UNIVERSIDADE DO EXTREMO SUL CATARINENSE - UNESC

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AMANDA VALNIER STECKERT

AVALIAÇÃO DOS EFEITOS COMPORTAMENTAIS E DE ESTRESSE OXIDATIVO APÓS A ADMINISTRAÇÃO DE BUTIRATO DE SÓDIO E TAMOXIFENO EM UM MODELO ANIMAL DE MANIA INDUZIDO POR D-ANFETAMINA

CRICIÚMA, DEZEMBRO DE 2010

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade do Extremo Sul Catarinense para obtenção do título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. João Quevedo
Co-orientador: Prof. Dr. Felipe Dal Pizzol

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(Albert Einstein)

RESUMO

O objetivo deste estudo é investigar o comportamento e os parâmetros de estresse oxidativo no cérebro de ratos submetidos ao modelo de mania induzido por d-anfetamina (d-AMPH), além de avaliar os efeitos de Butirato de Sódio (SB – inibidor de histonas deacetilases – HDAC) e de Tamoxifeno (TMX – inibidor de proteína quinase C – PKC) neste contexto. No modelo de reversão, os ratos receberam injeções intraperitoneais (i.p.) de d-AMPH (2 mg/kg) ou solução salina (1 mL/kg) uma vez ao dia, durante 14 dias. Do 8º ao 14º dia, os animais tratados com d-AMPH ou solução salina também receberam solução salina (1 mL/kg i.p. – duas vezes ao dia), SB (0,6 g/kg i.p. – duas vezes ao dia) ou TMX (1 mg/kg i.p. – duas vezes ao dia). No modelo de prevenção, os ratos receberam solução salina, SB ou TMX (concentrações citadas acima, i.p, duas vezes ao dia) por um período de 14 dias. Do 8º ao 14º dia, os animais também receberam solução salina ou d-AMPH. No 15º dia de tratamento (reversão e prevenção), os animais receberam uma única injeção de d-AMPH ou solução salina. A atividade locomotora foi avaliada 2h após a última injeção de d-AMPH ou solução salina através do teste do campo aberto e os parâmetros de estresse oxidativo foram mensurados nas estruturas cerebrais (córtex pré-frontal, amígdala, hipocampo e estriado). O tratamento com SB e TMX reverteu e preveniu a hiperatividade induzida por d-AMPH. Além disso, a d-AMPH aumentou significativamente o dano oxidativo (produção de superóxido, TBARS em partícula submitocondrial e em tecido total e proteína carbonil) nos animais tratados com solução salina no pré-frontal, amígdala, hipocampo e estriado em ambos os modelos experimentais, sendo que SB e TMX foram hábeis em reverter e proteger contra o dano oxidativo, porém o resultado variou conforme a estrutura cerebral. Adicionalmente, a d-AMPH diminuiu a atividade da SOD e aumentou a atividade da CAT em ambos os modelos experimentais. A administração de SB e TMX aumentou a atividade da SOD e normalizou a atividade da CAT nos dois protocolos de tratamento. O presente trabalho reforça a necessidade de estudos com inibidores de PKC e HDAC como possíveis alvos de novas medicações para o tratamento do Transtorno do Humor Bipolar.

Palavras-chave: Butirato de Sódio, Estresse oxidativo, Tamoxifeno, Transtorno do Humor Bipolar.

ABSTRACT

The objective of this study is to investigate the behavior and the oxidative stress parameters in brain of rats subjected to a model of mania induced by d-amphetamine (d-AMPH), and to evaluate the effects of Sodium Butyrate (SB – a histone deacetylase – HDAC – inhibitor) and Tamoxifen (TMX – a protein kinase C – PKC – inhibitor) in this context. In reversal model, rats received intraperitoneal (i.p) injection of either d-AMPH (2 mg/kg) or saline (1 mL/kg) once a day for 14 days. From the 8th to the 14th day, AMPH and saline treated animals also received saline (1 mL/kg i.p. - twice a day), SB (0,6 g/kg i.p. - twice a day) or TMX (1 mg/kg i.p. - twice a day). In prevention model, rats received either saline, SB or TMX (concentrations mentioned above, i.p, twice a day) for a period of 14 days. From the 8th to the 14th day, animals also received saline or AMPH. On the 15th day of reversion and prevention treatments, the animals received a single injection of AMPH or saline. The locomotor behavior was assessed 2h after the last injection of AMPH or saline using the open-field task, and the oxidative stress parameters were measured in brain structures (prefrontal, amygdala, hippocampus and striatum). The treatment with SB and TMX reversed and prevented d-AMPH-induced hyperactivity. Moreover, d-AMPH significantly increased oxidative damage (superoxide production, TBARS submitochondrial particle and total tissue and protein carbonyl) in animals treated with saline in the prefrontal cortex, amygdala, hippocampus and striatum in both experimental models (reversal and prevention), with SB and TMX were able to reverse and protect against oxidative damage, but the results varied according to brain structure. Additionally, d-AMPH decreased SOD activity and increased CAT activity in both experimental models (reversal and prevention). The administration of SB and TMX increased SOD activity and normalized the CAT activity in the two treatment protocols. The present study reinforces the need for the study with inhibitors of PKC and HDAC as possible targets for new medications in the treatment of bipolar disorder.

Keywords: Sodium Butyrate, Oxidative stress, Tamoxifen, Bipolar Disorder

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

BDNF – Fator Neurotrófico Derivado do Cérebro

CAT – Catalase

DA - Dopamina

d-AMPH – Dextroanfetamina

DNA – Ácido desoxirribonucléico

ERO – Espécies Reativas de Oxigênio

GAP-43 – *Growth Associated Protein 43*

GPx – Glutationa Peroxidase

HAT – Histona Acetiltransferase

HDAC – Histona Deacetilase

MAP quinases – *Mitogen Activated Protein Kinases*

MDA – Malondialdeído

PKC – Proteína Quinase C

SB – Butirato de sódio

SOD – Superóxido Dismutase

TBARS – Substâncias Reativas ao Ácido Tiobarbitúrico

THB – Transtorno do Humor Bipolar

TMX – Tamoxifeno

TSA – *Trichostatin A*

YMRS – *Young Mania Ratting Scale*

SUMÁRIO

1 INTRODUÇÃO

1.1 Transtorno do Humor Bipolar

O Transtorno do Humor Bipolar (THB) é uma condição clínica caracterizada pela presença de episódios recorrentes de mania e depressão (Belmaker, 2004) que acomete de 1 a 3% da população mundial (Grant et al., 2005). O curso clínico do THB é crônico, usualmente caracterizado por períodos de exacerbação dos sintomas (episódios agudos) intercalados por períodos subsindrômicos e períodos de remissão (eutimia) (Judd et al., 2003).

A ocorrência de pelo menos um episódio maníaco alternado com episódios depressivos durante a vida confere o diagnóstico de THB tipo I, enquanto a presença de hipomania intercalada com depressão caracteriza o THB tipo II (Belmaker, 2004; Grant et al., 2005). Entretanto, as fases maníacas não precisam necessariamente ser seguidas por fases depressivas, ou as depressivas por maníacas (DSM IV, 2003).

Durante os episódios maníacos, os pacientes exibem comportamento impulsivo, autoestima exagerada ou grandiosidade, hipersexualidade, diminuição da necessidade do sono, discurso eloquente, fuga de ideias, hiperatividade ou agitação psicomotora incontrolável que prejudicam a vida social e familiar destes indivíduos (Goodwin & Jamison, 1990; Weissman et al., 1996; Young et al., 2007).

A hipomania apresenta as mesmas características da mania, porém não há um prejuízo acentuado no funcionamento social ou ocupacional, bem como hospitalização ou sintomas psicóticos (Judd et al., 2003; Belmaker, 2004).

A depressão é caracterizada pelo humor deprimido, perda de interesse ou prazer nas atividades, insônia ou hipersonia, perda da libido, sentimento de inutilidade ou culpa, podendo ser acompanhado por pensamentos de morte ou ideação suicida. O número e a

gravidade dos sintomas possibilitam determinar três graus de um episódio depressivo: leve, moderado e grave (CID-10, 1993).

Devido ao seu curso crônico, à frequente reincidência e a gravidade dos sintomas de humor, o tratamento do THB baseia-se no manejo dos episódios agudos e no tratamento de manutenção como prevenção para ocorrência de novos episódios, ou seja, a conduta é para que os pacientes se mantenham eutímicos o maior tempo possível. A demora no diagnóstico e o número maior de crises refletem ou prognosticam uma piora cognitiva e clínica geral do paciente bipolar (Yatham et al., 2005).

Uma estratégia terapêutica para THB atualmente muito utilizada – inclusive nos grandes centros de tratamento – é a polifarmácia (Kupfer et al., 2002). Em seu estudo, Levine e colaboradores (2000) mostraram uma tendência ao tratamento de THB com polifarmácia, onde constataram que quase 50% dos pacientes recebiam três ou mais agentes psicotrópicos. Contudo, os índices de recorrência e de resistência aos fármacos de primeira linha ainda são muito elevados, visto que seus efeitos adversos são significativos, o que diminui a adesão dos pacientes ao tratamento (Post et al., 2003; Dennehy et al., 2005).

Embora os medicamentos de última geração possuam maior tolerabilidade e segurança em relação aos tradicionais, muito pouco se adicionou – no que diz respeito à eficácia – a estes medicamentos (Castrén, 2005). Possivelmente, este pequeno avanço no tratamento farmacológico do THB se deva, em grande parte, ao pouco conhecimento acerca dos mecanismos fisiopatológicos envolvidos neste transtorno (Zarate et al., 2006).

O lítio e o valproato são fármacos clássicos usados no tratamento do THB. Ambos são efetivos nos episódios de mania aguda, além de apresentarem uma modesta atividade antidepressiva (Keck & Manji, 2002: Davis et al., 2005). Muitos dos alvos bioquímicos em que o lítio e o valproato agem diretamente têm sido identificados atualmente, visto que desde a identificação dos efeitos antimaníacos de lítio, não foi desenvolvido nenhum outro fármaco

especificamente para o tratamento de THB, exigindo, portanto, novas abordagens terapêuticas (Gould et al., 2004).

1.2 Histonas Deacetilases e Transtorno do Humor Bipolar

O valproato, além de ser um estabilizador do humor é também um inibidor de histonas deacetilases (HDACs) (Eadie & Vadja, 2005; Chen et al., 2007). As histonas são as principais proteínas que compõem a cromatina, atuando como uma matriz na qual o DNA se enrola (Drummond et al., 2005).

A histona acetiltransferase (HAT) e a histona deacetilase (HDAC) são enzimas que influenciam na transcrição gênica por acrescentar (acetilação) ou remover (deacetilação), respectivamente, grupamentos acetil de um aminoácido ε-N-acetil lisina em uma histona (Figura 1). A acetilação da histona está ligada à atividade transcrissional, enquanto a deacetilação está ligada a inativação da transcrissão gênica (Langley et al., 2005).

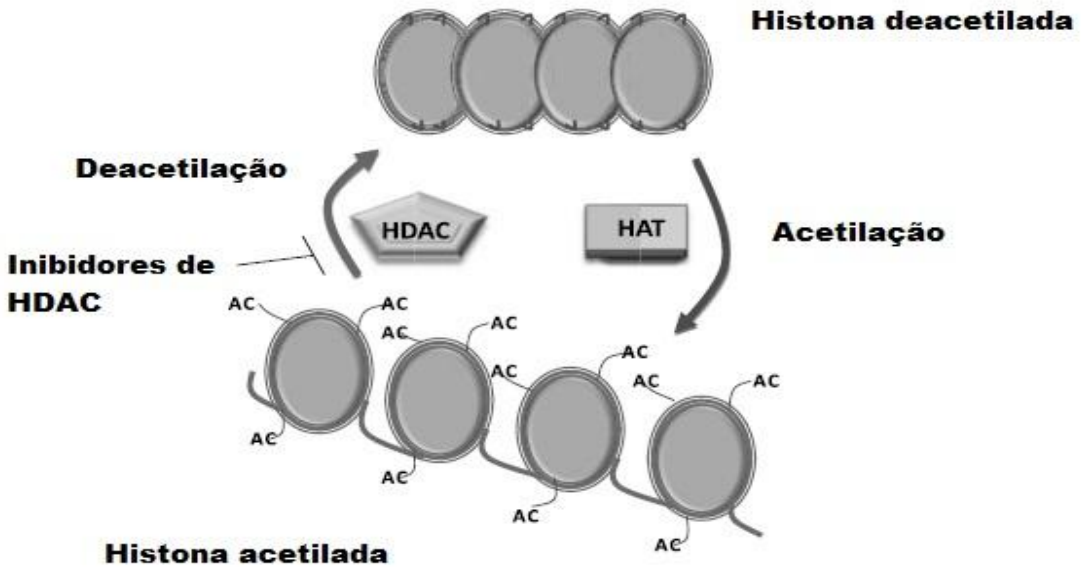

Figura 1. Efeitos dos inibidores de HDAC no remodelamento da cromatina. A acetilação das histonas é determinada pela ação das enzimas HAT e HDAC. Através do bloqueio da reação de deacetilação, os inibidores de HDAC alteram o equilíbrio dos níveis de acetilação das histonas. O aumento da acetilação causa modificações na conformação da cromatina, tornando-a menos condensada e aumentando a transcrição gênica. (Adaptado de Machado-Vieira, 2010).

A desregulação e atividade aberrante de HAT e HDAC têm sido implicadas na oncogênese e também no sistema nervoso central (Kazantsev & Thompson, 2008). Langley e colaboradores (2005) demonstraram que o aumento da atividade de HDACs parece estar envolvido na disfunção e degeneração neuronal. A inibição de HDACs, além de ser considerada um tratamento promissor para o câncer – visto que os inibidores de HDACs podem agir em vários processos que são desregulados em células neoplásicas (Drummond et al., 2005) – pode, inclusive, atenuar sequelas causadas por estressores no início da vida, o que é frequentemente observado em pacientes bipolares (Leverich et al., 2002).

Recentemente, estudos têm demonstrado que o butirato de sódio (SB) pode causar hiperacetilação de histonas através da inibição de HDACs (Gottlicher et al., 2001; Miller et al., 2003; Rodriguez et al., 2006; Sun et al., 2008; Wu et al., 2008). Neste contexto, Schröeder e colaboradores (2008) mostraram que o SB reverteu a hiperatividade induzida pelo consumo de cocaína em camundongos. Febo e colaboradores (2009) demonstraram que a inibição de HDACs resultou em uma diminuição de comportamentos induzidos por psicoestimulantes. Complementando estes achados, foi observado que inibidores de HDACs aumentam a expressão de fator neurotrófico derivado do cérebro (BDNF) em astrócitos, protegendo células dopaminérgicas (Yatham et al., 2002). Juntos, estes estudos sugerem um possível envolvimento de HDACs na fisiopatologia e tratamento do THB.

1.3 Proteína Quinase C e Transtorno do Humor Bipolar

A proteína quinase C (PKC) é um alvo bioquímico direto de lítio e de valproato. É encontrada principalmente no cérebro, sendo essencial nos aspectos de neurotransmissão pré e pós-sináptica, regulando a excitabilidade neuronal, a liberação de neurotransmissores e a plasticidade celular (Zarate & Manji, 2009).

Muitos autores sugerem o envolvimento da PKC na fisiopatologia do THB (Manji & Lenox, 1999; Chen et al., 2000; Kirshenboin et al., 2004; Einat et al., 2007). Em um estudo *post mortem* foi demonstrado um aumento da atividade da PKC no córtex pré-frontal de pacientes bipolares (Wang & Friedman, 1996). Além disso, Manji & Lenox (1999) mostraram que após o tratamento com lítio e valproato há um aumento e uma modulação na via de sinalização de PKC em pacientes bipolares. Adicionalmente, os psicoestimulantes – que induzem episódios maníacos em indivíduos susceptíveis e hiperatividade em roedores – parecem ativar PKC (Einat et al., 2007; Boudanova et al., 2008), sugerindo que a modulação desta tenha um papel fundamental no tratamento de mania (Chen et al., 2000; Kirshenboin et al., 2004; Hahn et al., 2005).

O tamoxifeno (TMX) é um antiestrogênio não-esteroidal sintético, podendo funcionar como agonista em alguns tecidos – o tecido mamário, por exemplo – e antagonista em outros – como os tecidos cardíaco, ósseo e endometrial (Riggins et al., 2007). Atualmente, o TMX é bastante utilizado no tratamento do câncer de mama (Jordan, 1994). Este fármaco consegue atravessar a barreira hematoencefálica, podendo ser administrado perifericamente, sendo eficaz no tratamento de gliomas malignos (Horgan et al., 1986).

Entretanto, o TMX apresenta outro mecanismo de ação importante, que é a inibição da atividade da PKC. Einat e colaboradores (2007) demonstraram o seu potencial efeito antimaníaco, onde o fármaco reduziu significativamente a hiperatividade e o comportamento de risco induzido pela anfetamina (d-AMPH).

