sináptico para a fenda). Além disso, a AMPH ainda é capaz de ligar-se de forma reversível ao DAT e bloquear a habilidade

do transportador de recaptar dopamina da fenda (Sulzer *et al*., 2005). Portanto, os altos níveis de dopamina são os principais responsáveis pelos efeitos farmacológicos da AMPH.

A dopamina é capaz de regular a expressão de proteínas essenciais para o estabelecimento de plasticidade neuronal duradoura e já foi associada a mecanismos de memória (Rossato *et al*., 2009). Embora os neurônios dopaminérgicos sejam projetados da área tegmental ventral e da substância *nigra* compacta, suas projeções alcançam quase todas as áreas do cérebro, incluindo o córtex pré-frontal, o lobo temporal medial e o hipocampo, regiões ativadas tipicamente durante a evocação da memória (Li *et al*., 2010). O desbalanço nos níveis de dopamina, resultante da perda de um dos alelos normais do gene que codifica o receptor DAT, causou um déficit na evocação de uma memória espacial associativa (Li *et al*., 2010). Além disso, esse prejuízo foi revertido por baixas doses de haloperidol, um antagonista dopaminérgico. Embora este mecanismo tenha sido descrito para um tipo específico de memória, ele sugere a necessidade de um controle dos níveis dopaminérgicos na fenda sináptica para um bom funcionamento dos mecanismos envolvidos na consolidação e evocação da memória.

Baseando-se nos achados em pacientes com THB, cujos níveis séricos de BDNF diminuem com a progressão da doença, resolvemos avaliar os níveis de BDNF no modelo animal como uma possível explicação para os déficits de memória encontrados. Os níveis protéicos de BDNF foram encontrados diminuídos no hipocampo dos ratos tratados com AMPH no grupo tardio e submetidos ao teste de habituação ao campo aberto. Nestes mesmos ratos, os níveis de mRNA do BDNF estavam aumentados no córtex pré-frontal. Por outro lado, a modulação parece ter sido inversa após o teste de esquiva inibitória, uma vez que os ratos tratados com AMPH no grupo tardio neste caso apresentaram níveis protéicos reduzidos

no córtex pré-frontal e níveis de mRNA aumentados no hipocampo. A nossa hipótese é a existência de uma modulação recíproca entre os níveis de BDNF no hipocampo e córtex pré-frontal, visto que ambos apresentam conexões neuroanatômicas e funcionais bem estabelecidas e são fundamentais para a consolidação de ambos os tipos de memória. Outros trabalhos já mostraram a capacidade da AMPH de diminuir níveis cerebrais de BDNF (Frey *et al.*, 2006; Angelucci *et al.*, 2007), mas novos estudos são necessários para explorar a possibilidade de uma modulação recíproca do BDNF entre áreas cerebrais diferentes.

A importância do BDNF nos processos de memória já foi consistentemente descrita (Cunha *et al.*, 2010). A sua expressão gênica, por exemplo, aumenta no hipocampo de ratos após o treino do labirinto aquático de Morris (Kesslak *et al.*, 1998), labirinto radial (Mizuno *et al.*, 2000), esquiva passiva (Ma *et al.*, 1998) e condicionamento ao medo contextual (Hall *et al.*, 2000). Além disso, a infusão intra-hipocampal de BDNF em ratos melhora o desempenho no labirinto aquático de Morris e na esquiva passiva. Da mesma forma, a infusão de um anticorpo anti-BDNF no córtex parietal prejudica a consolidação da memória de longa duração em uma série de protocolos comportamentais (revisado em Cunha *et al.*, 2010). Mais recentemente, um papel adicional do BDNF na manutenção da LTM após a consolidação foi sugerido (Bekinschtein *et al.*, 2007).

Uma das limitações do nosso estudo é a falta de dados a respeito dos níveis de BDNF em ratos que não foram submetidos aos testes comportamentais. De fato, como a modulação dos níveis foi diferente para cada teste, não foi possível avaliar o efeito da AMPH *per se* na modulação do BDNF. O que observamos é uma aparente associação entre o tratamento com AMPH no grupo tardio e o teste de habituação ao campo aberto, considerando a diferença significativa entre os grupos precoce e tardio após tratamento com AMPH. Nossa hipótese é

que a AMPH pode ter induzido modificações no gene *Bdnf*, alterando assim a modulação induzida pelo teste comportamental. Essas modificações podem incluir uma hiper- ou hipometilação dos promotores do gene *Bdnf* ou, ainda, modificações em histonas, todos os quais são mecanismos epigenéticos conhecidos de modulação da expressão gênica. Um estudo recente mostrou que drogas psicoestimulantes, como a cocaína, aumentam a metilação do DNA e os níveis de uma proteína de ligação ao DNA metilado, chamada proteína de ligação a CpG metilado 2 (MeCP2) (Anier *et al.*, 2010). Este achado, em conjunto com outras evidências que sugerem um efeito da ativação de receptores dopaminérgicos em mecanismos epigenéticos (Liu *et al.*, 2005), dá suporte à hipótese do envolvimento destes mecanismos no tratamento de longo prazo com AMPH e outros psicoestimulantes.

Os resultados obtidos neste trabalho, em conjunto com dados já discutidos nesta dissertação a respeito da neuroprogressão da doença, reforçam a extrema importância do tratamento de manutenção da eutimia em pacientes com THB. Além disso, nossos dados reforçam o envolvimento do BDNF no declínio cognitivo associado à esta progressão, possibilitando um maior entendimento acerca de sua neurobiologia.

