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Tese de Doutorado

DESENVOLVIMENTO DE UM MODELO ANIMAL DE MANIA: CORRELAÇÃO COM MARCADORES BIOQUÍMICOS

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“Um dia nós vamos descobrir a bipolarina.”

Prof. Dr. Ellis D’Arrigo Busnello (2000)

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Resumo

Embora há muito tempo se considere que alterações neurobiológicas tenham um papel central no transtorno bipolar (TB), os mecanismos moleculares ligados à sua fisiopatologia permanecem desconhecidos. A hipótese atual sugere que alterações nos circuitos cerebrais associados à regulação do humor e alterações em sistemas de sinalização intracelular associados à plasticidade e sobrevivência neuronal estão envolvidas no TB. Modelos animais são ferramentas úteis que nos permitam testar estas hipóteses e a resposta aos agentes farmacológicos. Um dos mais bem estabelecidos modelos animais de mania é o de hiperatividade induzida por psicoestimulantes. Portanto, o objetivo dos nossos estudos foi desenvolver um modelo animal de mania avaliando os efeitos do tratamento agudo e crônico com d-anfetamina (ANF) na atividade locomotora em ratos Wistar adultos. Em paralelo, nós investigamos o estresse oxidativo induzido pela ANF no córtex pré-frontal (CPF), hipocampo (HIPO) e estriado. A atividade locomotora foi avaliada através do teste de campo aberto e o malondialdeído (TBARS), proteinas carbonila, superóxido dismutase (SOD) e a catalase (CAT) foram usados como parâmetros de estresse oxidativo. O uso agudo e crônico de ANF aumentou a atividade locomotora e induziu um estado de estresse oxidativo no CPF, HIPO e estriado. Além disso, o tratamento crônico com ANF aumentou a formação de superóxido e TBARS em partículas submitocondriais no CPF e HIPO. Em conjunto, estes resultados sugerem que o tratamento crônico com ANF induz hiperatividade e aumenta o estresse oxidativo no cérebro de ratos. Em uma segunda etapa, estudamos se os estabilizadores de humor litio (Li) e valproato (VPT) previnem e revertem a hiperatividade e as alterações dos marcadores bioquímicos induzidos pela ANF no HIPO de ratos. Com este modelo, observamos que o Li e o VPT reverteram e preveniram a hiperatividade e o estresse oxidativo induzidos pela ANF. Ainda, observamos que o Li e o VPT aumentaram o fator neurotrófico derivado do cérebro e que este efeito pode estar envolvido com o comportamento locomotor. Entretanto, embora o Li tenha aumentado o fator de crescimento neural no HIPO dos ratos, este efeito foi independente ao comportamento locomotor. Nós também estudamos os níveis de SOD, CAT, TBARS e o dano em DNA no soro de duas gemas monozigóticas durante episódio maníaco. As gêmeas bipolares apresentaram elevação em SOD, TBARS e dano ao DNA e redução da CAT que o controle. Os níveis de SOD e de TBARS normalizaram após o tratamento com Li e antipsicóticos. Estes achados estão de acordo com os resultados do modelo animal e indicam que o estresse oxidativo pode estar associado à fisiopatologia do TB. Em conclusão, nossos estudos sugerem que nosso modelo animal apresenta uma boa validade aparente, interpretativa e preditiva como um modelo animal de mania.

Abstract

Although it has long been considered that neurobiological changes play a critical role in bipolar disorder (BD), the molecular mechanisms underlying its pathophysiology remain largely unknown. Current hypothesis suggests that changes within the brain circuits associated with mood regulation, and altered intracellular signaling system associated with neuronal plasticity and survival are involved in BD. Animal models are useful tools that allow us to test these hypotheses and the response to pharmacological agents. One of the best established animal models of mania is the psychostimulant-induced hyperactivity. Therefore, the aim of our studies was to develop an animal model of mania assessing the effects of acute and chronic treatment of d-amphetamine (AMPH) on locomotor activity in adult Wistar rats. In parallel, we investigated AMPH-induced oxidative stress in the prefrontal cortex (PFC), hippocampus (HIPPO), and striatum. Locomotor activity was assessed using the open field test and malondialdehyde (TBARS), protein carbonyl, superoxide dismutase (SOD), and catalase (CAT) were used as oxidative stress parameters. Acute and chronic AMPH exposure increased the locomotor activity and induced an oxidative stress status in the PFC, HIPPO, and striatum. Moreover, chronic AMPH treatment increased superoxide and TBARS formation in submitochondrial particles in the PFC and HIPPO. Taken together, these results suggest that chronic AMPH treatment induces hyperactivity and increased oxidative stress in rat brain. As a second step we studied whether the mood stabilizers lithium (Li) and valproate (VPT) prevent and reverse AMPH-induced hyperactivity and changes in biochemical markers in rat HIPPO. Using this model, we observed that Li and VPT reversed and prevented AMPH-induced hyperactivity and oxidative stress. Further, we found that Li and VPT increased brain-derived neurotrophic factor, and that this effect may be associated with the locomotor behavior. However, although Li was able to increase nerve growth factor level in rat HIPPO, this effect was independent on locomotor behavior. We also studied serum SOD, CAT, TBARS, and DNA damage levels in two monozygotic twins during a manic episode. The bipolar twins had higher SOD, TBARS and DNA damage, and lower CAT than the control. SOD and TBARS levels were normalized after treatment with Li and antipsychotics. These findings are in accordance with the results from the animal model, and further indicate that oxidative stress may be associated with the pathophysiology of BD. In conclusion, our studies suggest that our model presents adequate face, construct, and predictive validity as an animal model of mania.

Lista de Abreviaturas

AMPH = *d-amphetamine*

ANF = d-anfetamina

BD = *bipolar disorder*

BDNF = *brain-derived neurotrophic factor*

CAT = catalase

CPF = córtex pré-frontal

CREB = *cAMP response element binding*

DA = dopamina

GSK-3β = *glycogen synthase kinase 3-beta*

HIPO = hipocampo

HIPPO = *hippocampus*

Li = lítio

NGF = *nerve growth factor*

PET = tomografia por emissão de pósitrons

PFC = *prefrontal cortex*

SOD = superóxido dismutase

TB = transtorno bipolar

TBARS = *thiobarbituric acid reactive species*

VPT = valproato

Introdução

O Transtorno Bipolar (TB) acomete de 1-3% da população em todo o mundo e está associado a um alto índice de suicídio e desemprego (Weissman et al. 1996; Grant et al. 2005; Müller-Oerlinghausen et al. 2002). Segundo dados da Organização Mundial da Saúde, o TB é considerado uma das dez principais causas de incapacitação no mundo (Lopez e Murray 1998). O curso clínico do TB é crônico, usualmente caracterizado por períodos de exacerbação dos sintomas (episódios agudos) intercalados por períodos subsindrômicos e períodos de remissão (eutimia). Um estudo de seguimento que acompanhou pacientes bipolares tipo I por um período médio de 13 anos observou que os pacientes permaneceram metade deste período sintomáticos (Judd et al. 2002), enquanto que a persistência de sintomas subsindrômicos está associado a um maior risco de reagudização da doença (Perlis et al. 2006) e maior índice de incapacitação (Judd et al. 2005). O diagnóstico de TB baseia-se pela ocorrência de pelo menos um episódio maníaco ou hipomaníaco durante a vida, na qual a presença de episodio maníaco confere o diagnóstico de TB tipo I, enquanto a presença de episodio hipomaníaco confere o diagnóstico de TB tipo II (Belmaker 2004). A presença de um episódio maníaco é definida por uma elevação persistente do humor (humor eufórico ou irritável), acompanhado por pelo menos 3 dos seguintes sintomas (4 se humor irritável): aumento da autoconfiança ou grandiosidade, taquilalia ou pressão por falar, diminuição da necessidade do sono, pensamento acelerado ou fuga de idéias, distratibilidade, alteração do comportamento dirigido para atividades prazerosas, freqüentemente imprudentes ou perigosas, ou

agitação psicomotora. Além disso, o episódio deve ser suficientemente severo para causar prejuízo significativo no âmbito familiar, social ou ocupacional, ou necessidade de hospitalização ou ter presença de sintomas psicóticos (American Psychiatric Association 2000). Devido ao curso crônico e à alta recorrência e severidade dos sintomas de humor, o tratamento do TB baseia-se no manejo dos episódios agudos e no tratamento de manutenção como prevenção para ocorrência de novos episódios (Yatham et al. 2005). Entretanto, os índices de recorrência e de resistência aos medicamentos de primeira linha são bastante elevados. Dois estudos que avaliaram indivíduos bipolares tratados em instituições acadêmicas demonstraram que uma alta porcentagem dos pacientes permanece sintomática mesmo quando "adequadamente tratados" (Post et al. 2003; Dennehy et al. 2005). Embora os medicamentos de ultima geração possuam um melhor perfil de tolerabilidade e segurança que os medicamentos mais antigos, muito pouco se adicionou quanto à eficácia em relação aos primeiros medicamentos (Castrén 2005). De fato, o pouco avanço no tratamento do TB em termos de eficácia no controle da doença se deve ao pouco conhecimento sobre os mecanismos fisiopatológicos causadores deste transtorno (Zarate Jr et al. 2006).

Estudos familiares, estudos com gêmeos e estudos de adoção demonstram claramente que a herdabilidade genética é um dos fatores determinantes para o desenvolvimento do transtorno (Craddock e Jones 1999). Para se ter uma idéia em comparação com outras doenças, a herdabilidade do TB é maior do que a do câncer de mama e do diabetes tipo II, sendo que o risco de um familiar em primeiro grau de um individuo afetado desenvolver a doença é 10 vezes maior que o risco da população em geral (Craddock et al. 2005). Estudos neuroanatômicos

utilizando imagem por ressonância magnética têm demonstrado alterações do volume de determinadas regiões cerebrais envolvidas na regulação do humor. Achados que têm sido replicados no TB incluem diminuição do volume do córtex pré-frontal (CPF) subgenual e aumento do volume da amígdala e do estriado (Hajek et al. 2005; Strakowski et al. 2005). Estudos neurofuncionais com ressonância magnética funcional e tomografia por emissão de pósitrons (PET) apontam para uma diminuição significativa do metabolismo do CPF durante a depressão e subseqüente aumento em algumas regiões do CPF durante a fase maníaca (Malhi et al. 2004; Strakowski et al. 2005). Além disso, a diminuição do metabolismo do CPF parece estar acompanhada de um aumento no metabolismo da amígdala e do estriado, o que sugere que alterações no circuito que compreende o CPF, sistema límbico e gânglios da base estão associadas à fisiopatologia do TB. Embora a maioria dos estudos não encontrou alterações de volume do hipocampo no TB, três grupos independentes que utilizaram a técnica de espectroscopia por ressonância magnética observaram uma diminuição do N-acetil-aspartato (um marcador de viabilidade neuronal) no hipocampo de indivíduos bipolares (Atmaca et al. In press; Bertolino et al. 2003; Deicken et al. 2003), sugerindo uma diminuição do funcionamento dos neurônios hipocampais no TB. Nesta mesma linha, estudos conduzidos em tecido pós-mortem revelaram que indivíduos bipolares apresentam uma diminuição significativa de células neuronais e gliais no CPF dorsolateral e no CPF subgenual (Rajkowska et al. 2001; Öngur et al. 1998; Bouras et al. 2001). No hipocampo, indivíduos com TB apresentam diminuição da arborização de dendritos apicais, bem como diminuição da densidade de espinhas dendríticas em neurônios piramidais (Rosoklija et al.

2000), e diminuição do número de neurônios não-piramidais na região CA2 (Benes et al. 1998). Uma avaliação mais detalhada destas alterações neuropatológicas sugere que estas modificações seguem um padrão de alteração neurodesenvolvimental e de neuroplasticidade, e não um padrão de degeneração cerebral como previamente se pensava (Rajkowska 2003). Em paralelo, tem sido demonstrado que antidepressivos e estabilizadores de humor atuam modulando diversas cascatas de sinalização celular envolvidas em neuroplasticidade e sobrevivência neuronal (Manji et al. 2001). A primeira evidência surgiu dos trabalhos do laboratório do Prof. Ronald Duman demonstrando que o uso crônico de antidepressivos e de choques eletroconvulsivos aumentam a expressão do RNAm do fator neurotrófico derivado do cérebro (BDNF), do seu receptor trkB e do fator de transcrição nuclear CREB (*cAMP response element binding*) em hipocampo de ratos (Nibuya et al. 1995, 1996). Mais recentemente, estudos pré-clínicos têm demonstrado que o tratamento crônico com lítio ou valproato (medicamentos de primeira linha para o tratamento do TB) também são capazes de aumentar a expressão do BDNF no córtex frontal e hipocampo (Einat et al. 2003; Fukumoto et al. 2001). Além disso, os estabilizadores do humor possuem outros mecanismos de ação em comum, como inibição da GSK-3β (*glycogen synthase kinase 3-beta*), uma proteína que regula vias de sobrevivência e morte celular, e diminuição de inositol, um precursor da via de sinalização do fosfatidilinositol (Coyle e Manji 2002; Williams et al. 2002). Este recente corpo de evidências dá um passo adiante em relação à antiga “hipótese monoaminérgica”, a qual sugeria que os transtornos de humor seriam causados por uma deficiência

de monoaminas, particularmente de serotonina e noradrenalina (Schildkraut 1965). Atualmente acredita-se que os transtornos de humor são associados a alterações no sistema de comunicação entre os circuitos cerebrais reguladores do humor, e que antidepressivos e estabilizadores do humor ativam cascatas de sinalização que regulam a plasticidade e sobrevivência celular, com subseqüente melhora gradativa da transmissão da informação nestes circuitos cerebrais (Castrén 2005; Coyle e Duman 2003; Nestler et al. 2002; Manji et al. 2001).

Embora as evidências sejam mais fortes quando os resultados são em humanos, estudos em modelos animais permitem testar e levantar hipóteses em relação aos mecanismos fisiopatológicos dos diferentes transtornos mentais. Como conseqüência, estudos pré-clínicos são ferramentais fundamentais no desenvolvimento de novos agentes terapêuticos. A escolha de um modelo animal para pesquisa de transtornos mentais baseia-se fundamentalmente no quanto a fisiopatologia do determinado transtorno é conhecida, na especificidade e eficácia dos agentes farmacológicos e na capacidade de reproduzir as alterações comportamentais em animais de laboratório (Einat et al. 2000). Até o presente, não existe um modelo animal ideal para o TB (Einat e Manji 2006; Machado-Vieira et al. 2004), uma vez que o padrão crônico e oscilatório deste transtorno, com variações comportamentais extremas e clinicamente opostas é um verdadeiro desafio a ser modelado. Portanto, a resolução deste problema tem sido o desenvolvimento de modelos animais que reproduzem determinados aspectos do quadro clínico deste transtorno. A validação de um modelo animal de transtorno mental deve seguir os 3 seguintes critérios: validade aparente (*face validity*), validade preditiva (*predictive validity*) e validade interpretativa (*construct validity*)

(Willner 1986). Validade aparente representa o quanto o comportamento do animal consegue mimetizar os sintomas de um determinado transtorno. Validade interpretativa se refere aos aspectos fisiopatológicos que teoricamente explicariam as alterações encontradas no modelo e no correspondente transtorno testado. A validade preditiva, por sua vez, avalia o quanto as alterações comportamentais induzidas no modelo são revertidas ou prevenidas pelos agentes de primeira linha utilizados no tratamento do transtorno em questão.

Considerando que o TB caracteriza-se fundamentalmente pela presença de episódios maníacos e depressivos, e diversos modelos animais de depressão têm sido desenvolvidos (Nestler et al. 2002), modelos animais de TB têm focalizado no quadro clínico de mania. A dificuldade de desenvolver um modelo adequado de mania inicia na dificuldade de reproduzir o sintoma central, que é o humor elevado ou euforia (aqui cabe lembrar que esta mesma dificuldade existe para reproduzir o "humor depressivo"). Desta forma, modelos animais de mania geralmente avaliam atividade locomotora, agressividade, ou comportamento de risco ou recompensa (Machado-Vieira et al. 2004; Einat et al. 2000). Modelos que possuem relevância quanto ao quadro clínico do TB mas não têm sido explorados sistematicamente com este propósito incluem comportamento sexual, julgamento e diminuição do padrão de sono. Mais recentemente, com o avanço das técnicas de genética molecular têm sido desenvolvidos modelos baseados em "endofenótipos", ou seja, na expressão de um sinal, sintoma, ou comportamento que sejam intermediários entre os genes e a doença em questão (Lenox et al. 2002; Niculescu et al. 2000). O modelo de hiperatividade induzido por psicoestimulantes (usualmente anfetamina ou cocaína) é considerado o modelo animal de mania mais bem

estabelecido até o presente (Machado-Vieira et al. 2004; Gould et al. 2004; Nestler et al. 2002). Esse modelo é baseado em dois aspectos: um deles é a hiperatividade causada pelo tratamento agudo com psicoestimulantes, que pode ser prevenido com pré-tratamento com lítio (Berggren 1985), enquanto o segundo é o modelo de sensibilização comportamental (*behavioral sensitization*) induzida pelo tratamento crônico com psicoestimulantes (Robinson e Becker 1986). O modelo de sensibilização comportamental segue a teoria do *kindling* inicialmente proposto pelo Prof. Robert Post, no qual os repetidos episódios agudos de humor progressivamente seriam mais severos e temporalmente mais próximos, analogicamente similar ao padrão de episódios convulsivos observados na epilepsia (Post e Weiss 1996). Após extensa revisão da bibliografia sobre o assunto, nosso grupo decidiu por desenvolver um modelo animal de mania baseado na exposição crônica de d-anfetamina (ANF) em ratos.

A ANF atua principalmente facilitando a liberação de dopamina (DA) pelas vesículas pré-sinápticas, mas também age bloqueando a recaptação de DA pelos transportadores de DA pré-sinápticos (Sulzer et al. 1995). Desta forma, a ANF aumenta significativamente o conteúdo de DA na fenda sináptica, ativando a transmissão dopaminérgica cerebral. O envolvimento da via dopaminérgica no TB é apoiado por estudos farmacológicos, genéticos, pós-mortem e de neuroimagem. Há muito tempo se sabe que a administração de substâncias dopaminérgicas, como ANF ou L-DOPA, induz sintomas maníacos em indivíduos com TB (Muphy et al. 1971; Gerner et al. 1976). Mais tarde, Jacobs e Silverstone (1986) demonstraram que a ANF também é capaz de induzir sintomas maníacos em voluntários normais. Além disso, os medicamentos bloqueadores do receptor

dopaminérgico D2 são considerados agentes de primeira escolha no manejo da mania aguda (Yatham et al. 2005). Um estudo de PET demonstrou que o tratamento com divalproato de sódio reduziu a captação de ^{18}F-DOPA no estriado de pacientes bipolares em episódio maníaco, o que sugere uma diminuição da função dopaminérgica pré-sináptica após o uso de divalproato (Yatham et al. 2002). Alterações de receptores dopaminérgicos no TB têm sido demonstrados em dois estudos que observaram um aumento de 25% da expressão do RNAm do receptor D1 na região CA2 do hipocampo (Pantazopoulos et al. 2004) e uma menor expressão do receptor D3 em linfócitos de indivíduos bipolares (Vogel et al. 2004). Mais recentemente, tem sido sugerido que variações do gene do transportador de DA podem estar envolvidas na suscetibilidade para o desenvolvimento do TB (Greenwood et al. 2006). Em conjunto, estes estudos indicam que alterações do sistema dopaminérgico podem estar envolvidas nos mecanismos fisiopatológicos do TB e apóiam o uso da ANF como um modelo animal de mania (validade interpretativa).

Primeiro Modelo Animal

Como um primeiro passo no desenvolvimento do modelo animal de mania, estudamos um modelo inicial de hiperatividade induzida pelo uso agudo e crônico de ANF. Conforme comentado anteriormente não existe um modelo que reproduza todos os aspectos clínicos de um episódio maníaco, portanto, modelos animais de mania avaliam um determinado aspecto do transtorno. Considerando que um dos aspectos centrais do estado maníaco é o aumento da atividade motora (validade

aparente), primeiramente estudamos a resposta locomotora de ratos machos Wistar ao tratamento agudo (injeção única) e crônico (uma injeção por 7 dias) de três diferentes doses (1 mg/kg, 2 mg/kg ou 4 mg/kg) de ANF administradas via intraperitoneal (IP). A escolha de ratos machos se deve pela evidência de que ratas fêmeas apresentam diferentes respostas locomotoras ao tratamento com ANF de acordo com a fase hormonal (Becker e Cha 1989). Os protocolos de 1 e 7 dias para o tratamento agudo e crônico foram baseados em estudos prévios de que 1 e 7 dias de uso de ANF induzem um estado de hiperatividade locomotora em ratos (Panayi et al. 2002; Sams-Dodd 1998). A escolha das doses de ANF se deu a partir de experimentos demonstrando que doses entre 0,5 a 3 mg/kg de ANF aumentam progressivamente a atividade locomotora em ratos Wistar, enquanto doses acima de 3 mg/kg diminuem a locomoção e aumentam a ocorrência de estereotipias (Antoniou et al. 1998). A escolha da via IP se deu pela maior experiência do nosso grupo, uma vez que a grande maioria dos estudos que avaliaram a resposta locomotora com uso de ANF utilizou a via IP ou a via subcutânea (Einat et al. 2000). Como avaliação da atividade locomotora dos ratos, utilizamos o teste de campo aberto (*open field*) que é um teste universalmente aceito e validado para este propósito, no qual avaliamos a atividade horizontal (número de cruzamentos de um retângulo ao outro) e a atividade vertical (número de vezes em que o animal ergue as patas dianteiras e mantém-se sob as patas traseiras, ou *rearings*). Decidimos por avaliar a atividade locomotora dos ratos 2 horas após a última injeção de ANF pelo seguinte motivo: sabe-se que o efeito locomotor máximo do uso agudo de ANF em ratos se dá na primeira hora, sobretudo entre os primeiros 10-20 minutos (Kuczenski et al. 1991); portanto,

escolhemos avaliar a locomoção na segunda hora para que os resultados obtidos com o tratamento crônico não se confundam com o efeito agudo da última injeção. A atividade locomotora no teste de campo aberto foi medida durante 5 minutos. Este teste foi reproduzido a partir dos trabalhos publicados por diversos grupos do Departamento de Bioquímica da UFRGS demonstrando que este período é adequado para avaliar a atividade locomotora de ratos Wistar (Vinade et al. 2005; Barros et al. 2002).

Paralelamente ao comportamento locomotor dos animais, neste modelo preliminar estudamos índices de estresse oxidativo induzidos pelo uso agudo e crônico de ANF no CPF, hipocampo e estriado. As razões pelo estudo destas regiões cerebrais provêm das evidências de que estas regiões fazem parte do circuito de regulação do humor e parecem estar envolvidas na fisiopatologia do TB, conforme discutido anteriormente. O papel do estresse oxidativo no TB tem sido sugerido por estudos que observaram uma redução da atividade da superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (principais enzimas antioxidantes) e aumento de malondialdeído (um produto da peroxidação lipídica, também conhecido como uma das espécies reativas ao ácido barbitúrico, ou TBARS) em sangue periférico de indivíduos com TB (Ozcan et al. 2004; Ranjekar et al. 2003; Kuloglu et al. 2002). Portanto, nossos experimentos iniciais com este modelo deram origem aos dois primeiros trabalhos que serão apresentados na próxima seção, em que estudamos os efeitos do uso agudo e crônico de ANF na formação de produtos da oxidação lipídica e protéica (Frey et al. *Bipolar Disorders* 2006) e na atividade da SOD e da CAT (Frey et al. *Neurochemical Research* 2006) no CPF, hipocampo e estriado de ratos. No

terceiro trabalho deste modelo, estudamos a formação de superóxido ($O_2^{\cdot-}$) e de TBARS em partículas submitocondriais no CPF e hipocampo de ratos (Frey et al. *Brain Research* 2006). A justificativa para o estudo do estresse oxidativo em partículas submitocondriais surgiu fundamentalmente da corrente hipótese de disfunção mitocondrial no TB (Kato e Kato 2000; Konradi et al. 2004).