Em um estudo duplo-cego feito pelo Instituto Nacional de Doenças Mentais dos Estados Unidos foi verificado que os pacientes bipolares que receberam TMX apresentaram uma diminuição nos sintomas maníacos em relação ao grupo que recebeu placebo (Zarate et al., 2007). Corroborando estes achados, Bebchuk e colaboradores (2000) observaram que os pacientes que receberam TMX apresentaram uma melhora significativa dos sintomas maníacos, demonstrada pela redução na pontuação da escala de Young (*YMRS – Young*

Mania Ratting Scale), em comparação ao grupo controle. Estes estudos reforçam a hipótese de envolvimento da PKC na fisiopatologia e tratamento do THB.

1.4 Estresse oxidativo e Transtorno do Humor Bipolar

Apesar do grande impacto e incapacitação que o THB causa na população, pouco se sabe sobre a sua etiologia e neurobiologia. Trata-se de um quadro complexo de interação entre os múltiplos genes que causam susceptibilidade, bem como a relação destes com fatores ambientais (Shaltiel et al., 2007). Alguns estudos têm consistentemente demonstrado um aumento do estresse oxidativo e alterações nas enzimas antioxidantes em transtornos neuropsiquiátricos, especialmente no THB (Calabrese et al., 2001; Bem-Shachar, 2002; Kuloglu et al., 2002; Ranjekar et al., 2003; Ozcan et al., 2004; Frey et al., 2007; Machado-Vieira et al., 2007; Steckert et al., 2010).

O estresse oxidativo ocorre quando existe um desequilíbrio entre a geração de espécies reativas de oxigênio (ERO) e as defesas antioxidantes, ocasionando um potencial dano oxidativo em todas as biomoléculas, incluindo lipídios, proteínas e o DNA (Halliwell & Gutteridge, 1999; Dalle-Donne et al., 2006).

Todas as células aeróbicas podem sofrer dano oxidativo, porém o cérebro é particularmente susceptível a este dano. Colabora para isso o fato de o cérebro utilizar altas taxas de oxigênio quando comparado a outros órgãos; suas defesas antioxidantes serem modestas; muitos neurotransmissores serem auto-oxidantes; as mitocôndrias neuronais gerarem ânion superóxido e as membranas neuronais serem ricas em ácidos graxos poliinsaturados e os produtos da peroxidação lipídica podem causar dano cerebral (Halliwell, 1987; Kuloglu et al., 2002).

O alvo celular primário do estresse oxidativo pode variar conforme o tipo celular, as EROs geradas, o sítio de geração (intra ou extracelular) e a proximidade do oxidante à

estrutura celular. O ataque das EROs aos lipídios das membranas desencadeia um processo chamado lipoperoxidação, formando muitos produtos secundários, entre eles o malondialdeído (MDA) (Urso & Clarkson, 2003). As EROs podem modificar a conformação química inicial dos ácidos graxos poliinsaturados e, consequentemente, alterar a coesão, fluidez, permeabilidade e funções metabólicas das células (Chihuailaf et al., 2002).

A oxidação direta de proteínas por EROs produz derivados carbonilados altamente reativos, resultando na oxidação das cadeias laterais de diversos aminoácidos (Dalle-Donne et al., 2006). Adicionalmente, a quantificação de proteínas carboniladas apresenta uma vantagem sobre os produtos da lipoperoxidação como marcador de estresse oxidativo, pois as proteínas oxidadas geralmente são mais estáveis (Dalle-Donne et al., 2003).

Para se proteger do estresse oxidativo, o organismo dispõe de um elaborado sistema de defesa antioxidante não-enzimático e enzimático, este último sendo constituído por enzimas como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) (Mayne, 2003; Urso & Clarkson, 2003). A SOD, em condições normais, catalisa a reação de conversão do ânion superóxido em peróxido de hidrogênio, que é eliminado pela ação das enzimas CAT e GPx (Halliwell, 1987).

O aumento da atividade da SOD produz uma agressão endógena constante, pois acelera a reação de formação de peróxido de hidrogênio e, consequentemente, o desequilíbrio entre a atividade da SOD e da CAT induz a oxidação dos grupos sulfidrílicos e a peroxidação de lipídios insaturados, causando dano celular (Mayne, 2003).

Dados da literatura reforçam o possível envolvimento do estresse oxidativo na fisiopatologia do THB (Ranjekar et al., 2003; Ozcan et al., 2004; Machado-Vieira et al., 2007; Frey et al., 2007). Em um estudo clínico, Kuloglu e colaboradores (2002) avaliaram os níveis plasmáticos de TBARS (substâncias reativas ao ácido tiobarbitúrico) como marcador de dano oxidativo em lipídios e a atividade da SOD em pacientes com THB e encontraram um

aumento significativo em ambos os parâmetros estudados, em comparação ao grupo controle com indivíduos saudáveis.

Complementando os achados acima, Andreazza e colaboradores (2007) analisaram os níveis plasmáticos de TBARS e a atividade das enzimas antioxidantes SOD, CAT e GPx em pacientes bipolares nas diferentes fases do transtorno - mania, depressão e eutimia. Os resultados deste estudo indicaram um aumento na atividade da SOD, CAT e GPx nos pacientes nas fases de mania e depressão, porém os níveis de TBARS foram maiores nos indivíduos bipolares, independentemente da fase.

Estudos têm revelado o potencial antioxidante de SB e de TMX (Ryu et al., 2003; Obata, 2006). Ryu e colaboradores (2003) observaram que o SB e o valproato podem reduzir o estresse oxidativo e modular a transcrição de sequestradores de radicais livres. Neste contexto, Obata e colaboradores (2006) sugerem que o TMX possui atividade antioxidante, embora em algumas situações seja associado ao aumento do estresse oxidativo (Clarke et al., 2001).

1.5 Modelo animal de mania induzido por d-anfetamina

O desenvolvimento de modelos animais é uma ferramenta importante para o estudo dos processos envolvidos na manifestação dos quadros de transtornos de humor, inclusive para a elaboração de tratamentos farmacológicos adequados. Contudo, são poucos os modelos animais de mania atualmente existentes.

Um dos modelos animais mais aceitos é o que induz hiperlocomoção em animais com uma substância estimulante do sistema nervoso central (d-AMPH, por exemplo), o que confere ao modelo a validade de face (Frey et al., 2006a; Einat, 2007). Além do aumento da atividade locomotora, outros sinais semelhantes à mania podem ser observados, tais como redução do sono e comportamento de risco (Einat et al., 2007; Kato et al., 2007).

O envolvimento do sistema dopaminérgico no comportamento maníaco acrescenta a este modelo a validade de constructo (Manji et al., 2002). Um dos principais mecanismos de ação da d-AMPH é estimular a migração das vesículas que contêm dopamina em direção a membrana terminal, fazendo com que as vesículas se fundem à membrana terminal, liberando dopamina (Figura 2). Outro mecanismo é a liberação da dopamina no citoplasma do neurônio terminal, onde estas moléculas de dopamina são levadas para o exterior da célula via transporte reverso de transportador de dopamina, resultando em um importante aumento na concentração de dopamina na sinapse e, consequentemente, uma significativa estimulação do sistema dopaminérgico (Meyer & Quenzer, 2005).

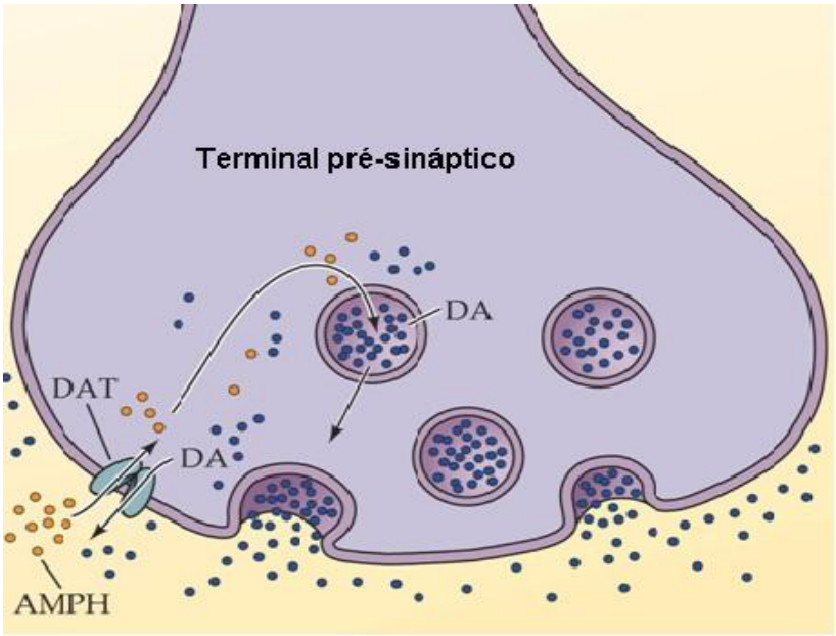

Figura 2. Mecanismo de ação da anfetamina (AMPH) sobre o sistema da dopamina (DA). (Adaptado de Meyer & Quenzer, 2005).

Por fim, este modelo promove alterações comportamentais e respostas terapêuticas que são semelhantes ao THB, onde a inibição dos efeitos comportamentais induzidos pela AMPH por estabilizadores do humor (lítio e valproato, por exemplo) garante ao modelo a validade preditiva (Einat, 2006).

Evidências indicam que a administração de d-AMPH em animais provoca alterações cerebrais semelhantes ao que é encontrado em pacientes bipolares, como aumento da lipoperoxidação, diminuição dos níveis de BDNF (Frey et al., 2006a,b) e inibição da atividade dos complexos da cadeia respiratória mitocondrial, parâmetros normalizados com o uso de lítio e valproato (Valvassori et al., 2010).

Considerando o exposto, somado às evidências encontradas na literatura de que a administração de d-AMPH é considerada um modelo animal de mania com todas as validades (face, preditiva e constructo), pesquisas com este modelo envolvendo a manipulação de alvos terapêuticos possivelmente envolvidos no THB são importantes para ajudar não só na compreensão da fisiopatologia, como no desenvolvimento de futuras alternativas de tratamento para este transtorno.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar os efeitos comportamentais e neuroquímicos da administração de Butirato de Sódio (SB) e Tamoxifeno (TMX) em um modelo animal de mania induzido por d-AMPH.

2.2 Objetivos específicos

1) Avaliar os efeitos da administração de Butirato de Sódio (SB, inibidor de HDAC) sobre a atividade locomotora, a atividade exploratória e o comportamento de risco em um modelo animal de mania induzido por d-AMPH.
2) Avaliar os efeitos da administração de Butirato de Sódio (SB, inibidor de HDAC) sobre a produção de ânion superóxido e de espécies reativas ao ácido tiobarbitúrico

(TBARS) em partícula submitocondrial de córtex pré-frontal, amígdala, hipocampo e estriado de ratos submetidos ao modelo animal de mania induzido por d-AMPH.

3) Avaliar os efeitos da administração de Butirato de Sódio (SB, inibidor de HDAC) sobre a produção de espécies reativas ao ácido tiobarbitúrico (TBARS) e a carbonilação de proteínas no córtex pré-frontal, amígdala, hipocampo e estriado de ratos Wistar submetidos ao modelo animal de mania induzido por d-AMPH.
4) Avaliar os efeitos da administração de Butirato de Sódio (SB, inibidor de HDAC) sobre a atividade das enzimas antioxidantes Superóxido dismutase (SOD) e Catalase (CAT) no córtex pré-frontal, amígdala, hipocampo e estriado de ratos submetidos ao modelo animal de mania induzido por d-AMPH.
5) Avaliar os efeitos da administração de Tamoxifeno (TMX, inibidor de PKC) sobre a atividade locomotora, a atividade exploratória e o comportamento de risco em um modelo animal de mania induzido por d-AMPH.
6) Avaliar os efeitos da administração de Tamoxifeno (TMX, inibidor de PKC) sobre a produção de ânion superóxido e de espécies reativas ao ácido tiobarbitúrico (TBARS) em partícula submitocondrial de córtex pré-frontal, amígdala, hipocampo e estriado de ratos submetidos ao modelo animal de mania induzido por d-AMPH.
7) Avaliar os efeitos da administração de Tamoxifeno (TMX, inibidor de PKC) sobre a produção de espécies reativas ao ácido tiobarbitúrico (TBARS) e a carbonilação de proteínas no córtex pré-frontal, amígdala, hipocampo e estriado de ratos Wistar submetidos ao modelo animal de mania induzido por d-AMPH.
8) Avaliar os efeitos da administração de Tamoxifeno (TMX, inibidor de PKC) sobre a atividade das enzimas antioxidantes Superóxido dismutase (SOD) e Catalase (CAT) no córtex pré-frontal, amígdala, hipocampo e estriado de ratos submetidos ao modelo animal de mania induzido por d-AMPH.

3 RESULTADOS

3.1 Artigo Científico:

Effects of Sodium Butyrate on d-amphetamine-induced oxidative stress in the rat brain

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Effects of Sodium Butyrate on d-amphetamine-induced oxidative stress in the rat brain

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Abstract

In this study, we investigated the effects of Sodium butyrate (SB, a histone deacetilase inhibitor) on oxidative stress in rats submitted to an animal model of mania induced by d-amphetamine (d-AMPH). In the reversal model, d-AMPH or saline (Sal) were administered to rats for 14 days, and between days 8-14, rats were treated with SB or Sal. In the prevention model, rats were pretreated with SB or Sal, and between days 8-14, d-AMPH or Sal were administrated. Locomotor activity and risk-taking behavior were assessed by open-field test and oxidative stress was measured in prefrontal cortex, amygdala, hippocampus and striatum, in both experiments. The results showed that SB reversed and prevented d-AMPH-induced behavioral effects. In these two models, the d-AMPH administration induced oxidative damage in all brain structures analyzed. Depending on the cerebral area and technique evaluated, SB was able to reverse and prevent this impairment. The present study reinforces the need for more studies of HDAC inhibitors as possible target for new medications in treatment for BD.

Keywords: Bipolar disorder; d-amphetamine; Histone deacetylase; Mania; Oxidative stress; Sodium butyrate

1. Introduction

Bipolar disorder (BD), characterized by recurrent manic and depressive episodes, is one of the most severely debilitating of all medical illnesses (Goodwin and Jamison, 1990). The pharmacological management of BD includes the use of lithium, valproate (VPA) and atypical antipsychotic drugs for the treatment of acute states and maintenance treatment in order to prevent new episodes (Keck, 2003; Evins et al., 2006).

Valproate is widely used for the treatment of seizure and BD and the long-term administration of this anticonvulsant results in neuroprotective effects (Dou et al., 2003; Chen et al., 2006; Leng and Chuang, 2006). These studies suggest that the neuroprotective mechanisms of VPA may involve, at least in part, an inhibition of histone deacetylase (HDAC) - an enzyme recently identified target of VPA and involved in the epigenetic regulation of gene expression - that is thought to be responsible for the control of long term changes in neuronal functions (Phiel et al., 2001).

Epigenetic mechanisms such as histones modification play a key role in regulation of gene expression during the processes of cell proliferation and differentiation (Li et al., 2007) and chromatin remodeling may also play a central role in cognitive impairments associated with psychiatric and neurodegenerative disorders (Abel and Zukin, 2008). The gene transcription is repressed by HDAC, which removes acetyl groups from histones (Kouzarides, 2007), therefore, substances targeting histone acetylation may provide benefits for the treatment of depression, schizophrenia and anxiety disorders (Xu et al., 2007).

Histone deacetylase inhibitors – such as phenylbutyrate, sodium butyrate (SB), trichostatin A and VPA – causes chromatin remodeling through histone hyperacetylation to regulate expression of neuroprotective/neurotrophic proteins and proapoptotic/proinflammatory proteins (Kruh, 1982; Buggy et al., 2000; Phiel et al., 2001; Langley et al., 2008). In this regard, SB was found to have a favorable effect in an animal

model of depression (Schröeder et al., 2007). Schröeder and colleagues (2008) and Febo and colleagues (2009) demonstrated that inhibition of HDAC activity results in a decrease of some psychostimulant-induced behaviors. Additionally, SB and VPA may directly reduce oxidative stress and modulate the transcription of free radical scavengers (Ryu et al., 2003).

Free radicals have a role in the pathogenesis of several diseases, including neuropsychiatric disorders, such as BD and Schizophrenia (Kuloglu et al., 2002; Ranjekar et al., 2003; Ozcan et al., 2004). The organism has developed various defense mechanisms in order to prevent the formation of free radicals and its consequent damage (Cochrane, 1991; De Vasconcelos et al., 2005). Several studies demonstrated the effects of free radicals in BD, indicating association with increased oxidative stress and changes in antioxidant enzymatic defense (Kuloglu et al., 2002; Ranjekar et al., 2003; Halliwell, 2006; Savas et al., 2006; Gergerlioglu et al., 2007; Selek et al., 2008; Yumru et al., 2009) and recently published review has shown that oxidative stress mechanisms may play a important role in the pathophysiology of BD (Steckert et al., 2010).