5. CONCLUSÕES

- O tratamento de ratos com AMPH no grupo precoce e no grupo tardio prejudicou a memória avaliada no teste de habituação ao campo aberto em comparação com ratos tratados com salina no mesmo período, sendo mais prejudicial no grupo tardio em comparação com o grupo precoce;

- O tratamento de ratos com AMPH no grupo precoce e no grupo tardio não aboliu a memória avaliada pelo teste de esquiva inibitória, mas a prejudicou em comparação com ratos tratados pelo mesmo período com salina;

- Ratos tratados com AMPH no grupo tardio apresentaram uma diminuição significativa nos níveis protéicos de BDNF no hipocampo e um aumento dos níveis de mRNA de BDNF no córtex pré-frontal após teste de habituação ao campo aberto;

- O tratamento com AMPH no grupo tardio diminuiu significativamente os níveis protéicos de BDNF no córtex pré-frontal e aumentou os níveis de mRNA de BDNF no hipocampo de ratos submetidos ao teste de esquiva inibitória;

- Os resultados obtidos neste trabalho sugerem o envolvimento do BDNF no hipocampo e no córtex pré-frontal na neuroprogressão do THB e reforçam a importância do tratamento de manutenção dos pacientes.

6. REFERÊNCIAS

Altshuler LL, Ventura J e van Gorp WG, Green MF, Theberge DC, Mintz J. Neurocognitive function in clinically stable men with bipolar I disorder or schizophrenia and normal control subjects. Biological Psychiatry. 56:560–569, 2004.

Andreazza AC, Kauer-Sant'Anna M, Frey BN, Stertz L, Zanotto C, Ribeiro L, Giasson K, Valvassori SS, Réus GZ, Salvador M, Quevedo J, Gonçalves CA, Kapczinski F. Effects of mood stabilizers on DNA damage in an animal model of mania. Journal of Psychiatry and Neuroscience. 33(6):516-24, 2008.

Angelucci F, Gruber SH, El Khoury A, Tonali PA, Mathé AA. Chronic amphetamine treatment reduces NGF and BDNF in the rat brain. Eur Neuropsychopharmacol. 17(12):756-62, 2007.

Anier K, Malinovskaja K, Aonurm-Helm A, Zharkovsky A, Kalda A. DNA methylation regulates cocaine-induced behavioral sensitization in mice. Neuropsychopharmacology. 35(12):2450-61, 2010.

Barbacid M. The Family of neurotrophin receptors. J Neurobiology. 25:1386-1403, 1994.

Barnett JH, Smoller JW. The genetics of bipolar disorder. Neuroscience. 164(1):331-43, 2009.

Bekinschtein P, Cammarota M, Izquierdo I, Medina JH. BDNF and memory formation and storage. Neuroscientist. 14(2):147-56, 2008.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH. Persistence of long-term memory storage requires a late protein synthesis- and BDNF-dependent phase in the hippocampus. Neuron. 53:261–277, 2007.

Belmaker, RH. Medical Progress: Bipolar Disorder. New England Journal of Medicine. 351:476-86, 2004.

Benabarre A, Vieta E, Martínez-Arán A, Garcia-Garcia M, Martín F, Lomeña F, Torrent C, Sánchez-Moreno J, Colom F, Reinares M, Brugue E, Valdés M. Neuropsychological disturbances and cerebral blood flow in bipolar disorder. The Australian and New Zealand Journal of Psychiatry. 39(4):227-234, 2005.

Berk M, Dodd S, Kauer-Sant'anna M, Malhi GS, Bourin M, Kapczinski F, Norman T. Dopamine dysregulation syndrome: implications for a dopamine hypothesis of bipolar disorder. Acta Psychiatrica Scandinavica Supp. (434):41-9, 2007.

Beyer JL, Krishnan KR. Volumetric brain imaging findings in mood disorders. Bipolar Disorders. 4:89–104, 2002.

Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes & Development. 14(23):2919-37, 2000.

Bisagno V, Ferguson D, Luine VN. Short toxic methamphetamine schedule impairs object recognition task in male rats. Brain Research. 940(1-2):95-101, 2002.

Bliss TVP, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 361:31-39, 1993.

Bramham CR. Local protein synthesis, actin dynamics, and LTP consolidation. Current Opinion in Neurobiology.18(5):524-31, 2008.

Caspi A, Moffitt TE. Gene-environment interactions in psychiatry: joining forces with neuroscience. Nature Reviews Neuroscience. 7(7):583-90, 2006.

Cavanagh JTO, van Beck M, Muir W, Blackwood DHR. Case–control study of neurocognitive function in euthymic patients with alcohol disorder: an association with mania. The British Journal of Psychiatry. 180:320–326, 2002.

Chalovich EM, Zhu JH, Caltagarone J, Bowser R, Chu CT. Functional repression of cAMP response element in 6-hydroxydopamine-treated neuronal cells. Journal of Biological Chemistry. 281(26):17870-81, 2006.

Cunha AB, Frey BN, Andreazza AC, Goi JD, Rosa AR, Gonçalves CA, Santin A, Kapczinski F. Serum brain-derived neurotrophic factor is decreased in bipolar disorder during depressive and manic episodes. Neuroscience Letters. 398(3):215-9, 2006.

Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? Frontiers in Molecular Neuroscience. 3:1, 2010.

de Oliveira GS, Ceresér KM, Fernandes BS, Kauer-Sant'Anna M, Fries GR, Stertz L, Aguiar B, Pfaffenseller B, Kapczinski F. Decreased brain-derived neurotrophic factor in medicated and drug-free bipolar patients. Journal of Psychiatric Research. 43(14):1171-4, 2009.

Deep-Soboslay A, Iglesias B, Hyde TM, Bigelow LB, Imamovic V, Herman MM, Kleinman JE. Evaluation of tissue collection for postmortem studies of bipolar disorder. Bipolar Disorders. 10(7):822-8, 2008.

Deicken RF, Peques MP, Anzalone S, Feiwell R, Soher B. Lower concentratin of hippocampal N-acetyleaspartate in familial bipolar I disorder. The American Journal of Psychiatry. 160:873–882, 2003.

