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ALTERAÇÕES COMPORTAMENTAIS E DO METABOLISMO ENERGÉTICO NO TRANSTORNO DO HUMOR BIPOLAR: EVIDÊNCIAS PRÉ-CLÍNICAS E CLÍNICAS

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências da Saúde para a obtenção do título de doutor em Ciências da Saúde.

Orientador: Prof. Dr. João Quevedo

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"Dedico este trabalho aos meus pais, Oswaldo e Maristela, por acreditarem em mim."

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"Vence, quem se vence."

Provérbio Latino

RESUMO

O modelo animal de mania induzido por dextroanfetamina (d-AMPH) é descrito na literatura como um bom modelo animal de transtorno bipolar (TB). Entretanto, tem sido relatado diferenças entre os tipos de anfetaminas em induzir alterações comportamentais e neuroquímicas. Além disso, vários estudos sugerem que o TB está associado à disfunções no metabolismo energético. No presente estudo foi avaliado a diferença entre d-AMPH e metanfetamina (m-AMPH) sobre o comportamento e disfunção no metabolismo energético no cérebro de ratos. Foi avaliado também os efeitos de lítio (Li) e valproato (VPA) sobre as alterações comportamentais e sobre metabolismo energético induzidos por m-AMPH. Finalmente, comparamos os níveis de creatina quinase (CK) no soro de pacientes bipolares nas fases depressiva, maníaca e eutímica. Para tanto, o trabalho foi dividido em três partes. Parte 1: Ratos Wistar receberam uma injeção intraperitoneal (i.p) de salina, d-AMPH (2mg/kg) ou m-AMPH (0,25, 0,5, 1 ou 2mg/kg) e foram submetidos ao teste do campo aberto para avaliação comportamental 2 h após. Foi avaliada também a atividade das enzimas do ciclo de Krebs (citrato sintase, succinato desidrogenase e malato desidrogenase), dos complexos da cadeia respiratória mitocondrial (I, II, II-III, IV) e da CK no cérebro dos ratos. Parte 2: Foi administrado m-AMPH ou salina i.p em ratos Wistar durante 14 dias e entre o 8º e o 14º dia os animais eram tratados com Li, VPA ou salina via i.p. Os animais foram submetidos aos mesmos testes comportamentais e bioquímicos descritos na Parte1. Parte 3: Foram avaliados os níveis de CK no soro de pacientes bipolares - eutímicos, depressivos e em mania – que foram comparados com voluntários saudáveis. Na primeira parte do trabalho foi demonstrado que d-AMPH e m-AMPH aumentaram a atividade locomotora nos ratos. As visitas ao centro do campo aberto aumentaram com a administração de ambas as drogas na dose de 2mg/kg. A administração de m-AMPH na dose de 2mg/kg aumentou o comportamento estereotipado dos animais (sniffing). A administração tanto de d-AMPH quanto de m-AMPH diminuiu a atividade das enzimas do ciclo de Krebs, dos complexos da cadeia respiratória mitocondrial e da CK; entretanto, estes efeitos variam de acordo com a região cerebral avaliada. Na segunda parte do trabalho foi demonstrado que Li e VPA revertem a hiperatividade e alterações no metabolismo energético induzida por m-AMPH. Por fim, na terceira parte do trabalho foi demonstrado que durante a mania, os níveis de CK estão aumentados no soro dos pacientes bipolares quando comparado com voluntários saudáveis. Estes dados demonstram que a m-AMPH, mas não d-AMPH, induz comportamento estereotípico em ratos; porém, as duas drogas parecem ter efeitos similares sobre o metabolismo energético; e que, assim como a d-AMPH, a m-AMPH é capaz de induzir hiperatividade e disfunção no metabolismo energético que são revertidos por Li e VPA. As fases maníaca, depressiva e eutímica do TB, além de apresentarem sintomatologia distinta, também podem ser diferenciadas pelo nível de CK presente no soro dos pacientes. Entretanto, mais estudos são necessários para entender as diferenças observadas na atividade da CK durante as fases do TB.