Segundo Modelo Animal

A partir deste primeiro modelo, nosso grupo partiu para o próximo passo que seria tratar os animais com lítio (Li) e valproato (VPT), que são os estabilizadores de humor mais utilizados na prática clínica, sob a hipótese de que as alterações comportamentais e bioquímicas causadas pelo uso crônico de ANF 2 mg/kg IP seriam revertidas e prevenidas pelos estabilizadores de humor. Este desenho, portanto, permitiria testar a validade preditiva do nosso modelo animal. A justificativa da escolha do uso de 2 mg/kg de ANF provem dos nossos resultados com o primeiro modelo em que observamos que esta foi a dose de ANF que provocou maior atividade locomotora nos animais, conforme será discutido mais adiante. Já a escolha das doses de Li (47,5 mg/kg 2 x/dia) e VPT (200 mg/kg 2 x/dia) foram baseadas em nossos próprios experimentos preliminares nos quais estas dosagens reverteram a hiperatividade induzida pela ANF sem causar efeitos sedativos ou tóxicos nos animais (dados não publicados). Além disso, uma vez que os resultados do primeiro modelo demonstraram que 7 dias de uso de ANF aumentou o estresse oxidativo e provocou um desequilíbrio nos níveis de SOD e CAT, desenhamos dois experimentos utilizando Li e VPT como tratamento. No

primeiro experimento, chamado “tratamento de reversão”, os animais foram tratados com ANF por 7 dias, seguidos por mais 7 dias de tratamento com ANF + estabilizador de humor (Li ou VPT). Já no segundo experimento, este chamado de “tratamento de prevenção”, os animais foram inicialmente tratados com estabilizador de humor (Li ou VPT) por 7 dias, seguidos por mais 7 dias de estabilizador de humor + ANF. Os desenhos destes experimentos foram propostos com o objetivo de mimetizar o manejo farmacológico do TB, que baseia-se no tratamento do episódio agudo (reversão) e no tratamento de manutenção (prevenção), conforme discutido anteriormente. Aqui cabe lembrar que em todos os experimentos realizados nós utilizamos animais “controles”, que utilizaram salina (NaCl 0,9%) nos mesmos tempos que os animais tratados com as substâncias ativas. Este modelo também deu origem a três publicações, no primeiro deles estudamos os efeitos do Li e do VPT na formação de produtos da oxidação lipídica e protéica, e na atividade da SOD e da CAT no hipocampo de ratos tratados com ANF (Frey et al. *Journal of Psychiatry and Neuroscience* 2006). No segundo trabalho, estudamos os efeitos do Li e do VPT nos níveis de BDNF (*brain-derived neurotrophic factor*) no hipocampo de ratos tratados com ANF (Frey et al. *Life Sciences* 2006). A razão pelo estudo do BDNF se deve pelo crescente corpo de evidências sugerindo que a via de sinalização do BDNF/trkB parece estar envolvida na fisiopatologia dos transtornos de humor, bem como na ação dos antidepressivos e dos estabilizadores de humor (Hashimoto et al. 2004). A hipótese de que alterações em cascatas de sinalização que regulam a sobrevivência e a plasticidade celular estão associadas ao TB e os achados preliminares sugerindo que o Li aumenta os níveis de NGF (*nerve growth factor*)

em ratos (Angelucci et al. 2003; Hellweg et al. 2002) instigou-nos a investigar os efeitos do Li e do VPT nos níveis de NGF no hipocampo de ratos tratados com ANF (Frey et al. *Behavioural Pharmacology* 2006).

Estudos em Humanos

Durante o período de desenvolvimento deste modelo animal de mania, nosso grupo também investigou a presença de alterações de marcadores bioquímicos em pacientes portadores de TB tipo I, isto é, em indivíduos com história atual ou passada de pelo menos um episódio maníaco durante a vida. No primeiro trabalho, investigamos os níveis de BDNF no soro de indivíduos bipolares durante episódio maníaco, depressivo e em eutimia (remissão) (Cunha et al. 2006). No segundo estudo, utilizamos a técnica do Cometa (*single cell gel electrophoresis assay*) que detecta quebras em ligas simples e duplas do DNA em sangue total de pacientes com TB (Andreazza et al. In press). O fato de que neste último trabalho encontramos um aumento muito significativo de dano ao DNA nos indivíduos com TB e que este dano pode ter sido causado por aumento do estresse oxidativo (Faust et al. 2004) nos levou a conduzir um estudo piloto no qual investigamos parâmetros de estresse oxidativo (TBARS, SOD e CAT) e de dano ao DNA (técnica do Cometa) em duas gêmeas monozigóticas durante episódio maníaco e os efeitos do tratamento com Li e antipsicóticos na reversão destes marcadores bioquímicos (Frey et al. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 2006).

Objetivos

Objetivo Geral

1. Desenvolver um modelo animal de mania em ratos baseado na hiperatividade locomotora induzida pela d-anfetamina.

Objetivos Específicos

1. Avaliar a formação de produtos da oxidação lipídica e protéica no córtex cerebral, hipocampo e estriado após tratamento agudo e crônico com d-anfetamina.
2. Avaliar os efeitos do tratamento agudo e crônico com d-anfetamina na atividade da superóxido dismutase e da catalase no córtex pré-frontal, hipocampo e estriado.
3. Avaliar os efeitos do tratamento agudo e crônico com d-anfetamina na formação de superóxido e de TBARS em partículas submitocondriais no córtex pré-frontal e hipocampo.
4. Avaliar os efeitos do lítio e do valproato nas alterações dos produtos de oxidação lipídica e protéica e da atividade da superóxido dismutase e da catalase induzidas pelo tratamento crônico com d-anfetamina no hipocampo.

5. Avaliar o perfil do estresse oxidativo e os efeitos do tratamento farmacológico em duas gêmeas monozigóticas durante episódio maníaco.
6. Avaliar se a administração de lítio e valproato reverte e previne os efeitos comportamentais do tratamento crônico com d-anfetamina e os efeitos da d-anfetamina e dos estabilizadores de humor nos níveis de BDNF no hipocampo.
7. Avaliar se o lítio e o valproato revertem e previnem os efeitos do tratamento crônico com d-anfetamina na atividade locomotora e nos níveis de NGF no hipocampo.

Na próxima seção, os trabalhos são apresentados no formato em que foram aceitos pelas revistas.

Parte II. Artigos publicados ou aceitos para publicação

Capítulo 1

Frey BN, Martins MR, Petronilho FC, Dal-Pizzol F, Quevedo J, Kapczinski F. Increased oxidative stress after repeated amphetamine exposure: possible relevance as a model of mania. *Bipolar Disorders* 8(3):275-280, 2006a.

Bipolar Disorders 2006: 8: 275–280

BIPOLAR DISORDERS

Original Article

Increased oxidative stress after repeated amphetamine exposure: possible relevance as a model of mania

Frey BN, Martins MR, Petronilho FC, Dal-Pizzol F, Quevedo J, Kapczinski F. Increased oxidative stress after repeated amphetamine exposure: possible relevance as a model of mania.
Bipolar Disord 2006: 8: 275–280. © Blackwell Munksgaard, 2006

Background: Acute mania can be modeled in animals using D-amphetamine (AMPH). Acute AMPH injections are associated with monoamine depletion, loss of neurofilaments and neurite degeneration. However, the precise mechanisms underlying AMPH-induced neurotoxicity are still unclear. Several studies have demonstrated that oxidative stress may play a role in the behavioral and neurochemical changes observed after AMPH administration.

Methods: The effects of a single and repeated injections (seven daily injections) of AMPH administered intraperitonially on locomotion and the production of lipid and protein oxidative markers in rat cortex, striatum and hippocampus were assessed. Locomotion was assessed in an open-field task and markers of oxidative stress were assessed in brain tissue.

Results: Both single and repeated injections of AMPH increased protein carbonyl formation in rat brain. Repeated exposure to AMPH induced an additional increase in thiobarbituric acid reactive species in brain tissue.

Conclusions: Longer periods of exposure to AMPH were associated with increased oxidative stress in rat brain. This adds to the notion that repeated manic episodes may be associated with greater brain damage and, therefore, poorer outcomes.

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Key words: amphetamine – animal model – bipolar disorder – mania – oxidative stress

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The use of amphetamine (AMPH) in rats has been put forward as a model of acute mania (1–3). AMPH-like psychostimulants promote acute and long-term changes in monoaminergic neurons (4), but the mechanisms underlying AMPH-induced neurotoxicity are not completely understood. Repeated AMPH administration induces long-term dopamine (DA) and serotonin (5HT) depletion (5), loss of neurofilaments (6), and neurite degeneration (7). In humans, brain-imaging studies demonstrated that AMPH can induce DA release in the striatum (8), and reduce striatal DA transporter density (9). Moreover, postmortem studies revealed that AMPH abusers presented reduction of striatal DA markers (10), increased copper–zinc superoxide dismutase, and uric acid in regions with more DA loss (11). However, data gathered from human and animal studies indicate that the severity of the AMPH-induced neurodegeneration may vary substantially across species and different brain regions (4).

Recent studies suggest that the formation of reactive oxygen species (ROS) and reactive

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nitrogen species (RNS) may play a role in the behavioral changes and neurodegeneration after AMPH use (12, 13). Fukami et al. (13) found that antioxidant *N*-acetyl-L-cysteine (NAC) reverses hyperlocomotion and behavioral sensitization in rats after methamphetamine administration, and that NAC protects against methamphetamine-induced neurotoxicity in rat striatum. It has been suggested that monoamine depletion precedes ROS-induced toxicity, and the formation of ROS is dependent of monoamine oxidation, whereas continued ROS formation is not (5).

The present study was designed to assess the formation of lipid and protein oxidation products in the cerebral cortex, striatum, and hippocampus of rats exposed to a single injection and repeated treatment using AMPH.

Methods

Animals

Experiments were performed on male Wistar rats (age, 3–4 months; weight, 220–310 g) obtained from our breeding colony. They were housed five to a cage with food and water available *ad libitum*, and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Experimental design

The animals were divided into four experimental groups: control, AMPH 1, AMPH 2, and AMPH 4 (12–15 animals per group). Animals received 1 mg/kg (AMPH 1), 2 mg/kg (AMPH 2), or 4 mg/kg (AMPH 4) intraperitoneal injections of D-amphetamine (Sigma, St Louis, MO, USA), either acute treatment (single injection), or repeated treatment (one injection daily for 7 days). Locomotor activity was measured 2 and 6 h after the last injection of AMPH, and the rats were sacrificed by decapitation right after the behavioral experiment. Striatum, hippocampus, and cortex were dissected, rapidly frozen, and stored at −80°C until assayed.

Locomotor activity

The locomotor activity was evaluated in the open-field task. This task was carried out in 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. Animals were gently placed on the left rear quadrant, and left to explore the arena for 5 min. Crossings of the black lines and rearings performed were counted.

Thiobarbituric acid reactive species (TBARS)

As an index of ROS production we used the formation of TBARS during an acid-heating reaction, which is widely adopted as a sensitive method for measurement of lipid peroxidation (14). Briefly, the samples were mixed with 1 mL of trichloroacetic acid 10% (TCA) and 1 mL of thiobarbituric acid 0.67% (TBA), then heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm. Results are expressed as malondialdehyde (MDA) equivalents (nmol/mg protein).

Measurement of protein carbonyls

The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described by Levine et al. (15). Briefly, proteins were precipitated by the addition of 20% TCA and redissolved in DNPH and the absorbance read at 370 nm.

Statistical analysis

All data are presented as mean ± SEM. Differences among experimental groups in experiments evaluating oxidative damage and behavioral parameters were determined by one-way ANOVA. Multiple comparisons were performed by a Newman–Keuls test. In all experiments, $p < 0.05$ were considered to indicate statistical significance.

Results

Fig. 1 shows that an acute (single injection) and repeated AMPH treatment significantly increased locomotion and rearing 2 h after the last AMPH injection, whereas no hyperactivity was seen 6 h after the last AMPH injection (data not shown). Furthermore, AMPH 2 group differs from both AMPH 1 and AMPH 4 groups in acute treatment. However, this difference was not seen in repeated treatment (Fig. 1), suggesting that AMPH might have induced behavioral sensitization after repeated administration (16).

Lipid peroxidation products (TBARS) are diminished in striatum and cortex 2 h after a single

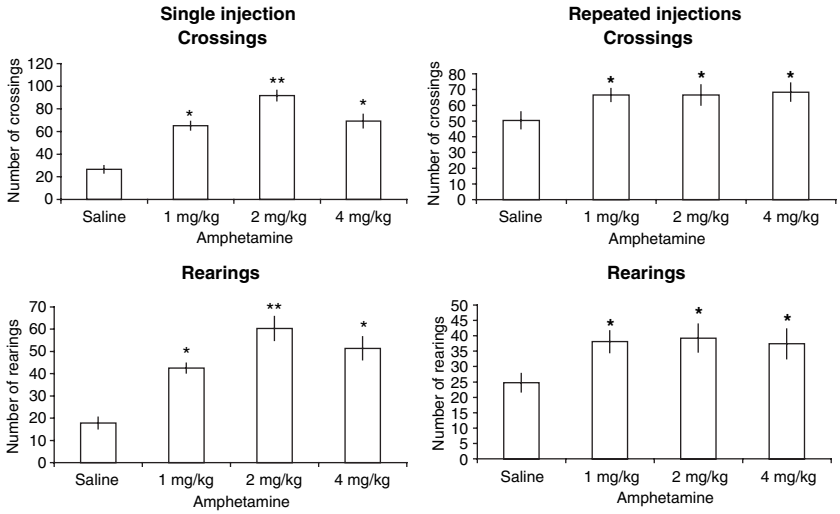

Fig. 1. Open-field task. Data are presented as mean ± SEM. Open-field assessment was carried out after a single injection and after seven daily injections of amphetamine. *p < 0.05 (saline versus active); **p < 0.05 when compared with other active groups.

AMPH injection, and these effects are absent 6 h after AMPH administration. No such changes were observed in the hippocampus after a single injection of AMPH. Conversely, repeated exposure to AMPH increased lipid peroxidation 2 h after the last AMPH injection in all brain regions (Fig. 2).

Fig. 3 demonstrates that a single dose of AMPH increased protein markers of oxidative stress (protein carbonyls) in striatum and hippocampus at 2 h, and after 6 h of MAPH injection this change was also detected. This suggests that striatum and hippocampus are susceptible to AMPH-induced oxidative stress. No changes were observed in the cortex. A 7-day administration of AMPH (repeated treatment) induced a significant increase in protein oxidation in all brain regions at 2 h after the last injection of AMPH. This change was detected after 6 h of AMPH injection in the striatum and cortex, but not in the hippocampus. These results suggest that after repeated use of AMPH (a) the hippocampus may develop sources of protection between 2 and 6 h after the last injection, (b) the cortex may become toxic, while (c) the striatum continues to be sensitive to AMPH-induced protein toxicity.

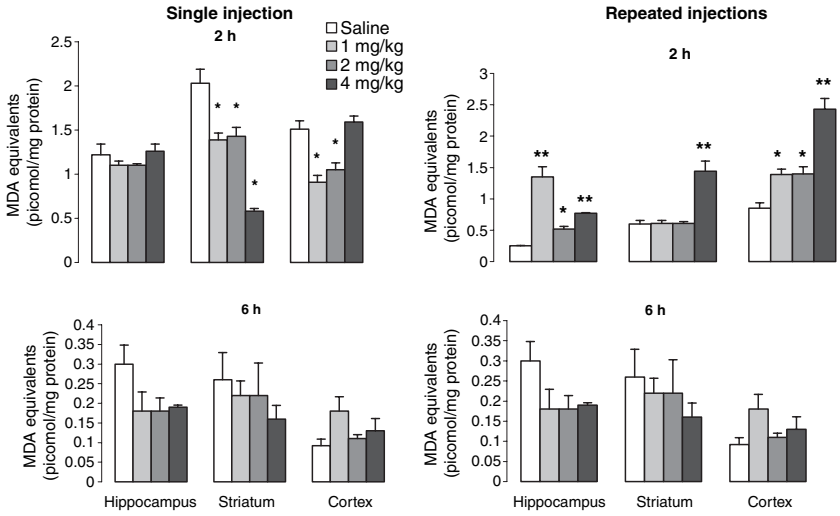

Fig. 2. Thiobarbituric acid reactive species (TBARS) assessment in brain. Data are presented as mean ± SEM. TBARS assessment 2 and 6 h after a single injection and after seven daily injections of amphetamine. MDA = malondialdehyde. *p < 0.05; **p < 0.01 when compared with control group (saline).

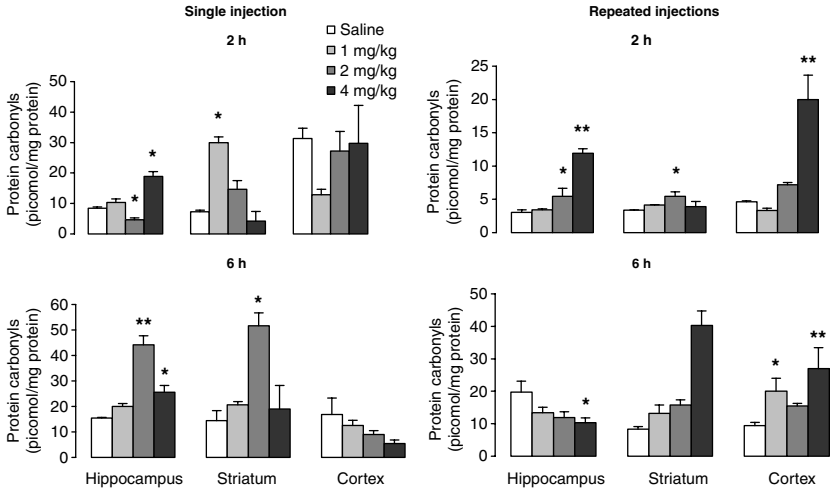

Fig. 3. Protein carbonyls assessment in brain. Data are presented as mean ± SEM. Protein carbonyls assessment 2 and 6 h after a single injection and after seven daily injections of amphetamine. *$p < 0.05$; **$p < 0.01$ when compared with control group (saline).

Discussion

The present study showed that, in a model of hyperactivity induced by AMPH, both single and repeated administration of AMPH increased protein carbonyl formation in rat brain. Repeated exposure to AMPH induced an additional increase in TBARS.

D-amphetamine-induced formation of lipid and protein oxidation products varies across brain regions and depends on the treatment regimen (single/repeated injections). Gluck et al. (5) reported that AMPH promote time-dependent and brain region-selective elevation of protein carbonyls and TBARS in mice. More specifically, the authors demonstrated elevated TBARS and protein carbonyl levels at 4 and 24 h after 40 mg/kg of AMPH in striatum and at 24 h in hippocampus; no changes in oxidative stress markers was observed in cortex (5). In rat striatum, it has been demonstrated that at least 15 mg/kg of AMPH is necessary to induce early (30 min) TBARS increase in a single dose regimen (17). Elevations in TBARS were also induced by the repeated administration of AMPH (5 mg/kg) (18). These results are in agreement with our study as we observed that repeated, but not a single injection of AMPH-induced lipid peroxidation in striatum, hippocampus, and cortex in rats. We also demonstrated that repeated AMPH exposure increased protein oxidation in all brain regions, and this effect persisted for, at least, 6 h after the last injection in striatum and cortex, but not in hippocampus.

Although the exact mechanisms of AMPH-induced neurotoxicity are unknown, it has been strongly suggested that oxidative stress may be involved (12). After AMPH administration, DA may accumulate in the intracellular compartment and predispose to DA-quinones formation (19) that can react with proteins and nucleic acids (7). In addition, Burrows et al. (20) demonstrated that AMPH may increase ROS formation by inhibiting mitochondrial electron transport chain complexes. It has been suggested that glutamatergic excitotoxicity may contribute to AMPH-induced neurodegeneration (4, 21). AMPH administration increases glutamate (GLUT) release in rat striatum (12, 22), and both ionotropic (23) and metabotropic (21) GLUT antagonists protected against AMPH-induced neurotoxicity. In a recent study, Jayanthi et al. (24) demonstrated that AMPH administration caused neuronal apoptosis through caspase-dependent and -independent pathways.

Kuloglu et al. (25) have demonstrated increased lipid peroxidation in blood from BD patients, suggesting that oxidative stress may play a role in the pathophysiology of BD. Two magnetic resonance spectroscopy studies have shown increased glutamate/glutamine peak in both adult (26) and juvenile (27) BD samples. Moreover, postmortem studies of bipolar patients have found decreased neuronal and glial cells in several brain regions, including prefrontal (28), anterior cingulate cortex (29), and hippocampus (30). These findings suggest that BD pathophysiology and AMPH-induced neurotoxicity may share common features, such as oxidative stress, glutamatergic toxicity, and cell death. In agreement with this model, antiglutamatergic drugs (such as valproate, carbamazepine, and lamotrigine) are extensively used in BD treatment. Additionally, lithium antagonizes both glutamatergic neurotoxicity (31) and AMPH-

mediated hyperlocomotion (32). In this regard, given the extensive neuroprotective effects of lithium and valproate against excitotoxicity mediated by NMDA receptors in cultured neurons (31), studies addressing the use of these mood stabilizers against AMPH-induced oxidative stress may further validate our animal model.

A previous study showed that pretreatment with lithium did not prevent AMPH-induced symptoms after a single dose of AMPH in healthy volunteers (33). Such findings raise a cautionary note on the validity of the use of AMPH injections as a model of mania. However, in the present study, not only a single injection, but several repeated injections of AMPH were administered. The use of several days of treatment with AMPH may be a better model of a manic episode, which is well known to last for several weeks or months.

This animal model study suggests that longer periods of induced manic-like hyperactivity are associated with more severe damages in brain. This adds to the notion that acute mania is a medical emergency and should be treated accordingly (34). Apart from the damages that acute mania can bring to patients and their relatives, clinicians might consider that the cumulative effect of repeated manic episodes may induce additional brain dysfunction, which might translate into poorer outcomes.

Acknowledgement

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Capítulo 2

Frey BN, Valvassori SS, Réus GZ, Martins MR, Petronilho FC, Bardini K, Dal-Pizzol F, Kapczinski F, Quevedo J. Changes in antioxidant defense enzymes after D-amphetamine exposure: implications as an animal model of mania. *Neurochemical Research* 31(5):699-703, 2006b.

Neurochem Res (2006) 31:699–703
DOI 10.1007/s11064-006-9070-6

ORIGINAL PAPER

Changes in Antioxidant Defense Enzymes after D-amphetamine Exposure: Implications as an Animal Model of Mania

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Abstract Studies have demonstrated that oxidative stress is associated with amphetamine-induced neurotoxicity, but little is known about the adaptations of antioxidant enzymes in the brain after amphetamine exposure. We studied the effects of acute and chronic amphetamine administration on superoxide dismutase (SOD) and catalase (CAT) activity, in a rodent model of mania. Male Wistar rats received either a single IP injection of D-amphetamine (1 mg/kg, 2 mg/kg, or 4 mg/kg) or vehicle (acute treatment). In the chronic treatment rats received a daily IP injection of either D-amphetamine (1 mg/kg, 2 mg/kg, or 4 mg/kg) or vehicle for 7 days. Locomotor behavior was assessed using the open field test. SOD and CAT activities were measured in the prefrontal cortex, hippocampus, and striatum. Acute and to a greater extent chronic amphetamine treatment increased locomotor behavior and affected SOD and CAT activities in the prefrontal cortex, hippocampus and striatum. Our findings suggest that amphetamine exposure is associated with an imbalance between SOD and CAT activity in the prefrontal cortex, hippocampus and striatum.

Keywords Animal model · Amphetamine · Bipolar disorder · Catalase · Oxidative stress · Superoxide dismutase

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Introduction

There is an emerging body of data indicating that major neuropsychiatric disorders, such as bipolar disorder (BD) and schizophrenia, are associated with increased oxidative stress and changes in antioxidant enzymatic defense [1–3]. A recent genetic study found that BD was significantly associated with a single-nucleotide polymorphism in the TRPM2 gene, which is involved in intracellular calcium homeostasis in response to oxidative stress [4]. In addition, chronic administration of lithium and valproate (first-line mood stabilizers) demonstrated robust antioxidant properties against glutamate-induced oxidative stress in vitro [5].