Therefore, the present study aims to investigate the effects of SB on oxidative stress parameters in the brain of rats using an animal model of mania induced by d-amphetamine (d-AMPH).

2. Materials and methods

2.1. Animals

We conducted the study using adult male Wistar rats (weighting 250–300g) obtained from our breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (lights on at 7:00 am), at a temperature of 23°C ± 1°C. These conditions were maintained constant throughout the experiments. All

experimental procedures were performed in accordance with the approval of the local Ethics Committee of Animals Use (Protocol 49/2009) and all efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

2.2. Treatments

Sodium butyrate (Sigma, St Louis, Mo.) and d-amphetamine (d-AMPH, Sigma, St Louis, Mo.) were directly dissolved in saline solution (NaCl 0.9%, w/v).

Reversal model: The reversal model was designed to reproduce the management of an acute manic episode. Animals received one daily intraperitoneally (i.p.) injection of either d-AMPH (2 mg/kg) or saline (1 mL/kg) for a period of 14 days. From the 8th to 14th day (treatment for 7 days), d-AMPH and Sal animals were randomly divided in two experimental groups: Sodium butyrate (SB) (1.2 g/kg i.p. twice a day) and saline (Sal) (1 mL/kg i.p. twice a day) treatments with 12 animals per group. Locomotor activity and visits to center of open-field were measured 2h after the last injection, and the rats were killed immediately after the open-field task.

Prevention model: The prevention model was designed to mimic the maintenance phase of BD treatment, when the drugs still are administrated even in periods of euthymia. Animals received either Sodium butyrate (SB) (1.2 g/kg i.p. twice a day) or saline (Sal) (1 mL/kg i.p. twice a day) for a period of 14 days. From the 8th to 14th day (treatment for 7 days), SB, and Sal-treated animals were randomly divided in two experimental groups: each treated group received one daily i.p. injection of either d-AMPH (2 mg/kg) or Sal (1 mL/kg), totalizing four groups with 12 animals per group. Locomotor activity and visits to center of open-field were measured 2h after the last injection, and the rats were killed immediately after the open-field task.

2.3. Behavioral assessment

The locomotor activity and visits to center of open-field were measured using the open-field task. The task was performed in a 40×60 cm open field surrounded by 50 cm high walls. The floor of apparatus was constructed with varnished wood and divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle, in order to explore the arena for 5 min. Crossings of the black lines, rearings and the visits to the center of an open field were counted. A center square of 30×30 cm was defined as the “center” area of the field.

2.4. Oxidative stress parameters

Animals were sacrificed by decapitation and the brain transferred within 1 min to ice-cold isolation buffer (0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl, and 1mM EDTA, pH 7.4). The prefrontal cortex, amygdala, hippocampus and striatum (n=5 animals per group) were dissected in ice-cold buffer in a Petri dish, and submitochondrial particles were prepared in parallel from the four brain regions of each animal. For biochemical analysis in total tissue, the brain structure were rapidly frozen and stored at -80°C.

2.4.1. Mitochondrial isolation

Rat brain homogenates were centrifuged at 700g for 10 min to discard nuclei and cell debris and the pellet was washed to enrich the supernatant that was centrifuged at 7000g for 10 min. The obtained pellet, washed and resuspended in the same buffer, was considered to consist mainly of intact mitochondria able to carry out oxidative phosphorylation. The operations were carried out at 0–2°C. Submitochondrial particles (SMP) were obtained by

freezing and thawing (three times) of isolated mitochondria. For superoxide production measurements, SMP were washed twice with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) and suspended in the same medium (Boveris et al., 1972).

2.4.2. Superoxide production in submitochondrial particles of the rat brain

Superoxide production was determined in washed brain SMP using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37°C (€ $_{480\ nm}$ = 4.0 mM^{-1} cm^{-1}). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), SMP (0.3–1.0 mg protein/ml), 0.1 µM catalase, and 1 mM epinephrine. NADH (50 µM) and succinate (7 mM) were used as substrates and rotenone (1 µM) and antimycin (1 µM) were added as specific inhibitors, respectively, to assay O^{-}_{2} production at the NADH dehydrogenase and at the ubiquinone–cytochrome b region. Superoxide dismutase (SOD) was used at 0.1–0.3 µM final concentration to give assay specificity (Boveris, 1984).

2.4.3. Thiobarbituric acid reactive species formation

To determine oxidative damage in lipid, we measured the formation of Thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described (Draper and Hadley, 1990). The samples were mixed with 1 ml of trichloroacetic acid 10% and 1ml of thiobarbituric acid 0.67%, and then heated in a boiling water bath for 30 min. Malondialdehyde equivalents were determined in tissue and in submitochondrial particles of the rat brain spectrophotometrically by the absorbance at 532 nm.

2.4.4. Carbonyls protein formation

The oxidative damage to proteins was assessed by the determination of carbonyl groups content based on the reaction with dinitrophenylhidrazine (DNPH), as previously described (Levine et al., 1994). Proteins were precipitated by the addition of 20% trichloroacetic acid and were redissolved in DNPH. The absorbance was monitored spectrophotometrically at 370 nm.

2.4.5. Superoxide dismutase activity

This method for the assay of superoxide dismutase (SOD) activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^{-2}*;* a substrate for SOD (Bannister and Calabrese, 1987). The inhibition of autoxidation of this compound thus occurs when SOD is present, and the enzymatic activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control. A calibration curve was performed using purified SOD as the standard, in order to calculate the specific activity of SOD present in the samples. A 50% inhibition of pyrogallol autoxidation is defined as 1 unit of SOD, and the specific activity is represented as units per mg of protein.

2.4.6. Catalase activity

The catalase (CAT) activity was assayed using a double-beam spectrophotometer with temperature control. This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1– 0.3 mg protein/ml (Aebi, 1984). One CAT unit is defined as 1 mol of

hydrogen peroxide consumed per minute, and the specific activity is reported as units per mg protein.

2.4.7. Protein determination

All biochemical measures were normalized to the protein content with bovine albumin as standard (Lowry et al., 1951).

2.5. Statistical analysis

All data are present as mean and standard error of the mean. Differences among experimental groups evaluated behavior parameters were determined by one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. The oxidative stress parameters were analyzed by one-way ANOVA and multiples comparisons were performed by the LSD test. In all comparisons, statistical significance was set at $P < 0.05$.

3. Results

Results for locomotor activity of reversion experiment are shown in Table 1 (on top). There was a significant effect of d-AMPH and SB in the number of crossings ($p < 0.001$). Further analysis with Tukey post-hoc test showed that administration of AMPH increased locomotion in saline-treated rats ($p < 0.001$) and this effect was reversed by SB ($p < 0.001$). SB alone did not alter behavioral measures, indicating that the effects of SB on d-AMPH-treated rats were not associated with sedation.

Results for locomotor activity of prevention experiment are shown in Table 1 (at the bottom). There was a significant effect of d-AMPH and SB in the number of crossings (p <

0.001). Tukey post-hoc test showed that crossings were significantly increased by d-AMPH in saline-treated rats ($p < 0.001$) and this effect was prevented by SB ($p < 0.001$). In accordance with the first experiment, the administration of SB alone did not affect behavioral measures.

We replicated here previous data from our group; a significant increased in superoxide in submitochondrial particles (SMP) (Fig. 1), TBARS in SMP (Fig. 2), TBARS in tissue (Fig. 3) and carbonyls protein content (Fig. 4) was detected in all brain structures analyzed in both the reversal (A) and prevention (B) models (Frey et al., 2006a, b, c).

As shown in Figure 1, SB reversed (A) and prevented (B) d-AMPH-induced increase in superoxide in SMP in all brain regions analyzed. SB alone increased superoxide formation in SMP only in the prefrontal in the prevention treatment.

According to Figure 2A, Administration of SB partially reverts in the prefrontal and reverts in the amygdala, hippocampus and striatum the formation of TBARS in SMP induced by d-AMPH. In Figure 2B; the pretreatment with SB prevent d-AMPH-induced increase in TBARS in SMP formation in all brain structures evaluated. In both experimental protocols, the administration of SB alone did not affect the TBARS in SMP formation in any brain structure evaluated.

Figure 3 shows the results of measurement of lipid damage – indicated by increased of TBARS. In the reversal model (Fig. 3A), administration of SB partially reverts in the hippocampus and striatum and reverts in the prefrontal and amygdala the d-AMPH-induced lipid damage. In the prevention model (Fig. 3B) the lipid damage induced by d-AMPH was partially reversed by SB in the prefrontal and amygdala and reversed by SB in the hippocampus and striatum. In addition, SB alone increased lipid damage in prefrontal, hippocampus and striatum in the prevention treatment.

Figure 4 shows the results of protein damage – indicated by increased levels of carbonyl groups content. In the reversal model (Fig. 4A) administration of SB diminished the d-AMPH-induced protein damage in the prefrontal and striatum. In the amygdala and

hippocampus SB reversed the protein damage induced by d-AMPH. In the prevention model (Fig. 4B); the d-AMPH-induced oxidative damage was prevented by SB treatment in the prefrontal, amygdala and striatum. In the hippocampus, administration of SB significantly diminished the protein damage induced by d-AMPH. In both experimental protocols, the SB regime in the control groups did not modify protein viability in any brain structures evaluated.

Next, we examined the activities of the antioxidant enzymes SOD (Fig. 5) and CAT (Fig. 6).

Figure 5A (reversal model) shows that SOD activity was decreased in the prefrontal and striatum with d-AMPH administration, however, this enzyme alteration was reverted by SB treatment. In addition, SB alone increased SOD activity in the amygdala, hippocampus and striatum. Moreover, the SB regime in the d-AMPH group also increased SOD activity in the amygdala and hippocampus. In the prevention model (Fig. 5B), treatment with d-AMPH decreased SOD activity in the hippocampus and striatum, which was prevented by pretreatment with SB. In addition, the pretreatment with SB in control and in the d-AMPH groups increased SOD activity in the prefrontal and amygdala.

According to Figure 6A (reversal model); treatment with d-AMPH increased CAT activity in all brain structures analyzed; which was reverted by SB treatment. SB alone increased CAT activity in the prefrontal and amygdala. Figure 6B shows the results of CAT activity in the prevention model. In accordance with the first experiment d-AMPH administration increased CAT activity in all brain structures evaluated. However, the pretreatment with SB prevented d-AMPH-induced increased in CAT activity, only in the amygdala. SB alone increased CAT activity in the striatum.

4. Discussion

Epigenetic modification such as histone deacetylation resulting in decreased gene expression is a proposed cause of several diseases, including psychiatric disorders (Petronis, 2004; Torrey et al., 2005). Therefore, the use of HDAC inhibitors as potential therapeutic agents in psychiatric patients and animal models is a promising area of research (Steffan et al., 2001; Hockly et al., 2003; Mattson, 2004; Sharma, 2005).

In this study, we showed that SB treatment reverted and prevented d-AMPH-induced hyperlocomotion and normalized d-AMPH-induced increase in the number of visits to the center of the open-field (risk behavior). Recently, VPA was characterized as an inhibitor of HDAC (Phiel et al., 2001) and also was demonstrated that it inhibits the hyperlocomotion induced by d-AMPH in animal models of mania (Frey et al., 2006 a,b). In addition, SB was shown to augment the increase in histone acetylation caused by exposure to cocaine (Kumar et al., 2005). Therefore, the behavioral results of the present study reinforce the importance of the histones regulation in the antimanic effect of VPA.

Various studies have showed that chromatin remodeling is an important regulatory mechanism underlying psychostimulants-induced neural and behavioral plasticity (Matsumoto et al., 2007; Schroeder et al., 2008; Sun et al., 2008). Additionally, in a study used blood-oxygen-level-dependent functional magnetic resonance imaging (to assess brain metabolic activation patterns) in awake rats demonstrated that SB modulates after repeated cocaine exposure emotion, motivation and memory, suggesting that inhibition of histone acetylation may contribute to drug-induced neural and behavioral alterations (Febo et al., 2009).

Evidences link bipolar disorder to a fundamental abnormality in oxidative energy generation (Kato, 2007). The mitochondrias are intracellular organelles that play a role in ATP and energy production, and also serve as calcium buffers and apoptosis regulators (Fattal et al., 2007). Free radicals and reactive oxygen species (ROS) are generated in the mitochondria and are detoxified by antioxidant enzymes. When the enzymatic antioxidant

systems are overwhelmed by elevated levels of ROS, oxidative damage may occur to lipids, proteins and DNA (Lenaz, 2001).

Here, we investigated the effects of SB on oxidative stress parameters in an animal model of mania. In this study, we reproduce previous results from our laboratory, in both experimental models d-AMPH induced increase in oxidative damage parameters (superoxide production, amount of TBARS in SMP, TBARS levels, protein carbonyl formation) in rat brain (Frey et al., 2006a, b, c). Also, in this study we demonstrated that SB reversed and prevented d-AMPH-induced oxidative damage in the brain of the rats. However, the effects of SB vary depending on the brain region. Regions of the CNS can differentially respond (Sullivan et al., 2005), and oxidative stress parameters were analyzed from different brain regions, that in part represent different cell types. Besides, within a homogeneous population of cells, there is heterogeneity in terms of physiological and metabolic characteristics (Lai et al.. 1977; Sims, 1991; Sonnewald et al., 1998).

Several studies have demonstrated that HDAC inhibitors may protect cells from a variety of toxic insults including inflammation and oxidative stress (Chen et al., 2006; Langley et al., 2008; Leng et al., 2008), and can increases the expression of neurotrophic factors and protect dopaminergic neurons during episodes of mania (Yatham et al., 2002). Additionally, a study using HDAC inhibitors demonstrated that SB and trichostatin A (TSA) promote DA neuronal survival and protect DA neurons from MPP+ in neuron-glia cultures (Wu et al., 2008). These mechanisms may also be linked with the neuroprotective effect of SB found in the current study.

Interesting enough, we also observed that the administration of SB in control groups can increase oxidative damage in some brain structures. Several reports have showed that various HDAC inhibitors stimulate ROS generation and that treatment with antioxidants reduces their anticancer activity (Ruefi et al., 2001; Butler et al., 2002; Rosato et al., 2003; Ungerstedt et al., 2005).

We demonstrated also that d-AMPH-induced oxidative damage was accompanied by decreased SOD activity and increased CAT activity, which was normalized by SB treatment in some brain structures. Human data of oxidative markers in BD demonstrated reduced SOD activity (Ranjekar et al., 2003; Gergerlioglu et al., 2007). Another study reported lowered SOD activity and elevated nitric oxide levels in bipolar depression (Selek et al., 2008). In addition, Machado-Vieira and colleagues (2007) related an increase in catalase activity in plasma of the bipolar patients in the manic phase. In addition, previous studies have demonstrated a decrease in the CAT activity in hippocampus of rats after treatment with lithium and valproate in animal model of mania induced by d-AMPH (Frey et al., 2006b). Ryu and colleagues (2003) demonstrated that SB may reduce oxidative stress and modulate the transcription of free radical scavengers, such MnSOD and CAT.

In conclusion, our results demonstrated that d-AMPH-induced hyperactivity and risk behavior was reverted and prevented by SB. In addition, we showed that SB could revert and protect the brain against d-AMPH-induced damage oxidative. However, the effects vary depending on the brain region evaluated. Then, we suggest that inhibition of histones deacetylases by SB, in addition to reverse and protect the d-AMPH-induced manic-like behavior also protecting against oxidative damage, two important milestones in BD. The present study reinforces the need for more studies of HDAC inhibitors as possible target for new medications in treatment for BD.

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Conflict of interest: None of the authors or funding sources has conflict of interest.

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Contributors: Amanda V. Steckert designed the study and made the first draft of the manuscript. Samira S. Valvassori undertook the statistical analysis and made the correction of the manuscript. Jéssica L. Borges, Roger B. Varela and Felipe Ornell were responsible for the pharmacological treatment and the behavioral assessment. Francielle Mina made the biochemical analysis. All authors contributed to and have approved the final manuscript.

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Legend to figures:

Figure 1A. Effects of Sodium butyrate administration on superoxide levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean $\pm$ S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 1B. Effects of Sodium butyrate administration on superoxide levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of

variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 2A. Effects of Sodium butyrate administration on TBARS levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 2B. Effects of Sodium butyrate administration on TBARS levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 3A. Effects of Sodium butyrate administration on TBARS levels in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 3B. Effects of Sodium butyrate administration on TBARS levels in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 4A. Effects of Sodium butyrate administration on protein carbonyl formation in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 4B. Effects of Sodium butyrate administration on protein carbonyl formation in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 5A. Effects of Sodium butyrate administration on superoxide dismutase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 5B. Effects of Sodium butyrate administration on superoxide dismutase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 6A. Effects of Sodium butyrate administration on catalase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data

were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Figure 6B. Effects of Sodium butyrate administration on catalase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 difference of SAL+SAL group. #P<0.05 difference of d-AMPH+SAL group.