Palavras-chave: mania; creatina quinase; metanfetamina; cadeia respiratória mitocondrial; transtorno bipolar; ciclo de Krebs

ABSTRACT

The animal model of mania induced by dextroamphetamine (d-AMPH) has been considered a good model for the study of bipolar disorder (BD). However, some studies have shown differences between amphetamines to induce both behavioral and biochemical changes. Besides, several studies have suggested that dysfunctional energy metabolism have a central role in BD. In the present study was investigated the potency of the d-AMPH and methamphetamine (m-AMPH) on the behavior and energetic dysfunction in the brain of rats. Were investigated also the effects of the lithium (Li) and valproate (VPT) on behavioral and energy metabolism changes in brain of rats undergoing treatment with the m-AMPH. Finally, was to compare serum creatine kinase (CK) levels between bipolar disorder patients, in the various phases (depressive, manic, and euthymic). The work was divided into three parts. Part 1: Wistar rats were given single intraperitoneal (i.p) injections of saline, d-AMPH (2 mg/kg) or m-AMPH (0.25, 0.5, 1 or 2 mg/kg). Locomotor behavior was assessed using the open-field task and activities of Krebs cycle enzymes (citrate synthase and succinate dehydrogenase), mitochondrial respiratory chain complexes (I, II, III and IV) and CK were measured in brain of rats. Part 2: Wistar rats were first given m-AMPH or saline for 14 days, and then, between days 8 and 14, rats were treated with Li, VPA or saline i.p. The animals were submitted to the same behavioral and biochemical tests described in Part 1. Part 3: Was compared serum CK levels between BD patients, in the various phases (depressive, manic, and euthymic), and healthy volunteers. In the first part of the study was demonstrated that d-AMPH and m-AMPH (all doses administered) increased locomotor activity of animals. The numbers of visits to the centre were increased by d-AMPH and m-AMPH at 2 mg/kg. The m-AMPH administration at 2mg/kg increased the amount of stereotypic behavior. The amphetamines significantly decreased the activities of Krebs cycle enzymes, mitochondrial respiratory chain complexes and CK; nevertheless, this effect varied depending on the brain region evaluated. In the second part of this study we found that Li and VPA reversed m-AMPH-induced hyperactivity. Besides, Li and VPA reversed m-AMPH-induced energetic metabolism dysfunction; however, the effects of Li and VPA were dependent on the brain region analyzed. Finally, in the third part of this study was demonstrated that CK levels were higher in the manic patients than in the controls. Together these data show that: 1) at high doses, m-AMPH increased stereotyped (sniffing) behavior in rats, but d-AMPH did not. However, this study shows that d-AMPH and m-AMPH seem to have similar effects on the brains energetic metabolism; and the d-AMPH, m-AMPH is able to induced hyperactivity and energetic metabolism dysfunction, both seen in BD. In addition, Li and VPA reversed m-AMPH's effects on locomotor activity and energetic metabolism. The clinical differences among the depressive, manic, and euthymic phases of BD are paralleled by contrasting levels of CK. However, further studies are needed in order to understand the state-dependent differences observed in serum CK activity.

Key-words: mania; creatine kinase; methamphetamine; mitochondrial respiratory chain; bipolar disorder; Krebs cycle.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ADP - Adenosina Difosfato