Del Arco A, González-Mora JL, Armas VR, Mora F. Amphetamine increases the extracellular concentration of glutamate in striatum of the awake rat: involvement of high affinity transporter mechanisms. Neuropharmacology. 38(7):943–54, 1999.

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 Disorders. 11(6):663-71, 2009.

Drevets WC, Gautier C, Price JC. Amphetamine-induced dopamine release in human ventral striatum correlates with euphoria. Biological Psychiatry. 49(2):81–96, 2001.

Dudai Y. The neurobiology of consolidations, or, how stable is the engram? Annual Reviews of Psychology. 55:51-86, 2000.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 112(2):257-69, 2003.

Elshahawi HH, Essawi H, Rabie MA, Mansour M, Beshry ZA, Mansour AN. Cognitive functions among euthymic bipolar I patients after a single manic episode versus recurrent episodes. Journal of Affective Disorders. 2010. *In press*.

Finkbeiner S. CREB couples neurotrophin signals to survival messages. Neuron. 25:11-14, 2005.

Frey BN, Valvassori SS, Réus GZ, Martins MR, Petronilho FC, Bardini K, Dal-Pizzol F, Kapczinski F, Quevedo J. Effects of lithium and valproate on amphetamine-induced oxidative stress generation in an animal model of mania. Journal of Psychiatry and Neuroscience. 31(5):326-32, 2006.

Gelenberg AJ, Kane JM, Keller MB, Lavori P, Rosenbaum JF, Cole K, Lavelle J. Comparison of standard and low serum levels of lithium for maintenance treatment of bipolar disorder. New England Journal of Medicine. 321:1489–1493, 1989.

Goodwin FK, Jamison KR. Manic-Depressive Illness: Bipolar and Recurrent Unipolar Disorders. 2.ed. Nova York: Oxford University Press, 2007.

Grande I, Kunz M, Potter W, Balanzá-Martínez V, Vieta E, Kapczinski F. Should bipolar disorder be considered a systemic illness? 2010. (submitted)

Hajek T, Carrey N, Alda M. Neuroanatomical abnormalities as risk factors for bipolar disorder. Bipolar Disorders. 7(5):393-403, 2005.

Hall J, Thomas KL, Everitt BJ. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nature Neuroscience. 3:533–535, 2000.

Huxley N, Baldessarini RJ. Disability and its treatment in bipolar disorder patients. Bipolar Disorders. 9(1-2):183-196, 2007.

Izquierdo I, Quillfeldt JA, Zanatta MS, Quevedo J, Schaeffer E, Schmitz PK, Medina JH. Sequential role of hippocampus and amygdala, entorhinal cortex and parietal cortex in formation and retrieval of memory for inhibitory avoidance in rats. The European Journal of Neuroscience. 9(4):786-93, 1997.

Kapczinski F, Vieta E, Andreazza AC, Frey BN, Gomes FA, Tramontina J, Kauer-Sant'anna M, Grassi-Oliveira R, Post RM. Allostatic load in bipolar disorder: implications for pathophysiology and treatment. Neuroscience Biobehaviors Reviews. 32(4):675-92, 2008a.

Kapczinski F, Frey BN, Kauer-Sant'Anna M, Grassi-Oliveira R. Brain-derived neurotrophic factor and neuroplasticity in bipolar disorder. Expert Reviews of Neurotherapeutics. 8(7):1101-13, 2008b.

Kauer-Sant'Anna M, Kapczinski F, Andreazza AC, Bond DJ, Lam RW, Young LT, Yatham LN. Brain-derived neurotrophic factor and inflammatory markers in patients with early- vs. late-stage bipolar disorder. The International Journal of Neuropsychopharmacology. 12(4):447-58, 2009.

Kessing LV, Andersen PK, Mortensen PB. Predictors of recurrence in affective disorder. A case register study. Journal of Affective Disorders. 49:101–108, 1998.

Kesslak JP, So V, Choi J, Cotman CW, Gomez-Pinilla F. Learning upregulates brain-derived neurotrophic factor messenger ribonucleic acid: a mechanism to facilitate encoding and circuit maintenance? Behavioral Neuroscience. 112:1012–1019, 1998.

Kilbourne AM, Cornelius JR, Han X, Pincus HA, Shad M, Salloum I, Conigliaro J, Haas GL. Burden of general medical conditions among individuals with bipolar disorder. Bipolar Disorders. 6(5):368-73, 2004.

Korte M, Carroll P, Wolf E, Brem E, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proceedings of the National Academy of Sciences USA. 92:8856–8860, 1995.

Lent R. Neurociência da mente e do comportamento. 1.ed. Rio de Janeiro: Guanabara Koogan, 2008.

Lessmann V, Gottmann K, Malcangio M. Neurotrophin secretion: current facts and future prospects. Progress in Neurobiology. 69:341-374, 2003.

Li F, Tsien JZ. Memory and the NMDA receptors. New England Journal of Medicine. 361(3):302-303, 2009.

Li F, Wang LP, Shen X, Tsien JZ. Balanced dopamine is critical for pattern completion during associative memory recall. PLoS One. 27;5(10):e15401, 2010.

Liu JC, Baker RE, Chow W, Sun CK, Elsholtz HP. Epigenetic mechanisms in the dopamine D2 receptor-dependent inhibition of the prolactin gene. Molecular Endocrinology. 19(7):1904-1917, 2005.

Lopez-LarsonMP, DelBello MP, Zimmerman ME, Schwiers ML, Strakowski SM. Regional prefrontal gray and white matter abnormalities in bipolar disorder. Biological Psychiatry. 52:93–100, 2002.

Lyoo IK, Kim MJ, Stoll AL, Demopulos CM, Parow AM, Dager SR, Friedman SD, Dunner DL, Renshaw PF. Frontal lobe gray matter density decreases in bipolar I disorder. Biological Psychiatry. 55:648–651, 2004.