Table 1. Effects of Sodium butyrate (SB) on d-amphetamine-induced behavioral alteration in animal model of mania. The administration of d-AMPH increased locomotion in saline-treated rats (p< 0.001) and this effect was reversed (p<0.001) and prevented (p<0.001) by SB.

Table 1. Effects of Sodium Butyrate on d-amphetamine-induced behavioral alteration in animal model of mania

			Crossings		Rearings		Visits to Center	
	Treatment	n	Mean ± Error of Mean	p values	Mean ± Error of Mean	p values	Mean ± Error of Mean	p values
Reversal Model	SAL+SAL	8	36.37 ± 2.14		20.5 ± 2.59		2.64 ± 0.35	
	SAL+SB	8	41.62 ± 3.45	0.416	19.25 ± 3.75	0.77	2.12 ± 0.35	0.674
	d-AMPH+SAL	7	84.57 ± 5.26	<0.001	36.14 ± 3.83	<0.001	17.27 ± 1.89	<0.001
	d-AMPH+SB	7	44.42 ± 7.41	0.23	15.71 ± 2.92	0.284	2.21 ± 0.44	0.739
Prevention Model	SAL+SAL	7	37.42 ± 2.54		16.71 ± 2.26		0.85 ± 0.23	
	SB+SAL	8	50 ± 10.75	0.25	19.12 ± 2.75	0.617	2.14 ± 0.47	0.388
	SAL+d-AMPH	7	84.86 ± 4.79	<0.001	33.28 ± 2.27	0.002	9.28 ± 2.13	<0.001
	SB+d-AMPH	7	47.85 ± 7.72	0.359	18.14 ± 3.83	0.77	0.6 ±0.45	0.870