It has long been recognized that the administration of dopaminergic drugs induces manic symptoms in individuals with BD [6, 7]. Further, the use of amphetamine in healthy volunteers induced manic symptoms, such as enhanced mood, racing thoughts, high energy and restlessness [8]. Considering the difficulty of modeling the highly complex mood swinging nature of BD, the psychostimulant-induced

hyperactivity is the best established animal model of mania [9–11]. We have recently found that longer administration of amphetamine was associated with increased protein and lipid oxidative damage in rat brain [12]. As an extension of this latter study, we assessed the effects of acute and chronic amphetamine administration on superoxide dismutase and catalase activity (two major antioxidant enzymes) in a rodent model of mania.

Experimental procedure

Adult male Wistar rats, obtained from our breeding colony, were maintained on a 12-h light/dark cycle, with free access to food and water. All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. Rats received 1 mg/kg, 2 mg/kg, or 4 mg/kg IP injections of D-amphetamine (Sigma, St Louis, USA) either as an acute treatment (single injection) or chronic treatment (once daily injection for 7 days). Locomotor activity was measured 2 h after the last injection, and the rats were sacrificed by decapitation right after the behavioral experiment. The prefrontal cortex, hippocampus and striatum were dissected, rapidly frozen, and stored at −80°C until assayed.

Locomotor activity was assessed using the open-field task. This task was carried out in 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 rectangle, and allowed to explore the arena. Crossings of the black lines and rearings performed were counted for 5 min. To determine CAT activity, the brain tissue was sonicated in 50 mM phosphate buffer and the resulting suspension was centrifuged at 3,000*g* for 10 min. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm [13]. SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described [14]. Differences among groups were performed using one-way ANOVA and multiple comparisons were performed by a Newman-Keuls test. Behavioral data are presented as mean ± SEM. and biochemical data are presented as mean ± SD. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

Results

As expected, both acute and repeated amphetamine administration significantly increased locomotor activity (Figs. 1, 2). Figures 3 and 4 illustrate that a single amphetamine injection increased SOD activity in the prefrontal cortex, whereas repeated amphetamine exposure increased SOD activity in the hippocampus and decreased striatal SOD activity. No effects on CAT activity were observed after a single amphetamine injection in any brain region (Fig. 5). Repeated amphetamine administration increased CAT activity in the prefrontal cortex and decreased striatal CAT activity (Fig. 6). Higher amphetamine dosage (4 mg/kg) increased CAT activity, whereas a lower dosage (2 mg/kg) decreased CAT activity in the hippocampus after repeated amphetamine exposure.

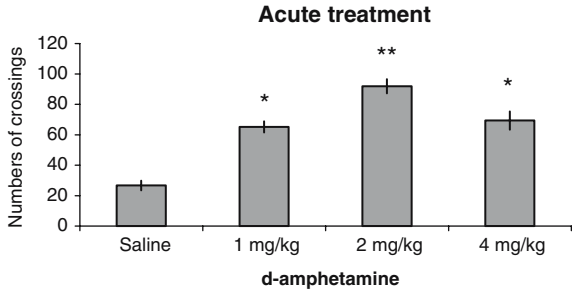

Fig. 1 Numbers of crossings in the acute treatment

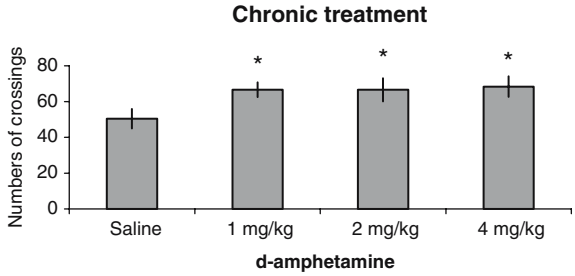

Fig. 2 Numbers of crossings in the chronic treatment

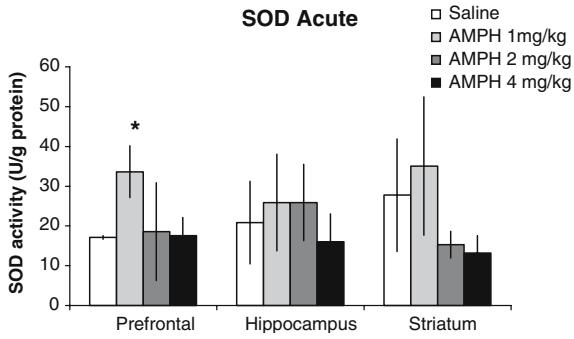

Fig. 3 SOD activity in the acute treatment SOD: superoxide dismutase

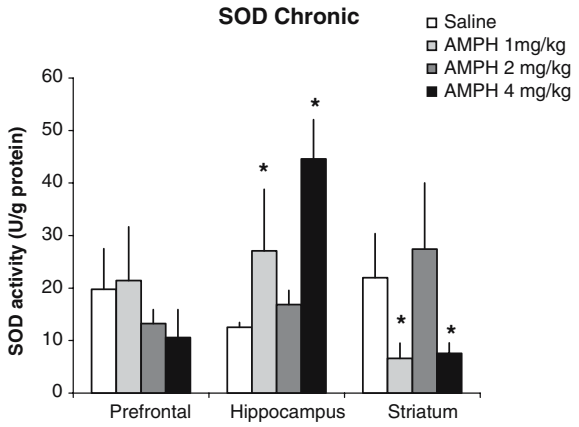

Fig. 4 SOD activity in the chronic treatment SOD: superoxide dismutase

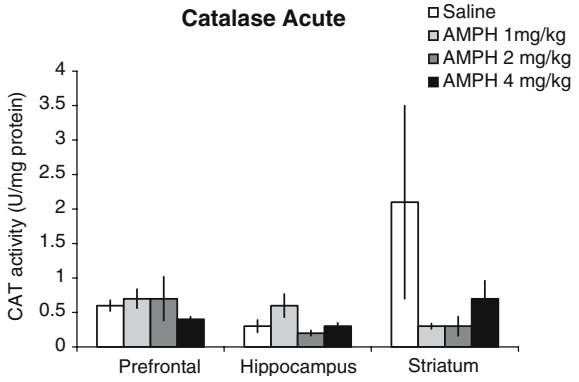

Fig. 5 Catalase activity in the acute treatment

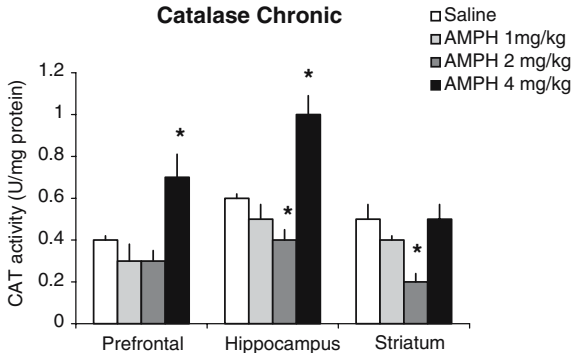

Fig. 6 Catalase activity in the chronic treatment

Discussion

In the present study, we demonstrated that acute and, to a greater extent, repeated amphetamine exposure modified SOD and CAT activities in the prefrontal cortex, hippocampus and striatum. Using the same animal model, we have recently reported that repeated amphetamine administration increased oxidative stress in a greater extent than single amphetamine use [12]. SOD acts by metabolizing the excess of superoxide anion ($O_2^{\cdot-}$) generation and by producing hydrogen peroxide (H_2O_2). CAT metabolizes the excess of H_2O_2 producing $O_2 + H_2O$, thereby decreasing the intracellular redox status. The brain is particularly prone to oxidative damage due to its relative high content of peroxidizable fatty acids and limited antioxidant capacity [15]. Previous studies have demonstrated that alterations on the redox state can lead to an imbalance between SOD and CAT activities and to oxidative stress [16, 17]. In situations which SOD levels are increased without a concomitant CAT increase, the intermediate product H_2O_2 may accumulate and generate hydroxyl radicals, which may lead to lipid and protein oxidation (damage).

Even though there is compelling data indicating that oxidative stress plays a major role in amphetamine-induced neurotoxicity [18, 19], there is a paucity of data assessing the effects of amphetamine on antioxidant enzymes. In a model of neurotoxicity (4 × 10 mg/kg), Jayanthi et al. [20] found that methamphetamine exposure decreased SOD activity in the frontal cortex and decreased CAT activity in the striatum of CD-1 mice. Using a different model (20 mg/kg for 14 days), Carvalho et al. [21] showed that amphetamine decreased SOD activity in the striatum and increased CAT activity in the prefrontal cortex of Wistar rats. On the other hand, D'Almeida et al. [22] found no changes in SOD and CAT activity after chronic methamphetamine treatment (2.5 mg/kg for 5 months) in Wistar rats. The profound methodological differences between studies make it difficult to draw conclusions, and therefore further studies are necessary to clarify the importance of these antioxidant changes. Interestingly, it has been reported that the dopamine D2 receptor agonist ropinirole protected mouse striatal neurons against 6-hydroxydopamine (6-OHDA) toxicity, by increasing SOD, CAT, and glutathione activity [23], whereas the dopamine agonist cabergoline demonstrated robust antioxidant effects against 6-OHDA-neurotoxicity [24]. In addition, the stimulation of D2 presynaptic autoreceptors may exert neuroprotective effects by a negative feedback mechanism, reducing the release of dopamine for oxidation by monoamine oxidase [25]. Studies assessing the effect of D2 blockers on amphetamine-induced oxidative stress would help to increase our knowledge on the dopamine-mediated neuroprotection and neurotoxicity.

Acute and chronic amphetamine exposure modulated SOD and CAT activity with a distinct pattern for each brain region and dosage regimen. These findings may explain, in part, why different areas of the brain are differentially susceptible to the toxic effects of amphetamine [26, 27]. One reason for these discrepancies may be fact the basal

activities of various antioxidant enzymes, such as SOD, CAT, glutathione reductase, glutathione peroxidase, and glutathione-*S*-transferase are highly variable across brain regions [21]. It has been recently demonstrated that amphetamine regulates the expression of SOD mRNA via c-fos/c-jun activation in the hypothalamus [28]. Although speculative, it is possible that the regulation of antioxidant's gene expression by amphetamine in other brain regions may be relevant to the differences observed. Future studies addressing the genes regulated by amphetamine exposure may help us clarify this issue.

We also found that during acute treatment, 4 mg/kg of amphetamine increased the locomotor behavior to a lesser extent than 2 mg/kg. During chronic treatment all of the dosages induced the same level of locomotor activation. This is in line with previous reports showing that the locomotor activity after acute amphetamine challenge reduces with increasing dosage, due to the emergence of stereotypic behavior [29, 30]. In conclusion, our findings suggest that acute and, to a greater extent, chronic amphetamine administrations are associated with an imbalance between SOD and CAT activities. This is possibly due to changes in the intracellular redox state, in the prefrontal cortex, hippocampus, and striatum. Such an imbalance may increase the predisposition to the generation of free radicals and therefore increase the susceptibility to oxidative damage. Given the recent evidence that oxidative stress may play a role in the pathophysiology of BD [1–4], this animal model may be a useful tool to further test the molecular underpinnings underlying amphetamine-induced oxidative stress.

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Capítulo 3

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Research Report

Increased oxidative stress in submitochondrial particles after chronic amphetamine exposure

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Abbreviations:
BD, bipolar disorder
ETC, electron transport chain
ROS, reactive oxygen species
SCZ, schizophrenia
SMP, submitochondrial particles
TBARS, thiobarbituric acid reactive substances

ABSTRACT

Previous studies have suggested that reactive oxygen species (ROS) production may play a role in the pathophysiology of many neuropsychiatric disorders, such as bipolar disorder (BD) and schizophrenia (SCZ). In addition, there is an emerging body of data indicating that BD and SCZ may be associated with mitochondrial dysfunction. We studied the effects of acute and chronic d-amphetamine on ROS production in submitochondrial particles of rat brain. Male Wistar rats were divided in two experimental groups: acute and chronic treatment. In the acute treatment, rats received one single IP injection of d-amphetamine (1, 2 or 4 mg/kg) or saline (control group). In the chronic treatment, rats received one daily IP injection of d-amphetamine (1, 2 or 4 mg/kg) or saline for 7 days. Locomotor activity was assessed with the open field task, and thiobarbituric acid reactive substances (TBARS) and superoxide production were measured in submitochondrial particles of the prefrontal cortex and hippocampus. Both acute and chronic amphetamine treatment increased locomotor behavior. Chronic amphetamine exposure induced a 3- to 6-fold increase of TBARS and a 1.5- to 2-fold increase of superoxide production in submitochondrial particles of prefrontal cortex and hippocampus ($P < 0.05$). No effects on superoxide or TBARS were observed with acute treatment. These findings suggest that amphetamine-induced mitochondrial ROS generation may be a useful model to investigate the hypothesis of altered brain energy metabolism associated with BD and SCZ. Further studies assessing the effects of mood stabilizers and antipsychotics in preventing mitochondrial oxidative stress are necessary.

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1. Introduction

There is an emerging body of data indicating that impaired energetic metabolism due to mitochondrial dysfunction may play a role in the pathophysiology of major mental disorders, such as bipolar disorder (BD) and schizophrenia (SCZ) (Ben-Shachar, 2002; Kato and Kato, 2000). Brain magnetic resonance spectroscopy studies have demonstrated decreased N-acetyl-aspartate (a marker of mitochondrial energy production) (Clark, 1998) and lower pH and phosphocreatine levels in BD and SCZ subjects (Gangadhar et al., 2004; Stork and Renshaw, 2005), further suggesting altered brain energy metabolism in vivo. Moreover, recent postmortem studies have reported changes in mitochondrial-related gene expression in BD and SCZ (Iwamoto et al., 2005; Munakata et al., 2005).

The central nervous system requires a high-energy supply due to its intense ATP-consuming processes. Thus, abnormal cellular energy metabolism may impair neuronal function and plasticity. Under normal conditions, mitochondria are the major source of reactive oxygen species (ROS), which are produced in the complexes of the electron transport chain (ETC) (Mattiasson, 2004). On the other hand, a shift in the antioxidant/pro-oxidant balance towards oxidative stress may inhibit ETC complexes, leading to decrease in ATP production and cellular dysfunction (Calabrese et al., 2001). It has been reported that amphetamines inhibited ETC complexes (Burrows et al., 2000), thereby impairing mitochondrial functioning. However, the effects of amphetamines on mitochondrial function are not fully understood (Brown and Yamamoto, 2003).

It is well known that dopamine (DA) antagonists are first-line agents in the treatment of manic and psychotic episodes (Falkai et al., 2005; Yatham et al., 2005). Given the proposed role of dopamine in the pathophysiology of BD and SCZ (Greenwood et al., 2006; Ben-Shachar, 2002), we studied the effects of acute and chronic amphetamine exposure on the generation of ROS in submitochondrial particles in the rat brain. More specifically, we decided to investigate the prefrontal cortex and hippocampus because alterations in these brain regions are thought to be associated with BD and SCZ (Soares and Mann, 1997; Antonova et al., 2004).

2. Results

Both acute and chronic d-amphetamine administration significantly increased locomotion and rearing behavior ($P < 0.05$; all active groups vs. saline; Figs. 1 and 2). As previously reported (Frey et al., in press), a single injection of 2 mg/kg of d-amphetamine induced higher locomotor activity than 1 or 4 mg/kg ($P < 0.05$; 2 mg/kg vs. 1 and 4 mg/kg). This difference between active groups was not observed in the chronic treatment.

Repeated d-amphetamine exposure increased superoxide ($F = 5.08$; $P = 0.017$; one-way ANOVA; Fig. 3) and TBARS ($F = 12.20$; $P = 0.001$; one-way ANOVA; Fig. 4) generation in the hippocampus (chronic treatment). We also found that repeated d-amphetamine treatment increased superoxide ($F = 5.27$; $P = 0.015$; one-way ANOVA; Fig. 5) and TBARS ($F = 9.29$; $P = 0.002$; one-way ANOVA; Fig. 6) generation in the prefrontal cortex. No significant effects on ROS generation were observed after a single d-amphetamine administration (acute treatment).

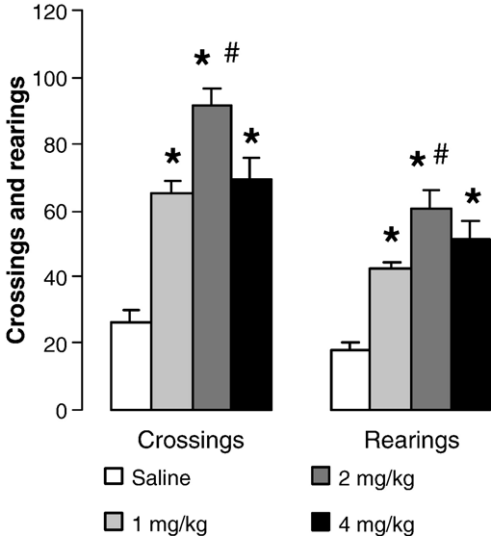

Fig. 1 – Numbers of crossings and rearings after acute d-amphetamine exposure ($n = 10$ per group). $^{*}P < 0.05$ (active groups vs. saline; Newman–Keuls test). $^{\#}P < 0.05$ (2 mg/kg vs. 1 and 4 mg/kg; Newman–Keuls test).

3. Discussion

We demonstrated that repeated d-amphetamine exposure lead to increased superoxide and TBARS formation in prefrontal and hippocampal submitochondrial particles in vivo. Amphetamine-induced generation of superoxide and products of lipid peroxidation (TBARS) from mitochondria may originate from several sources (Brown and Yamamoto, 2003). One potential source is the inhibition of mitochondrial ETC complexes (Burrows et al., 2000). Moreover, because amphetamine increases DA release from cytoplasmic vesicles, increased DA metabolism via monoamine oxidase (which is located in the outer mitochondrial membrane) may produce hydrogen peroxide and dihydroxyphenylacetic acid (Berman and Hastings, 1999). In addition, DA may undergo spontaneous auto-oxidation and form highly reactant DA quinones (LaVoie and Hastings, 1999). It has been postulated that mutations in mitochondrial DNA (mtDNA) could lead to increased ROS generation and vice versa (de Grey, 2005). Interestingly, recent studies have suggested that ROS generation may play a role in the increased mtDNA mutations observed in neuropsychiatric disorders, such as BD and SCZ (Marchbanks et al., 2003; Munakata et al., 2005).

We also found no effects on ROS production in submitochondrial particles after a single d-amphetamine administration. Recent reports have demonstrated that a single injection of larger doses of AMPH (5–7.5 mg/kg) increased lipid peroxidation in rat cortex and striatum (Bashkatova et al., 2002; Wan et al., 2000). Differences in drug regimens and animal strains may account for this discrepancy. Using

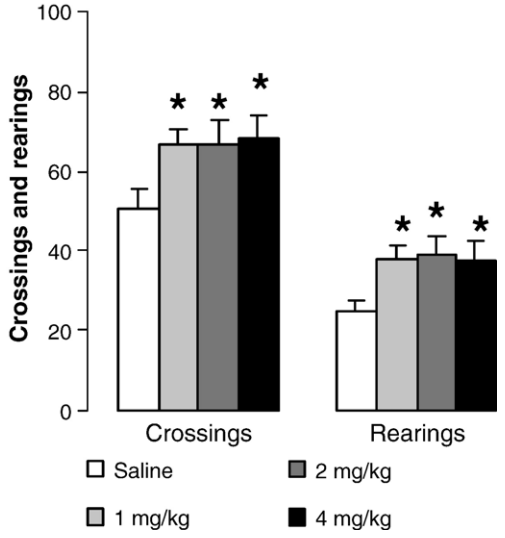

Fig. 2 – Number of crossings and rearings after chronic d-amphetamine exposure (n = 10 per group). *P < 0.05 (active groups vs. saline; Newman–Keuls test).

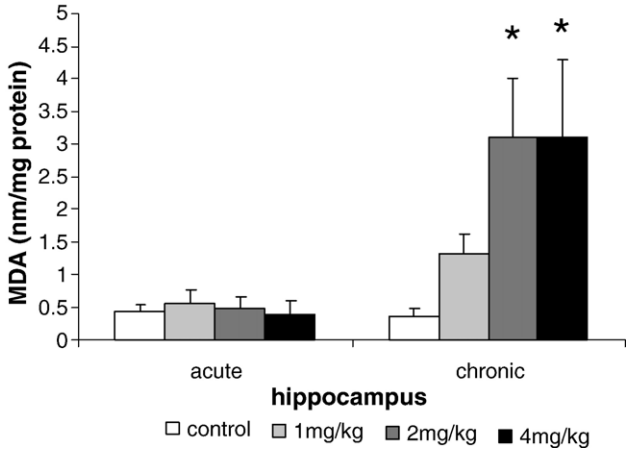

Fig. 4 – TBARS levels in submitochondrial particles after acute and chronic d-amphetamine exposure (n = 4 per group). *P < 0.001 (vs. saline; Newman–Keuls test). TBARS: thiobarbituric acid reactive substances.

dopaminergic cultured neurons, Lotharius and O'Malley (2001) found a rapid increase in ROS generation after d-amphetamine incubation. However, the authors found that only prolonged amphetamine exposure induced oxidative protein damage (Lotharius and O'Malley, 2001). Further evidence supporting the role of oxidative stress in AMPH-induced neurotoxicity is that the antioxidants α-phenyl-*N*-*tert*-butyl nitrone and *N*-acetylcysteine partially prevented amphetamine-induced DA depletion and lipid peroxidation in rat striatum (Wan et al., 2006). In humans, it has been reported increased lipid peroxidation and decreased SOD and CAT activity in erythrocytes of 3,4-methylenedioxymethamphetamine abusers (Zhou et al., 2003). In a postmortem study, Mirecki et al. (2004) showed moderate changes in SOD and oxidized glutathione in the caudate of methamphetamine users.

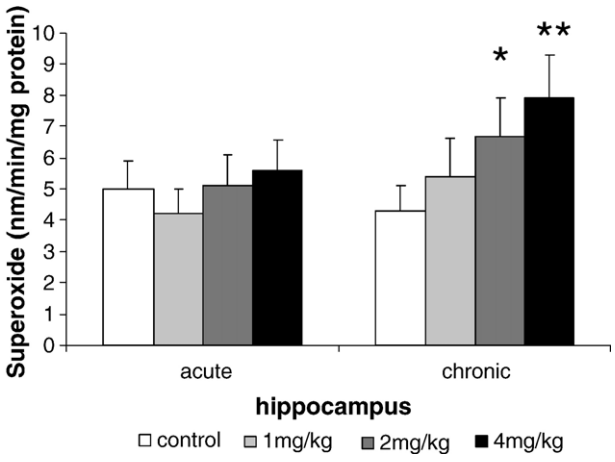

Fig. 3 – Superoxide levels in submitochondrial particles after acute and chronic d-amphetamine exposure (n = 4 per group). *P = 0.032 (vs. saline; Newman–Keuls test). **P = 0.004 (vs. saline; Newman–Keuls test).

In the present study, we have used a relative low-dose d-amphetamine regimen (1–4 mg/kg) and previous data have demonstrated that much higher doses of amphetamine are necessary to induce cellular death in vivo (Ryan et al., 1990; Davidson et al., 2001; Krasnova et al., 2005). Interestingly, recent studies have found that ROS might act as intracellular messengers to mediate neuroprotective effects after brain ischemia (Liang et al., 2005; Mattiasson et al., 2003). More specifically, it has been demonstrated that superoxide produced in the mitochondria activates uncoupling proteins (Echtay et al., 2002) through the generation of lipid peroxidation products (Murphy et al., 2003), thereby lowering mitochondrial membrane potential and decreasing intramitochondrial ROS levels, like a negative feedback (Echtay et al., 2002; Mattiasson et al., 2003; Murphy et al., 2003). Using the same model, we previously showed that chronic, but not acute, d-amphetamine administration increased TBARS in brain homogenates in vivo (Frey et al., in press). Although speculative, we hypothesize that this initial, but limited (Calabrese et al., 2001), brain's ability to withstand oxidative stress might be overwhelmed after accumulate (chronic) amphetamine-induced ROS generation. Further studies are warranted to test this hypothesis. In this same vain, it has been demonstrated that chronic amphetamine exposure altered the activity of the major antioxidant enzymes in rat brain (Carvalho et al., 2001).