Lyoo IK, Sung YH, Dager SR, Friedman SD, Lee JY, Kim SJ, Kim N, Dunner DL, Renshaw PF. Regional cerebral cortical thinning in bipolar disorder. Bipolar Disorders. 8:65–74, 2006.

Ma YL, Wang HL, Wu HC, Wei CL, Lee EH. Brain-derived neurotrophic factor antisense oligonucleotide impairs memory retention and inhibits long-term potentiation in rats. Neuroscience. 82:957–967, 1998.

Malhi GS, Ivanovski B, Szekeres V, Olley A: Bipolar disorder: it's all in your mind? The neuropsychological profile of a biological disorder. Canadian Journal of Psychiatry. 49(12):813-819, 2004.

Mamelak M. An amphetamine model of manic depressive illness. International Pharmacopsychiatry. 13:193–208, 1978.

Marshall JF, Belcher AM, Feinstein EM, O'Dell SJ. Methamphetamine-induced neural and cognitive changes in rodents. Addiction. 102 Suppl 1:61-9, 2007.

Martínez-Arán A, Vieta E, Reinares M, Colom F, Torrent C, Sánchez-Moreno J, Benabarre A, Goikolea JM, Comes M, Salamero M. Cognitive function across manic or

hypomanic, depressed, and euthymic states in bipolar disorder. American Journal of Psychiatry. 161(2):262-270, 2004.

Martinez-Aran A, Vieta E, Torrent C, Sanchez-Moreno J, Goikolea JM, Salamero M, Malhi GS, Gonzalez-Pinto A, Daban C, Alvarez-Grandi S, Fountoulakis K, Kaprinis G, Tabares-Seisdedos R, Ayuso-Mateos JL. Functional outcome in bipolar disorder: the role of clinical and cognitive factors. Bipolar Disorders. 9(1-2):103-113, 2007.

McGaugh JL. Time-dependent processes in memory storage. Science. 153:1351-1358, 1966.

McIntosh AM, Moorhead TW, McKirdy J, Sussmann JE, Hall J, Johnstone EC, Lawrie SM. Temporal grey matter reductions in bipolar disorder are associated with the BDNF Val66Met polymorphism. Molecular Psychiatry. 12:902–903, 2007.

Merikangas KR, Akiskal HS, Angst J, Greenberg PE, Hirschfeld RM, Petukhova M, Kessler RC. Lifetime and 12-month prevalence of bipolar sprectrum disorder in the National Comorbidity Survey replication. Archives of General Psychiatry. 64(5):543-552, 2007.

Minichiello L. TrkB signalling pathways in LTP and learning. Nature reviews: Neuroscience. 10:850-860, 2009.

Mizuno M, Yamada K, Olariu A, Nawa H, Nabeshima T. Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. Journal of Neuroscience. 20:7116–7121, 2000.

Mur M, Portella MJ, Martínez-Arán A, Pifarré J, Vieta E. Persistent neuropsychological deficit in euthymic bipolar patients: executive function as a core deficit. Journal of Clinical Psychiatry. 68(7):1078-1086, 2007.

Murer MG, Yan Q, Raisman-Vozari R. Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. Progress in Neurobiology. 63:71–124, 2001.

Pang PT, Lu B. Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Research Reviews. 3(4):407-30, 2004.

Peet M, Peters S. Drug-induced mania. Drug Safety. 12(2):146-53, 1995.

Post RM. Kindling and sensitization as models for affective episode recurrence, cyclicity, and tolerance phenomena. Neuroscience Biobehaviors Reviews. 31(6):858-73, 2007.

Quillfeldt JA. Behavioral Methods to Study Learning and Memory in Rats. In: Andersen MA, Tufik SA. Animal Models as Tools in Ethical Biomedical Research. Editora FAPESP, 2010.

Rajkowska G. Postmortem studies in mood disorders indicate altered numbers of neurons in glial cells. Biological Psychiatry. 48:766–777, 2000.

Robinson LJ, Thompson JM, Gallagher P, et al.: A meta-analysis of cognitive deficits in euthymic patients with bipolar disorder. Journal of Affective Disorders. 93(1-3):105-115, 2006a.

Robinson LJ, Ferrier IN. Evolution of cognitive impairment in bipolar disorder: a systematic review of cross-sectional evidence. Bipolar Disorders. 8(2):103-116, 2006b.

Rossato JI, Bevilaqua LR, Izquierdo I, Medina JH, Cammarota M. Dopamine controls persistence of long-term memory storage. Science. 325(5943):1017-1020, 2009.

Rybakowski JK, Borkowska A, Skibinska M, Hauser J. Illness-specific association of val66met BDNF polymorphism with performance on Wisconsin Card Sorting Test in bipolar mood disorder. Molecular Psychiatry. 11(2):122-124, 2006.

Sassi RB, Nicoletti M, Brambilla P, Mallinger AG, Frank E, Kupfer DJ, Keshavan MS, Soares JC. Increased gray matter volume in lithium-treated bipolar disorder patients. Neuroscience Letters. 329(2):243-5, 2002.

Schröder N, O'Dell SJ, Marshall JF. Neurotoxic methamphetamine regimen severely impairs recognition memory in rats. Synapse. 49(2):89-96, 2003.

Schuepbach D, Novick D, Haro JM, Reed C, Boeker H, Noda S, Angst J, Hell D, EMBLEM Advisory Board. Determinants of voluntary vs. Involuntary admission in bipolar disorder and the impact of adherence. Pharmacopsychiatry. 41:29–36, 2008.

Shaltiel G, Chen G, Manji HK. Neurotrophic signaling cascades in the pathophysiology and treatment of bipolar disorder. Current Opinions in Pharmacology. 7(1):22-6, 2007.

Squire LR, Kandel ER. Memory: from mind to molecules. 2.ed. Colorado: Roberts & Company, 2009.