Fig. 1A

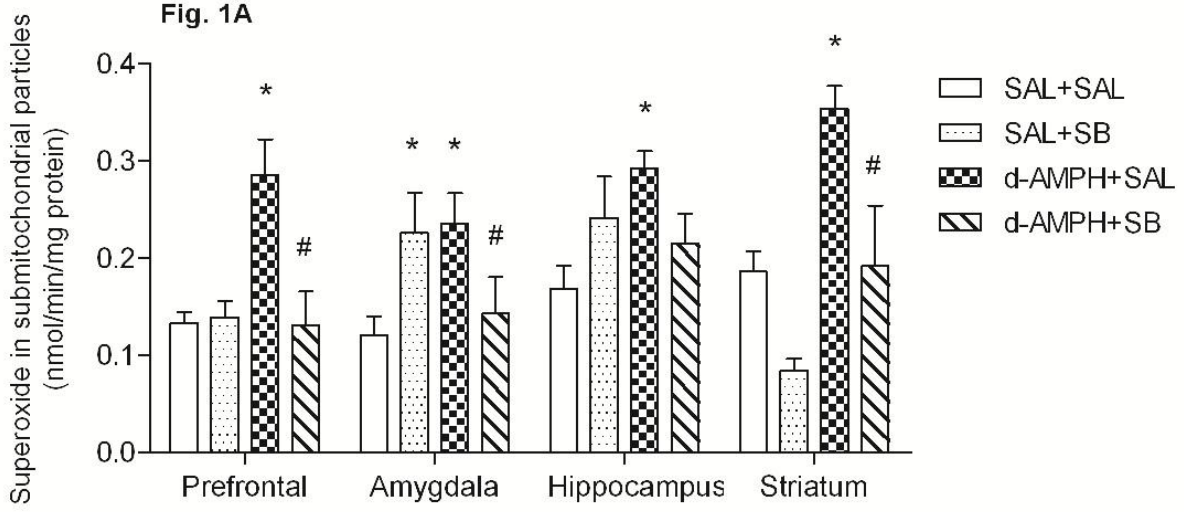


Fig. 1B

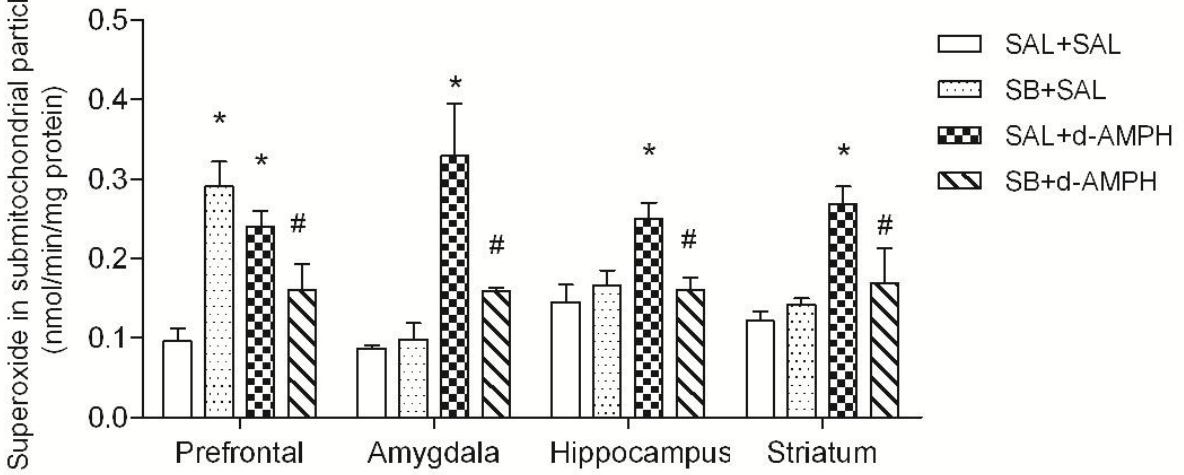


Fig. 2A

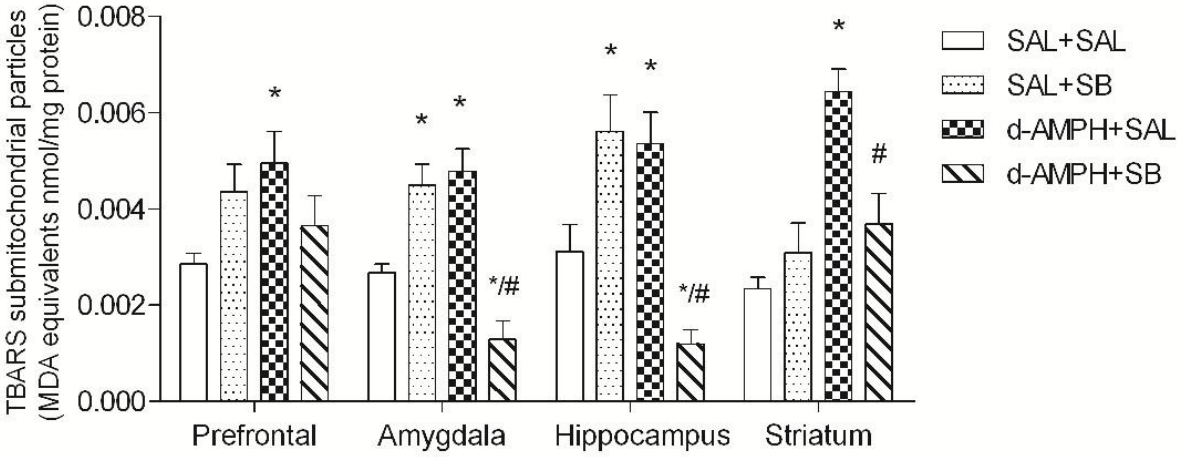


Fig. 2B

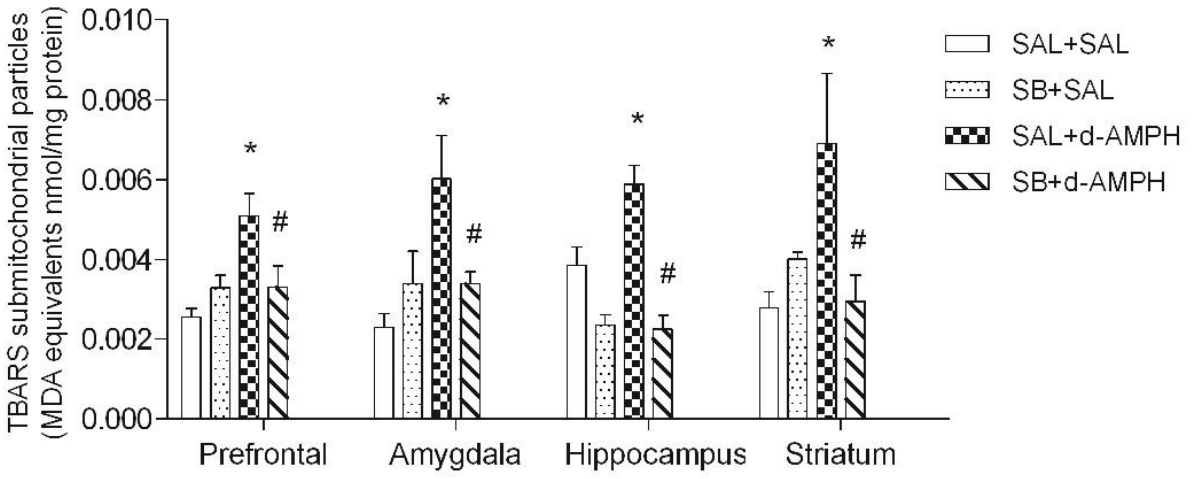


Fig. 3A

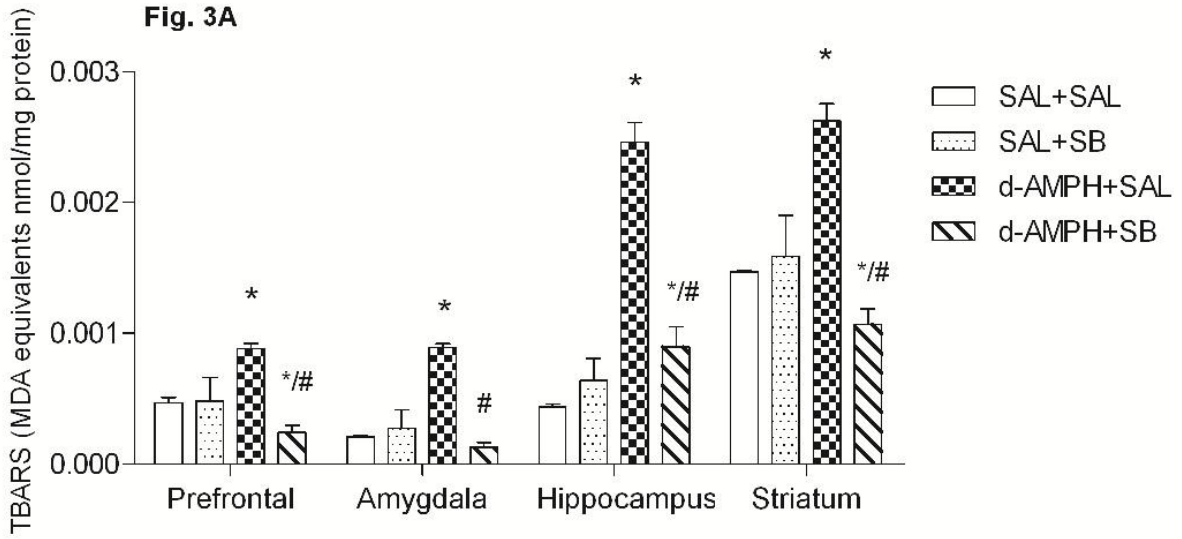


Fig. 3B

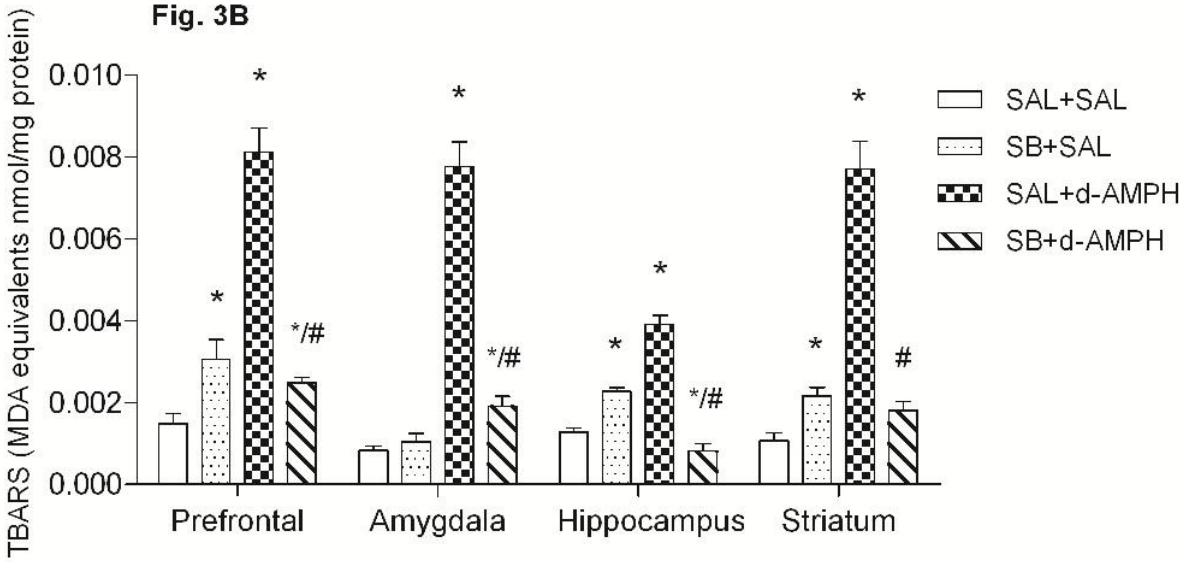


Fig. 4A

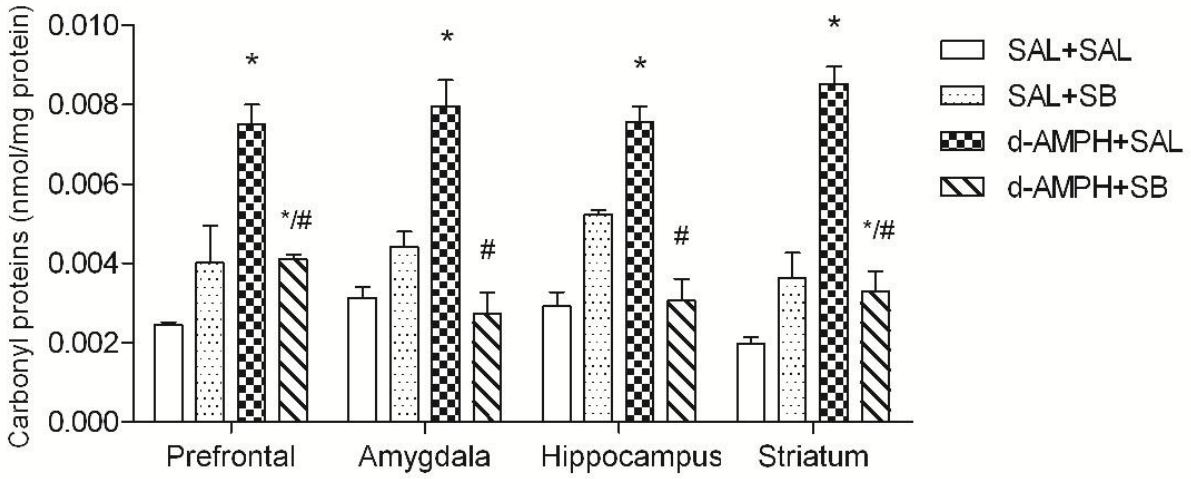


Fig. 4B

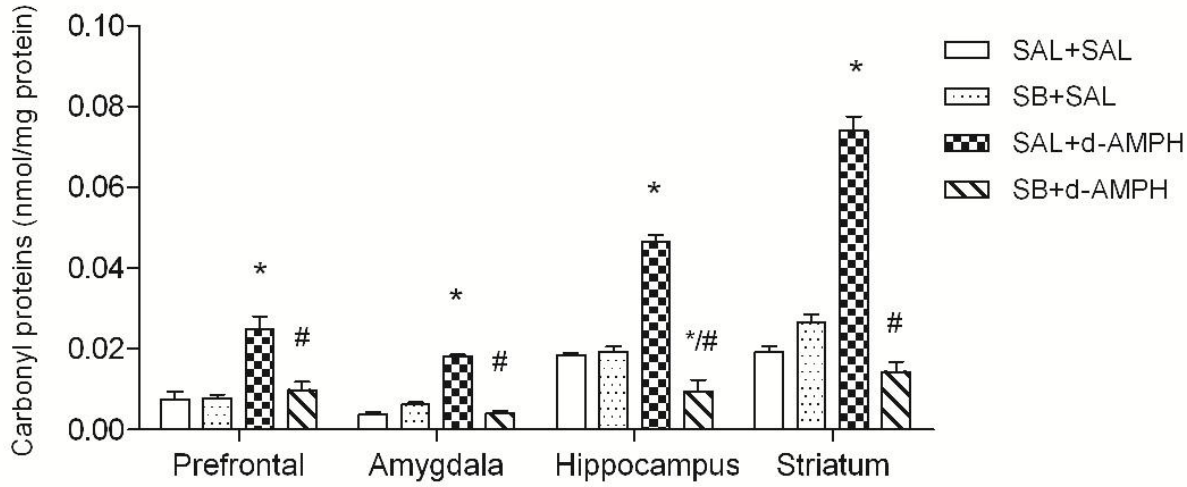


Fig. 5A

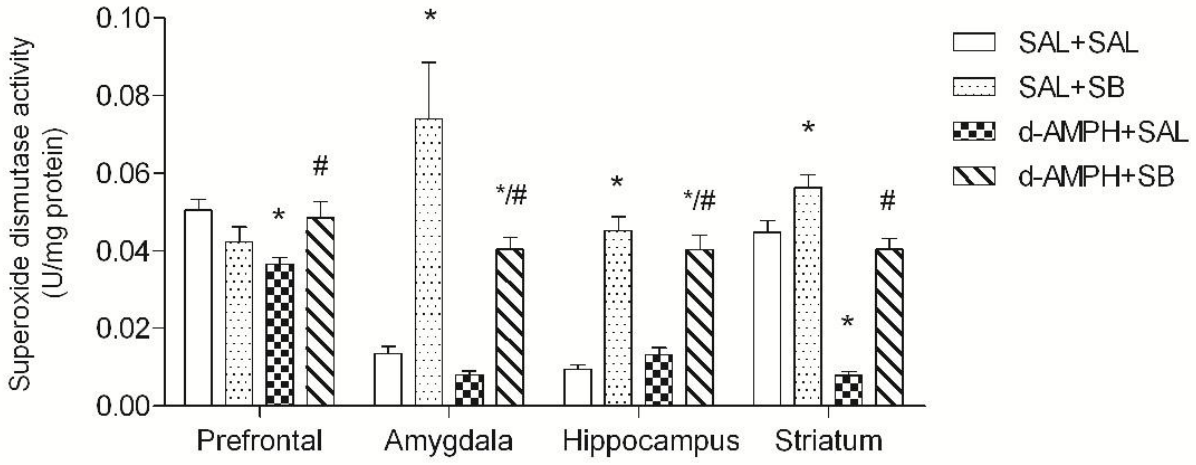


Fig. 5B

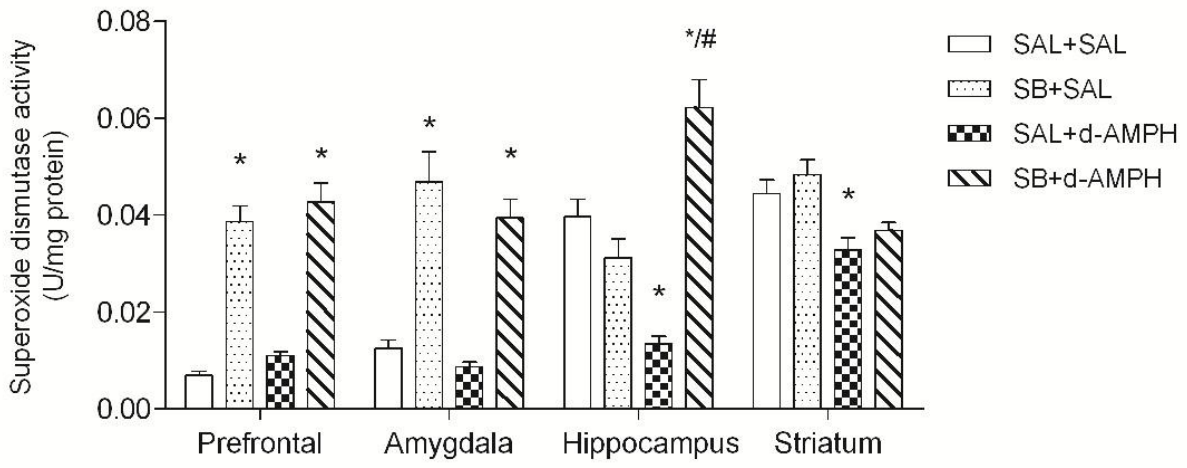


Fig. 6A

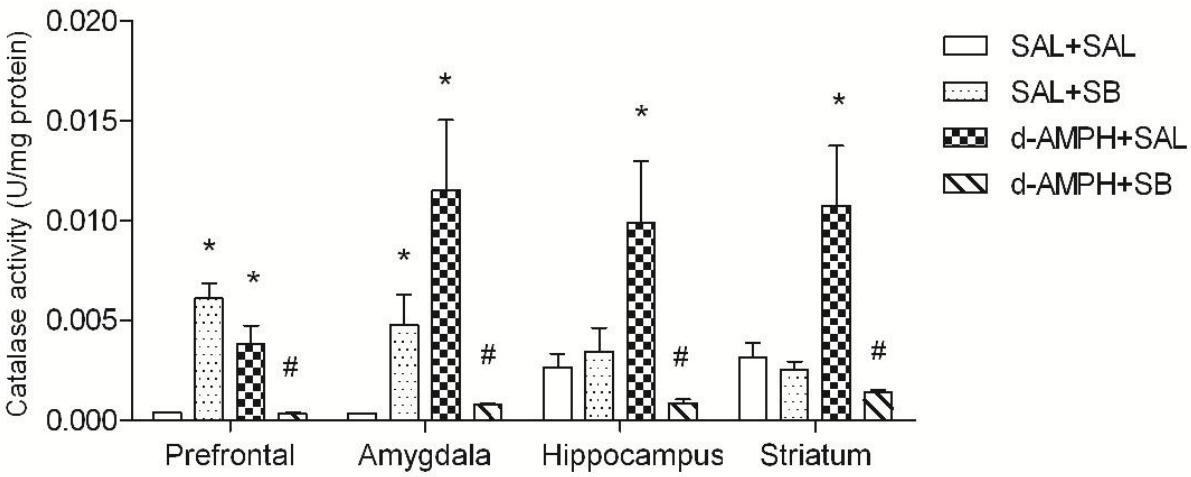
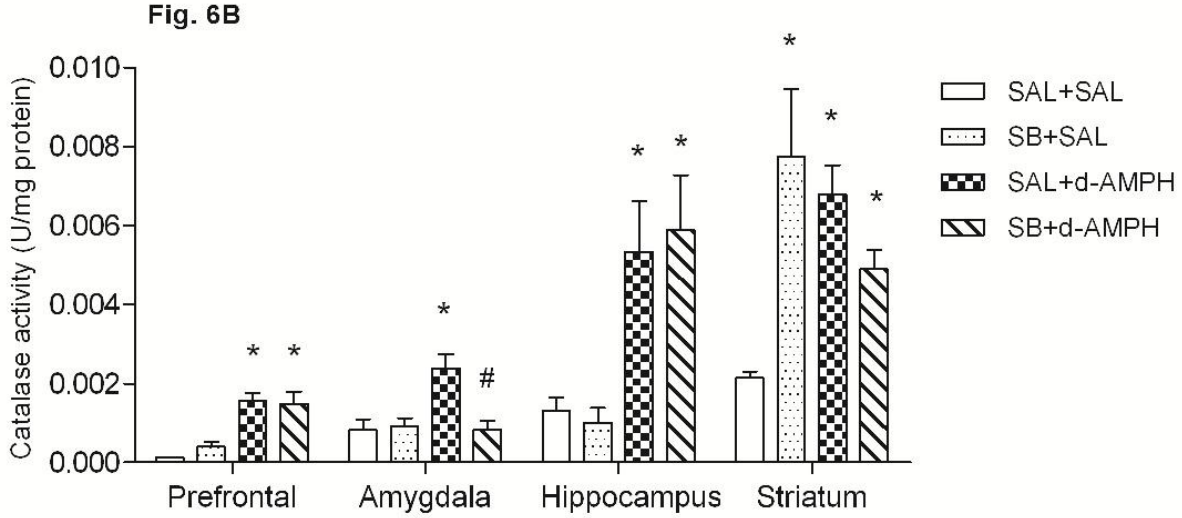


Fig. 6B



3.2 Artigo Científico:

Effects of Tamoxifen on oxidative stress parameters in an animal model of mania

Amanda V. Steckert; Samira S. Valvassori; Francielle Mina; Camila L. Ferreira; Edemilson S. Mariot; Flávio Kapczinski; Felipe Dal-Pizzol; João Quevedo

Submetido ao periódico Neuropharmacology

Effects of Tamoxifen on oxidative stress parameters in an animal model of mania

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Abstract

The present study aims to investigate the effects of Tamoxifen (TMX, a protein kinase C inhibitor) on oxidative stress in rats submitted to an animal model of mania induced by d-amphetamine (d-AMPH). In the reversal model, d-AMPH or saline (Sal) were administered to rats for 14 days, and between days 8-14, rats were treated with TMX or Sal. In the prevention model, rats were pretreated with TMX or Sal, and between days 8-14, d-AMPH or Sal were administrated. In both experiments locomotor activity and risk-taking behavior were assessed by open-field test and oxidative stress was measured in prefrontal, amygdala, hippocampus and striatum. The results showed that TMX reversed and prevented d-AMPH-induced behavioral effects. In addition, the d-AMPH administration induced oxidative damage in both structures tested in two models. The TMX was able to reverse and prevent this impairment, however in a way dependent of cerebral area and technique evaluated. These findings reinforce the hypothesis that PKC play an important role in the pathophysiology of BD and the need for the study of inhibitors of PKC as a possible target for treatment the BD.

Keywords: Bipolar disorder; d- amphetamine; Oxidative stress; Tamoxifen

1. Introduction

Bipolar disorder (BD) is a prevalent and chronic psychiatric disorder associated with higher rates of suicide and disability (Belmaker, 2004; Kupfer, 2005), clinically characterized by mood disturbances with recurrent episodes of mania, hypomania and depression (American Psychiatric Association, 1994). The pharmacological management of BD includes the treatment of acute states - with lithium, valproate and atypical antipsychotic drugs - and maintenance treatment in order to prevent new episodes (Keck, 2003; Evins et al., 2006). Since the identification of the antimanic effects of lithium, do not have development a new treatment specifically for BD (Zarate et al., 2007).

Recent data suggest that mania is associated with overactive protein kinase C (PKC) (Friedman et al., 1993). The PKC is a family of structurally related isozyme subspecies with a heterogeneous distribution throughout the body (Tanaka and Nishizuka, 1994; Casabona, 1997). In the brain, PKC has an important function in regulation both pre and pos-synaptic aspects of neurotransmission (Zarate et al., 2006). The psychostimulants induced manic-like behaviors in susceptible individuals and in rodents (Goodwin and Jamison 1990; Iwata et al., 1997) and known to activate PKC (Giambalvo 1992; Gnegy et al., 1993). The potential involvement of PKC in BD have been demonstrated by several authors and that represent a novel direct biochemical target for the treatment of mania (Chen et al., 1994; Browman et al., 1998; Young et al., 1999).

Tamoxifen, a synthetic anti-estrogen, has been widely used in the treatment of breast cancer (Jordan, 1994). Recently, some studies were showed that Tamoxifen is also potent and selective PKC inhibitor, such as lithium and valproate (Horgan et al., 1986; Couldwell et al., 1993). Einat and colleagues (2007) demonstrated that the Tamoxifen significantly reduced amphetamine-induced hyperactivity and risk-taking behavior in rodents, suggesting that PKC inhibitor may be antimanic. Preliminary studies of Tamoxifen in the treatment of acute mania

showed antimanic effects compared to placebo (Bebchuk et al., 2000; Kulkarni et al., 2006). The antimanic properties of tamoxifen were confirmed in double-blind, placebo controlled clinical studies (Zarate et al., 2007; Yildiz et al., 2008). Thus, these data indicates that PKC signaling may play an important factor in the pathophysiology and treatment of BD.

Oxidative stress mechanisms also have been implicated in the pathophysiology of neuropsychiatric disorders, such as BD (Kuloglu et al., 2002; Ranjekar et al., 2003; Ozcan et al., 2004; Savas et al., 2006; Selek et al., 2008). When the generation of free radicals exceeds the capacity of antioxidant defense, the oxidative stress may cause damage to cellular proteins and lipids, thereby affecting cellular function (Cochrane, 1991; De Vasconcelos et al., 2005). There is an emerging body of data indicating that major neuropsychiatric disorders are associated with increased oxidative stress and changes in antioxidant enzymatic defense (Kuloglu et al., 2002; Ranjekar et al., 2003; Halliwell, 2006; Yumru et al., 2009). Recently published review has shown the role of oxidative stress in the pathophysiology of bipolar disorder (Steckert et al., 2010).

Therefore, considering that oxidative stress may be involved in the pathophysiology of BD, the objective of this study was to investigate the effects of Tamoxifen on oxidative stress parameters in the brain of rats using an animal model of mania induced by d-amphetamine.

2. Methods

2.1. Animals

Adult male Wistar rats (weighting 250–300g) were obtained from our breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (lights on at 7:00 am), at a temperature of 23°C ± 1°C. These conditions were maintained constant throughout the experiments. All experimental

procedures were performed in accordance with the approval of the local Ethics Committee of Animals Use (Protocol 49/2009).

2.2. Drugs and treatments

Tamoxifen citrate (Salutas Pharma GmbH, Barleben, Ger.) and d-amphetamine (d-AMPH, Sigma, St Louis, Mo.) were directly dissolved in saline solution (NaCl 0.9%, w/v).

Reversal model: The reversal model was designed to reproduce the management of an acute manic episode. Animals (n=48) received one daily intraperitoneally (i.p.) injection of either d-AMPH (2 mg/kg) or saline (Sal) (1 mL/kg) for a period of 14 days. From the 8th to 14th day (treatment for 7 days), d-AMPH and Sal animals were randomly divided in two experimental groups: Tamoxifen (TMX) (1 mg/kg i.p. twice a day) and Sal (1 mL/kg i.p. twice a day) treatments with 12 animals per group. Locomotor activity and visits to center of open-field were measured 2h after the last injection, and the rats were killed right after the open-field task.

Prevention model: The prevention model was designed to mimic the maintenance phase of BD treatment, when the drugs still are administrated even in periods of euthymia. Animals (n=48) received either Tamoxifen (TMX) (1 mg/kg i.p. twice a day) or saline (Sal) (1 mL/kg i.p. twice a day) for a period of 14 days. From the 8th to 14th day (treatment for 7 days), TMX and Sal treated animals were randomly divided in two experimental groups: each treated group received one daily i.p. injection of either d-AMPH (2 mg/kg) or Sal (1 mL/kg) with 12 animals per group. Locomotor activity and visits to center of open-field were measured 2h after the last injection, and the rats were killed right after the open-field task.

2.3. Behavioral assessment

The locomotor activity and visits to center of open-field were measured using the open-field task. The task was performed in a 40×60 cm open field surrounded by 50 cm high walls. The floor of apparatus was constructed with varnished wood and divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle, in order to explore the arena for 5 min. Crossings of the black lines, rearings and the visits to the center of an open field were counted. A center square of 30×30 cm was defined as the “center” area of the field.

2.4. Biochemical analysis

Animals were sacrificed by decapitation and the brain transferred within 1 min to ice-cold isolation buffer (0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl, and 1mM EDTA, pH 7.4). The prefrontal cortex, amygdala, hippocampus and striatum (n=5 animals per group) were dissected in ice-cold buffer in a Petri dish, and submitochondrial particles were prepared in parallel from the four brain regions of each animal. For biochemical analysis in total tissue, the brain structure were rapidly frozen and stored at -80°C.

2.4.1. Mitochondrial isolation

Rat brain homogenates were centrifuged at 700g for 10 min to discard nuclei and cell debris and the pellet was washed to enrich the supernatant that was centrifuged at 7000g for 10 min. The obtained pellet, washed and resuspended in the same buffer, was considered to consist mainly of intact mitochondria able to carry out oxidative phosphorylation. The operations were carried out at 0–2°C. Submitochondrial particles (SMP) were obtained by

freezing and thawing (three times) of isolated mitochondria. For superoxide production measurements, SMP were washed twice with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) and suspended in the same medium (Boveris et al., 1972).

2.4.2. Superoxide production in submitochondrial particles of the rat brain

Superoxide production was determined in washed brain SMP using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37°C (€ $_{480\ nm}$ = 4.0 mM^{-1} cm^{-1}). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), SMP (0.3–1.0 mg protein/ml), 0.1 µM catalase, and 1 mM epinephrine. NADH (50 µM) and succinate (7 mM) were used as substrates and rotenone (1 µM) and antimycin (1 µM) were added as specific inhibitors, respectively, to assay O^{-}_{2} production at the NADH dehydrogenase and at the ubiquinone–cytochrome b region. Superoxide dismutase (SOD) was used at 0.1–0.3 µM final concentration to give assay specificity (Boveris, 1984).