AMPHs - Anfetaminas

ATP - Adenosina-5'-Trifosfato

BDNF - Fator Neurotrófico Derivado do Cérebro

CK - Enzima Creatina Quinase

CS - Citrato Sintase

DA - Dopamina

d-AMPH - Dextroanfetamina

FADH2 - Dinucleotídeo Flavina Adenina Reduzida

FDA - Food and Drug Administration

GSK-3 - Glicogênio Sintase Quinase 3

GTP – Guanosina 5'Trifosfato

i.p - Injeção Intraperitoneal

Li - Lítio

m-AMPH - Metanfetamina

MD - Enzima Malato Desidrogenase

NADH - Dinucleotídeo Nicotinamida Adenina Reduzida

OMS - Organização Mundial de Saúde

PBI – Transtorno Bipolar do tipo I

PBII – Transtorno Bipolar do Tipo II

Pi - Fosfato Inorgânico

PKC - Proteína Quinase C

SD - Enzima Succinato Desidrogenase

SNC - Sistema Nervoso Central

TB - Transtorno Bipolar

UNODC – United Nations Office on Drugs and crime Reports

VPA – Valproato

SUMÁRIO

1. Introdução

1.1. Dependência Química a Anfetaminas

Com o mundo à beira da guerra, e seus efeitos tóxicos ainda não bem descritos, os efeitos clínicos das anfetaminas (AMPHs) foram pensados para serem ideais para os soldados em combate: atenção aumentada, agressão, além de diminuição da fome e da necessidade de dormir. Durante a Segunda Guerra Mundial, os Estados Unidos, Alemanha e Japão empregaram o uso de AMPHs nas suas tropas (Suwaki et al., 1997; Anglin et al, 2000; Rusyniak, 2011). Após a guerra, o Japão experimentou o abuso generalizado como excedentes do exército inundando o mercado (Rusyniak, 2011). Embora o uso de AMPHs no Japão declinasse na década de 1960, ele ressurgiu na década de 1970 e continua a ser um problema de saúde publica até hoje (Suwaki et al., 1997; Hunt et al., 2006).

Os tipos mais comuns de AMPH são a dextroanfetamina (d-AMPH) e a metanfetamina (m-AMPH). Esses dois tipos de AMPH têm estrutura química similar, sendo que a m-AMPH é um análogo n-metilado da d-AMPH (Hoffman & Lefkowitz, 1996) (Figura 1).

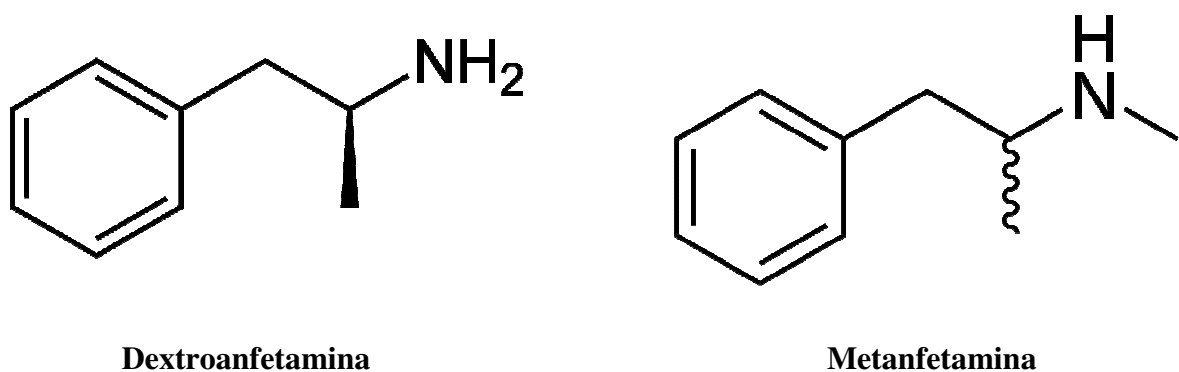**Dextroanfetamina**   **Metanfetamina**

Figura 1. Estrutura molecular da dextroanfetamina e da metanfetamina.

Apesar da similaridade estrutural, alguns estudos mostram diferenças entre as duas drogas, principalmente na indução de alterações comportamentais e neuroquímicas. Estudos

epidemiológicos mostram que a m-AMPH apresenta maiores taxas de abuso em relação à d-AMPH (Samhsa, 2007).