Strakowski SM, Adler CM, Holland SK, Mills NP, DelBello MP, Eliassen JC. Abnormal FMRI brain activation in euthymic bipolar disorder patients during a counting Stroop interference task. American Journal of Psychiatry. 162(9):1697-1705,2005.

Strakowski SM, DelBello MP, Zimmerman ME, Getz GE, Mills NP, Ret J, Shear P, Adler CM. Ventricular and periventricular structural volumes in first- versus multiple-episode bipolar disorder. *American Journal of Psychiatry*. 159(11):1841-1847, 2002.

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

Sulzer D, Sonders MS, Poulsen NW, Galli A. Mechanisms of neurotransmitter release by amphetamines: a review. Progress in Neurobiology. 75(6):406–33, 2005.

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. American Journal of Psychiatry. 156:1264–1266, 1999.

Tai-Xiang Xu, Qi Ma, Roger D. Spealman, Wei-Dong Yao. Amphetamine modulation of long-term potentiation in the prefrontal cortex: dose dependency, monoaminergic contributions, and paradoxical rescue in hyperdopaminergic mutant. Journal of Neurochemistry. 115(6):1643–1654, 2010.

Thompson JM, Gallagher P, Hughes JH, Watson S, Gray JM, Ferrier IN, Young AH.. Neurocognitive impairment in euthymic patients with bipolar affective disorder. British Journal of Psychiatry. 186:32–40, 2005.

Tohen M, Waternaux CM, Tsuang MT. Outcome in mania. A 4-year prospective follow-up of 75 patients utilizing survival analysis. Archives of General Psychiatry 47:1106–1111, 1990.

Torres IJ, Boudreau VG, Yatham LN. Neuropsychological functioning in euthymic bipolar disorder: a meta-analysis. Acta Psychiatrica Scandinavica Suppl. 434:17-26, 2007.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nature Neuroscience. 9(4):519-25, 2006.

Tyler WJ, Perrett SP, Pozzo-Miller LD. The role of neurotrophins in neurotransmitter release. Neuroscientist. 8(6):524-31, 2002.

Valvassori SS, Moretti M, Kauer-Sant'Anna M, Roesler R, Petronilho F, Schwartsmann G, Kapczinski F, Dal-Pizzol F, Quevedo J. Effects of a gastrin-releasing peptide receptor

antagonist on D-amphetamine-induced oxidative stress in the rat brain. Journal of Neural Transmission. 117(3):309-16, 2010.

Vianna MR, Izquierdo LA, Barros DM, de Souza MM, Rodrigues C, Sant'Anna MK, Medina JH, Izquierdo I. Pharmacological differences between memory consolidation of habituation to an open field and inhibitory avoidance learning. Brazilian Journal of Medical and Biological Research. 34(2):233-40, 2001.

Vieta E, Popovic D, Rosa A. The clinical implications of cognitive impairment and allostatic load in bipolar disorder. Psychother Psychosom (submitted), 2010.

Walz JC, Frey BN, Andreazza AC, Ceresér KM, Cacilhas AA, Valvassori SS, Quevedo J, Kapczinski F. Effects of lithium and valproate on serum and hippocampal neurotrophin-3 levels in an animal model of mania. Journal of Psychiatric Research. 42(5):416-21, 2008.

Weissman MM. Affective disorders. In: Robins LN, Regier DA. Psychiatric disorders in America: the epidemiologic catchment area study. New York: Free Press, 1991.

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

Xu TX, Sotnikova TD, Liang C, Zhang J, Jung JU, Spealman RD, Gainetdinov RR, Yao WD. Hyperdopaminergic tone erodes prefrontal long-term potential via a D2 receptor-operated protein phosphatase gate. Journal of Neuroscience. 29:14086–14099, 2009.

Yamada K, Nabeshima T. Brain-derived neurotrophic factor/TrkB signaling in memory processes. Journal of Pharmacological Sciences. 91(4):267-70, 2003.

Yatham LN, Liddle PF, Shiah IS, Lam RW, Ngan E, Scarrow G, Imperial M, Stoessl J, Sossi V, Ruth TJ. PET study of [(18)F]6-fluoro-L-dopa uptake in neuroleptic- and mood-stabilizer-naive first-episode nonpsychotic mania: effects of treatment with divalproex sodium. American Journal of Psychiatry. 159(5):768-74, 2002.

Yatham LN, Lyoo IK, Liddle P, Renshaw PF, Wan D, Lam RW, Hwang J. A magnetic resonance imaging study of mood stabilizer- and neuroleptic-naive first-episode mania. Bipolar Disorders. 9:693–697, 2007.

Yoshii A, Constantine-Paton M. Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Developmental Neurobiology. 70(5):304-22, 2010.

ANEXO

Instruções para a submissão de manuscritos na revista *The International Journal of Neuropsychipharmacology*.

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INSTRUCTIONS TO AUTHORS

Mission Statement

The International Journal of Neuropsychopharmacology (*IJNP*) is the official scientific journal of the Collegium Internationale Neuro-psychopharmacologicum (CINP). *IJNP* aims to serve as a major forum for the rapid publication and wide dissemination of high quality, influential research in neuropsychopharmacology, in the basic and clinical domains. The central focus of *IJNP* is on research that (i) advances understanding of existing and new neuropsychopharmacological agents including their mode of action and clinical application, or (ii) provides insights into the biological basis of neuropsychiatric disorders and thereby advances their pharmacological treatment. Such research may derive from the full spectrum of biological and psychological fields of inquiry encompassing classical and novel techniques in neuropsychopharmacology as well as strategies such as neuroimaging, genetics, psychoneuroendocrinology and neuropsychology. Emphasis will be given to original research that is of wide interest and clearly advances the field.

Categories of papers

Regular research articles - This category is intended for full-scale basic or clinical studies including large controlled trials. Articles may contain up to 5,000 words (not including references, figures and tables) and should include an abstract of up to 250 words and 3–5 key words. (Exceptions to the length limitation will be considered for unusually large or complex studies.)