2.4.3. Thiobarbituric acid reactive species (TBARS) levels

We measured the formation of TBARS during an acid-heating reaction, as previously described (Draper and Hadley, 1990) as a marker of lipid peroxidation. Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1ml of thiobarbituric acid 0.67%, and then heated in a boiling water bath for 30 min. Malondialdehyde equivalents were determined in tissue and in submitochondrial particles of the rat brain spectrophotometrically by the absorbance at 532 nm.

2.4.4. Carbonyls protein content

The oxidative damage to proteins was assessed by the determination of carbonyl groups content based on the reaction with dinitrophenylhidrazine (DNPH), as previously described (Levine et al., 1994). Proteins were precipitated by the addition of 20% trichloroacetic acid and were redissolved in DNPH. The absorbance was monitored spectrophotometrically at 370 nm.

2.4.5. Superoxide dismutase (SOD) activity

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^{-2}*;* a substrate for SOD (Bannister and Calabrese, 1987). The inhibition of autoxidation of this compound thus occurs when SOD is present, and the enzymatic activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control. A calibration curve was performed using purified SOD as the standard, in order to calculate the specific activity of SOD present in the samples. A 50% inhibition of pyrogallol autoxidation is defined as 1 unit of SOD, and the specific activity is represented as units per mg of protein.

2.4.6. Catalase (CAT) activity

The CAT activity was assayed using a double-beam spectrophotometer with temperature control. This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1– 0.3 mg protein/ml (Aebi, 1984). One CAT unit is defined as 1 mol of

hydrogen peroxide consumed per minute, and the specific activity is reported as units per mg protein.

2.4.7. Protein determination

All biochemical measures were normalized to the protein content with bovine albumin as standard (Lowry et al., 1951).

2.5. Statistical analysis

All data are present as mean and standard error of the mean. Differences among experimental groups evaluated behavior parameters were determined by one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. The oxidative stress parameters were analyzed by one-way ANOVA and multiples comparisons were performed by the LSD test. In all comparisons, statistical significance was set at $P < 0.05$. All analyses were performed using the Statistical Package for the Social Science (SPSS) software.

3. Results

3.1. Behavioral assessment (Table 1)

In the first experiment (reversal treatment) we found that d-AMPH increased crossings, rearings and risk behavior (visits to center of open-field) in rats treated with saline; TMX reverted d-AMPH related hyperactivity behavior. The administration of TMX in saline treated animals did not change behavioral measures, indicating that the effects of TMX in rats treated with d-AMPH were not associated with sedation.

Behavioral measures of second experiment (prevention treatment) demonstrated that TMX pretreatment were also able to prevent d-AMPH related hyperactivity – crossings, rearings and risk behavior. Saline administration in rats pretreated with TMX did not affect locomor behavior.

3.2. Superoxide production in submitochondrial particles (SMP) of the rat brain (Figure 1)

Administration of d-AMPH significantly increased the superoxide production in SMP of rat brain (prefrontal, amygdala, hippocampus and striatum) at both reversal (Fig. 1A) and prevention (Fig. 1B) models.

In the reversal model (Fig. 1A); the TMX regime in the control group increased the superoxide production in SMP in the prefrontal of rats. Also in the prefrontal, the administration of TMX did not change d-AMPH-induced increased in superoxide formation in SMP. Already in the amygdala and hippocampus, TMX treatment partially reversed superoxide production in SMP. However, in the striatum, the formation of superoxide in SMP induced by d-AMPH was totally reversed by TMX.

In the prevention model (Fig. 1B); the use of TMX alone did not change superoxide production in SMP in the rat brain structures (prefrontal, amygdala, hippocampus and striatum). The formation of superoxide in SMP induced by d-AMPH was prevented by TMX in the prefrontal, amygdala and striatum. In the hippocampus, TMX partially prevented d-AMPH-induced increased in superoxide formation in SMP.

3.3. Thiobarbituric acid reactive species (TBARS) levels in SMP of the rat brain (Figure 2)

We found that the administration of d-AMPH in saline-treated animals increased TBARS levels in SMP in all structures assessed at both reversal (2A) and prevention (2B) models.

In the reversal model (Fig. 2A); the treatment with TMX alone increased TBARS levels in SMP in the prefrontal and striatum of animals. TMX reversed d-AMPH-induced TBARS levels in SMP in the amygdala, hippocampus and striatum, but not in prefrontal.

In the prevention model (Fig 2B); the TMX regime in the control group increased TBARS concentration in SMP in the hippocampus of rats. In addition, TMX prevented d-AMPH-induced TBARS levels in SMP in all brain regions analyzed.

3.4. Thiobarbituric acid reactive species (TBARS) levels in tissue of the rat brain (Figure 3)

We found that d-AMPH increased lipid peroxidation in all brain structure evaluated – as indicated by increased levels of TBARS – in both models (reversion = Fig. 3A; prevention = Fig. 3B).

In reversal model (Fig. 3A); the administration of TMX alone did not alter the TBARS levels in any brain region analyzed. In addition, TMX partially reversed AMPH-induced lipid peroxidation in prefrontal, hippocampus and striatum. In the amygdala, the TBARS formation induced by d-AMPH was totally prevented by TMX.

In prevention model (Fig. 3B); the pretreatment with TMX in saline-treated animals increased TBARS levels in prefrontal and hippocampus of rats. TMX significantly diminished AMPH-induced protein damage in all brain structures evaluated.

3.5. Carbonyls protein content (Figure 4)

In our study, we observed also that d-AMPH increased protein damage in all brain regions analyzed – as indicated by increased levels of carbonyl groups content – in both reversion (Fig. 4A) and prevention (Fig. 4B) models.

In reversal model (Fig. 4A); TMX regime in the control group did not change the carbonyls protein content in any brain structure analyzed. TMX treatment partially reversed d-AMPH-induced protein damage in the rat prefrontal, hippocampus and striatum. On the other hand, in the amygdala TMX reversed the d-AMPH-induced protein damage.

In prevention model (Fig. 4B); as in the reversal treatment, no changes were observed in protein carbonyl formation in the brain of animals treated with TMX alone. TMX pretreatment partially prevented d-AMPH-induced protein damage in the hippocampus and striatum of animals. In the prefrontal and amygdala, TMX prevented totally the protein carbonyl formation induced by d-AMPH.

3.6. Superoxide dismutase (SOD) activity (Figure 5)

In the reversal model (Figure 5A); the use of TMX alone significantly increased SOD activity in amygdala, hippocampus and striatum, but not in the prefrontal. The administration of d-AMPH plus TMX also increased SOD activity in amygdala and hippocampus of rats in this experimental model. The d-AMPH administration in saline-treated animals decreased SOD activity in prefrontal and striatum, which was partially reversed by TMX treatment in the striatum, but not in prefrontal.

In the prevention model (Fig 5B); the TMX pretreatment significantly increased SOD activity in the prefrontal and amygdala of saline-treated animals. The administration of d-AMPH in TMX-pretreated animals also increased SOD activity in prefrontal and amygdala.

However, in the hippocampus and striatum, the SOD activity was significantly decreased in saline plus d-AMPH group, and TMX was able to prevent the d-AMPH-induced decreased of the SOD activity in both hippocampus and striatum.

3.7. Catalase (CAT) activity (Figure 6)

Treatment with d-AMPH alone increased CAT activity in all structures analyzed in both reversal (Fig. 6A) and prevention (Fig. 6B) models.

In the reversal experiment (Fig. 6A); TMX alone increased CAT activity in the amygdala of rats. In the prefrontal d-AMPH-induced increased in CAT activity was significantly increased by TMX. In the amygdala, TMX partially reversed the d-AMPH-induced increased of the CAT activity. However, in the hippocampus and striatum, TMX reverts totally d-AMPH-induced increased of CAT activity.

In the prevention model (Fig. 6B); TMX regime in the control group did not alter the CAT activity in any brain regions analyzed. TMX prevented the d-AMPH-induced increased in CAT activity in prefrontal and amygdala. In the amygdala and hippocampus, TMX partially prevented the increased in CAT activity induced by d-AMPH.

4. Discussion

Evidences in the literature have demonstrated that activation of PKC enhances release of dopamine (Robinson, 1991; Cowell et al., 2000), and that inhibition of PKC reduces amphetamine-induced dopamine release (Giambalvo, 1992; Kantor and Gnegy, 1998). In addition, psychostimulants facilitate the release of dopamine in large part by activation of PKC (Giambalvo, 1992; Gnegy et al., 1993).

The present study showed that TMX reversed and prevented d-AMPH-induced hyperactivity and increased risk taking behavior associated to an animal model of mania. These findings are in accordance with literature which demonstrated that inhibition of PKC activity decreases some psychostimulant-induced behaviors. In previous study, Einat and colleagues (2007) showed that TMX reduced d-AMPH-induced hyperactivity in an open-field and normalized d-AMPH-induced increase risk taking behavior. A pilot study with bipolar patients, manic or mixed, with or without psychotic features that received TMX for three weeks demonstrated that subjects on TMX showed marked improvement in mania compared to placebo as early as five days, an effect that remained significant throughout the three-week trial (Zarate et al., 2007). Additionally, in a double-blind, randomized, placebo-controlled 6-week study on the efficacy and safety of the TMX adjunctive to lithium in acute bipolar mania of the Amrollahi and colleagues (2010), the authors demonstrated that the combination of TMX with lithium was superior to lithium alone for the rapid reduction of manic symptoms and it was well tolerated in these acutely manic patients. Likewise, Sabioni and colleagues (2008) analyzed the effects of TMX, chelerythrine (a PKC inhibitor) and medroxyprogesterone (an antiestrogenic drug) in AMPH-induced hyperlocomotion of mice, and found that lithium, TMX and chelerytrine completely blocked the AMPH-induced hyperlocomotion, but while the intermediate medroxyprogesterone dose (3.0 mg/kg) partially reduced the AMPH-induced hyperlocomotion, lower (1.0 mg/g) and higher (6.0 mg/kg) doses produced no effect.

We demonstrated also that in both experimental models d-AMPH induced oxidative damage by increasing the superoxide production in SMP, amount TBARS in SMP and in tissue brain and protein carbonyl in prefrontal, amygdala, hippocampus and striatum of rats. Here, we confirmed previous results that d-AMPH-induced oxidative damage in the rat brain (Frey et al., 2006; Andreazza et al., 2008; Kunz et al., 2008).

In the present study TMX prevented d-AMPH-induced changes in oxidative damage parameters (superoxide in SMP, TBARS in SMP and in tissue and carbonyl proteins). However, the effects of TMX vary depending on the brain region in the reversion treatment. Therefore, we suggest that TMX is more effective against oxidative damage when given long term. Moreover, our findings are in line with previous reports that described heterogeneity of oxidative stress parameters across brain regions and treatment regimens (Musavi and Kakkar, 1998, 2000, 2003).

The mechanism of how TMX exerts neuroprotection is unclear. However, Kimelberg and colleagues (2000) have shown that TMX can inhibit amino acid release and nitric oxide synthase activity after temporal cerebral ischemia in male rodents. Several studies have suggested that TMX possesses free radical-scavenging and antioxidant activity *in vitro* and *in vivo* (Cardoso et al., 2002, 2004; Obata, 2006) and shown to improve mitochondrial respiratory function and enhance superoxide-scavenging activity mitochondria in the heart (Zhao et al., 2006). It is know that AMPH induces generation of free radicals via the oxidative catabolism of dopamine and the subsequent dysfunction of mitochondrial respiration (Burrows et al. 2000; Cadet et al. 2005; Deng et al. 2002, Valvassori et al., 2010). PKC plays a major role in the regulation of neuronal excitability and neurotransmitter release – as dopamine (see Zarate et al., 2006). In this context, pharmacological inhibition of PKC by TMX results in the attenuation of dopamine release, which may be protecting brain tissue against oxidative damage.

Conversely, in a few situations, TMX alone increased oxidative damage parameters in some brain regions in both experimental protocols. Despite considerable amount of biochemical data supports the potential involvement of PKC in the pathophysiology of mental disorders (Manji and Lenox 1999; Young et al 1999), in normal situation PKC plays an important role in long-term alterations in gene expression and plasticity. Together these data suggest that the decrease of PKC may leave the brain tissue vulnerable to oxidative damage.

Nazarewicz and colleagues (2007) showed that TMX induces production of reactive oxygen species, exceeding the antioxidant capacity thereby resulting in cell injury. It has been shown that TMX releases cytochrome c from liver mitochondria and increases lipid peroxidation. Those effects of TMX were prevented when mitochondrial nitric oxide synthase was inhibited and antioxidant (glutathione) was supplemented (Nazarewicz et al., 2007). Moreover, is important to note that TMX also interacts with the estrogen receptor and affects other intracellular mechanisms including MAP kinases, respiratory chain complexes and other parameters which were not evaluated in this work.

Another important fact shown in this article was that d-AMPH-induced oxidative damage was accompanied by decreased superoxide dismutase activity and increased CAT activity. Interestingly, TMX increased SOD activity – decreased by d-AMPH - and normalized the activity of CAT, which was increased by d-AMPH. SOD is a protective enzyme that can selectively scavenge the superoxide anion radical (O_2^-) by catalyzing its dismutation to hydrogen peroxide (H_2O_2) and CAT metabolizes the excess of H_2O_2 producing $O_2 + H_2O$, decreasing the intracellular redox status (Andreazza et al., 2008). Reduced levels of the major antioxidant enzymes, SOD, CAT and glutathione peroxidase have also been found in patients with schizophrenia compared with controls (Ranjekar et al., 2003; Li et al., 2006). In bipolar patients, Benes and colleagues (2006) demonstrated lowered gene expression of several antioxidants enzymes in hippocampus, including SOD, glutathione peroxidase and glutathione S-transferase. In addition, Herken and colleagues (2001) related an increase of catalase activity in erythrocyte in different forms of schizophrenia.

5. Conclusion

Our results showed that d-AMPH-induced hyperactivity and risk behavior was reverted and prevented by TMX, which previous studies had already reported. On the other

hand, we demonstrated that TMX could revert and protect against d-AMPH-induced damage through oxidative stress. However, the effects in the reversal model depend on the brain region analyzed. Then we suggest that inhibition of PKC by TMX, in addition to reverse the d-AMPH-induced manic-like behavior also protecting against oxidative damage, two important milestones in BD.

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pharmacological treatment and the behavioral assessment. Francielle Mina made the biochemical analysis. All authors contributed to and have approved the final manuscript.

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Legend to figures:

Figure 1A. Effects of Tamoxifen administration on superoxide levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 1B. Effects of Tamoxifen administration on superoxide levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 2A. Effects of Tamoxifen administration on TBARS levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 2B. Effects of Tamoxifen administration on TBARS levels in submitochondrial particles in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 3A. Effects of Tamoxifen administration on TBARS levels in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 3B. Effects of Tamoxifen administration on TBARS levels in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 4A. Effects of Tamoxifen administration on protein carbonyl formation in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 4B. Effects of Tamoxifen administration on protein carbonyl formation in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 5A. Effects of Tamoxifen administration on superoxide dismutase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 5B. Effects of Tamoxifen administration on superoxide dismutase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 6A. Effects of Tamoxifen administration on catalase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the reversal model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Figure 6B. Effects of Tamoxifen administration on catalase activity in the prefrontal, amygdala, hippocampus and striatum of rats in the prevention model (n=5 for each group). Data were analyzed by one-way analysis of variances followed by LSD test when *F* was significant. Values are expressed as mean ± S.E.M. *P<0.05 diference of SAL+SAL group. #P<0.05 diference of d-AMPH+SAL group.

Table 1. Effects of Tamoxifen on d-amphetamine-induced behavioral alteration in animal model of mania. [a]difference of SAL+SAL group (reversal and prevention model). [b]difference of d-AMPH+SAL group (reversal model) and difference of SAL+d-AMPH group (prevention model).