There is growing evidence supporting that glutamate may play a role in amphetamine-induced oxidative stress. It has been demonstrated that amphetamine augmented cortical glutamate efflux in rodents (Stephans and Yamamoto, 1994). Overstimulation of NMDA receptors induces abnormal Ca^{+2} influx, mitochondrial dysfunction and excessive free radical generation (Schinder et al., 1996). Interestingly, the administration of ionotropic (Sonsalla et al., 1989; Wan et al., 2000) and metabotropic receptor-5 antagonists (Battaglia et al., 2002) prevented amphetamine-induced neurotoxicity. In addition, the excess of free radicals generated by amphetamine exposure may interact with glutamate transporters in astrocytes and further increase glutamate concentration by

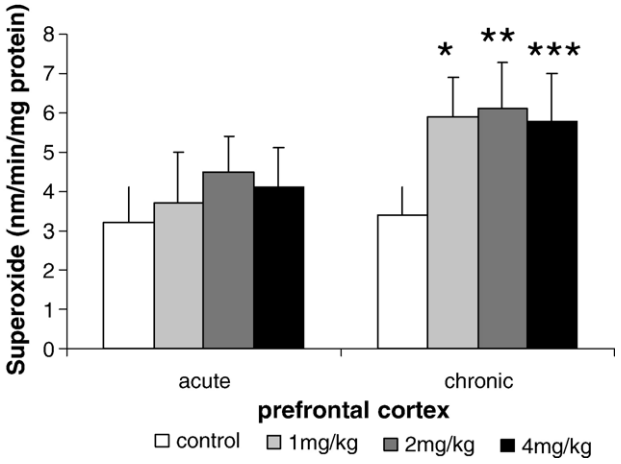

Fig. 5 – Superoxide levels in submitochondrial particles after acute and chronic d-amphetamine exposure (n = 4 per group). $^{*}P$ = 0.009 (vs. saline; Newman–Keuls test). $^{**}P$ = 0.004 (vs. saline; Newman–Keuls test). $^{***}P$ = 0.012 (vs. saline; Newman–Keuls test).

inhibiting its uptake (Volterra et al., 1994). Although the activity of glutamate decarboxylase and choline acetyltransferase were not altered after high doses of amphetamine suggesting that GABAergic and cholinergic neurons may be spared (Hotchkiss et al., 1979), the role of other neurotransmitters in amphetamine-induced neurotoxicity remains to be elucidated (Ricaurte and McCann, 1992).

Thus, amphetamine-induced mitochondrial ROS generation may provide a useful model to test the hypothesis of altered brain energy metabolism associated to neuropsychiatric disorders. Studies addressing the effects of mood stabilizers and antipsychotics may provide new insights about their mechanisms of action. Additionally, a better understanding of how ROS generation impairs mitochondrial function may help to design novel therapeutics to reduce the cognitive decline observed in severe mentally ill subjects.

4. Experimental procedures

4.1. *Animals*

Adult male Wistar rats were obtained from our breeding colony. They were housed five to a cage with free access to food and water and were maintained on a 12-h light/dark cycle (lights on at 7:00 AM). All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

4.2. *Experimental model*

The animals were divided into two groups: acute and chronic treatment. In the acute treatment, rats received a single intraperitoneal injection of saline (control) or d-amphetamine (Sigma, St. Louis, MO, USA) 1, 2 or 4 mg/kg. In the chronic treatment, rats received one daily injection of vehicle (control) or d-amphetamine 1, 2 or 4 mg/kg for 7 days. It has been previously demonstrated that at low-intermediate doses, amphetamine increased locomotor behavior, which was progressively reduced with higher doses due to the emergence of stereotyped behavior (Antoniou et al., 1998). Locomotor activity was measured 2 h (Bashkatova et al., 2002) after the last injection of AMPH, and the rats were sacrificed by decapitation immediately after the behavioral experiment. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80 °C for posterior biochemical analysis.

4.3. *Locomotor activity*

The apparatus and procedures were described elsewhere (Barros et al., 2002; Frey et al., in press). The open field task was carried out in 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. Animals were gently placed on the left rear rectangle and were left to explore the arena for 5 min. Crossings of the black lines and rearings performed were counted.

4.4. *Oxidative stress in submitochondrial particles*

As an index of uncoupling of electron transporter chain (ETC), the generation of mitochondrial superoxide ($O_2^{\cdot-}$) was measured as previously described (Poderoso et al., 1996). In brief, superoxide anion production was determined in washed submitochondrial particles (SMP) using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37 °C (E480 nm = 4.0 mM cm). Mitochondria (1 mg/ml) were treated for 10 min, at 37 °C. SMP were obtained by freezing and thawing (three times) the mitochondria solution, washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) and suspended in the same medium. The reaction medium consisted of 230 mM mannitol, 70 mM

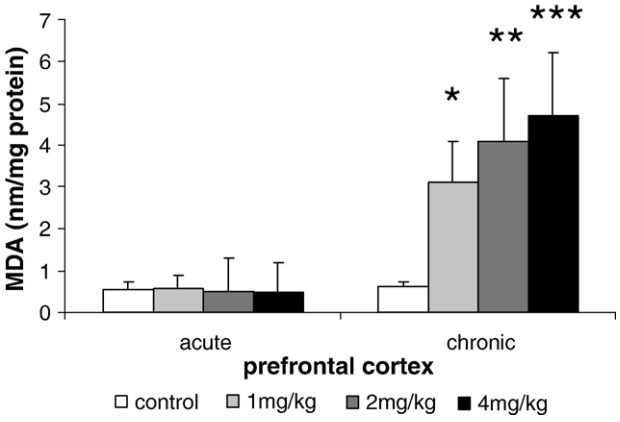

Fig. 6 – TBARS levels in submitochondrial particles after acute and chronic d-amphetamine exposure (n = 4 per group). $^{*}P$ = 0.011 (vs. saline; Newman–Keuls test). $^{**}P$ = 0.001 (vs. saline; Newman–Keuls test). $^{***}P < 0.001$ (vs. saline; Newman–Keuls test). TBARS: thiobarbituric acid reactive substances.

sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, SMP (1.0 mg protein/ml), 0.1 μM catalase and 1 mM epinephrine. Superoxide dismutase (E.C. 1.15.1.1.) was used at 0.1–0.3 μM final concentration as a negative control to confirm assay specificity. As a marker of lipid peroxidation, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described (Esterbauer and Cheeseman, 1990). Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, and then heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm. All the results were normalized by the protein content, using bovine albumin as standard (Lowry et al., 1951).

4.5. *Statistical analysis*

Results were presented as mean ± SEM. Differences among experimental groups were determined by one-way ANOVA. Multiple comparisons were performed by a Newman–Keuls test. P values <0.05 were considered to indicate statistical significance.

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Capítulo 4

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):326-332, 2006d.

Effects of lithium and valproate on amphetamine-induced oxidative stress generation in an animal model of mania

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Objective: Previous studies have suggested that oxidative stress may play a role in the pathophysiology of bipolar disorder (BD). Moreover, recent studies indicate that lithium and valproate exert neuroprotective effects against oxidative stress. We studied the effects of the mood stabilizers lithium and valproate on amphetamine-induced oxidative stress in an animal model of mania. **Methods:** In the first model (reversal treatment), adult male Wistar rats received d-amphetamine or saline for 14 days, and between the 8th and 14th days, they were treated with lithium, valproate or saline. In the second model (prevention treatment), rats were pretreated with lithium, valproate or saline, and between the 8th and 14th days, they received d-amphetamine or saline. We assessed locomotor activity with the open-field task. We measured thiobarbituric acid reactive substances (TBARS) and protein carbonyl formation, as parameters of oxidative stress, and superoxide dismutase (SOD) and catalase (CAT), the major antioxidant enzymes, in the prefrontal cortex and hippocampus. **Results:** Lithium and valproate reversed (reversal treatment model) and prevented (prevention treatment model) amphetamine-induced hyperactivity and reversed and prevented amphetamine-induced TBARS formation in both experiments. However, the co-administration of lithium or valproate with amphetamine increased lipid peroxidation, depending on the brain region and treatment regimen. No changes in protein carbonyl formation were observed. SOD activity varied with different treatment regimens, and CAT activity increased when the index of lipid peroxidation was more robust. **Conclusion:** Our findings suggest that lithium and valproate exert protective effects against amphetamine-induced oxidative stress in vivo, further supporting the hypothesis that oxidative stress may be associated with the pathophysiology of BD.

Objectif : Des études antérieures ont indiqué que le stress oxydant peut jouer un rôle dans la pathophysiologie du trouble bipolaire. Des études récentes indiquent de plus que le lithium et le valproate ont un effet neuroprotecteur contre le stress oxydant. Nous avons étudié les effets du lithium et du valproate, agents thymorégulateurs, sur le stress oxydant provoqué par des amphétamines dans un modèle animal de la manie. **Méthodes :** Dans le premier modèle (traitement d'inversion), des rats Wistar mâles adultes ont reçu de la d-amphétamine ou une solution physiologique pendant 14 jours et, entre le 8e et le 14e jour, on leur a administré du lithium, du valproate ou une solution physiologique. Dans le deuxième modèle (traitement de prévention), on a traité au préalable les rats en leur administrant du lithium, du valproate ou une solution physiologique, et ils ont reçu, entre le 8e et le 14e jour, de la d-amphétamine ou une solution physi-

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Medical subject headings: animal models; bipolar disorder; dopaminergic system; lithium; psychopharmacology.

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ologique. Nous avons évalué l'activité locomotrice au moyen de la tâche en champ ouvert. Nous avons mesuré les substances réactives à l'acide thiobarbiturique (SRATB) et la formation du complexe protéine carbonyle comme paramètres du stress oxydant, ainsi que la superoxyde-dismutase (SOD) et la catalase (CAT), les principales enzymes anti-oxydantes, dans le cortex préfrontal et l'hippocampe. **Résultats :** Le lithium et le valproate ont inversé (modèle du traitement d'inversion) et évité (modèle du traitement de prévention) l'hyperactivité causée par l'amphétamine et inversé et évité la formation de SRATB provoquée par l'amphétamine au cours des deux expériences. L'administration simultanée de lithium et de valproate avec l'amphétamine a toutefois accru la peroxydation des lipides, selon la région du cerveau et le traitement. On n'a observé aucun changement de la formation du complexe protéine carbonyle. L'activité de la SOD a varié en fonction des différents traitements et celle de la CAT a augmenté lorsque l'indice de peroxydation des lipides était plus élevé**. Conclusion :** Nos constatations indiquent que le lithium et le valproate ont un effet protecteur contre le stress oxydant provoqué par les amphétamines in vivo, ce qui appuie encore l'hypothèse selon laquelle il peut y avoir un lien entre le stress oxydant et la pathophysiologie du trouble bipolaire.

Introduction

Recent studies have consistently reported increased products of lipid peroxidation and alterations of the major antioxidant enzymes in people with bipolar disorder (BD).[1–3] It has been widely demonstrated that the generation of reactive oxygen species (ROS) plays a critical role in the pathophysiology of several neuropsychiatric disorders.[4,5] The brain is particularly vulnerable to ROS production because it metabolizes 20% of total body oxygen and has a limited amount of antioxidant capacity.[6] In situations where the generation of free radicals exceeds the capacity of antioxidant defence, oxidative stress may lead to membrane degradation, cellular dysfunction and apoptosis. This might be relevant for the pathogenesis of BD, because in-vivo magnetic resonance spectroscopy studies have demonstrated changes in brain compounds related to oxidative phosphorylation, energy production and phospholipid metabolism.[7] In addition, it has been hypothesized that BD is associated with mitochodrial dysfunction,[8,9] and abnormalities in respiratory complex activity and energy production may lead to cellular degeneration.[5]

Additional associations between oxidative and antioxidative systems in BD have been demonstrated in pharmacological studies. In primary cultured neuronal cells, lithium and valproate (first-line mood stabilizers) prevent glutamate-induced oxidative stress[10] and increase mRNA and protein levels of glutathione S-transferase M1 isoenzyme;[11] and valproate inhibits $FeCl_3$-induced lipid peroxidation and protein oxidation.[12] More recently, King and Jope[13] showed that lithium protects against caspase activation that is induced by intrinsic and extrinsic sources of oxidative stress in human neuroblastoma SH-SY5Y cells. Together, these findings strongly suggest that the modulation of ROS generation may be relevant in the mood-stabilizing effects of lithium and valproate; however, the effects of lithium and valproate on oxidative stress have not been studied in vivo. We recently found that repeated amphetamine exposure increases thiobarbituric acid-reactive species and protein carbonyl formation in the rat hippocampus and cerebral cortex, 2 brain regions related to mood regulation.[14] In addition, recent genetic and postmortem studies suggest that BD may be associated with altered dopaminergic transmission.[15–17] Because of these findings, we designed the present study to investigate the effects of lithium and valproate on lipid and protein oxidation levels (markers of oxidative stress) and on superoxide dismutase (SOD) and catalase (CAT) activities (the major antioxidant enzymes) in a dopaminergic model of mania.

Methods

We conducted the study, using adult male Wistar rats obtained from our breeding colony. The animals were housed 5 to a cage, on a 12-hour light/dark cycle (lights on at 7:00 am), with free access to food and water. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behaviour (SBNeC) recommendations for animal care. This study was approved by the local ethics committee (Comitê de Ética em Pesquisa da Universidade do Extremo Sul Catarinense).

Reversal treatment

In this model, we reproduced the treatment of an acute manic episode. Rats received either a daily injection of d-amphetamine, 2 mg/kg (Sigma, St. Louis, Mo.), or saline for 14 days. Between the 8th and the 14th days, the animals were divided into 3 experimental groups (12–15 animals per group): 1 group received lithium, 47.5 mg/kg intraperitoneal (IP), twice a day; the second group received valproate, 200 mg/kg IP, twice a day; and the third group received saline IP twice a day. Locomotor activity was assessed 2 hours after the last injection, and rats were sacrificed by decapitation immediately following the open-field task. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80°C until assayed.

Prevention treatment

In this model, we reproduced the maintenance treatment of BD. Rats received either lithium, 47.5 mg/kg; valproate, 200 mg/kg; or saline IP twice a day for 14 days. The animals were then divided into 2 groups (12–15 animals per group). Between the 8th and the 14th days, each group received one daily IP injection of d-amphetamine, 2 mg/kg, or saline. Locomotor activity was assessed 2 hours after the last injection,

and rats were sacrificed by decapitation immediately following the open-field task. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80°C until assayed. All lithium-treated animals presented lithium levels between 0.6–1.2 mEq/L in plasma, as recommended in the treatment of patients with BD.

Locomotor activity

We used the open-field task to assess locomotor activity. The task was performed 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 rectangle and were allowed to explore the arena. Crossings of the black lines and rearings were counted for 5 minutes.

Oxidative stress parameters

To determine oxidative damage, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described.[18] The samples were mixed with 1 mL of trichloroacetic acid (TCA) 10% and 1 mL of thiobarbituric acid (TBA) 0.67% and were then heated in a boiling water bath for 15 minutes. TBARS were determined by the absorbance at 535 nm. Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH), as previously described.[19] Proteins were precipitated by the addition of 20% trichloroacetic acid and were redissolved in DNPH; the absorbance was read at 370 nm.

To determine CAT activity, the brain tissue was sonicated in 50 mmoL/L phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at 3000 *g* for 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm.[20] SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described[21] All biochemical measures were normalized to the protein content, with bovine albumin as standard.[22]

All data are presented as mean and standard error of the mean. Differences among the experimental groups evaluating exploratory behaviour were determined by 1-way analysis of variance (ANOVA), followed by the Tukey post hoc test. Biochemical data were analyzed by 1-way ANOVA, and multiple comparisons were performed with the Newman-Keuls test. In all comparisons, statistical significance was set at $p < 0.05$.

Results

Behaviour

In the reversal and prevention models, amphetamine increased locomotor and rearing behaviours, and the administration of lithium and valproate reversed and prevented amphetamine-induced hyperactivity (Fig. 1, Fig. 2). Lithium and valproate did not affect behavioural measures in animals treated with saline, suggesting that the effects of mood stabilizers on animals treated with amphetamine

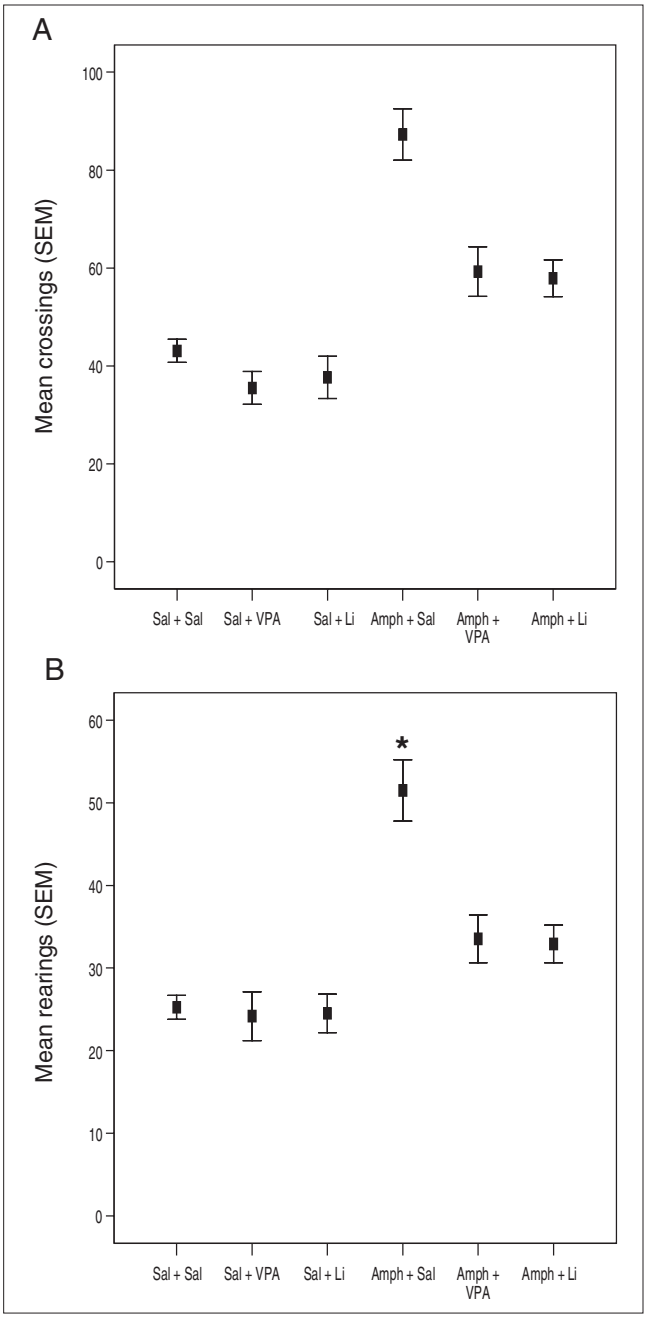

Fig. 1: Numbers of crossings (A) and rearings (B) in the reversal model (n = 12 for each group). Bars represent means; error bars represent standard error of the means (SEM). Crossings = 1-way analysis of variance (ANOVA); $F_{5,66} = 22.68$; $p < 0.001$; *different from all groups (Tukey's post hoc; $p < 0.001$). Rearings = 1-way ANOVA; $F_{5,66} = 15.28$; $p < 0.001$; *different from all groups (Tukey's post hoc; $p < 0.001$). Rats were pretreated with amphetamine (Amph) for 7 days and then treated with Amph plus mood stabilizers between the 8th and the 14th days. Li = lithium, Sal = saline, VPA = valproate.

were not related to sedation. These results replicate our recent findings[23] and confirm the reproducibility of this behavioural model.

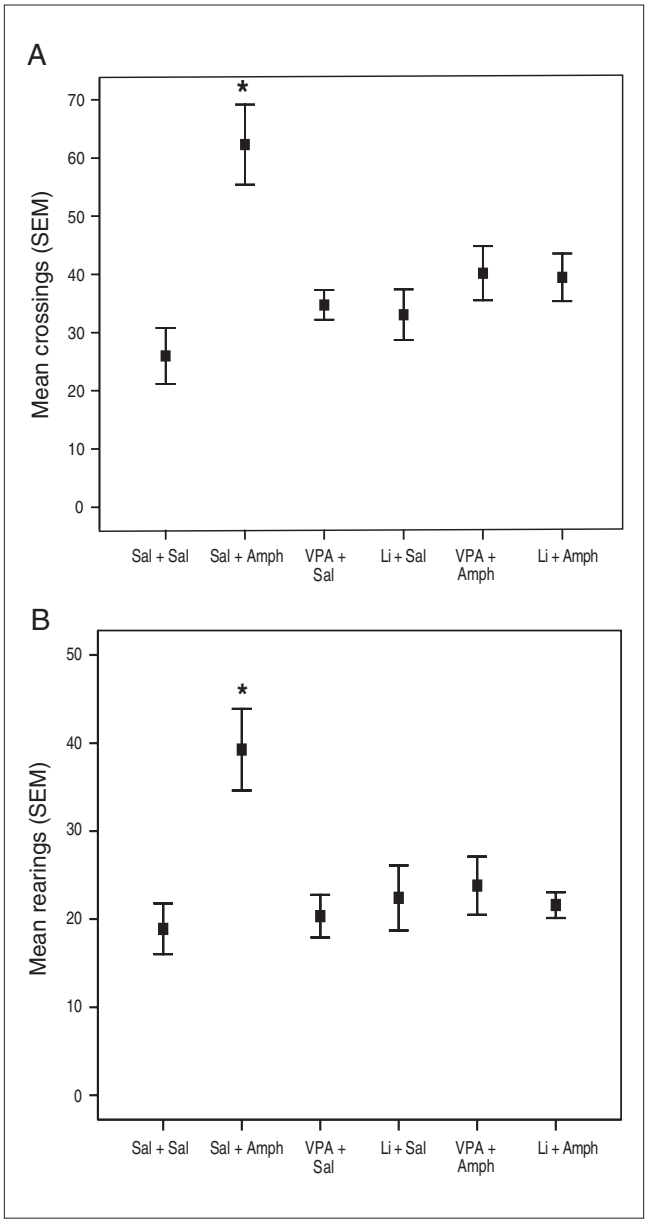

Fig. 2: Numbers of crossings (A) and rearings (B) in the prevention model (n = 12 for each group). Bars represent means; error bars represent standard error of the means (SEM). Crossings = 1-way analysis of variance (ANOVA); $F_{5,66}$ = 7.04; p < 0.001; *different from all groups (Tukey's post hoc; p < 0.001 from Sal + Sal and Li + Sal; p < 0.001 from VPA + Sal; p = 0.018 from Li + Amph; p = 0.023 from VPA + Amph). Rearings = 1-way ANOVA; $F_{5,66}$ = 4.80; p < 0.001; *different from all groups (Tukey's post hoc; p < 0.001 from Sal + Sal and VPA + Sal; p = 0.006 from Li + Sal and VPA + Amph; p = 0.022 from Li + Amph). Rats were pretreated with mood stabilizers for 7 days and then treated with mood stabilizers plus amphetamine (Amph) between the 8th and the 14th days. Sal = saline, VPA = valproate.