Nas últimas seis décadas, o abuso de AMPHs passou a ocorrer em todo o mundo (Baberg et al., 1996; Shaw, 1999). As AMPHs são as substâncias ilícitas mais usadas no mundo ficando atrás apenas da *cannabis sativa*. Em 2008, o *United Nations Office on Drugs and Crime Reports* (UNODC)- uma agencia dos Estados Unidos que controla drogas e previne crimes - estimou em 25 milhões os abusadores de AMPHs no mundo, superando o número de usuários tanto para a cocaína (14 milhões) quanto para a heroína (11 milhões). Uma das razões que levou o consumo de AMPHs a ultrapassar o de cocaína em todo o mundo é que ela tem uma meia-vida de 12 horas, permitindo que o viciado tenha um longo e sustentado período de ação da droga (Rusyniak, 2011). As AMPHs podem ser sintetizadas a partir de uma ampla variedade de matérias-primas e métodos em laboratórios clandestinos. Em contraste, a cocaína deve ser extraída da planta, convertida em sua forma de uso, exportada, para depois ser distribuída através de traficantes para os usuários (Streatfeild, 2001).

Baseado em informações do UNODC 2011, não há dados atualizados na prevalência de substâncias do grupo das anfetaminas na América do Sul. Informações existentes mostram que a prevalência anual do uso de substâncias do grupo das AMPHs, na América do Sul, continua próxima da média mundial, com estimativas entre 0,5% e 0,7% da população entre 15-64 anos ou entre 1,34 e 1,89 milhões de pessoas nesse grupo de idade que fizeram uso dessas substâncias no ano anterior. Porem em uma pesquisa nacional feita entre estudantes universitários no Brasil em 2009 mostra que a prevalência anual do uso de anfetaminas entre estudantes foi relatada com 10,5%. A prevalência anual foi maior entre estudantes mulheres (14,1%) do que entre estudantes homens (5,5%), e também foi maior entre estudantes mais velhos, isto é, aqueles de 35 anos ou mais (18,6%), seguidos por estudantes entre 25-34 anos (13,7%). O uso de substâncias como AMPH é relatada como sendo mais comum entre

mulheres devido aos efeitos anoréxicos e a uma cultura predominante de uso de medicamentos para propósitos de perda de peso (UNODC, 2011).

Os efeitos agudos das AMPHs são respostas do tipo luta ou fuga: aumento da freqüência cardíaca, aumento da pressão arterial, vasoconstrição, broncodilatação e hiperglicemia (Cruickshank et al., 2009; Rusyniak, 2011). As alterações decorrentes tanto dos efeitos agudos como dos crônicos das AMPHs são resultados de sua farmacologia e toxicologia, sendo que usuários crônicos desses psicoestimulantes desenvolvem sintomas que são muito semelhantes aos sintomas de mania idiopática que ocorrem em condições de intoxicação, abstinência ou ambos (Smith & Davis, 1977; Brauer & de Wit 1996).

A estimulação do sistema nervoso central (SNC) induzida por essas drogas podem também resultar em euforia, energia acentuada, estado de alerta aumentado, intensa curiosidade e auto-estima elevadas (Cruickshank et al., 2009; Rusyniak, 2011), sintomas estes também encontrados na fase maníaca do transtorno bipolar (TB). Em usuários crônicos de AMPHs estes estados psiquiátricos são por vezes prolongados com o surgimento de sintomas residuais, que são facilmente exacerbados pelo uso de outros anfetaminoides, por sua reutilização ou ainda por estresse psicológico (Sato et al., 1992; Konuma et al., 1994; Buffenstein et al., 1999). Segundo o DSM-IV-TR, o início do TB induzido por AMPHs pode ocorrer durante a intoxicação ou a abstinência. De modo geral, a intoxicação está associada a características maníacas ou mistas; a abstinência, a característica de humor depressivo (Kaplan & Sadock, 2007) produzindo sintomatologia semelhante à encontrada nas distintas fases do TB.