Brief reports/clinical reports - This category is for smaller, self-contained laboratory or clinical studies including series of cases illustrating a novel therapeutic approach or clinical observation, or alerting readers to important adverse drug effects. (Single cases will not be considered in this category and should be submitted as Letters to the Editor.) Papers in this category may contain up to 2,500 words (not including references, figures and tables) and should include a maximum of 25 references, up to 2 illustrations (figures or tables), an abstract of up to 150 words and 3–5 key words.

Rapid communications - This category is for 'fast-breaking' new work, which is of great potential interest and can be succinctly presented.

*Authors who wish to submit a rapid communication must first send an abstract to the Editor in Chief, Alan Frazer (*frazer@uthscsa.edu*) for approval of submission in this category. Submissions that do not have prior approval will be reviewed on the regular track.*

Papers in this category may contain up to 2,500 words (not including references, figures and tables) and should include a maximum of 25 references, up to 2 illustrations (figures or tables), an abstract of up to 150 words and 3–5 key words. Rapid communications will be reviewed and published on a “fast track”.

Reviews - *IJNP* will publish a limited number of scholarly, comprehensive reviews that summarize and critically evaluate research in the field addressed and identify future implications. Reviews will be invited by the Editors but may also be submitted. Authors wishing to submit papers in this category are advised to contact either the Editor-in-Chief or appropriate Field Editor before doing so. Reviews may contain up to 5,000 words (not including references, figures and tables) and should include an abstract of up to 250 words and 3–5 key words. (Exceptions to the length limitation will be considered if justified by the scope of the Review).

Trends and perspectives - Papers in this section provide readers of *IJNP* with focused coverage of topical issues in neuropsychopharmacology and related disciplines which are of high current interest and potential. Hypothesis and Opinion papers, Updates, Commentaries and Notes on New Techniques will also be included. Articles of this type will be invited but may also be submitted. Papers in this section may contain up to 2500 words and should include an abstract of up to 150 words and 3–5 key words. Authors wishing to submit articles for this section are asked to consult with the Editor-in-Chief or appropriate Field Editor.

Letters to the Editor - These should briefly report single experiments and cases of clinical interest or respond to recent articles in *IJNP*. The body of the Letter may contain a maximum of 1,000–1,500 words plus one Figure or Table and up to 10 references.

Focus Papers – These papers focus attention on a research paper published in the same issue in the journal. Focus Papers should highlight, discuss and amplify the issues addressed in the research paper adding perspectives derived the author's own work and the literature and should consider the implications of the findings. Focus papers need not necessarily agree with the paper they address. Focus Papers may contain up to 1500 words (not including references, figures and tables) and 3–5 key words. No abstract is needed. Focus Papers are invited by the Editor-in-Chief.

Papers in all categories, whether invited or submitted, will be peer reviewed.

Clinical Trials

As a condition of consideration for publication, registration of clinical trials in a public trials registry is required. A clinical trial is defined by the International Committee of Medical Journal Editors (in accordance with the definition of the World Health Organisation) as any research project that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes. Trials must be

registered before the start of patient enrollment. The registry must be accessible to the public at no charge. It must be open to all prospective registrants and managed by a not-for-profit organization. There must be a mechanism to ensure the validity of the registration data, and the registry should be electronically searchable. An acceptable registry must include at minimum a unique trial number, trial registration date, secondary identification information if assigned by sponsors or others, funding source(s), primary and secondary sponsor(s), responsible contact person, research contact person, official scientific title of the study, research ethics review, the medical condition being studied, intervention(s), key inclusion and exclusion criteria, study type, anticipated trial start date, target sample size, recruitment status, primary outcome, and key secondary outcomes. Registration information must be provided at the time of submission. Trial registry name, registration identification number, and the URL for the registry should be included at the end of the abstract.

Manuscripts reporting the results of randomized controlled trials should include a "CONSORT" flow diagram as a figure in the manuscript to illustrate the progress of all patients in the study (See: Schulz KF, Altman D, for the CONSORT Group. The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomized trials. JAMA. 2001;285(15):1987-1991.)

Originality and copyright

To be considered for publication in *IJNP*, a manuscript cannot have been published previously, nor can it be under review for publication elsewhere. (Previously published figures may be sparingly used in Reviews, with appropriate permission.) The posting of a brief summary of clinical trial outcomes on a pharmaceutical website (such as the PhRMA-sponsored database < www.clinicalstudyresults.org >) will not necessary count as prior publication nor impede full consideration of a manuscript: *IJNP* will look at this on a case-by-

case basis to determine the extent of overlap between the trial data posted and the manuscript as submitted, and will decide whether the manuscript contains a sufficiently new perspectives or sufficient additional data for it to count as original. Authors should declare when submitting manuscripts that such data have already been posted and *IJNP* will review this sympathetically.

Papers with multiple authors are reviewed with the assumption that all authors have contributed materially to the research reported, have approved the submitted manuscript and concur with its submission to *IJNP*. A Copyright Transfer Agreement, with certain specified rights reserved by the author, must be signed and returned to the Editor by the senior author of accepted manuscripts (Signing on behalf of all the other authors), prior to publication. This is necessary for the wide distribution of research findings, and the protection of both author and the CINP under copyright law.

Authorship

All individuals included as authors of papers must have contributed substantially to the scientific process leading up to the writing of the paper. Such contribution includes the conception and design of the project, the performance of experiments and the analysis and interpretation of data. In addition the author should have made a substantial contribution to drafting or critical revision of the manuscript for important intellectual content. The first author or designated corresponding author should add a statement to this effect to the cover letter and should sign on behalf of the other authors.

The statement regarding authorship must be included in the cover letter.

If there is no statement regarding authorship, the paper will not be reviewed.

We are aware that authors sometimes receive assistance from technical writers, language editors and/or writing agencies in preparing manuscripts for publication. Such assistance must

be noted in the cover letter and in the Acknowledgements section along with a declaration that the author(s) are entirely responsible for the scientific content of the paper.