Oxidative stress parameters

Reversal treatment

Amphetamine increased TBARS levels in the prefrontal cortex, and this effect was reversed by both mood stabilizers (Fig. 3a). The administration of lithium and valproate

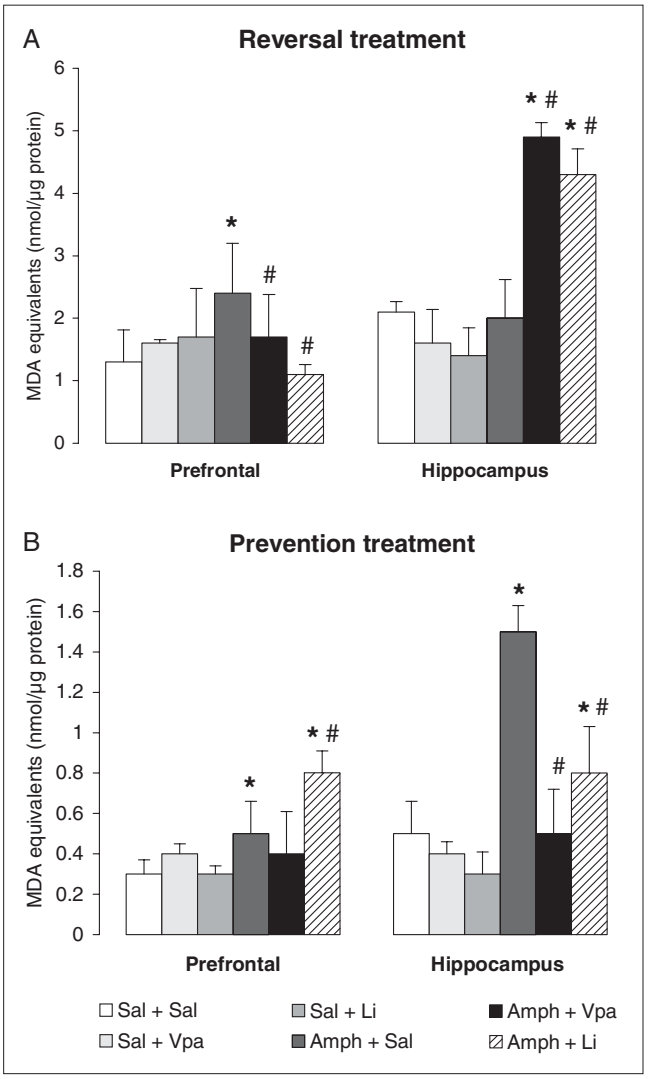

Fig. 3: TBARS levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments (n = 5 for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); $F_{5,24}$ = 3.14; p = 0.02. Hippocampus in the reversal treatment group = 1-way ANOVA; $F_{5,24}$ = 22.44; p < 0.001. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 7.78; p = 0.001. Hippocampus in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 15.66; p < 0.001. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; p < 0.05); # different from the Amph + Sal group (Newman-Keuls post hoc; p < 0.05). Amph = amphetamine, Li = lithium, MDA = malondialdehyde, Sal = saline, TBARS = thiobarbituric acid reactive species.

increased TBARS formation in the hippocampus of rats pretreated with amphetamine. No changes were observed in protein carbonyl formation (data not shown).

Prevention treatment

Amphetamine increased TBARS levels in the prefrontal cortex and hippocampus, and these effects were prevented by valproate pretreatment (Fig. 3b). Lithium pretreatment partially prevented amphetamine-induced lipid peroxidation in the rat hippocampus but augmented amphetamine-induced lipid peroxidation in the prefrontal cortex. As in the reversal treatment, no changes were observed in protein carbonyl formation (data not shown). Taken together, our findings suggest that the neuroprotective effects of lithium and valproate on amphetamine-induced oxidative stress vary with brain region and treatment regimen. When interpreting the results, it

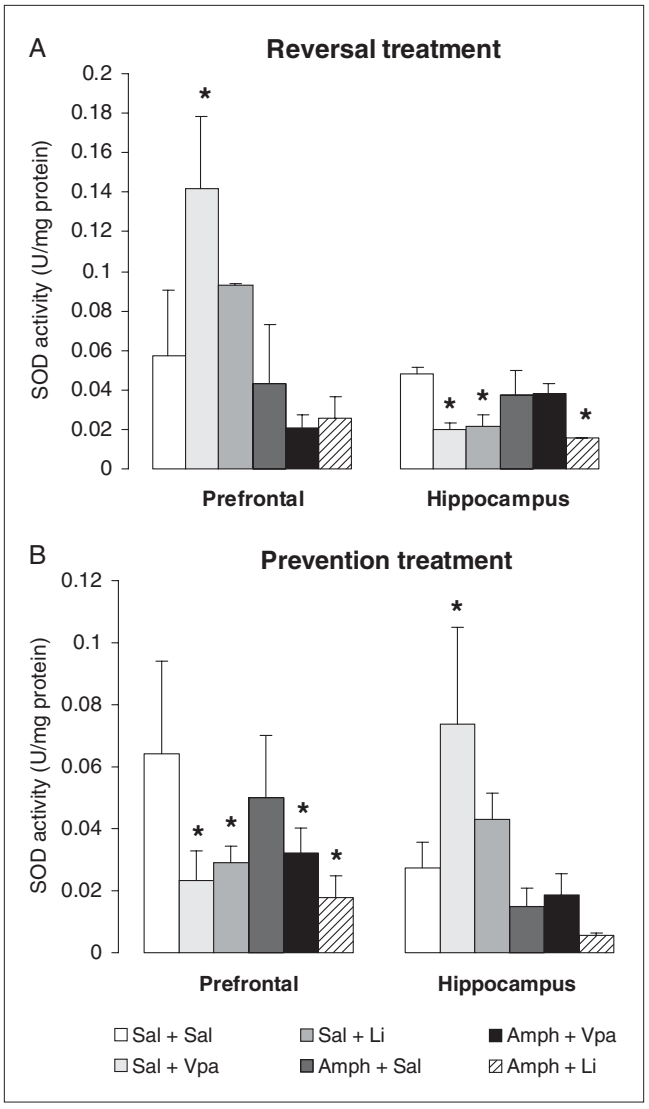

Fig. 4: Superoxide dismutase (SOD) levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments (n = 5 for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); $F_{5,24}$ = 8.58; p = 0.02. Hippocampus in the reversal treatment group = 1-way ANOVA; $F_{5,24}$ = 8.94; p = 0.01. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 4.48; p = 0.011. Hippocampus in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 8.36; p = 0.001. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; $p < 0.05$); Amph = amphetamine, Li = lithium, Sal = saline, Val = valproate.

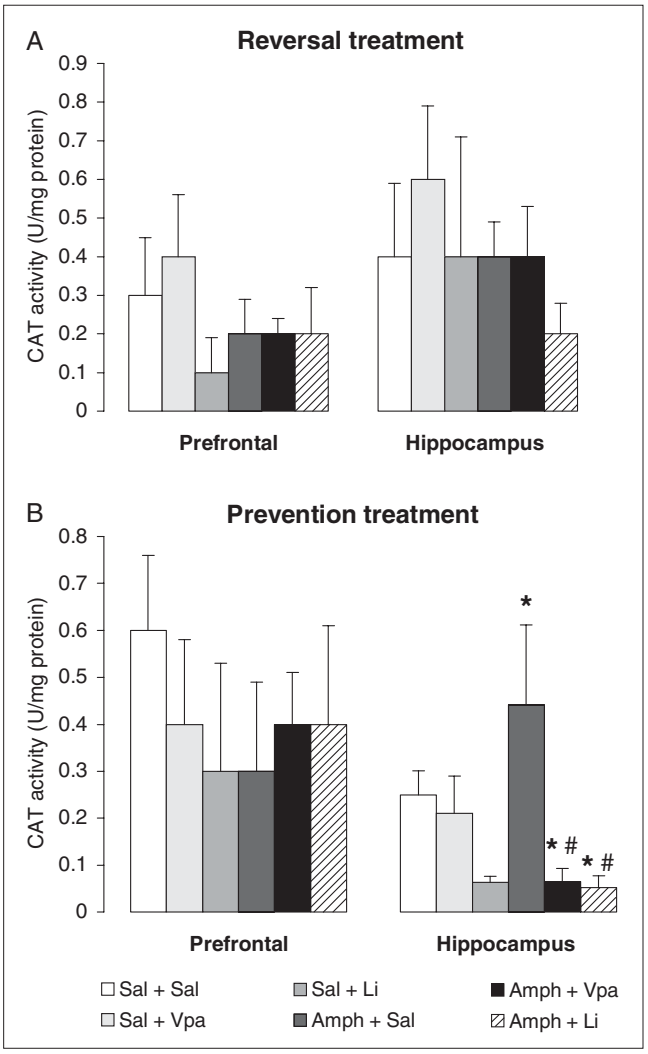

Fig. 5: Catalase (CAT) levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments (n = 5 for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); $F_{5,24}$ = 2.57; p = 0.069. Hippocampus in the reversal treatment group = 1-way ANOVA; $F_{5,24}$ = 2.35; p = 0.088. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 0.63; p = 0.679. Hippocampus in the prevention treatment group = 1-way ANOVA; $F_{5,24}$ = 8.91; p = 0.001. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; $p < 0.05$); #different from the Amph + Sal group (Newman-Keuls post hoc; $p < 0.05$). Amph = amphetamine, Li = lithium, Sal = saline, Vpa = valproate.

is worth noting that the administration of lithium and valproate alone had no effect on oxidative stress.

Antioxidant enzyme activity

Reversal treatment

In this model, valproate increased SOD levels in the prefrontal cortex, whereas lithium, valproate and lithium plus amphetamine decreased SOD in the rat hippocampus (Fig. 4a). No changes were observed on CAT activity (Fig. 5a).

Prevention treatment

Lithium, valproate, lithium plus amphetamine and valproate plus amphetamine decreased SOD levels in the prefrontal cortex, whereas valproate increased SOD in the hippocampus (Fig. 4b). Amphetamine increased CAT levels, whereas lithium plus amphetamine, and valproate plus amphetamine decreased CAT levels in the rat hippocampus (Fig. 5b).

Discussion

In this study, we demonstrated that lithium and valproate reversed amphetamine-induced lipid peroxidation in the prefrontal cortex and prevented amphetamine-induced lipid peroxidation in the hippocampus. Using the present model, we were able to reproduce previous findings of the neuroprotective effects of mood stabilizers in response to oxidative stress.[10–13] Our results are consistent with existing evidence that mood stabilizers share significant neuroprotective properties.[24] However, we also found that the co-administration of lithium or valproate with amphetamine can increase TBARS formation, suggesting that their effects on oxidative stress vary depending on the brain region and treatment regimen. Because the administration of lithium or valproate alone did not induce oxidative damage, it is conceivable that they might augment amphetamine-induced oxidative stress in some situations. Amphetamine can enhance ROS formation through several pathways, for example, through autooxidation of dopamine with the formation of highly reactive quinones,[25] direct inhibition of mitochondrial electron transport chain complexes[26] and increased glutamate release.[27] Although the mechanisms by which mood stabilizers decrease ROS generation are poorly understood, it has been suggested that they might involve the induction of the molecular chaperone GRP78,[28] buffering $[Ca^{2+}]i$ levels,[10] and by stabilizing mitochondrial function.[10] Interestingly, Carli and colleagues[29] demonstrated that chronic lithium administration modifies the affinity of dopamine transporters, thereby decreasing overactive dopamine transmission. In addition, divalproex sodium has been shown to decrease [^{18}F]-dopa uptake constants in the striatum of patients with mania, suggesting that divalproex sodium treatment decreases aromatic amino acid decarboxilase activity, which should decrease the rate of dopamine synthesis.[30] Thus, lithium and valproate could decrease amphetamine-induced ROS generation, at least in part, by decreasing the amount of dopamine available for release from presynaptic neurons.

We did not find any change in protein carbonyl formation in the present model. This is contrary to our previous finding that repeated d-amphetamine administration increases protein carbonyl levels in the rat hippocampus.[14] Differences in experimental design may account for this discrepancy. The effects of amphetamine, lithium and valproate on antioxidant enzymes were highly variable in our experiments. In fact, there is a paucity of data assessing the effects of these drugs on antioxidant systems. D'Almeida and others[31] administered methamphetamine, 2.5 mg/kg daily, for 5 months and found no differences in SOD or CAT activity in the rat brain. More recently, Carvalho and colleagues[32] administered d-amphetamine, 20 mg/kg daily, for 14 days and reported increased CAT activity in medial prefrontal cortex but no effect on SOD or CAT activity in the rat hippocampus. During physiological states, SOD metabolizes superoxide anion (O_2^-), producing hydrogen peroxide (H_2O_2), which can react with iron to generate highly reactant hydroxyl radicals via the Fenton reaction. CAT is the most important peroxidase in detoxifying excess hydrogen peroxide to prevent hydroxyl production. Thus an increase in SOD or CAT levels per se does not necessarily indicate increased oxidative stress, whereas an imbalance between SOD and CAT activities could lead to an excessive generation of free radicals.[33,34] In the context of the present model, we specifically found increased CAT activity when the degree of lipid peroxidation was more robust (Fig. 3b, Fig. 5b). It is likely that CAT activity increased in response to increased H_2O_2 production induced by amphetamine metabolism. We also found changes in SOD activity without evidence of lipid or protein oxidation when lithium and valproate were administered alone. This is in line with a previous finding that electroconvulsive shocks increased SOD and CAT activity in the rat hippocampus but did not induce oxidative stress.[35]

In conclusion, we have demonstrated that lithium and valproate can reverse and prevent amphetamine-induced oxidative stress in vivo. Our findings further support the notion that neuroprotection may be one of the mechanisms of action of mood stabilizers and that oxidative stress may play a role in the pathophysiology of BD.

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Competing interests: None declared for Dr. Frey, Ms. Valvassori, Ms. Réus, Mr. Martins, Ms. Petronilho, Ms. Bardini, Dr. Dal-Pizzol and Dr. Quevedo. Dr. Kapczinski has received speaker fees and educational grants from Lilly and Abbot and travel assistance from Lilly.

Contributors: Drs. Frey, Kapczinski and Quevedo and Mr. Martins designed the study. Mses. Valvassori, Réus, Petronilho and Bardini and Dr. Dal-Pizzol acquired and analyzed the data. Drs. Frey, Dal-Pizzol and Kapczinski and Mr. Martins wrote the article; Drs. Frey and Quevedo and Mses. Valvassori, Réus, Petronilho and Bardini critically reviewed it. All authors gave final approval for publication.

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Capítulo 5

Frey BN, Andreazza AC, Kunz M, Gomes FA, Quevedo J, Salvador M, Gonçalves CA, Kapczinski F. Increased oxidative stress and DNA damage in bipolar disorder: a twin-case report. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* In press, 2006e.

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Short communication

Increased oxidative stress and DNA damage in bipolar disorder: A twin-case report

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Abstract

Objective: There is an emerging body of data suggesting that oxidative stress may be associated with the pathophysiology of bipolar disorder (BD). In the present study we investigated the oxidative stress profile in two monozygotic twins during a manic episode.
Methods: Two monozygotic twins diagnosed as currently manic by the Structured Clinical Interview for DSM-IV were studied. Serum thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and catalase (CAT) were measured as parameters of oxidative stress. DNA damage was assessed using the single cell gel electrophoresis technique (Comet Assay). All biochemical measures were conducted at baseline and after a 6-week treatment.
Results: Bipolar twins had higher TBARS, SOD and DNA damage, and lower CAT than the healthy control. TBARS and SOD were normalized after mood stabilization, whereas CAT and DNA damage remained altered at week 6.
Conclusions: These findings support that oxidative stress may play a role in the pathophysiology of BD and that pharmacological treatment may exert antioxidant effects. Studies with larger samples are warranted to further clarify this issue.

Keywords: Bipolar disorder; DNA damage; Oxidative stress; Pathophysiology

1. Introduction

Studies have consistently reported increased lipid peroxidation and changes in the major antioxidant enzymes in individuals with bipolar disorder (BD) (Ozcan et al., 2004; Ranjekar et al., 2003; Kuloglu et al., 2002), suggesting that oxidative stress may play a role in the pathophysiology of BD. The excessive generation of reactive oxygen species, such as hydroxyl radicals, can lead to lipid and protein oxidation, with consequent membrane and DNA damage. Moreover, there is recent evidence that the mood stabilizing agents lithium and valproate exert robust antioxidant effects *in vitro* (Shao et al., 2005). We have recently demonstrated that BD subjects have increased DNA damage, possibly due to increased oxidative stress (Andreazza et al., in press). As an extension of this latter study, we prospectively investigated the oxidative stress profile and DNA damage in two medication-free monozygotic twins during a manic episode. Because genetic inheritance increases the risk for the development of BD, we hypothesized that the BD twins would present increased oxidative stress and DNA damage and these changes would be reversed after mood stabilization.

2. Case report

Two identical female twins, 59 years old, were assessed. One subject (patient 1) was admitted in the hospital for inpatient treatment, while the other (patient 2) refused treatment. The

Abbreviations: BD, bipolar disorder; CAT, catalase; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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diagnosis of Bipolar I Disorder, current manic episode was carried out using the Structured Clinical Interview for DSM-IV-Axis I (First et al., 1998), and the severity of manic and depressive symptoms were assessed using the Young Mania Rating Scale (Young et al., 1978) and the Hamilton Depression Rating Scale (Hamilton, 1960), respectively. A healthy 58 year-old female was used as control. Blood samples were collected from each subject by venipuncture at baseline (time 0) and after 6 weeks (time 1). During this period, patient 1 used lithium carbonate 1500 mg/day (serum levels = 0.9 mEq/l) + chlorpromazine 300 mg/day for 2 weeks. Then, chlorpromazine was switched to haloperidol 10 mg/day until the discharge. As parameters of oxidative stress, serum thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation, and superoxide dismutase (SOD) and catalase (CAT), two major antioxidant enzymes were measured. All biochemical procedures were run in duplicate. To assess DNA damage we used the single cell gel electrophoresis technique, also known as the Comet Assay (CA), as previously described (Andreazza et al., in press). Under alkaline conditions the CA detects DNA single- and double-strand breaks and alkali-labile sites (Tice et al., 2000). Negative and positive controls were used for each electrophoresis assay in order to ensure the reliability of the procedure. All subjects provided written informed consent and this study was approved by the local ethics committee (Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil).

3. Results and discussion

At baseline (time 0), bipolar subjects had higher SOD and TBARS, and lower CAT levels than the healthy control (see Table 1). These findings are in accordance with previous studies (Ozcan et al., 2004; Ranjekar et al., 2003; Kuloglu et al., 2002), and indicate that the oxidative stress status was increased in the bipolar twins. It is intriguing that patient 1 had similar TBARS and SOD levels as the control after mood stabilization (time 1), whereas these oxidative stress parameters remained altered in the untreated twin (patient 2). These findings suggest that SOD and TBARS may be normalized by pharmacological treatment. CAT activity remained lower in both patients. Under normal conditions SOD metabolizes the excess of superoxide anion (O_2^{-}) producing hydrogen peroxide (H_2O_2), which can spontaneously generate highly reactant hydroxyl radicals (OH^{-}). CAT and glutathione peroxidase detoxify the excess of H_2O_2 to prevent OH^{-} generation. Therefore, an imbalance within this physiological antioxidant system may increase the production of OH^{-} and, consequently, lead to lipid and protein damage (Mahadik and Mukherjee, 1996). In this context, lithium has been shown to possess antioxidant properties (Shao et al., 2005), haloperidol seems to increase oxidative stress (Pillai et al., in press), and studies conducted with chlorpromazine have reported controversial results (Pillai et al., in press; Roy et al., 1984). Thus, it is not clear which of these medications are associated with the partial improvement of the parameters of oxidative stress observed in the treated patient.

We also found that DNA damage was markedly higher in BD twins as compared with the healthy control at both time points, suggesting that DNA damage may be a trait rather than a state in BD. Interestingly, a recent study found that genes encoding the DNA repair enzyme, PARP, and several antioxidant enzymes, including SOD and CAT were decreased in the hippocampus of BD subjects (Benes et al., 2006). These results are in line with the present report, suggesting that the generation of ROS associated with the oxidative stress may induce lipid and protein oxidation, and consequently increasing DNA damage. Although the only study that directly assessed DNA fragmentation in BD showed no changes in the anterior cingulated cortex, the authors did not rule out the possibility that oxidative stress might be present (Benes et al., 2003). While the present study should be interpreted with the limitations of a case report, further studies are warranted to better determine the role of oxidative stress in the pathophysiology of BD.

Table 1
Clinical and oxidative stress parameters in patients and control

	YMRS score	HAMD score	TBARS (nmol/ml)	SOD (U/mg protein)	CAT (U/mg protein)	DNA damage (arbitrary units)
Healthy control	N/A	N/A	3.12±0.25	3.01±0.14	1.54±0.19	35.77±5.4
Patient 1						
Time 0	36	0	4.39±0.20	5.64±0.15	0.31±0.05	108.0±6.5
Time 1	4	3	3.45±0.31	2.62±0.14	0.31±0.06	118.6±6.8
Patient 2						
Time 0	19	6	6.36±0.38	4.98±0.21	0.55±0.05	106.8±4.6
Time 1	15	6	5.78±0.25	3.98±0.18	0.62±0.02	129.0±7.5

YMRS = Young Mania Rating Scale; HAMD = Hamilton Depression Rating Scale; TBARS = thiobarbituric acid reactive substances; SOD = superoxide dismutase; CAT = catalase.

4. Conclusion

These findings further substantiate that oxidative stress may play a role in the pathophysiology of BD and suggest that some measures of oxidative stress might be corrected by pharmacological treatment. Prospective studies with larger samples are warranted to investigate the effects of mood stabilizers on oxidative stress, as well as the clinical impact of oxidative stress in BD patients.

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Capítulo 6

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Effects of mood stabilizers on hippocampus BDNF levels in an animal model of mania

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Abstract

There is an emerging body of data suggesting that mood disorders are associated with decreased brain-derived neurotrophic factor (BDNF). The present study aims to investigate the effects of the mood stabilizers lithium (Li) and valproate (VPT) in an animal model of bipolar disorder. In the first experiment (acute treatment), rats were administered D-amphetamine (AMPH) or saline for 14 days, and then between day 8 and 14, rats were treated with either Li, VPT or saline. In the second experiment (maintenance treatment), rats were pretreated with Li, VPT or saline, and then between day 8 and 14, rats were administered AMPH or saline. In both experiments, locomotor activity was measured using the open-field test and BDNF levels were measured in rat hippocampus by sandwich-ELISA. Li and VPT reversed AMPH-induced behavioral effects in the open-field test in both experiments. In the first experiment, Li increased BDNF levels in rat hippocampus. In the second experiment, AMPH decreased BDNF levels and Li and VPT increased BDNF levels in rat hippocampus. Our results suggest that the present model fulfills adequate face, construct and predictive validity as an animal model of mania.

Keywords: Amphetamine; Bipolar disorder; Brain-derived neurotrophic factor; Lithium; Mania; Valproate

Introduction

Bipolar disorder (BD) is a devastating major mental illness associated with higher rates of suicide and work loss (Belmaker, 2004; Kupfer, 2005). Although there have been recent advances in genetic, neurobiological and pharmacological methodologies, its pathophysiology remains largely unknown. The development of animal models has been an important tool in investigating new intracellular systems that may be involved in BD (Einat et al., 2003; Manji and Chen, 2002) and new pharmacological approaches (Lamberty et al., 2001). However, BD presents a complex alternating clinical course, with recurrent mood switches including manic, depressive, and mixed episodes, which makes the development of an adequate animal model challenging (Machado-Vieira et al., 2004). Ellenbroek and Cools (1990) have proposed that the validity of animal models in psychiatric disorders should demonstrate the following three major criteria: face, construct and predictive validity. Face validity represents how similar the model can mimic the symptoms of a determinate illness, whereas construct validity is related to the ability of the model to reproduce some pathophysiological aspects of the illness. Finally, the predictive validity evaluates if the therapeutical agents used in the treatment of an illness can reverse the symptoms induced in the animal model.

The clinical hallmark in diagnosing BD is the presence of manic symptoms (Belmaker, 2004; APA, 1994), thus an adequate animal model of BD should resemble some features of a manic episode such as euphoria, irritability, aggressiveness,

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hyperactivity, insomnia or increased sexual drive (face validity). Recent studies have demonstrated that changes in intracellular pathways that regulate neuronal transmission, plasticity and survival are associated with the pathophysiology of BD (Coyle and Duman, 2003; Bezchlibnyk and Young, 2002; Manji et al., 2001) and a reasonable animal model of bipolar disorder should both reproduce some of the molecular changes (construct validity) and response to antimanic agents, such as antipsychotics and mood stabilizers (predictive validity). A number of animal models of mania have used hyperlocomotion induced by psychostimulants (Frey et al., in press; reviewed in Machado-Vieira et al., 2004). Dopaminergic drugs, such as amphetamine, are able to induce manic symptoms in both normal human volunteers (Strakowski and Sax, 1998) and BD subjects (Anand et al., 2000). Interestingly, higher urinary dopamine levels have been associated with the emergence of manic symptoms (Joyce et al., 1995) and recent studies have demonstrated dopamine receptor changes in BD patients (Pantazopoulos et al., 2004; Vogel et al., 2004). Taken together, these studies suggest that the dopaminergic system may play a role in the pathophysiology of BD.