1.2. Mecanismo de Ação das Anfetaminas

Uma vez no SNC a AMPH entra no neurônio através dos transportadores de dopamina (DA). Dentro do neurônio a AMPH se liga a transportadores vesiculares de monoaminas, facilitando a exocitose vesicular e conseqüentemente aumentando a liberação de DA na fenda sináptica. Em concentrações mais elevadas, as AMPHs também podem atravessar as membranas celulares independentemente da vinculação a um transportador (Rusyniak, 2011). Além disso, o alto pKa da AMPH dificulta o gradiente de prótons - que normalmente mantém as monoaminas dentro da vesícula - fazendo com que as monaminas sejam liberadas da vesícula e se acumulem no citoplasma, onde serão transportadas para fora da célula através dos transportadores reversos de dopamina (Brandle et al., 1992; Fleckenstein et al., 2007; Schep et al., 2010; Rusyniak, 2011). Além de aumentar a liberação de DA, as AMPHs também diminuem a sua recaptação e degradação (Suzuki et al., 1980), provocando um aumento rápido e prolongado nas concentrações extracelulares desse neurotransmissor (Rusyniak, 2011).

Estudos sugerem que os déficites cognitivos devido ao uso crônico das AMPHs podem ser ainda devido a alterações no sistema glutamatérgico (Simões et al., 2007; Gross & Marshall, 2009). Alguns trabalhos salientam a existência de projeções glutamatérgicas no circuito de recompensa mesolímbico-dopaminérgico, o que possibilita uma base anatômica de interação entre os dois sistemas (Gass & Olive, 2008). Foi relatado também que o aumento dos níveis extracelulares de DA estriatal pode causar uma libertação secundária de glutamato na via corticostriatal, através do circuito estriato-tálamo-corticostriatal (Mark et al., 2004; 2007). Juntos esses estudos sugerem que o aumento dos níveis de glutamato desempenha um papel importante na neurotoxicidade induzida pelas AMPHs (Nash & Yamamoto, 1992; Mark et al., 2004, 2007; Caligiuri & Buitenhuys, 2005).

Os efeitos clínicos subjacentes associados ao abuso das AMPHs envolvem a estimulação excessiva do sistema nervoso simpático, sendo esta ativação a principal responsável pela toxicidade dessa droga (Schep et al., 2010; Rusyniak, 2011). Foi demonstrado em estudos clínicos e pré-clinicos que a administração crônica de AMPH provoca danos aos terminais nervosos, levando ao esgotamento de DA, que por sua vez faz com que o usuário crônico tenha a capacidade diminuída em sentir prazer (Anedonia), contribuindo, conseqüentemente, para o abuso da substância. A administração repetida de AMPH em animais resulta em sensibilização comportamental, manifestada por aumento da locomoção e comportamento estereotipado (Rusyniak, 2011). Essa sensibilização parece envolver tanto o glutamato quanto a DA, e, mais recentemente, a DA mediando diminuição de acetilcolina (Pierce & Kalivas, 1997; O'Sullivan et al., 2009; Aliane et al., 2010).Esses danos neurais induzidos pelas AMPHs são eventos complexos, que envolvem o aumento das concentrações intracelulares e extracelulares, principalmente, de DA e glutamato, os quais induzem uma cascata de eventos que inclui o estresse oxidativo a neuroinflamação e neurotoxicidade excitatória (Yamamoto et al., 2010).

1.3. Transtorno Bipolar

O Transtorno Bipolar (TB) é um dos mais graves transtornos psiquiátricos, caracterizado pela presença de episódios recorrentes de mania e depressão (Belmaker, 2004). Apesar da importância desse transtorno, a precisa origem do TB não foi estabelecida. Sabe-se que múltiplos fatores podem estar envolvidos (genéticos, bioquímicos, psicodinâmicos e sócio-ambientais).