Failure to acknowledge assistance from technical writers, language editors and/or writing agencies in preparing manuscripts for publication in the cover letter and in the Acknowledgements section may lead to disqualification of the paper.

Under no circumstances will IJNP accept submissions by writing or editorial agencies on behalf of authors and there will be no correspondence with writing or editorial agencies regarding submitted or revised manuscripts.

Manuscript submission

All manuscripts must be submitted online via the website:

http://mc.manuscriptcentral.com/IntJNP

Author Submission Instructions

A generic Author Quick Start Guide is available to download from the Manuscript Central website:

http://mchelp.manuscriptcentral.com/tutorials/Author.pdf

There is also a chapter on manuscript submission in the online interactive guide to Manuscript Central:

http://mcv3help.manuscriptcentral.com/stalkjddfesd/MC4Help.htm

The Editor-in-Chief will acknowledge receipt of the manuscript, provide it with a manuscript reference number and assign it to a Field Editor and to reviewers. The reference number of the manuscript should be quoted in all correspondence with the *IJNP* Office and Publisher.

Initial Submission

The following instructions must be followed carefully:

- The preferred file format for submission is Microsoft Word; however you can also submit Adobe Acrobat (.pdf) files readable with Acrobat Reader. There is no need to include figures in the manuscript document for initial submission. Figures should be submitted as separate files. The system creates a single PDF of all uploaded files as the final stage of author submission.
- Word Perfect or other word-processor files or Macintosh-based files are not acceptable.
- Tables should be placed at the end of the document and not within the text.
- Do not use "enter" in order to start a new page. "Hard" page or section breaks must be used.
- A cover letter should be attached as a separate file. In the cover letter, the category under which the manuscript is submitted should be indicated and the corresponding author identified, including phone number, fax number and electronic mail address. The cover letter must include a statement regarding authorship (see Authorship section above).
- File-names should indicate the name of the first author of the paper or an abbreviated version thereof and the content of the file (text, tables, figures).
- Printed copies of the cover letter, manuscript, tables and figures are not required and should not be sent.

Please note that correspondence regarding submitted and revised manuscripts will be with the Corresponding Author only.

Revised\Final Submission

- The uploaded manuscript must be in the form of a Word for Windows file with figures (prepared as instructed below) in separate files. Word Perfect or other word-processor files or Macintosh-based files are not acceptable.
- Do not use "enter" in order to start a new page. "Hard" page or section breaks must be used.
- The cover letter and responses to reviewers should be attached as a separate file.
- Figures should be prepared using appropriate formats and saved as TIFF or JPEG files. It is essential that JPEGs are greater than 320dpi. PowerPoint files or figures "pasted" into Word files are not acceptable for revised or final submissions.
- File names should indicate the manuscript number assigned by the journal and the content of the file (text, figures).

Review process

Manuscripts submitted to *IJNP* including those for supplements will be reviewed by at least two external reviewers and evaluated by a Field Editor. Authors are required to suggest a minimum of 3 reviewers who are especially qualified to referee the work and would not have a conflict of interest. Please provide the first and second names, email addresses, institution or affiliation, and phone number of the suggested reviewers. If authors would prefer that a particular reviewer(s) not evaluate the paper, they may indicate this request in the cover letter, which will be treated confidentially. Suggestions and requests regarding reviewers will be considered by the Editor without obligation to accept them. Authors should note that manuscripts might be returned after initial review by the Editors if the paper is deemed unlikely to be reviewed favorably. This rapid rejection process enables the author to submit promptly for publication elsewhere.

Every effort will be made to provide the author with a review within 6 weeks of receipt of the manuscript. For Rapid Communications the target review time is 3 weeks. If the Editor requests that minor revisions be made to a manuscript these should be resubmitted within 30 days. A maximum of 90 days shall be allowed for preparation of major revisions, except in unusual circumstances.

Manuscript preparation and style

The manuscript should be typed double-spaced throughout on 'Letter' or A4 paper. Pages should be numbered sequentially beginning with the Title Page. Margins should not be less than 2.5cm on all sides and the font should be clearly legible and uniform throughout.

The *Title Page* should contain the full titles of the manuscript, the category under which the manuscript is submitted, the full names and affiliations of all authors, a contact name and address with telephone number, fax number and electronic mail address (if available) for the corresponding author and reprint requests, and a Statistical Summary containing the Word Count of the abstract and the body of the manuscript (separately), the number of references and the number of figures and tables.

A S*hort Title* of up to fifty characters should be provided on the title page and should be repeated at the top right of every following page. The names of the authors (e.g. Smith et al. or Smith and Jones) should be given at the top left of every page besides the title page.

The *Abstract* (page 2) should be unstructured (i.e., no sub-headings) but must provide the reader with a self-contained summary of the paper. It should include a brief introduction to the paper, the method, the key findings, and the conclusions. A list of 3–5 key words or terms for indexing should follow the abstract.

The *Body of the Manuscript* should begin on page 3. For Regular Research Articles, Brief and Clinical Reports and Rapid Communications, the formal should include: Introduction,

Method, Results, Discussion and Acknowledgements. These should be followed on a new page by the References.

Tables should be consecutively numbered as they appear in the text (Table 1, etc.). Each Table should be typed on a separate sheet with the Table number and heading above and any note below.

Figures should be consecutively numbered as they appear in the text (Figure 1, 2 etc). Use italic letters for parts a, b, c etc. Use abbreviation (Fig), except where starting a new sentence. Legends should be provided for each Figure. Scale bars should be added to photomicrographs and other similar images. Figures should be supplied as either TIFF or EPS files where possible at approximately the size in which they are to reproduce. Line artwork should be supplied in black and white mode at a resolution of 1200 dpi; combination artwork (line/tone) at a resolution of 800 dpi; black and white halftone artwork should be saved in 'grayscale' mode at a resolution of 300dpi; colour halftone artwork should be saved in CMYK mode at a resolution of 400 dpi.