The pharmacological management of bipolar disorder includes the treatment of acute states and maintenance treatment in order to prevent new episodes. Mood stabilizing drugs, particularly lithium and valproate, are considered first line agents for both acute mania and maintenance treatment (Yatham et al., 2005). Several studies have suggested that the neuroprotective effects of lithium and valproate may be responsible for their therapeutical effects (Chuang et al., 2002; Li et al., 2002) and one of the mechanisms implicated is the induction of brain-derived neurotrophic factor (BDNF)/TrkB signaling pathway (Hashimoto et al., 2002; Manji et al., 2001). Recent magnetic resonance spectroscopy studies have demonstrated reduced *N*-acetyl-aspartate (a marker of neuronal viability) in the hippocampus of BD patients (Deicken et al., 2003; Bertolino et al., 2003), suggesting that BD may be associated with hippocampal dysfunction.

It is intriguing that animal models have focused primarily in the acute treatment and we are not aware of a study assessing both acute and maintenance treatments. Thus, the present study aims to investigate (a) if the administration of lithium and valproate reverses and prevents the behavioral effects of chronic use of D-amphetamine in rats, and (b) the effects of D-amphetamine and mood stabilizers on BDNF expression in rat hippocampus.

Materials and methods

Animals

The experiments were performed in male Wistar rats (age: 3–4 months; weight: 220–310 g), obtained from our breeding

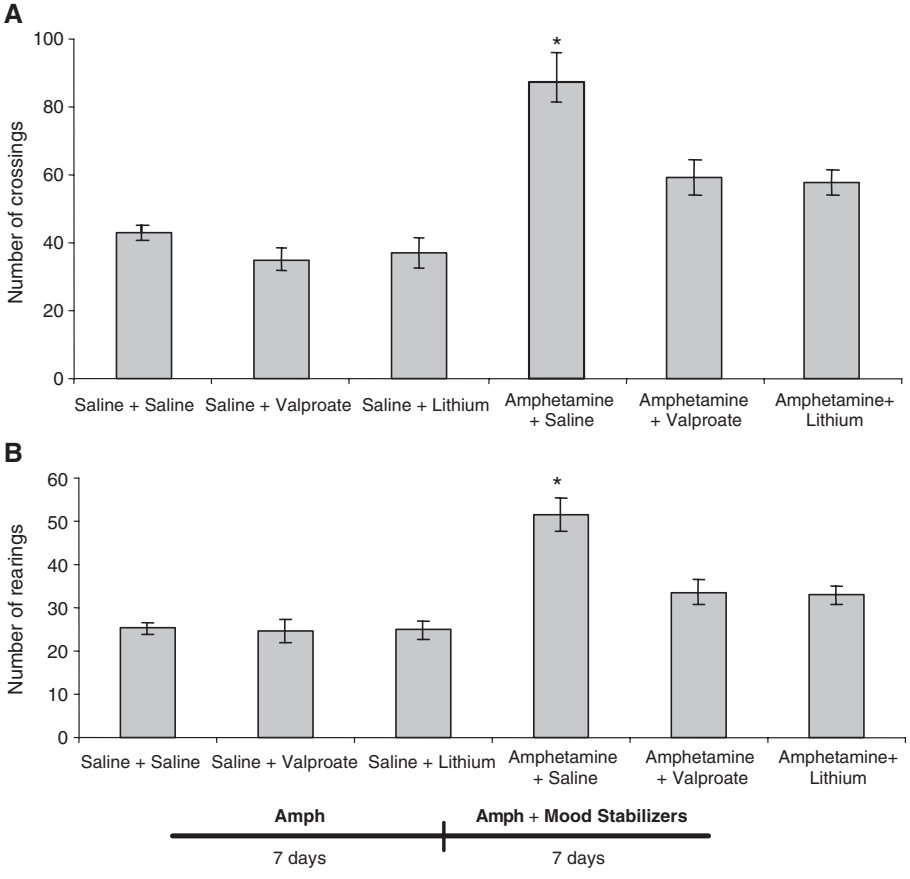

Fig. 1. Open-field test after seven days of treatment with amphetamine + seven days of amphetamine and mood stabilizers. *ANOVA and Post-test of Tukey; $p<0.05$. Legend: Results are presented as mean±S.E.M.

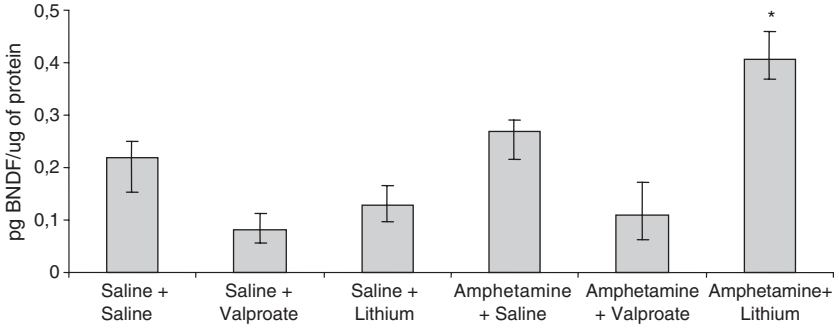

Fig. 2. Brain-derived neurotrophic factor in hippocampus after seven days of treatment with amphetamine + seven days of amphetamine and mood stabilizers. *ANOVA and Post-test of Tukey; $p<0.05$. Legend: Results are presented as mean±S.E.M.

colony. Rats were housed five to a cage, on a 12-h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.), and food and water were available ad libitum. All experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Acute treatment

The first model was designed in order to reproduce the management of an acute manic episode (reversal treatment — Fig. 1). Animals received one daily IP injection of either D-amphetamine (AMPH-Sigma, St Louis, USA) 2 mg/kg or saline for 14 days (45 animals per group). Between the 8th and the 14th day, saline and AMPH animals were divided in three experimental groups (15 animals per group): lithium (Li) treatment, valproate (VPT) treatment and saline (SAL) treatment. Li-treated animals received Li 47.5 mg/kg IP twice a day, and VPT-treated animals received VPT 200 mg/kg IP twice a day. We have previously found that Li 47.5 mg/kg IP bid and valproate 200 mg/kg IP bid did not alter locomotor behavior in male Wistar rats (unpublished results). Locomotor activity was measured 2 h after the last injection, and the rats were

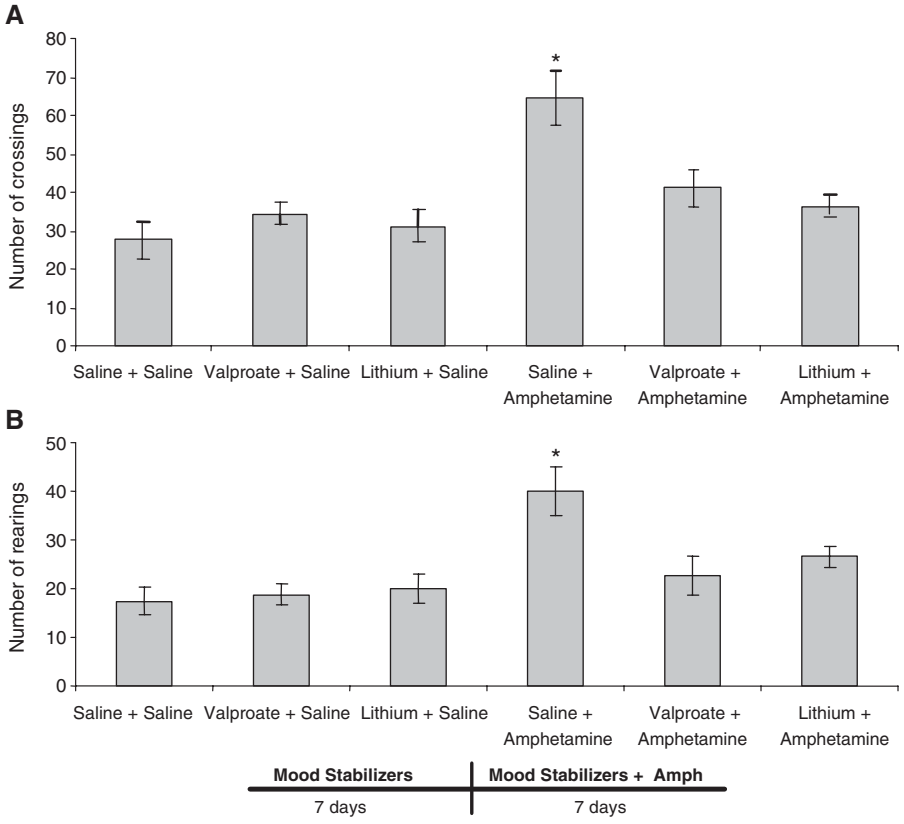

Fig. 3. Open-field test after seven days of treatment with mood stabilizers + seven days of mood stabilizers and amphetamine. *ANOVA and Post-test of Tukey; $p<0.05$. Legend: Results are presented as mean±S.E.M.

sacrificed by decapitation right after the open-field task. The hippocampus was dissected, rapidly frozen, and stored at −80 °C until assayed.

Maintenance treatment

The second model was designed to mimic the maintenance phase of BD treatment (prevention treatment — Fig. 3). Animals received either Li 47.5 mg/kg IP twice a day, VPT 200 mg/kg IP twice a day or saline for 14 days (30 animals per group). Between the 8th and the 14th day, Li-, VPT- and saline-treated animals were divided in two experimental groups (15 animals per group): each treated group received one daily IP injection of either AMPH 2 mg/kg or saline. Locomotor activity was measured 2 h after the last injection, and the rats were sacrificed by decapitation right after the open-field task. The hippocampus was dissected, rapidly frozen, and stored at −80 °C until assayed.

Locomotor activity

The locomotor activity was assessed using the open-field task. The task was performed 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, in order to explore the arena for 5 min. Crossings of the black lines and rearings were counted.

Biochemical measures

BDNF levels in hippocampus were measured by anti-BDNF sandwich-ELISA, according to the manufacturer instructions (Chemicon, USA). Briefly, brain slices were homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM (EGTA). Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent and standard curve ranged from 7.8 to 500 pg/ml of BNDF. The plates were then washed four times with sample diluent and a monoclonal anti-BNDF rabbit antibody diluted 1:1000 in sample diluent was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After addition of streptavidin-enzyme, substrate and stop solution, the amount of BDNF was determined by absorbance in 450 nm. The standard curve demonstrates a direct relationship between Optical Density (OD) and BDNF concentration. Total protein was measured by Lowry's method using bovine serum albumin as a standard. Serum Li levels were measured by a commercial laboratory blind to the experiments.

Statistical analysis

All data are presented as mean±S.E.M. Differences among experimental groups in experiments evaluating BNDF levels were determined by ANOVA. Multiple comparisons were performed by a Tukey test. In all experiments, p values less than 0.05 were considered to indicate statistical significance.

Results

In the first experiment (reversal treatment), AMPH increased locomotor and rearing behavior in saline-treated rats and both Li and VPT reversed AMPH-related hyperactive behavior (Fig. 1). The administration of Li or VPT in saline-treated animals did not change behavioral measures, indicating that the effects of mood stabilizers in AMPH-treated rats were not associated with sedation. AMPH administration had no effect on BDNF levels in rat hippocampus (Fig. 2). However, Li treatment increased BDNF expression after AMPH administration. VPT had no effect on BDNF levels in AMPH- or saline-pretreated animals.

Fig. 3 summarizes the behavioral measures of the second experiment (prevention treatment). Both Li and VPT pretreatment were able to prevent AMPH-related hyperactivity. Saline administration in mood stabilizer-pretreated animals demonstrated no behavioral effect. In this experiment, AMPH decreased BDNF levels in saline pretreated rats, while both Li and VPT pretreatment increased BDNF in rat hippocampus after AMPH administration (Fig. 4). Chronic use of Li and VPT in saline-treated animals had no influence in BDNF levels. Taken together, these results suggest that the effect of mood stabilizers on BDNF expression may be associated with the

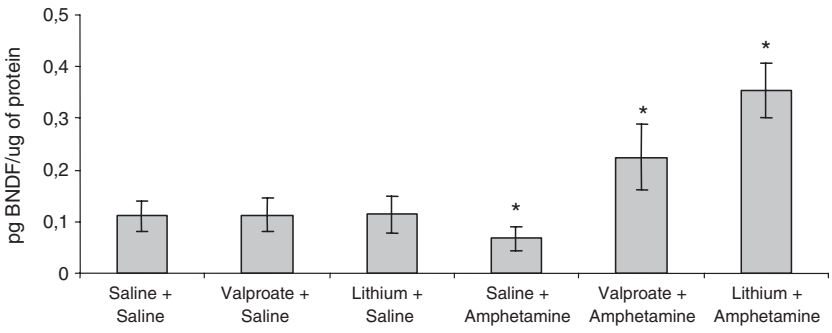

Fig. 4. Brain-derived neurotrophic factor in hippocampus after seven days of treatment with mood stabilizers + seven days of mood stabilizers and amphetamine. *ANOVA and Post-test of Tukey; $p<0.05$. Legend: Results are presented as mean±S.E.M.

Table 1
Serum lithium (mEq/L) in reversal and prevention experiments

Experiment/group	Serum lithium (mean-mEq/L)	S.E.M.
Reversal/saline + lithium	0.785	0.069
Reversal/amphetamine + lithium	0.772	0.053
Prevention/lithium + saline	0.926	0.088
Prevention/lithium + amphetamine	0.829	0.069

reversal and, more likely, prevention of hyperactive behavior in AMPH-treated rats. All Li-treated animals had Li plasmatic levels between 0.6 and 1.2 mEq/L (Table 1), as recommended in the treatment of BD patients.

Discussion

In the present study we investigated, for the first time, the effects of the mood stabilizing agents Li and VPT in an animal model of both acute and maintenance treatment for BD. According to our model, both mood stabilizers reversed and prevented hyperactivity induced by AMPH, indicating a good predictive validity. Despite of the well-recognized limitations of animal models of bipolar disorder in terms of face validity (Machado-Vieira et al., 2004; Einat et al., 2000), it is well known that AMPH induces manic symptoms in BD patients (Anand et al., 2000) as well as in healthy volunteers (Strakowski and Sax, 1998). Using fMRI, Bell et al. (2005) reported that Li and VPT attenuated AMPH-induced changes in human brain during neuropsychological tasks. In addition, Beaulieu et al. (2004) recently found that the hyperactivity induced by AMPH is partially mediated by Akt/glycogen synthase kinase-3β (GSK-3β) signaling pathway and that Li was able to reverse this effect. It is worth noting that both Li and VPT exert inhibitory effects over GSK-3β signaling pathway (Gould and Manji, 2002). Furthermore, it has been demonstrated that the blockade of extracellular signal-regulated kinase (ERK) pathway induces hyperactive behavior similar to that produced by AMPH (Einat et al., 2003). Interestingly, Mai et al. (2002) found that Li augmented BDNF-induced phosphorylation of ERK 1/2 in GSK-3β overexpressing cells. Thus, our findings reinforce the idea that BDNF (Narita et al., 2003; Guillin et al., 2001) and mood stabilizers (Beaulieu et al., 2004) may have adjunctive effects on the modulation of dopamine-dependent behavior.

Even though the precise pathophysiology of BD is far from being fully understood, recent studies have demonstrated that BD is associated with changes in intracellular signaling pathways that modulate neuronal plasticity and survival (Manji and Chen, 2002; Manji and Lenox, 2000). It has also been suggested that BDNF may be involved in the neurobiology of mood disorders (Duman, 2002). In the present model, we demonstrated that Li increased BDNF levels in rat hippocampus when administered both before and after AMPH, and VPT increased BDNF levels when used before AMPH (prevention treatment). This discrepancy suggests that, when co-administered with AMPH, Li may increase BDNF content in an earlier time course than VPT. We also found that neither 7 nor 14 days of administration of Li or VPT alone altered BDNF levels in rat hippocampus. Contrary to our results, Fukumoto et al. (2001) found that 14 days of Li and VPT increased BDNF expression in rat hippocampus, although they did not find changes in the expression of catalytic or truncated form of TrkB receptor. Differences in time for decapitation, dosage regimens or age of animals may account to the discrepancies between the studies. Previous studies have reported that a single administration of Li caused no significant inhibition on AMPH-induced behavioral changes (Smith, 1981; Aylmer et al., 1987; Okada et al., 1990). In addition, Aylmer et al. (1987) showed that 9 days of Li pretreatment did not inhibit AMPH-induced hyperactivity, measured on a Y-maze apparatus in female hooded rats. Differences may be due to distinct experimental design, drug dosage and animal strain used. Using in vivo microdialysis, Narita et al. (2003) demonstrated that the administration of BDNF-antibody and TrkB-antibody in the nucleus accumbens decreased dopamine release and methamphetamine-induced hyperactivity. The authors also suggested that these effects may be mediated, at least in part, by protein kinase C (PKC) modulation. In fact, several evidences have pointed out that PKC modulation seems altered in BD patients (Pandey et al., 2002; Soares et al., 2000; Wang and Friedman, 1996). In this regard, Bebchuk et al. (2000) reported significant antimanic properties of tamoxifen, a PKC inhibitor, in a small sample of manic BD patients.

Conclusion

In conclusion, we propose a new design of animal model of mania, focusing both acute and prophylactic treatments. In the present model, we were able to demonstrate that (a) the mood stabilizers Li and VPT reversed and prevented AMPH-induced hyperactivity and that (b) these effects may be associated with BDNF increase, which reinforces the notion that the neurotrophic effects of BDNF may play a role in the therapeutic effects of Li and VPT (Hashimoto et al., 2004). Studies focusing the modulation of Akt/GSK-3β and PKC signaling cascades in reversal and prevention models of BD are warranted to further clarify the molecular effects of the mood stabilizing agents.

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Capítulo 7

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Lithium increases nerve growth factor levels in the rat hippocampus in an animal model of mania

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Pharmacological studies suggest that neurotrophins may play a role in the effects of lithium and valproate on mood regulation. In this study, we tested the hypotheses that lithium and valproate would reverse and prevent the behavioral and biochemical effects of amphetamine, using a rat model of mania. In the reversal treatment, male Wistar rats were first administered D-amphetamine or saline for 14 days, and then, between days 8–14, rats were treated with lithium, valproate or saline. In the prevention treatment, rats were pretreated with lithium, valproate or saline, and then, between days 8–14, rats were administered D-amphetamine or saline. Locomotor behavior was assessed using the open-field task and hippocampal nerve growth factor levels were determined by enzyme-linked immunosorbent assay. Both lithium and valproate reversed and prevented D-amphetamine-induced hyperactivity. Lithium increased nerve growth factor content in rat hippocampus in both experiments, but this effect was blocked with the co-administration of D-amphetamine. No significant effects on nerve growth factor levels were observed with valproate or D-amphetamine alone. These findings suggest that nerve growth factor may play a role in the neurotrophic effects of lithium but do not support the hypotheses that the nerve growth factor/TrkA pathway is involved in the pathophysiology of bipolar disorder. *Behavioural Pharmacology* 17:311–318 © 2006 Lippincott Williams & Wilkins.

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Introduction

Bipolar disorder (BD) is a major psychiatric illness, characterized by the presence of recurrent manic and depressive episodes (Belmaker, 2004). It has been recently suggested that abnormalities of intracellular signaling pathways may underlie the pathophysiology of BD (Bezchlibnyk and Young, 2002). Genetic studies found a positive association of a brain-derived neurotrophic factor (BDNF) gene polymorphism (val66met) in BD family samples (Craddock *et al.*, 2005). Anatomical and functional neuroimaging findings have suggested that the interconnection between cortical and limbic structures, mainly the prefrontal cortex, amygdala and hippocampus, may be altered in mood-disordered subjects (Soares and Mann, 1997; Manji *et al.*, 2001). A recent study of pediatric BD patients suggested that reduction of hippocampal volume may be present early in the course of the bipolar illness (Frazier *et al.*, 2005). In addition, postmortem studies have demonstrated that the neuropathological abnormalities found in bipolar and unipolar (depressive) disorders are associated with impaired neuronal plasticity and resilience rather than with a neurodegenerative pattern (Rajkowska, 2003). Given the well known effects of neurotrophic factors in synaptic plasticity and neuronal survival (Black, 1999; Finkbeiner, 2000), it has been proposed that neurotrophins may play an important role in the pathophysiology of mood disorders (Altar, 1999; Hashimoto *et al.*, 2004).

Lithium (Li) and valproate (VPT) are the major therapeutic options for BD. Their mechanism of action in mood regulation, however, is still unknown. Preclinical studies demonstrated that both Li and VPT increased BDNF expression in the frontal cortex and hippocampus (Fukumoto *et al.*, 2001; Einat *et al.*, 2003; Frey *et al.*, 2006). In this same vein, Hashimoto *et al.* (2002) found that the induction of the BDNF/tyrosine kinase B pathway by Li treatment protected rat cortical neurons against glutamate excitotoxicity. There is an important body of evidence demonstrating effects of antidepressants, mood

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stabilizers and electroshock on BDNF expression (Nibuya *et al.*, 1995, 1996; Duman, 2002). There is, however, a paucity of data focusing on other neurotrophins, such as the nerve growth factor (NGF). Angelucci *et al.* (2003b) reported that chronic Li treatment increased NGF in the hippocampus of Flinders' sensitive line ('depressed') rats. Li also increased NGF levels in the frontal cortex, hippocampus, amygdala, and limbic forebrain of Sprague–Dawley rats (Hellweg *et al.*, 2002). To date, there is no appropriate animal model for BD. Various animal models of BD have been proposed, and the chronic administration of D-amphetamine (AMPH) has been considered the best established animal model for mania (Einat *et al.*, 2000; Machado-Vieira *et al.*, 2004). In this context, recent studies have reported dopaminergic alterations in BD patients (Pantazopoulos *et al.*, 2004; Vogel *et al.*, 2004).

The animal models used in this study were designed to mimic both acute and maintenance treatment with Li and VPT. We studied the effects of these mood stabilizers on hippocampal NGF levels in an animal model of mania. In the reversal model, we tested the hypotheses that the mood stabilizers would reverse the behavioral and biochemical effects of AMPH. In the prevention model, we hypothesized that the mood stabilizers would prevent the behavioral and biochemical effects of AMPH.

Methods

Subjects

One hundred and thirty-seven male Wistar rats (age: 3–4 months; weight: 220–310 g), obtained from our breeding colony, were used in the experiments. Rats were housed five to a cage, on a 12-h light/dark cycle (lights on 07.00–19.00 h), with free access to food and water. All experimental procedures were carried out in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* and the Brazilian Society for Neuroscience and Behavior recommendations for animal care.

Reversal treatment

In the reversal model, we simulated the treatment of an acute manic episode. In this model, rats received repeated AMPH administration, which resulted in a sensitized response (Yetnikoff and Arvanitogiannis, 2005). The animals received one daily intraperitoneal injection of AMPH 2 mg/kg (Sigma, St Louis, Missouri, USA) or saline for 14 days. Between the 8th and the 14th days, saline and AMPH-treated animals additionally received either Li 47.5 mg/kg intraperitoneally twice a day, VPT 200 mg/kg intraperitoneally twice a day or saline (11–12 animals per group). Locomotor activity was assessed 2 h after the last injection, and the rats were killed by decapitation immediately after the open-field task. The hippocampi were dissected, rapidly frozen and stored at –80°C until assayed.