A Organização Mundial de Saúde (OMS) estima que o TB seja a 5ª maior causa de incapacitação no mundo (Murray & Lopez, 1997). Mais de dois milhões de adultos

americanos, ou aproximadamente 1% da população, tem TB (Spearing, 2001; Hirschfeld et al., 2003). Segundo o Estudo da Área de Captação Epidemiológica do Instituto Nacional de Saúde Mental (ECA-NIMH), conduzido nos Estados Unidos, a taxa anual de incidência para TB foi de 0,5%, muito próximo da prevalência anual de 0,6% (Lima et al., 2005). A alta prevalência de TB é particularmente digna de nota por causa do significativo prejuízo associado a esta condição, não somente aos pacientes, mas também aos parentes e cuidadores (Ten Have et al., 2002; Calabreze et al., 2003). Pacientes bipolares passam metade de suas vidas doentes, sendo que a maioria dos dias em depressão (Judd et al., 2003).

Talvez esta seja a doença mental com uma das maiores taxas de suicídio para casos não tratados com índice 30 vezes maior do que o encontrado na população geral, o que representa um enorme desafio para pacientes, familiares e médicos. A taxa de suicídio no TB foi estimada em 19%, podendo igualar-se (e talvez superando) a de transtorno depressivo maior (Goodwin & Jamison, 1990) e cerca de um terço dos indivíduos afetados pelo TB admite, pelo menos, uma tentativa de suicídio (Müller-Oerlinghausen et al., 2002). Embora a fase de mania, especificamente, identifique a doença, é na fase depressiva onde ocorre o maior índice de suicídios (Baldessarini, 1999; Judd et al., 2002).

O termo bipolar expressa dois pólos de humor ou dois estados afetivos que se alternam nesse transtorno: a depressão e seu oposto a hipomania ou mania dependendo da gravidade (Lara, 2004). O TB é subdividido em vários tipos que compõem o espectro bipolar, porém a classificação mais utilizada o divide em três principais tipos: TB do tipo I apresenta transtornos de humor recorrentes, com um ou mais episódios maníacos ou mistos, ou ambos maníacos e episódios mistos e pelo menos um episódio depressivo maior. O TB do tipo II é caracterizado por um ou mais episódios de depressão maior e pelo menos um episódio hipomanico. Finalmente, o TB do tipo III é diagnosticado como hipomania associada a antidepressivos, caracterizada por pacientes que apresentam episódios de hipomania ou mania

quando em uso de antidepressivos ou psicoestimulantes. Usualmente ocorre em pacientes com temperamento ciclotímico prévio (Müller-Oerlinghausen et al., 2002; Alcantara et al., 2003).

No episódio maníaco, o indivíduo apresenta o humor elevado, expansivo ou irritável, autoestima exagerada ou grandiosidade, diminuição da necessidade do sono, pensamento e fala acelerados, geralmente com fuga de ideias, distratibilidade, aumento da libido, hiperatividade ou agitação psicomotora praticamente incontrolável e aumento da exposição a riscos (ex: gastos excessivos, hipersexualidade) (CID-10, 1993). Nos episódios típicos de cada um dos três graus de depressão – leve, moderado ou grave - o paciente apresenta o humor deprimido, perda de interesse ou prazer nas atividades, aumento ou perda de peso, insônia ou hipersonia, lentidão psicomotora importante, agitação, perda de apetite, perda da libido, fadiga ou perda da energia, sentimento de inutilidade ou culpa, podendo ser acompanhada por delírios, diminuição da capacidade de pensar ou concentração e pensamentos de morte ou ideação suicida (CID-10, 1993).

O diagnóstico precoce e tratamento podem ajudar a reduzir a gravidade associada a esta condição. Entretanto, pacientes com TB - quando buscam tratamento - geralmente se apresentam no estágio depressivo e assim eles freqüentemente são diagnosticados apenas como depressão maior (Manning, 1997; Hirschfeld et al., 2003). Um diagnóstico errado pode levar a uma terapia inapropriada, ou seja, a monoterapia com antidepressivos, que pode levar o paciente bipolar ao estado de mania aguda, estados mistos ou estados cíclicos rápidos (Peet, 1994; Ghaemi et al., 2004).