Table 1. Effects of Tamoxifen on d-amphetamine-induced behavioral alteration in animal model of mania

			Crossings		Rearings		Visits to Center	
	Treatment	n	Mean ± Error of Mean	p values	Mean ± Error of Mean	p values	Mean ± Error of Mean	p values
Reversal Model	SAL+SAL	8	41.37 ± 2.41		23 ± 2.41		4,83 ± 1.51	
	SAL+TMX	8	41.25 ± 5.08	0.985	19.5 ± 3.32	0.411	3.67 ± 0.89	0.38
	d-AMPH+SAL	7	90.57 ± 6.67[a]	<0.001	36.14 ± 3.83[a]	0.005	15.99 ± 1.46[a]	<0.001
	d-AMPH+TMX	7	31.14 ± 3.47[b]	0.152	11.14 ± 1.22[ab]	0.010	2.7 ± 0.67[b]	0.134
Prevention Model	SAL+SAL	8	40.5 ± 1.54		16 ± 1.1		0.5 ± 0.3	
	TMX+SAL	8	51.5 ± 8.65	0.411	22 ± 5.25	0.066	1.12 ± 1.02	0.24
	SAL+d-AMPH	8	84 ± 6.22[a]	<0.001	33 ± 3.83[a]	<0.001	10 ± 1.85[a]	<0.001
	TMX+d-AMPH	7	62 ± 7.5[b]	0.279	20 ± 1.93[b]	0.767	2 ± 0.89[b]	0.203

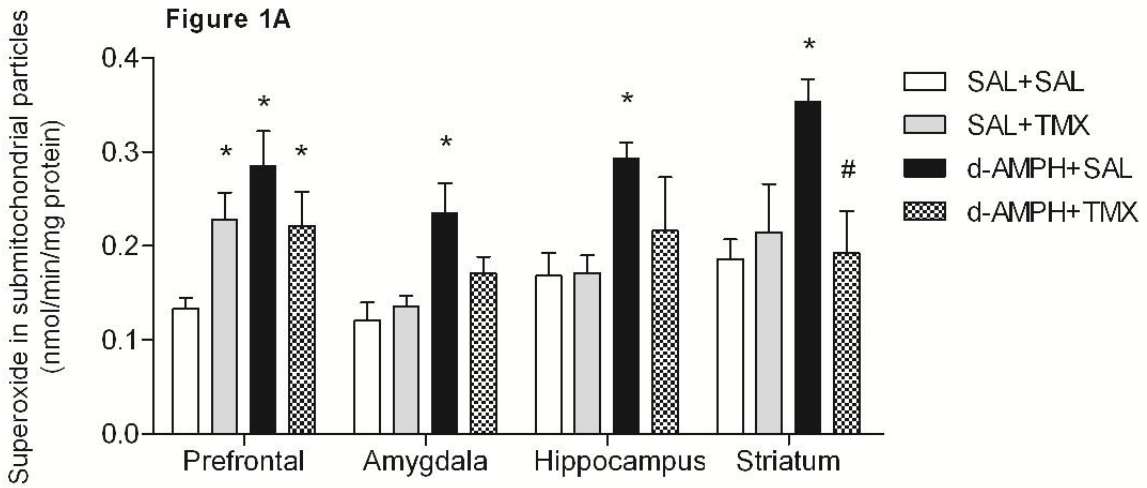


Figure 1A
Superoxide in submitochondrial particles
(nmol/min/mg protein)
0.4
0.3
0.2
0.1
0.0
Prefrontal
Amygdala
Hippocampus
Striatum
SAL+SAL
SAL+TMX
d-AMPH+SAL
d-AMPH+TMX

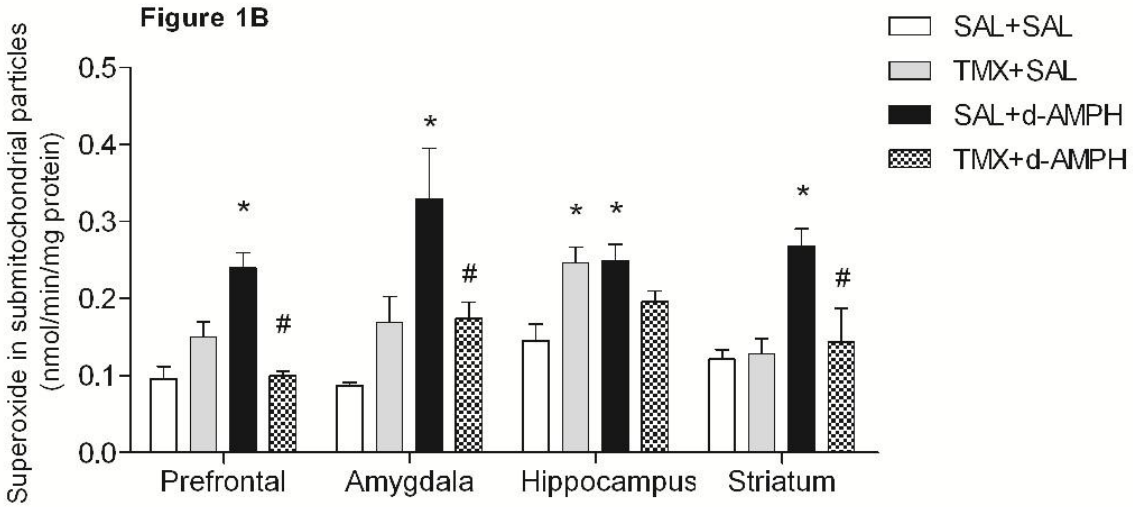


Figure 1B
Superoxide in submitochondrial particles
(nmol/min/mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
Prefrontal
Amygdala
Hippocampus
Striatum
SAL+SAL
TMX+SAL
SAL+d-AMPH
TMX+d-AMPH

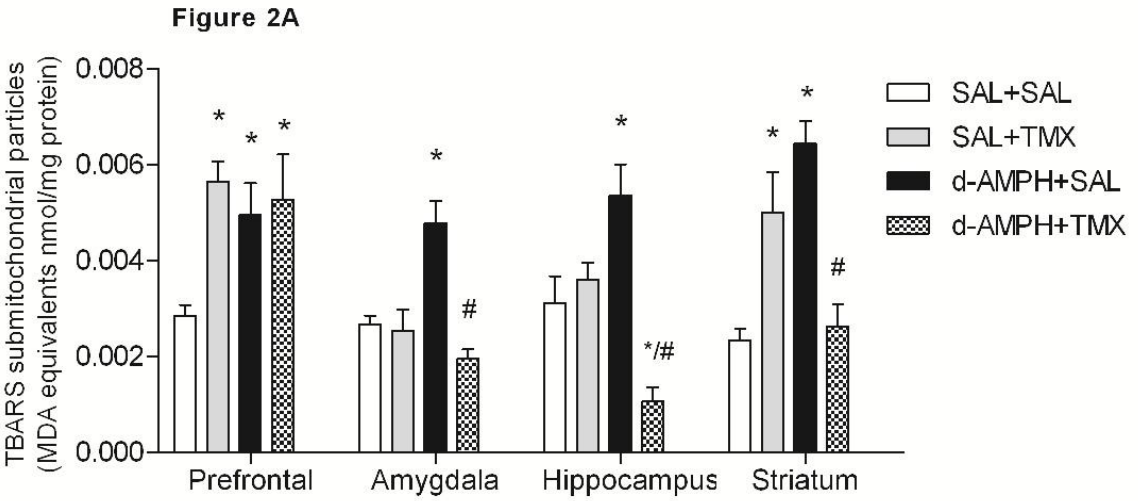


Figure 2A
TBARS submitochondrial particles
(MDA equivalents nmol/mg protein)
0.008
0.006
0.004
0.002
0.000
Prefrontal
Amygdala
Hippocampus
Striatum
SAL+SAL
SAL+TMX
d-AMPH+SAL
d-AMPH+TMX