Prevention treatment

In the prevention model, we simulated the maintenance phase of BD treatment. Animals were treated with either Li 47.5 mg/kg intraperitoneally twice a day, VPT 200 mg/kg intraperitoneally twice a day or saline for 14 days. Between the 8th and the 14th days, Li, VPT and saline-treated animals additionally received one daily intraperitoneal injection of either AMPH 2 mg/kg or saline (10–13 animals per group). Locomotor activity was measured 2 h after the last injection, and the rats were sacrificed by decapitation immediately after the open-field task. The hippocampi were dissected, rapidly frozen and stored at –80°C until assayed. All Li-treated animals attained serum Li levels between 0.6 and 1.2 mEq/l, as recommended in the treatment of BD.

Open-field task

The locomotor activity task was performed 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 rectangle, and permitted to explore the arena freely for 5 min. Crossings of the black lines and rearings were counted.

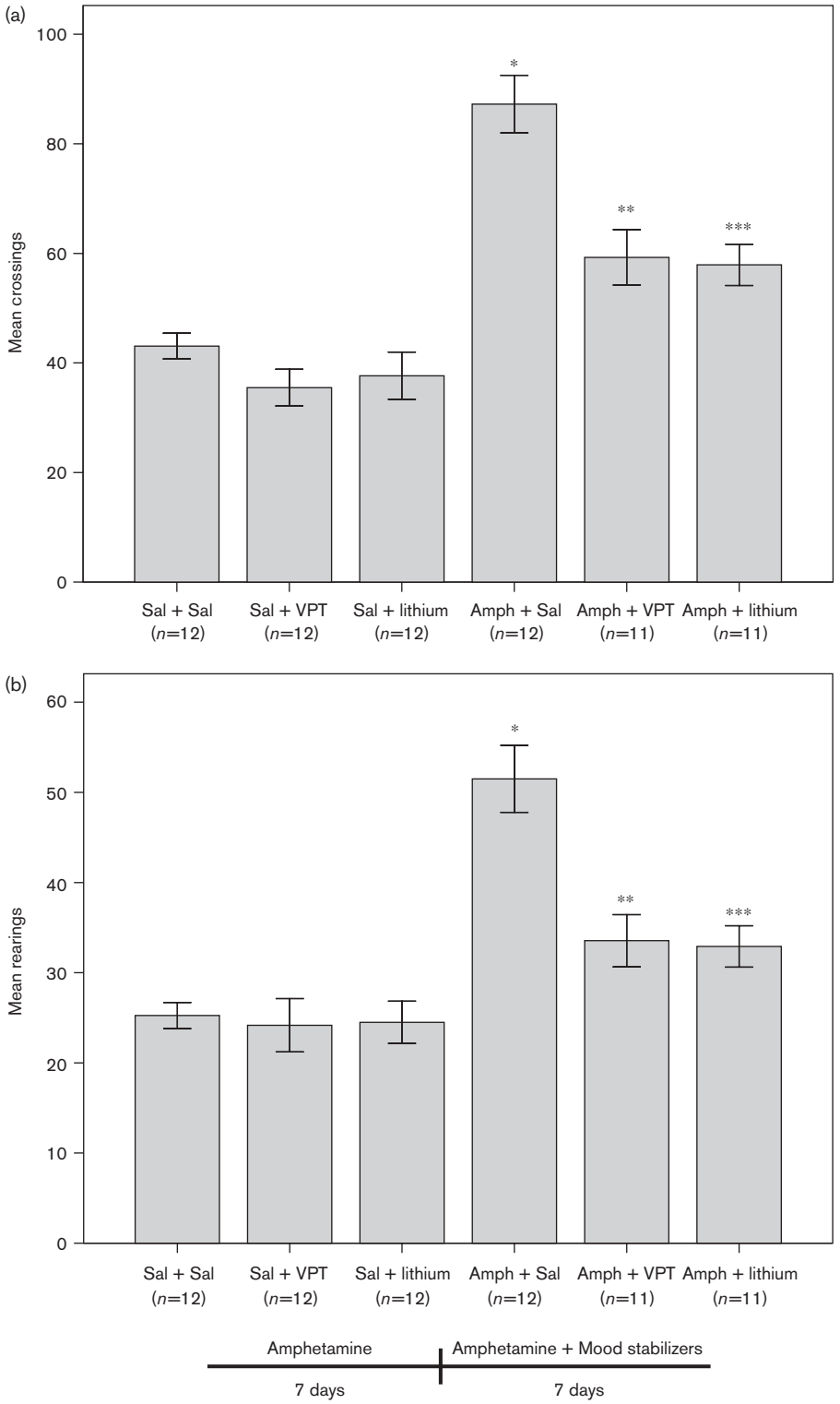
Serum lithium and nerve growth factor assessments

Serum Li levels were assessed in a commercial laboratory blind to the experiments. NGF levels in the hippocampus were measured with sandwich enzyme-linked immunosorbent assay, using a commercial kit according to the manufacturer's instructions (Chemicon, Temecula, California, USA). Briefly, brain slices were homogenized in phosphate buffer solution with 1 mmol/l phenylmethylsulfonyl fluoride and 1 mmol/l ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid. Microtiter plates (96-well flat bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent. The standard curve ranged from 7.8 to 500 pg of NGF. Then, plates were washed four times with sample diluents and monoclonal anti-NGF rabbit antibody diluted 1:1000 in sample diluent was incubated for 3 h at room temperature. After washing, a second incubation was carried out with anti-rabbit antibody peroxidase conjugated, diluted 1:1000 for 1 h at room temperature. After addition of streptavidin enzyme, substrate and stop solution the amount of NGF was determined for absorbance in 450 nm. The standard curve demonstrated a direct relationship between optical density and NGF concentration. Total protein was measured by Lowry's method using bovine serum albumin as a standard.

Statistical analysis

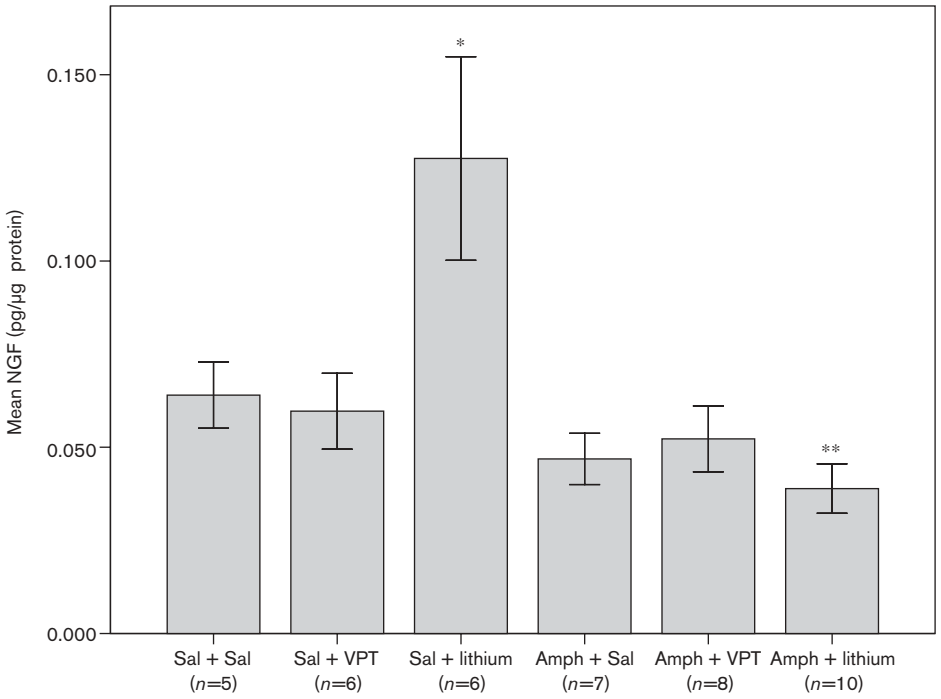
Raw NGF data exhibited non-normality and heterogeneity of variances and therefore were transformed using power transformations before analysis. Normality and homogeneity of variances in the transformed variables

Fig. 1



Crossings (a) and rearings (b) after 7 days of amphetamine +7 days of amphetamine and mood stabilizers (mean ± SEM). *Different from Sal + Sal ($P < 0.001$); **different from AMPH + Sal ($P < 0.001$); ***different from AMPH + Sal ($P < 0.001$); post-hoc pairwise comparisons. AMPH, amphetamine; Sal, saline; VPT, valproate.

Fig. 2



Nerve growth factor (NGF) levels in the reversal model (mean ± SEM). *Different from Sal + Sal ($P = 0.046$); **different from Sal + lithium ($P < 0.001$); post-hoc pairwise comparisons. Sal, saline; VPT, valproate.

were confirmed using the Shapiro–Wilks test and the Levin test, respectively ($P > 0.05$). Transformed data were submitted to a two-way factorial analysis of variance with one factor having two levels corresponding to AMPH versus saline treatment and the second factor having three levels corresponding to saline versus Li versus VPT treatment. All factors were modeled as fixed effects. The interaction effect of this model tests the hypothesis that the mood stabilizers moderate the effects of AMPH treatment. The statistically significant interaction between AMPH and mood stabilizers was deconstructed by performing post-hoc pairwise comparisons between the AMPH and saline treatments at each of the three levels of mood stabilizer treatment. Six independent groups were studied. Behavioral data were first submitted to a factorial multivariate analysis of variance. Following a significant multivariate test, univariate analysis of variance and post-hoc pairwise comparisons were carried out as described for NGF. All analyses were performed using SPSS software (SPSS Inc., Chicago, Illinois, USA) release 14.0.1. All data are shown as untransformed values. All testing was two-sided at a significance level of $P < 0.05$.

Results

Reversal treatment

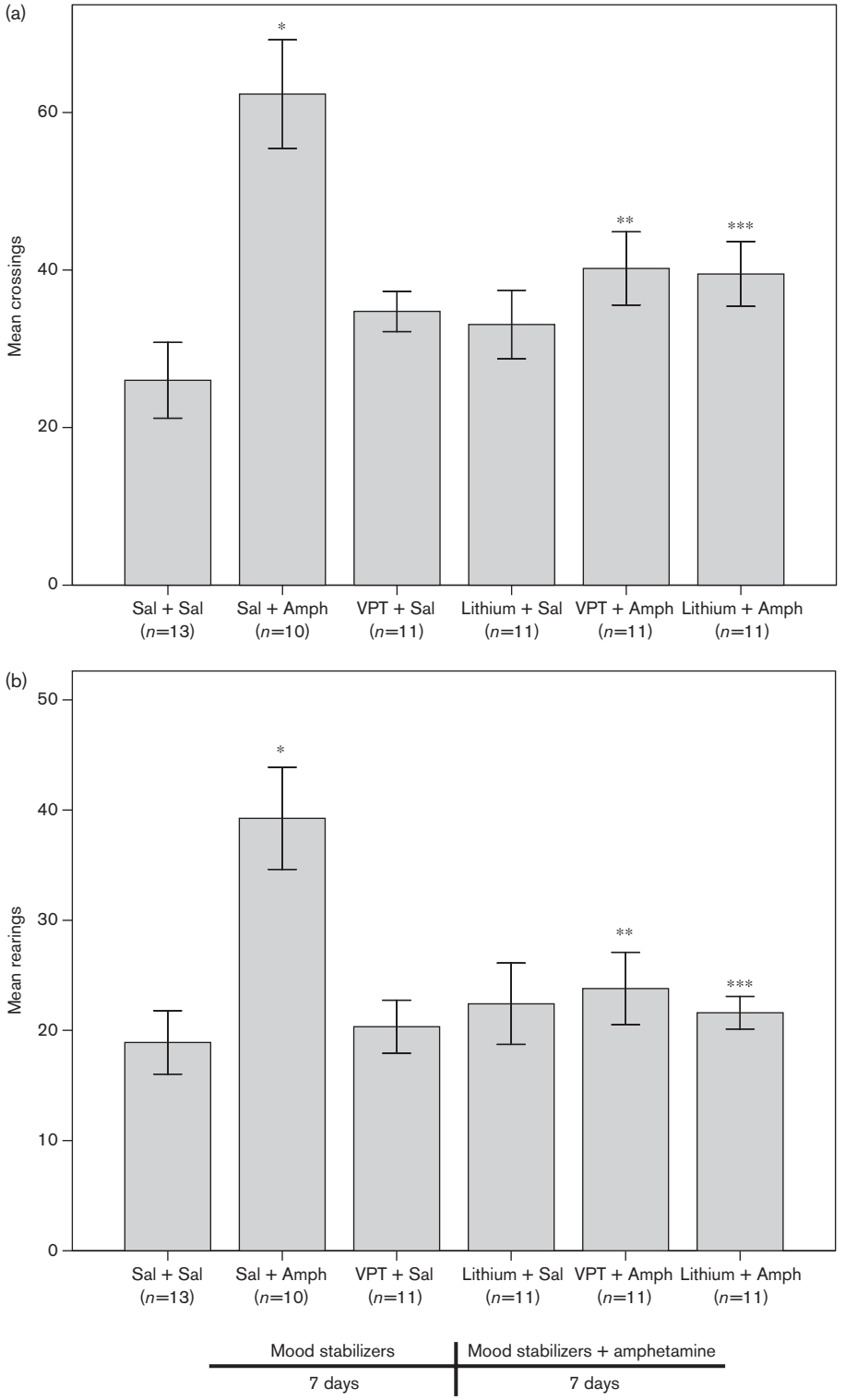
In the reversal experiment (Fig. 1a and b), there was a significant main effect of AMPH [$F(2,63) = 42.0$, $P < 0.001$] and a significant interaction between AMPH and the mood stabilizers on locomotor and rearing behaviors [$F(4,126) = 3.8$, $P < 0.005$]. Crossings were significantly increased by AMPH in saline-treated rats ($P < 0.001$), and this effect was reversed by Li ($P < 0.001$) and VPT ($P < 0.001$). Rearings were significantly increased by AMPH in saline-treated rats ($P < 0.001$), and this effect was reversed by Li ($P < 0.001$) and VPT ($P < 0.001$). Li or VPT alone did not alter behavioral measures, indicating that the effects of mood stabilizers on AMPH-treated rats were not associated with sedation.

A significant main effect of AMPH [$F(1,36) = 12.4$, $P < 0.001$] and a significant interaction between AMPH and the mood stabilizers on NGF levels [$F(2,36) = 4.5$, $P < 0.02$] were observed. A pairwise comparison showed that Li administration increased NGF levels in saline-pretreated animals ($P < 0.05$; Fig. 2) but this effect was blocked in AMPH-pretreated animals ($P < 0.001$). No significant difference was noted between the NGF levels of the VPT-treated subjects that received AMPH or saline pretreatment.

Prevention treatment

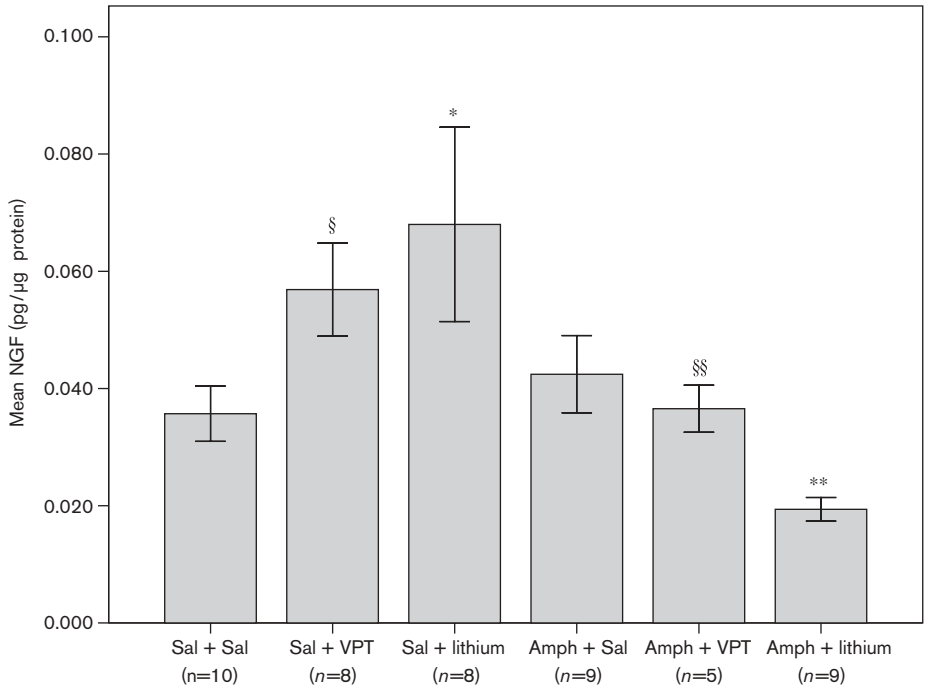
In the prevention experiment (Fig. 3a and b), there was a significant main effect of AMPH [$F(2,60) = 8.2$,

Fig. 3



Crossings (a) and rearings (b) after 7 days of mood stabilizers +7 days of mood stabilizers and amphetamine (mean ± SEM). *Different from Sal + Sal ($P < 0.001$); **different from Sal + AMPH ($P < 0.01$); ***different from Sal + AMPH ($P < 0.01$); post-hoc pairwise comparisons. AMPH, amphetamine; Sal, saline; VPT, valproate.

Fig. 4



Nerve growth factor (NGF) levels in the prevention model (mean ± SEM). *Different from Sal + Sal ($P<0.001$); **different from Sal + lithium ($P<0.001$); §different from Sal + Sal ($P=0.052$); §§different from Sal + VPT ($P=0.059$); post-hoc pairwise comparisons. Sal, saline; VPT, valproate.

$P < 0.001$] and a significant interaction between AMPH and the mood stabilizers on locomotor and rearing behaviors [$F(4,120) = 3.6$, $P < 0.01$]. Crossings were significantly increased by AMPH in saline-treated rats ($P < 0.001$), and this effect was prevented by Li ($P < 0.005$) and VPT ($P < 0.01$). Rearings were significantly increased by AMPH in saline-treated rats ($P < 0.001$), and this effect was prevented by Li ($P < 0.001$) and VPT ($P < 0.01$). In accordance with the first experiment, the administration of Li and VPT alone did not affect behavioral measures.

In this model, there was a significant main effect of AMPH [$F(1,43) = 18.2$, $P < 0.001$] and mood stabilizers [$F(2,43) = 6.1$, $P < 0.005$], and a significant interaction between AMPH and the mood stabilizers on NGF levels [$F(2,43) = 13.7$, $P < 0.001$]. In the Li pretreated group, NGF levels were significantly higher in saline-treated animals ($P < 0.001$; Fig. 4) but this effect was blocked in AMPH-treated animals ($P < 0.001$). In the VPT-pretreated animals, the mean NGF level was non-significantly higher in the saline-treated group ($P = 0.052$) and this effect was non-significantly blocked in the AMPH-treated group ($P = 0.059$).

Discussion

In the present study, we showed that 7 and 14 days of Li administration, within the therapeutic window, significantly increased NGF levels in the rat hippocampus. These results are in line with previous studies demonstrating increased NGF levels after 14 and 42 days of Li treatment (Hellweg *et al.*, 2002; Angelucci *et al.*, 2003b). In contrast, we found no significant effects of VPT on NGF levels, with a dosage sufficient to reverse and prevent AMPH-induced hyperactivity. As far as we are aware, this is the first study to assess VPT effects on NGF levels *in vivo*. It has been reported that NGF promotes local axonal sprouting via mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK1/2 and phosphatidylinositol 3-kinase signaling pathways (Atwal *et al.*, 2000), and increases neuronal survival by a retrograde signaling to the cell body through a cAMP response element-binding protein-dependent transcription of Bcl-2 (Finkbeiner, 2000). Thus, it is reasonable to suppose that the effects of Li on NGF expression may be related to the neurotrophic effects of Li. In fact, the neurotrophic effects of Li have been associated with increased gray matter content observed after chronic Li treatment in humans (Moore *et al.*, 2000; Sassi *et al.*, 2002). Further, it has also been reported that chronic Li administration induces neurogenesis in rat hippocampus (Chen *et al.*, 2000).

We also found that AMPH blocked the ability of Li to increase NGF. This finding was somewhat unexpected as

AMPH alone had no effects on NGF concentration. To date, there are no studies assessing in-vivo effects of AMPH on NGF expression. It has been demonstrated that the BDNF/tyrosine kinase B pathway is implicated in the modulation of dopamine-related behaviors (Guillin *et al.*, 2001; Narita *et al.*, 2003). Our results, however, suggest that the NGF/TrkA pathway is not, as we found a clear dissociation between the behavioral effects and the changes in NGF levels in both experiments. Both Li and VPT reversed AMPH-induced hyperactivity but only Li altered NGF levels, while AMPH increased locomotor behavior but had no effects on NGF levels.

Previous studies suggest that alternative treatments for BD, such as electroconvulsive shock and antipsychotics, also modulate NGF expression. Specifically, electroconvulsive shock increases NGF mRNA (Follesa *et al.*, 1994) and NGF concentration in the rat hippocampus (Angelucci *et al.*, 2003a). Further, risperidone and haloperidol were shown to decrease, whereas olanzapine was shown to increase hippocampal NGF levels (Angelucci *et al.*, 2000). Studies of patients with schizophrenia demonstrated that plasma NGF was reduced in this disorder (Bersani *et al.*, 1999; Parikh *et al.*, 2003), but that chronic cannabis or multiple substance abuse, and the use of atypical antipsychotics, may increase NGF levels in schizophrenic subjects (Jockers-Scherubl *et al.*, 2003; Parikh *et al.*, 2003). Although there is no study to date assessing NGF levels in BD subjects, the present findings negate rather than support that NGF is involved in the pathophysiology of BD. Future studies are needed to further clarify this issue.

In conclusion, we found that Li and VPT reversed and prevented AMPH-induced hyperactivity. Li increased NGF levels in the rat hippocampus, whereas this effect was blocked by AMPH. VPT had no significant effect on NGF levels. Our findings suggest that NGF may be associated with the neurotrophic effects of Li but do not support the hypothesis that the NGF/TrkA pathway is involved in the pathophysiology of BD.

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Parte III. Discussão

No primeiro modelo, demonstramos que o uso agudo e o uso crônico de ANF induzem um estado de hiperatividade locomotora em ratos. Ainda, observamos que a injeção aguda de ANF 2 mg/kg aumenta significativamente a locomoção em relação às doses de 1 e 4 mg/kg (Frey et al. 2006a,b,c). Esses resultados replicam estudos prévios demonstrando que doses mais elevadas de ANF diminuem o comportamento locomotor devido à emergência de estereotipias (Antoniou et al. 1998). Tais diferenças não foram observadas nos animais que utilizaram ANF cronicamente, possivelmente devido a alterações neuroplásticas no sistema dopaminérgico meso-límbico causada pelo uso repetido da ANF (Koob e Swerdlow 1988). Concomitante ao estado de hiperatividade motora, o tratamento agudo com ANF aumentou a formação de grupos protéicos carbonila em hipocampo e estriado, que é um indicativo de oxidação protéica (dano) nessas regiões cerebrais. Além disso, a exposição repetida à ANF causou um aumento da formação de proteína carbonila e de TBARS em todas as regiões cerebrais avaliadas, o que sugere um dano oxidativo adicional às estruturas lipídicas causado pelo tratamento crônico (Frey et al. 2006a). Estes resultados estão de acordo com o desequilíbrio causado pela ANF sobre as enzimas antioxidantes SOD e CAT (Frey et al. 2006b). Aqui cabe lembrar que um simples aumento ou diminuição de uma determinada enzima antioxidante não necessariamente indica um estado de estresse oxidativo. No entanto, um desequilíbrio entre a atividade da SOD e da CAT podem levar ao acúmulo de radicais livres. Fisiologicamente, o radical superóxido ($O_2^{\cdot -}$) é produzido na cadeia de transporte de elétrons durante a

respiração mitocondrial. A SOD atua metabolizando o excesso de $O_2^{\cdot -}$, formando peróxido de hidrogênio (H_2O_2). O excesso de H_2O_2 é metabolizado pela CAT, produzindo O_2 + H_2O, o que diminui o estado redox intracelular. Entretanto, em situações de desequilíbrio entre a SOD e a CAT pode ocorrer um acúmulo do intermediário H_2O_2, o qual pode reagir espontaneamente com o ferro e produzir radicais hidroxila (OH^-). Os radicais OH^-, por sua vez, são altamente reativos e podem gerar danos irreversíveis a estruturas protéicas e lipídicas, como o DNA e a membrana celular.