The use of Abbreviations, except those that are widely used, is strongly discouraged. They should be used only if they contribute to better comprehension of the manuscript. Acronyms should be spelled out at first mention. Metric system (SI) units should be used.

IJNP encourages submissions from all over the world. Authors who are not fluent in written English are encouraged to seek assistance in this regard before submitting their manuscripts.

Acknowledgements

In this section authors should acknowledge support for the work that is reported in the paper from granting agencies and other sources. This is particularly important in the case of research that is supported by industry. Support from industry not only includes direct financial support for the study but also support in kind such as provision of medications, equipment, kits or reagents without charge or at reduced cost and provision of services such as statistical analysis. The contribution of individuals who assisted with the research but are not included as authors of the paper may be acknowledged in this section.

The Acknowledgements should be placed after the main body of the text before the references. If there are no Acknowledgements, the title should be inserted followed by "None". Papers that do not include an Acknowledgement section will not be reviewed.

Statement of Interest

Conflict of interest exists when an author has interests that might inappropriately influence his or her judgement, even if that judgement is not influenced. Because of this, authors must disclose potentially conflicting interests so that others can make judgments about such effects. At the time of submission authors should disclose any financial arrangements or connections they may have that are pertinent to the submitted manuscript and that may be perceived as potentially biasing their paper. Non-financial interests that could be relevant in this context should also be disclosed. If no relevant interests exist, this should be stated. This requirement applies to all the authors of a paper and to all categories of papers including letters to the editor. Authors may consult with the Editor-in-Chief regarding material to be included in this disclosure (by email to frazer@uthscsa.edu). Such consultation will be held in strict confidence. All submissions must contain a Statement of Interest.

The Statement of Interest should be placed after the Acknowledgements before the references.

If there are no interests to declare, the title should be inserted followed by "None".

Papers that do not include a Statement of Interest will not be reviewed.

Supplemental online material

The online platform gives authors the opportunity to include data that would be impossible or impractical to include in the printed version. These data might substantially enhance the importance of the research and might also be of benefit to readers. Authors may include tables and figures as well as data such as videos, 3-D structures/images, extensive datasets and any other supplementary material not suitable for print duplication. All supplementary material must be submitted with the original manuscript. Supplementary data should be referred to in the text with the prefix "S" (e.g. Supplementary Table S1, Supplementary Figure S1). Supplementary files will not be copy-edited, and will be published as supplied.

References

Text references should be cited as follows: 'The findings replicate the results of an earlier study (Stahl et al., 1993),' or 'The method used followed that of De Montigny and Aghajanian (1978)'. Multiple references should be cited alphabetically: 'Previous studies (Farde et al., 1980; Lerer and Newman, 1987)'. If multiple works by the same author(s) in the same year, are cited, use a, b, c, in the order these appear in the text (Frazer et al., 1992a, 1992b, etc.). Note that if reference is to a work with more than two authors use et al. to replace the second and any subsequent authors' names. All listed references should be cited in the text.

In the References List, authors should be included using **bold** font and the name of the journal should be given in full, in *italic* font. If there are more than four authors, then the first four

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Scientific Article:

Baldessarini RJ, Hennen J, Wilson M, Calabrese J, *et al.* (2003*a*). Olanzapine versus placebo in acute mania: treatment responses in subgroups. *Journal of Clinical Psychopharmacology* **23**, 370-376.

Baldessarini RJ, Tondo L, Hennen J (2003*b*). Lithium treatment and suicide risk in major affective disorders: update and new findings. *Journal of Clinical Psychiatry* **64** (Suppl.), 44-52.

Ball JR, Mitchell PB, Corry JC, Skillecorn A, *et al* (2006). A randomized controlled trial of cognitive therapy for bipolar disorder: focus on long-term change. *Journal of Clinical Psychiatry* **67**, 277-286.

Bauer MS, Wisniewski SR, Marangell LB, Chessick CA, *et al.* (2006). Are antidepressants associated with new-onset suicidality in bipolar disorder? A prospective study of participants in the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD). *Journal of Clinical Psychiatry* **67**, 48-55.

For work that is 'in press' i.e., accepted for publication but not yet published, '(In Press)' should be written in parenthesis and not the year of expected publication.

Up to four authors names can be listed in a given reference. For papers with more than four co-authors, provide the first-named author only, followed by et al.

Book

Rosenberg DR, Holttum J, Gershon S (1994). *Textbook of Pharmacotherapy for Child and Adolescent Psychiatric Disorders.* New York: Brunner/Mazel.

Chapter in Book

Maes M, Meltzer HY (1995) The serotonin hypothesis of major depression. In: Bloom FE, Kupfer DJ (Eds.), *Psychopharmacology: The Fourth Generation of Progress* (pp. 933-944) New York: Raven Press.

Manual, Diagnostics Scheme, etc.

American Psychiatric Association (1994*) Diagnostic and Statistical Manual of Mental Disorders*, (4th ed.) Washington DC: American Psychiatric Association.

Unpublished work

Unpublished work by the authors including papers submitted for publication but not yet accepted, should not be included in the Reference List but referred to in the text as '(Jones et al., unpublished observations).' Personal Communications made in writing to the author(s) may be sparingly cited as (Smith J, personal communication: June 1998).

Ethical considerations

Papers reporting experiments involving human subjects must contain the statement that the research was approved by an Internal Review Board, Helsinki Committee or similar body and that subjects gave informed consent. Papers describing animal experiments must indicate that the research was approved by a Review Committee or clearly state that the experiments were performed in accordance with accepted guidelines such as 'Guiding principles in the care and use of animals (DHEW Publications, NIH, 80-23). Papers that do not contain an Ethics Statement will not be reviewed.

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