Figure 2B

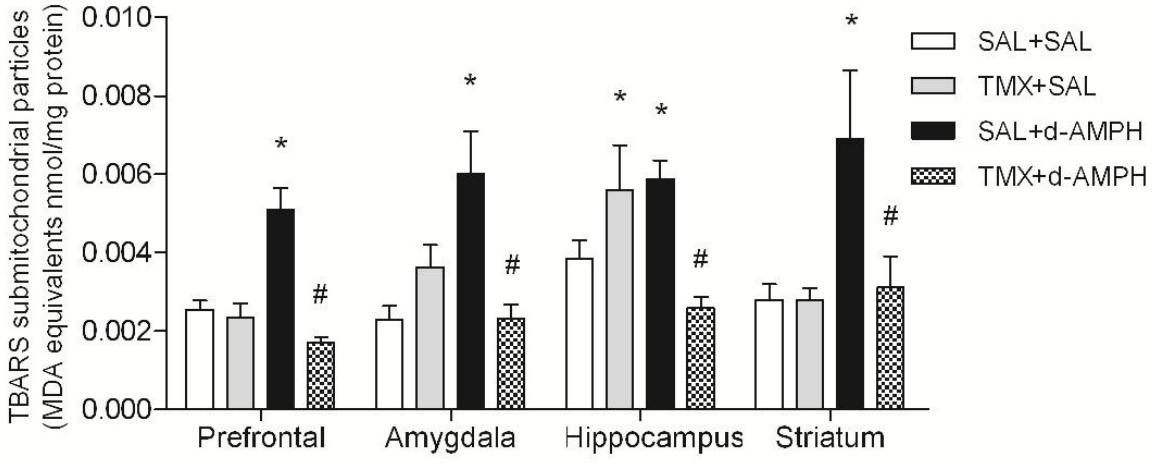


Figure 3A

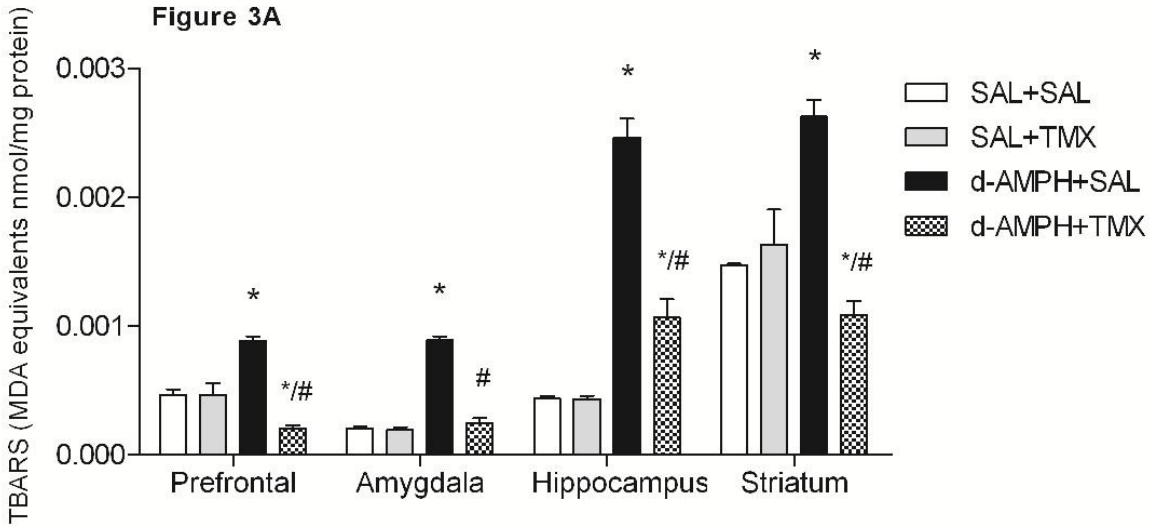


Figure 3B

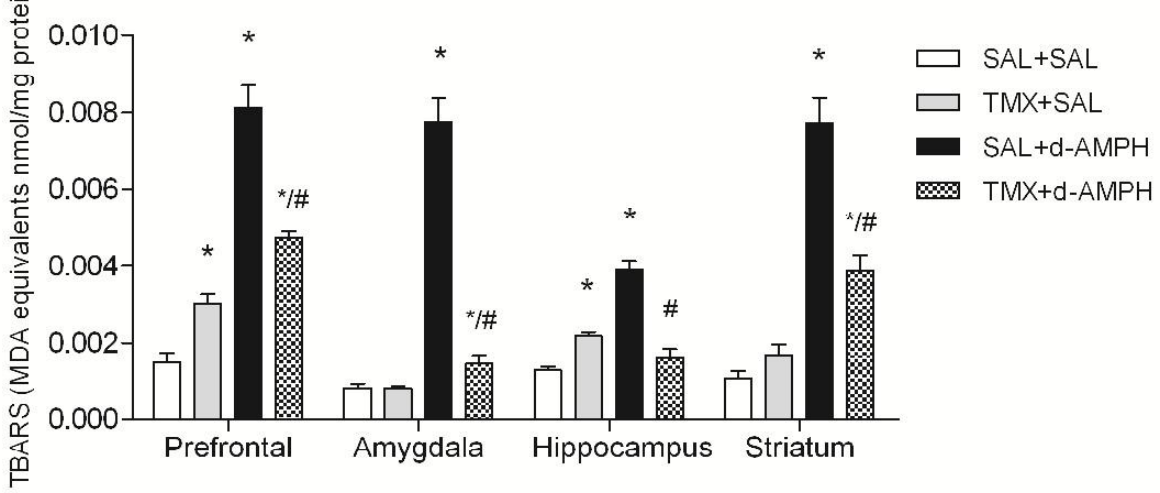


Figure 4A

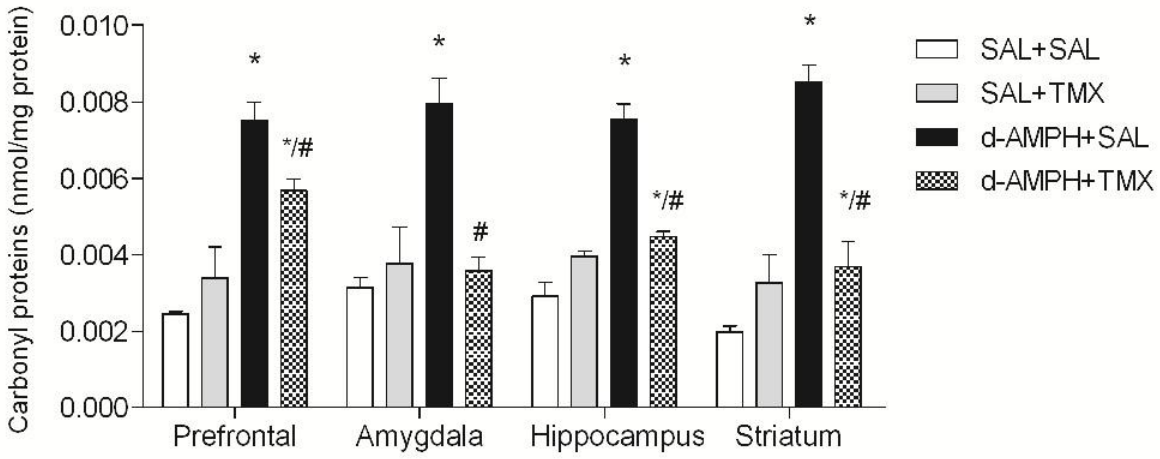


Figure 4B

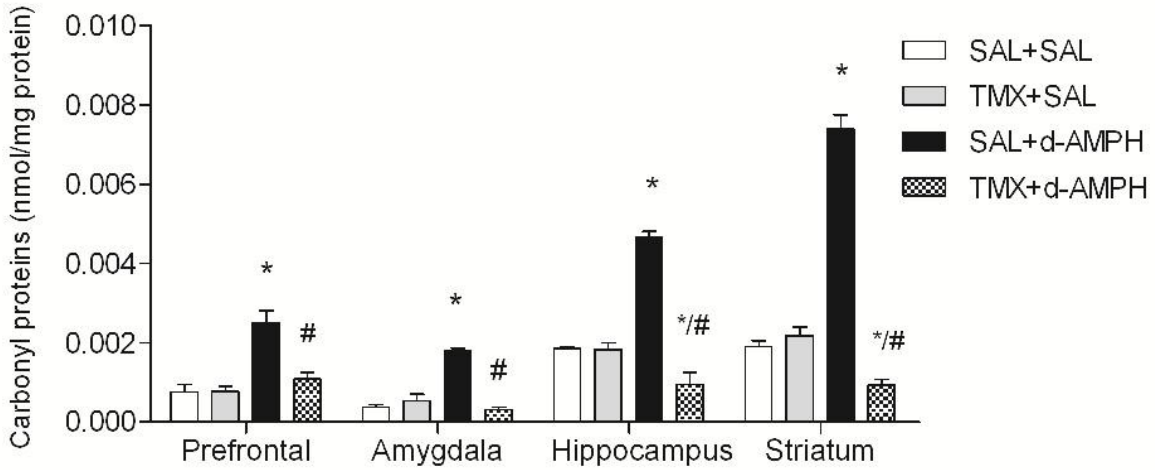


Figure 5A

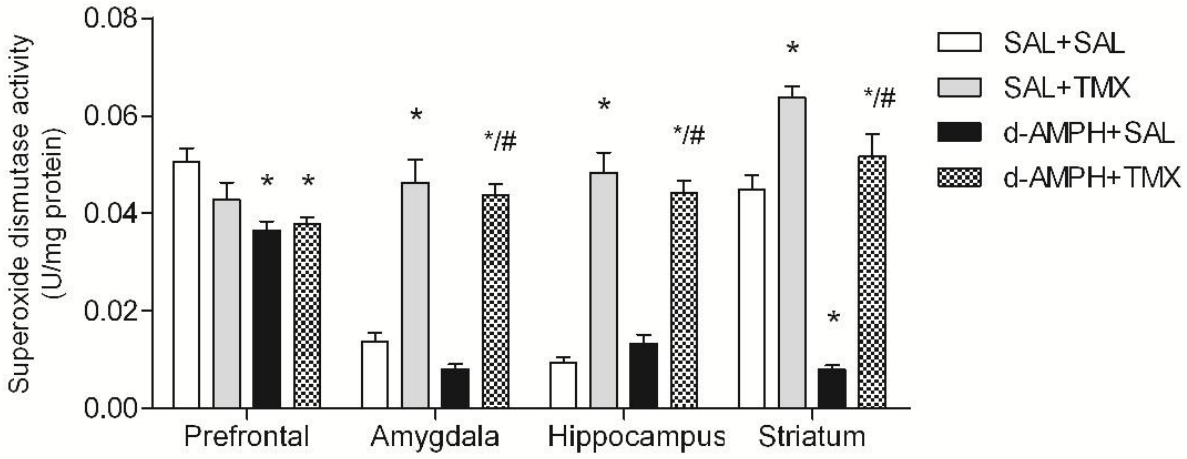


Figure 5B

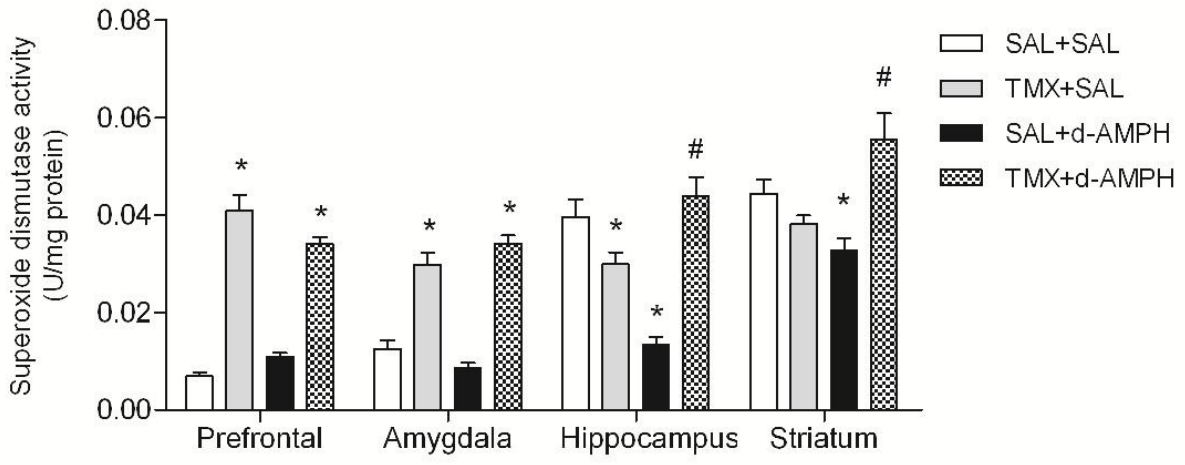


Figure 6A

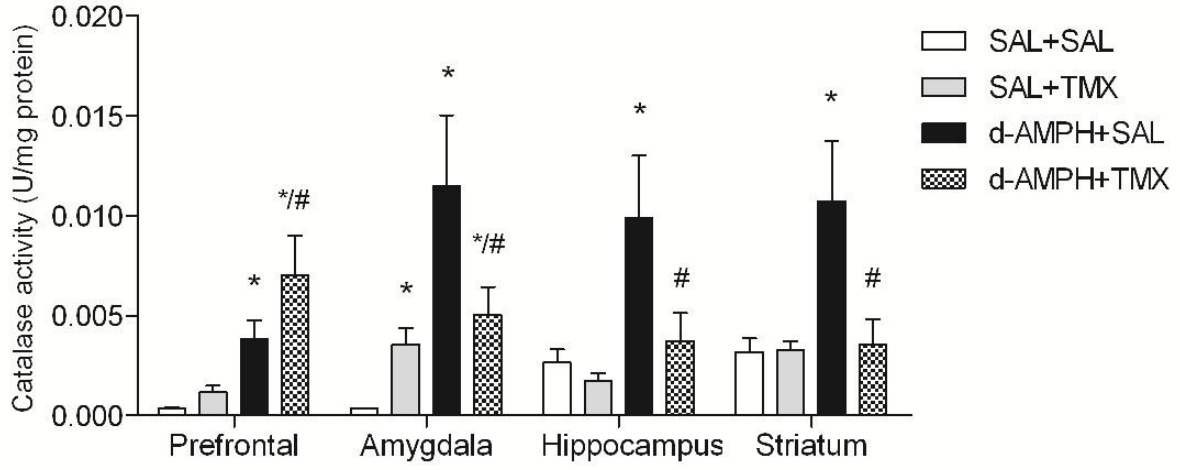
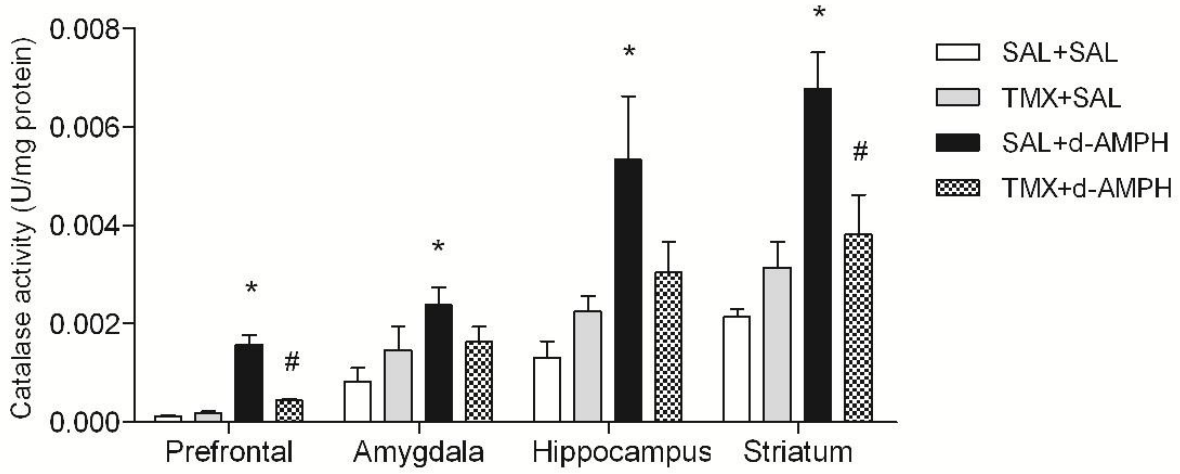


Figure 6B



4 DISCUSSÃO

Os resultados comportamentais deste estudo mostram que a administração de d-AMPH aumentou a atividade locomotora, exploratória e o comportamento de risco dos animais tratados com solução salina em ambos os modelos experimentais (reversão e prevenção). Estes achados corroboram com resultados de estudos prévios que descreveram um efeito hiperlocomotor induzido pela administração de d-AMPH em ratos (Frey et al., 2006a,b,c; Mavrikaki et al., 2010).

Por ser um agonista indireto do sistema catecolaminérgico, a d-AMPH exerce seu efeito aumentando a liberação de catecolaminas das terminações nervosas no cérebro (Meyer & Quenzer, 2005). Seiden e colaboradores (1993) mostraram que o aumento da atividade locomotora observado nos animais tratados com d-AMPH reflete a hiperatividade do sistema dopaminérgico, o que aumenta de forma significativa a concentração de dopamina nas sinapses, motivo pelo qual este psicoestimulante provoca aumento da locomoção tanto em ratos quanto em outros animais.

A administração de SB reverteu e preveniu a hiperatividade e o comportamento de risco induzidos pela d-AMPH. Está descrito na literatura que a remodelação da cromatina é um importante mecanismo regulatório subjacente à plasticidade neuronal e ao comportamento (Schröeder et al., 2007). Além disso, mudanças na expressão gênica que afetam circuitos dopaminérgicos podem resultar em adaptações neuroquímicas e comportamentais em resposta a um estímulo. Dessa forma, a inibição de HDAC implica na diminuição de alguns comportamentos induzidos por psicoestimulantes (Schröeder et al., 2007; Febo et al., 2009).

Além de ser amplamente utilizado no tratamento do THB, o valproato foi recentemente caracterizado como um potente inibidor de HDAC (Eadie & Vadja, 2005; Chen et al., 2007). Tal fármaco também é capaz de reverter a hiperlocomoção induzida por d-AMPH em modelos animais de mania (Frey et al., 2006a,b). Em adição, Kumar e

colaboradores (2005) mostraram que o SB aumentou a acetilação de histonas causada pela exposição a cocaína. Assim, os resultados comportamentais deste estudo reforçam a importância da regulação das histonas no efeito antimaníaco do valproato.

O comportamento induzido por d-AMPH foi atenuado após o tratamento com TMX, cuja ação é através da inibição direta de PKC. Este resultado é consistente com os achados de outros estudos que demonstraram a eficácia de inibidores de PKC, diminuindo a resposta da d-AMPH (Browman et al., 1998; Einat et al., 2004; 2007), particularmente em relação ao TMX (Einat et al., 2007). Um estudo piloto com pacientes bipolares (maníacos e mistos), com ou sem sintomas psicóticos, que receberam TMX durante três semanas mostrou uma melhora significativa da mania já nos primeiros cinco dias, comparados ao grupo placebo (Zarate et al., 2007). Adicionalmente, Browman e colaboradores (1998) observaram que quando administrado Ro31-8220 – inibidor de PKC – no núcleo accumbens ocorre uma diminuição da resposta motora produzida pela d-AMPH.

Dados da literatura mostram que a PKC, por estar envolvida na regulação de aspectos relacionados à neurotransmissão, apresenta um potencial envolvimento no THB, representando um novo alvo bioquímico no tratamento da mania (Browman et al., 1998; Young et al., 1999). A ativação de PKC aumenta a liberação de dopamina – neurotransmissor envolvido na mania (Cowell et al., 2000) e a inibição da mesma reduz a liberação de dopamina induzida pela d-AMPH (Giambalvo, 1992; Kantor & Gnegy, 1998), o que contribui para os efeitos antimaníacos do TMX, reforçando a importância da modulação da PKC no tratamento da mania.

A administração de d-AMPH, em ambos os modelos experimentais (reversão e prevenção) induziu dano oxidativo através do aumento da produção de superóxido e de TBARS em partícula submitocondrial, assim como de TBARS e de carbonil no córtex pré-frontal, amígdala, hipocampo e estriado de ratos. Estes dados confirmam resultados prévios da literatura onde a d-AMPH induziu dano oxidativo em tecido cerebral de ratos (Frey et al.,

2006; Andreazza et al., 2008; Kunz et al., 2008).

Neste estudo foi demonstrado que o SB reverteu e preveniu o dano oxidativo induzido por d-AMPH no tecido cerebral de ratos. Entretanto, os efeitos de SB dependem da estrutura cerebral analisada. Regiões do sistema nervoso central podem responder diferentemente (Sullivan et al., 2005) e neste estudo, os parâmetros de estresse oxidativo foram analisados em diversas regiões cerebrais, representando assim, diferentes tipos celulares. Além disso, dentro de uma população homogênea de células há uma heterogeneidade em condições fisiológicas e características metabólicas (Lai et al.. 1977; Sims, 1991; Sonnewald et al., 1998).

Muitos estudos têm demonstrado que inibidores de HDACs podem proteger as células de alguns insultos tóxicos, incluindo inflamação e estresse oxidativo (Chen et al., 2006; Langley et al., 2008; Leng et al., 2008) e podem, inclusive, aumentar a expressão de fatores neurotróficos e proteger neurônios dopaminérgicos durante os episódios de mania (Yatham et al., 2002). Complementando estes dados, Wu e colaboradores (2008) demonstraram que SB e TSA (*Trichostatin A*) – dois inibidores de HDACs – promovem a sobrevivência de neurônios dopaminérgicos. Estes mecanismos podem ter ligação com o efeito neuroprotetor de SB encontrado no presente trabalho.

Outro resultado interessante encontrado foi que a administração de SB nos grupos controles aumentou o dano oxidativo em algumas estruturas cerebrais. Diversos estudos têm mostrado que vários inibidores de HDACs estimulam a geração de EROs e que o tratamento com antioxidantes reduz a atividade anticâncer destes inibidores (Butler et al., 2002; Rosato et al., 2003; Ungerstedt et al., 2005).

O tratamento com TMX preveniu o dano oxidativo induzido por d-AMPH (produção de superóxido, TBARS em partícula submitocondrial e em tecido total e proteína carbonil), porém, os efeitos de TMX também variam conforme a região cerebral no modelo de reversão. Dessa forma, sugere-se que o TMX é mais efetivo contra o dano oxidativo

quando administrado num longo período. Estes achados estão de acordo com a literatura que descreve uma heterogeneidade nos parâmetros de estresse oxidativo sobre regiões cerebrais e tratamentos (Musavi & Kakkar, 1998, 2000, 2003).

O mecanismo pelo qual o TMX exerce neuroproteção ainda é pouco conhecido. Kimelberg e colaboradores (2000) mostraram que o TMX pode inibir a atividade da óxido nítrico sintase após uma isquemia cerebral temporal em ratos. Diversos estudos têm sugerido que o TMX possui atividade antioxidante *in vivo* e *in vitro* (Cardoso et al., 2002; 2004; Obata et al., 2006). Entretanto, está descrito na literatura que a d-AMPH induz a geração de radicais livres - via oxidação de dopamina - e subsequente disfunção mitocondrial (Burrows et al. 2000; Cadet et al. 2005; Deng et al. 2002, Valvassori et al., 2010). Sabe-se também que a PKC está envolvida na regulação da excitabilidade neuronal e na liberação de neurotransmissores, como a dopamina (Zarate et al., 2006). Neste contexto, a inibição farmacológica de PKC por TMX resulta na diminuição da liberação de dopamina, que pode estar protegendo o cérebro contra o dano oxidativo induzido por d-AMPH

Em determinadas situações, o TMX aumentou os parâmetros de dano oxidativo em algumas regiões cerebrais, em ambos os modelos experimentais. Apesar de uma considerável quantidade de dados bioquímicos indicarem um possível envolvimento da PKC na fisiopatologia de transtornos psiquiátricos (Manji and Lenox 1999; Young et al., 1999), em condições normais, a PKC apresenta um importante papel nas alterações a longo prazo na plasticidade e expressão gênica. Juntos, estes resultados sugerem que a inibição de PKC pode deixar o tecido cerebral vulnerável ao dano oxidativo. Reforçando estes achados, Nazarewicz e colaboradores (2007) demonstraram que o TMX induz a produção de radicais livres, excedendo a capacidade antioxidante, resultando no dano celular. Os efeitos de TMX foram prevenidos quando a óxido nítrico sintase foi inibida e a glutationa foi suplementada como antioxidante.

É importante considerar, entretanto, que o mecanismo de ação do TMX não se

restringe apenas a inibição de PKC, pois o mesmo pode interagir com receptores de estrogênio e afetar outros mecanismos intracelulares – incluindo MAP quinases, complexos da cadeia respiratória mitocondrial e outros parâmetros que não foram avaliados neste estudo.

Outro importante resultado encontrado neste estudo foi uma diminuição na atividade da SOD e um aumento na atividade da CAT induzidos por d-AMPH. A administração de SB e de TMX aumentou a atividade da SOD e normalizou a atividade da CAT em ambos os protocolos de tratamento (reversão e prevenção). Adicionalmente, Ryu e colaboradores (2003) demonstraram que SB pode reduzir o estresse oxidativo e modular a transcrição de sequestradores de radicais livres, como a MnSOD e a CAT. Paralelamente, uma atividade antioxidante também foi observada em relação ao TMX (Obata et al., 2006).

Como descrito anteriormente, a SOD é uma enzima que catalisa a reação de conversão do ânion superóxido em peróxido de hidrogênio, que é então eliminado pela ação da CAT (Halliwell, 1987). Níveis reduzidos de SOD, CAT e GPx tem sido encontrados em pacientes esquizofrênicos, comparados com indivíduos controles (Ranjekar et al., 2003; Li et al., 2006). Em pacientes bipolares, Benes e colaboradores (2006) encontraram baixa expressão de genes de diversas enzimas antioxidantes – incluindo SOD e GPx – no hipocampo. Em adição, Herken a colaboradores (2001) relataram um aumento na atividade da CAT em eritrócitos de pacientes com esquizofrenia.

Contudo, os resultados do presente estudo permitem concluir que o SB e o TMX são hábeis em reverter e prevenir a hiperatividade e o comportamento de risco induzidos por d-AMPH. A administração de ambos os fármacos reverte e tem ação protetora contra o dano oxidativo induzido por d-AMPH, porém os efeitos dependem da estrutura cerebral analisada. Dessa forma, este trabalho reforça a necessidade de estudos com inibidores de HDAC e de PKC como possíveis alvos de novas medicações para o tratamento do THB.

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