A formação de radicais livres pelo uso de ANF pode ocorrer através de diversos mecanismos. Em condições normais, a mitocôndria é a principal fonte de espécies reativas ao oxigênio, que originam-se nos complexos da cadeia respiratória localizados na matriz mitocondrial. Utilizando a técnica de histoquímica, Burrows et al. (2000) demonstraram que a ANF inibe os complexos da cadeia respiratória, o que potencializa a formação de espécies reativas ao oxigênio. Outra fonte de formação de radicais livres é a própria degradação da ANF pela enzima monoamino oxidase, que localiza-se na membrana mitocondrial externa, o que favorece a produção de H_2O_2 (Brown e Yamamoto 2003). Como a ANF aumenta a liberação de DA das vesículas pré-sinápticas, a DA pode sofrer um processo de auto-oxidação, via reação de Fenton que utiliza o ferro como cofator, formando substâncias altamente reativas chamadas DA quinonas (La Voie e Hastings 1999). Ainda, tem sido demonstrado que a ANF pode aumentar a liberação de glutamato (Sonsalla et al. 1989), que sabidamente pode potencializar o estado de estresse oxidativo (Coyle e Puttfarcken 1993). Tendo em vista o papel central da mitocôndria na formação de espécies reativas de oxigênio, estudamos

os efeitos do tratamento agudo e crônico com ANF nos níveis de $O_2^{\cdot -}$ e TBARS em partículas submitocondriais (Frey et al. 2006c). Neste estudo, a administração crônica de ANF aos animais causou um aumento nos níveis de $O_2^{\cdot -}$ e de TBARS no CPF e hipocampo, mas não houve diferenças na administração aguda. Estes resultados indicam que o uso repetido de ANF aumentou a formação de $O_2^{\cdot -}$ provavelmente pela inibição dos complexos da cadeia respiratória, e este excesso de $O_2^{\cdot -}$ levou à produção de radicais livres, com conseqüente aumento da peroxidação lipídica (aumento da TBARS). Aqui cabe ressaltar que o cérebro é particularmente suscetível ao dano oxidativo: embora corresponda a 2% do peso corporal, o cérebro consome 20% do oxigênio circulante. Além disso, o cérebro é rico em ferro e em ácidos graxos facilmente peroxidáveis, e tem limitada reserva de defesas antioxidantes (Floyd 1999). Este fato ressalta a importância de um melhor entendimento do papel do estresse oxidativo nas doenças neuropsiquiátricas e das propriedades pró/antioxidantes dos medicamentos psicotrópicos.

Com este intuito, avaliamos os efeitos dos estabilizadores de humor Li e VPT nas alterações comportamentais e nos marcadores de dano e defesa de estresse oxidativo causado pela administração crônica de ANF (Frey et al. 2006d). Conforme esperado, a administração de ANF por 14 dias aumentou significativamente a atividade locomotora horizontal e vertical. Embora não tenha sido diretamente avaliado, as medidas médias de locomoção entre o tratamento de reversão (14 dias de ANF) e o tratamento de prevenção (7 dias de ANF) indicam a ocorrência de sensibilização comportamental (*behavioral sensitization*). Ambos os estabilizadores de humor reverteram e preveniram o estado de

hiperatividade induzida pela ANF, o que demonstra que este modelo tem uma boa validade preditiva. Além disso, o uso de Li ou VPT nos animais controle (salina) não alterou as medidas comportamentais, confirmando que os efeitos dos estabilizadores nos animais tratados com ANF não estavam associados à sedação. Embora os mecanismos pelos quais o Li e o VPT regulam as mudanças comportamentais causadas pela ANF ainda sejam desconhecidos, alguns efeitos na neurotransmissão dopaminérgica têm sido considerados. Pert et al. (1978) demonstraram que o uso crônico de Li inibiu a sensibilização comportamental causada pelo haloperidol (bloqueador dos receptores D2) e que este efeito estava associada a uma inibição da *upregulation* dos receptores D2 no estriado de ratos. Além disso, outro estudo demonstrou que o tratamento crônico com Li aumenta a afinidade dos transportadores de DA em diversas subregiões do estriado, sem causar efeito na expressão do RNAm que codifica os transportadores (Carli et al. 1997). Mais recentemente, um estudo com PET demonstrou que o divalproato de sódio diminuiu a captação de ^{18}F-DOPA (precursor da DA) no estriado de indivíduos bipolares durante episódio maníaco (Yatham et al. 2002). Em conjunto, estes trabalhos sugerem que o Li e o VPT podem reduzir a quantidade de DA disponível na fenda sináptica, através da redução da síntese ou do aumento da recaptação de DA, ou ainda através da modulação da afinidade dos receptores dopaminérgicos. O resultado final destes efeitos seria uma diminuição da transmissão dopaminérgica no estriado, uma região cerebral que possui um papel central sobre a regulação da atividade locomotora (Groenewegen 2003). Em relação aos efeitos sobre os marcadores de estresse oxidativo, tanto o Li quanto o VPT reverteram os índices de peroxidação lipídica no CPF e preveniram os

índices de peroxidação lipídica induzida pela ANF no hipocampo. Estes resultados estão de acordo com trabalhos anteriores demonstrando que o Li e o VPT possuem propriedades antioxidantes *in vitro* (Shao et al. 2005; Wang et al. 2003). O nosso estudo é a primeira evidência de efeitos antioxidantes destes estabilizadores de humor *in vivo*. No entanto, nós também observamos que o uso de Li e VPT associados à ANF aumentou os índices de peroxidação lipídica no hipocampo (tratamento de reversão) e o uso de Li + ANF aumentou a peroxidação lipídica no CPF (tratamento de prevenção). Estes dados indicam que os efeitos neuroprotetores dos estabilizadores de humor variam de acordo com a região cerebral e com o esquema terapêutico empregado. Porém, uma vez que em nenhum dos experimentos o uso de Li e VPT alterou os índices de peroxidação lipídica nos animais tratados com salina, o mais provável é que eles tenham potencializado o dano causado pela ANF nestas circunstâncias. Ao contrário do que se esperava, não encontramos alterações nos níveis de proteína carbonila, indicando que não houve dano oxidativo significativo em proteínas no presente modelo. Este resultado é oposto ao que encontramos no primeiro modelo (Frey et al. 2006a), sendo que as diferenças metodológicas entre os estudos (que serão discutidas em detalhe nas limitações) devem responder por esta discrepância. Conforme abordado previamente, alterações na atividade das enzimas antioxidantes devem ser interpretadas dentro de um contexto. No presente estudo, é digno de nota que apenas observamos um aumento significativo da CAT quando a oxidação lipídica causada pela ANF foi mais intensa, o que sugere que a ANF provocou um excesso na produção de H_2O_2. Em resumo, este estudo demonstrou que o Li e o VPT podem reverter e prevenir a hiperatividade locomotora e o estado

de estresse oxidativo causado pelo uso crônico de ANF *in vivo*. O quanto estes resultados do modelo animal podem ser generalizados para os humanos e, fundamentalmente, para o TB ainda está para ser determinado.

Conforme vimos anteriormente o estado de estresse oxidativo, que recentemente tem sido associado ao TB e ao mecanismo de ação do Li e VPT, pode resultar em dano em estruturas lipídicas e protéicas, como a membrana celular e o DNA, respectivamente. Com o objetivo de avaliar o possível dano ao DNA em pacientes bipolares, utilizamos a técnica do Cometa que detecta quantitativamente quebras em ligas simples e duplas do DNA (Andreazza et al. In press). Este estudo demonstrou que indivíduos bipolares apresentam um aumento marcante de dano em DNA no sangue periférico em relação a controles normais. Além disso, o grau de dano no DNA apresentou uma correlação positiva com a intensidade de sintomas maníacos e depressivos. Embora a relevância clínica destes achados e os mecanismos pelos quais os indivíduos com TB apresentam um elevado dano sistêmico ao DNA ainda são desconhecidos, tem sido demonstrado que o dano ao DNA detectado pela técnica do Cometa parece estar associado ao dano por estresse oxidativo (Migliore et al. 2005; Faust et al. 2004). Como uma investigação preliminar das hipóteses levantadas por este trabalho, avaliamos prospectivamente parâmetros de estresse oxidativo (TBARS, SOD, CAT) e de dano ao DNA (Cometa) em duas gêmeas monozigóticas durante episódio maníaco, sendo uma paciente com e uma sem tratamento. Antes do tratamento, ambas as pacientes apresentavam maior SOD e TBARS e menor atividade da CAT que o controle normal. Esses achados estão de acordo com estudos anteriores que demonstraram alterações dos níveis de SOD e CAT e

aumento da TBARS no sangue de pacientes bipolares, indicando um aumento sistêmico do estresse oxidativo no TB (Ozcan et al. 2004; Ranjekar et al. 2003; Kuloglu et al. 2002). Além disso, estes resultados também são similares aos encontrados nos nossos modelos animais, em que um desequilíbrio entre as enzimas antioxidantes SOD e CAT estavam associadas a um maior índice de peroxidação lipídica (Frey et al. 2006a,b,d). Um dos achados mais interessantes deste trabalho é que a gêmea que recebeu tratamento com Li e antipsicóticos apresentou níveis de SOD e TBARS comparáveis ao controle após 6 semanas de tratamento, enquanto a gêmea que recusou tratamento continuou com estes índices alterados. Ainda que preliminar, este é o primeiro relato na literatura de que alguns parâmetros de estresse oxidativo podem ser corrigidos com tratamento farmacológico em indivíduos com TB. Logicamente, estudos prospectivos com amostras maiores são necessários para melhor elucidar esta questão. Além disso, de acordo com os nossos resultados anteriores (Andreazza et al. In press) observamos que os índices de dano ao DNA encontravam-se bastante aumentados em ambas as pacientes, mantendo se aumentados mesmo após o tratamento medicamentoso. Embora ainda especulativo, este estudo sugere que um estado de estresse oxidativo crônico pode estar associado a um aumento do dano em DNA, conforme discutido previamente. Entretanto, o quanto este marcado dano sistêmico reflete alterações cerebrais em indivíduos com TB ainda está para ser determinado. Dentro deste contexto, um estudo pós-mortem não encontrou dano em DNA no córtex cingulado anterior (Benes et al. 2003), enquanto outro estudo encontrou uma diminuição da expressão de genes que codificam a PARP-1 (enzima de reparação ao dano em DNA) e que codificam

enzimas antioxidantes como a SOD e a CAT no hipocampo de pacientes bipolares (Benes et al. 2006), o que indica um potencial pró-oxidativo nesta determinada região cerebral.

Esta crescente busca de um melhor entendimento sobre o papel do estresse oxidativo na fisiopatologia do TB é apoiado pela corrente hipótese de disfunção mitocondrial e alteração do metabolismo energético neste transtorno (Kato e Kato 2000; Stork e Renshaw 2005). De acordo com esta hipótese, mutações ou polimorfismos do DNA mitocondrial ou de determinados locus cromossomiais podem alterar a expressão de genes que regulam o funcionamento da mitocôndria (Kato e Kato 2000). Recentemente, três estudos independentes demonstraram uma diminuição significativa da expressão de genes mitocondriais (incluindo genes que codificam componentes da cadeia respiratória) no CPF e hipocampo de indivíduos com TB (Sun et al. 2006; Iwamoto et al. 2005; Konradi et al. 2004). Considerando que o dano mitocondrial está intimamente associado à indução de cascatas de sinalização que levam à apoptose (Nicholls e Budd 2000), é interessante notar que indivíduos bipolares apresentam diminuição de células neuronais e gliais nestas mesmas regiões cerebrais (Rajkowska et al. 2001; Benes et al. 1998). Além disso, as evidências de que o Li e o VPT apresentam efeitos neuroprotetores através da redução do excesso de cálcio intracelular (Shao et al. 2005) e que o Li atenua a indução das proteínas pró-apoptóticas Bax e caspase-3 (King e Jope 2005) sugerem que a regulação de cascatas de sinalização intracelular associadas à apoptose podem ser relevantes na terapêutica a longo prazo.

Uma das vias que sabidamente previnem contra a apoptose é a cascata de sinalização do BDNF/trkB (Barde 1994), sendo que diversos estudos têm sugerido que a indução da via do BDNF/trkB é um dos mecanismos responsáveis pelos efeitos terapêuticos dos estabilizadores do humor e dos antidepressivos (Coyle e Duman 2003; Nibuya et al. 1995). Por exemplo, tem sido demonstrado que o uso do Li modula a fosforilação (atividade) do receptor trkB e da CREB, esta que regula a transcrição gênica de uma série de proteínas associadas com sobrevivência e plasticidade neuronal (Einat et al. 2003; Rantamäki et al. 2006). Além disso, estudos familiares demonstraram uma associação positiva de um polimorfismo no gene do BDNF, que substitui uma valina por metionina no códon 66 (val66met), em indivíduos portadores de TB (Lohoff et al. 2005; Sklar et al. 2002). Desta forma, nosso grupo investigou os efeitos reversivos e preventivos do Li e do VPT nas alterações comportamentais e na expressão do BDNF após o uso crônico de ANF em hipocampo de ratos (Frey et al. 2006f). Conforme comentado anteriormente, ambos os estabilizadores de humor reverteram e preveniram a hiperatividade induzida pela ANF. Além disso, o Li aumentou os níveis de BDNF quando administrado antes e depois da ANF, enquanto o VPT aumentou os níveis de BDNF quando administrado antes da ANF (modelo de prevenção). Estes achados sugerem que a ação dos estabilizadores de humor sobre o BDNF pode estar associada aos efeitos comportamentais modulados pelo sistema dopaminérgico. Neste contexto, Beaulieu et al. (2004) demonstraram que os efeitos do Li sobre o comportamento induzido pela ANF ocorrem em parte através da regulação da via de sinalização da Akt/GSK-3β, enquanto que Narita et al.

(2003) sugeriram que os efeitos do BDNF/trkB sobre o comportamento induzido pela ANF parecem envolver a ativação da PKC. Evidências diretas de alteração dos níveis de BDNF em humanos provêm de estudos que observaram uma diminuição significativa dos níveis séricos de BDNF em pacientes com depressão maior (unipolar) (Karege et al. 2002; Shimizu et al. 2003) e em indivíduos bipolares durante episódio maníaco (Machado-Vieira et al. 2005). Portanto, nosso grupo avaliou os níveis séricos de BDNF em pacientes com TB tipo I durante episódio maníaco, depressivo e na fase de eutimia (remissão) (Cunha et al. 2006). Com este estudo demonstramos que os níveis séricos de BDNF encontravam-se diminuídos durante os episódios agudos de mania e depressão, enquanto indivíduos em remissão apresentaram valores comparáveis aos controles normais. Este estudo sugere que os níveis de BDNF circulantes diminuem especificamente durante os episódios agudos no TB e que a normalização do BDNF pode estar associada com a estabilização do humor. Ainda, o polimorfismo val66met do gene do BDNF parece estar associado com déficit cognitivo (Rybakowski et al. 2003), suscetibilidade para o desenvolvimento de ciclagem rápida (Green et al. 2006) e melhor resposta à profilaxia com Li (Rybakowski et al. 2005). Em conjunto à crescente evidência de que a regulação da via do BDNF/trkB parece estar envolvida na fisiopatologia dos transtornos de humor (Hashimoto et al. 2004), nossos estudos indicam que o BDNF pode modular a atividade locomotora dependente do sistema dopaminérgico (modelo animal) e que o BDNF pode estar associado aos efeitos terapêuticos dos estabilizadores de humor (modelo animal e humanos).

A partir dos primeiros indicativos de que o tratamento crônico com Li aumenta os níveis de NGF no CPF, hipocampo e amígdala (Hellweg et al. 2002; Angelucci et al. 2003) e da escassez de dados acerca do potencial envolvimento de outras neurotrofinas na regulação do humor, avaliamos os efeitos do Li e do VPT nos níveis de NGF neste mesmo modelo animal de mania induzido pela ANF (Frey et al. 2006g). Além dos resultados comportamentais já discutidos anteriormente, nós replicamos os achados de que o Li aumenta os níveis de NGF no hipocampo de ratos. Entretanto, embora o VPT tenha demonstrado uma mesma tendência em um dos experimentos, não encontramos alterações significativas do VPT sobre os níveis de NGF. Além disso, apesar de causar efeitos significativos sobre a atividade locomotora, a ANF não alterou os níveis de NGF. Essas discrepâncias entre os efeitos comportamentais e os níveis de NGF sugerem que a cascata de sinalização do NGF/trkA não está envolvida na regulação da atividade motora induzida pela ANF, bem como não apóia o envolvimento desta via de sinalização na fisiopatologia do TB. Mesmo assim, estudos em humanos podem ser úteis para uma compreensão mais aprofundada do possível papel desta via na regulação do humor.

Limitações e Pontos Altos do Modelo

As limitações do nosso modelo animal também devem ser consideradas. Em relação à validade aparente, obviamente o modelo representa apenas uma das diversas facetas do quadro clínico do TB. De fato um transtorno mental caracterizado pela recorrência de sintomas extremos e opostos é muito difícil de

ser modelado em animais de laboratório. Além disso, por definição os episódios de humor são autônomos, isto é, não são causados por uma condição médica geral ou pelo uso de substâncias (American Psychiatric Association 2000). Além da hiperatividade locomotora, modelos animais de mania podem utilizar parâmetros como agressividade, diminuição da necessidade do sono, prejuízo no julgamento, ou hipersexualidade nos animais. Mais interessante ainda seria integrar todas estas diversas facetas clínicas em um só modelo. Quanto à validade interpretativa, no presente modelo investigamos fundamentalmente o hipocampo e, nos experimentos iniciais (primeiro modelo), estudamos também o CPF e o estriado. Portanto além de não ter investigado o CPF e o estriado no segundo modelo, no qual utilizamos os estabilizadores de humor, o papel de outras regiões cerebrais como a amígdala e o cerebelo também têm sido associadas ao TB. Já em relação à validade preditiva do modelo, uma lacuna importante a ser estudada é o efeito dos antipsicóticos atípicos nos efeitos comportamentais e neuroquímicos causado pela exposição crônica à ANF, uma vez que os antipsicóticos atípicos são medicamentos de primeira linha no tratamento da mania aguda (Yatham et al. 2005). Nosso modelo também apresenta algumas limitações técnicas que são dignas de nota. Uma vez que trabalhamos com fatias de cérebro homogenadas, esta técnica não nos permitiu investigar as alterações dos marcadores bioquímicos nas diferentes sub-regiões cerebrais, como as regiões CA1, CA2 e giro denteado do hipocampo, por exemplo. Outra limitação é que não dosamos os níveis séricos de VPT nos animais, apenas de Li. Desta forma, não podemos determinar se a dosagem de VPT utilizada nos animais encontrava-se dentro dos valores considerados terapêuticos para os humanos. Finalmente, existem

evidências de que as variações de temperatura causadas pela ANF estão associadas aos seus efeitos deletérios e que o controle da temperatura pode ter efeito neuroprotetor (Bowyer et al. 1994; Fleckenstein et al. 1997). Embora dosagens de ANF entre 0,3 a 3 mg/kg não alteram a temperatura corporal em ratos Wistar e ratos Sprague-Dawley adultos (Phillis et al. 2001; Jaehne et al. 2005), o que sugere que esse fator não deve ter influenciado significativamente nossos resultados, nós não controlamos esta variável no nosso modelo.

Alguns pontos altos do presente modelo animal também merecem ser destacados. Nosso modelo foi o primeiro a mimetizar em paralelo um modelo de tratamento de episódio agudo (reversão) e um modelo de tratamento de manutenção (prevenção). Este desenho pode ajudar a levantar novas hipóteses que podem ser testadas para o desenvolvimento de novos medicamentos para o tratamento do TB. Além disso, pela primeira vez os efeitos antioxidantes dos estabilizadores de humor foram demonstrados *in vivo*. Se estudos futuros replicarem nossos achados, o desenvolvimento de novos agentes com propriedades antioxidantes podem ser potenciais promissores no tratamento do TB. Além disso, o fato de termos conduzido estudos em pacientes bipolares utilizando os mesmos marcadores bioquímicos do modelo animal pode auxiliar na busca de um melhorar conhecimento sobre a fisiopatologia de um transtorno ainda pouco esclarecido. Nossa perspectiva futura é melhorar ainda mais esse modelo animal, controlando outros fatores como a temperatura e a dosagem sérica de VPT, além de testar os efeitos de outros medicamentos e substâncias neuroprotetoras no modelo. Pretendemos também testar agonistas e inibidores de determinadas vias de sinalização celular que podem estar envolvidas na regulação

do humor. Além disso, a idéia de conduzir paralelamente estudos em modelos animais e em humanos deve ser mantida. No presente, estamos finalizando uma série de experimentos com esse modelo animal avaliando o dano em DNA através da técnica do Cometa no sangue e no cérebro de ratos. Nosso objetivo é testar a hipótese de que o marcado dano em DNA observado no sangue de indivíduos bipolares (Andreazza et al. In press) pode correlacionar com dano cerebral.

Conclusões

Com o primeiro modelo, demonstramos que o uso agudo e crônico de ANF induz um estado de hiperatividade locomotora em ratos adultos. Além disso, observamos que a administração aguda e crônica de ANF aumentam o dano oxidativo em estruturas protéicas e lipídicas (Frey et al. 2006a). Esse estado aumentado de estresse oxidativo parece ser reforçado pelo desequilíbrio causado na SOD e na CAT, duas das principais enzimas antioxidantes (Frey et al. 2006b). Considerando que a mitocôndria é a principal fonte de espécies reativas do oxigênio, encontramos que o uso repetido, mas não agudo, de ANF aumentou a produção de $O_2^{\cdot -}$ em partículas submitocondriais, o que foi acompanhado de um aumento da peroxidação lipídica (Frey et al. 2006c). A seguir, demonstramos que o estado de hiperatividade locomotora e o aumento do estresse oxidativo causados pela administração crônica de ANF podem ser revertidos e prevenidos pelo uso de Li e VPT (Frey et al. 2006d). Além disso, em nossos estudos em humanos observamos que indivíduos portadores de TB apresentam um marcado aumento de dano em DNA em sangue periférico, possivelmente causado por um

estado de estresse oxidativo (Andreazza et al. In press), e que algumas medidas de estresse oxidativo podem ser normalizadas com o tratamento farmacológico (Frey et al. 2006e). Os efeitos do Li e do VPT sobre a hiperatividade causada pela ANF podem estar associados a um aumento da expressão do BDNF (Frey et al. 2006f), o que parece ser relevante na estabilização do humor em pacientes bipolares (Cunha et al. 2006). Embora os efeitos neuroprotetores do Li podem estar associados a um aumento dos níveis de NGF, as variações dos níveis de NGF não estão associadas aos efeitos do Li e do VPT na hiperatividade induzida pela ANF, o que não apóia o envolvimento da via do NGF/trkA na fisiopatologia do TB (Frey et al. 2006g).

Em conclusão, este conjunto de estudos indica que o presente modelo animal de mania apresenta características que permitem testar hipóteses que podem ser relevantes para a fisiopatologia do TB. O modelo apresenta uma parcial validade aparente pois reflete apenas uma das facetas do quadro clínico de mania. No entanto a hiperatividade induzida pela exposição crônica à ANF foi prevenida e revertida pelo Li e pelo VPT, o que indica uma boa validade preditiva. Além disso os indicativos de aumento de estresse oxidativo e de um potencial efeito do tratamento estabilizador do humor demonstrado em pacientes bipolares também puderam ser reproduzidos no modelo animal. Desta forma, nossos resultados apóiam a hipótese de que o aumento do estado de estresse oxidativo está associado à fisiopatologia do TB e de que a via de sinalização do BDNF/trkB (mas não a via do NGF/trkA) deve estar envolvida aos efeitos benéficos dos estabilizadores de humor. Estudos futuros abrangendo outras facetas do quadro clínico da mania e testando a resposta a outras classes de medicamentos podem

ajudar no desenvolvimento de novos agentes para o tratamento de um transtorno severo e incapacitante como o TB.

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