Mania ou hipomania são tratadas com fármacos antipsicóticos, anticonvulsivantes, ou sais de Lítio (Li) e algumas vezes suplementados com um potente sedativo em curto prazo. Os sais de Li e certos anticonvulsivantes, como Valproato (VPA), têm propriedades de estabilizadores do humor, e são usados para prevenção em longo prazo de recorrências (Baldessarini, 2001).

O tratamento de indivíduos com TB é uma tarefa altamente complexa, envolvendo estratégias distintas nas diferentes fases da doença: mania, depressão e eutimia (Cheniaux, 2011). Atualmente, tem-se dado uma grande valorização dos sintomas depressivos no TB. A grande questão, entretanto, após o diagnóstico correto, é saber quando, se e como se institui uma terapêutica antidepressiva na depressão bipolar, pois o risco de virada hipomaníaca ou maníaca é sempre uma incógnita com a introdução de um antidepressivo (Shansis & Cordioli, 2005). Aproximadamente 25 a 40% dos pacientes bipolares apresentam pelo menos uma virada deste tipo ao longo da vida associada ao uso de antidepressivos e há uma tendência de que esta ocorra no mesmo paciente independentemente da classe de antidepressivo que ele esteja usando (Goldberg & Truman, 2003). Até o momento, receberam aprovação do *Food and Drug Administration* (FDA) - a agência americana responsável pelo controle de medicamentos e alimentos - a combinação olanzapina-fluoxetina e a quetiapina para o tratamento da depressão bipolar (Cheniaux, 2011).

1.4. Lítio e Valproato

O Li e o VPA são fármacos clássicos usados no tratamento do TB e ambos possuem boa ação antimaníaca e modesta ação antidepressiva. Os mecanismos de ação de Li e VPA ainda não estão bem descritos, porém sabe-se que eles agem sobre diversos sistemas de neurotransmissores e cascatas de sinalização intracelulares. Alguns sistemas em que Li e VPA agem estão descritos abaixo.

Evidências indicam que o TB está associado a uma desregulação dopaminérgica. Em apoio a esta hipótese, temos que os fármacos que inibem a transmissão dopaminérgica exercem uma ação anti-maníaca no TB, enquanto drogas que estimulam a síntese de DA, ativam os receptores de DA ou inibem a recaptação de DA, muitas vezes desencadeando

sintomas de mania (Yatham 2002; Schatzberg 2004; Silverstone & Silverstone, 2004). Porém, enquanto neurolépticos convencionais e anti-psicóticos, que também são eficazes no tratamento da mania aguda (McElroy e Keck 2000), agem diretamente inibindo os receptores DA (Seeman & Lee, 1975; Creese et al 1976), os estabilizadores de humor Li e VPA agem indiretamente nestes mesmos receptores por um mecanismo de ativação de segundos mensageiros ativados por receptores do tipo D2 (Yatham et al. 2002).

Além disso, ambos os estabilizadores do humor, Li e VPA, atenuam a função glutamatérgica atravez de múltiplos mecanismos. A administração crônica desses fármacos promove o aumento da recaptação do glutamato (Dixon & Hokin, 1998), atenuando a função dos seus receptores (Nonaka et al., 1998; Du et al., 2004) e, consequentemente, reduzindo a cascata de sinalização intracelular que são ativadas pela ligação do glutamato ao seu receptor (Manji & Lenox, 1999). Outro mecanismo de ação do VPA que é bem descrito na literatura é em relação ao metabolismo de GABA, em diferentes fases da transmissão gabaérgica, aumentando significativamente os níveis desse neurotransmissor, bloqueando a entrada de sódio e facilitando a saída de potássio (Post et al., 1992).

Vários estudos têm demonstrado que os estabilizadores do humor, Li e VPA, também exercem efeitos neurotróficos. Tanto Li quanto VPA ativam o promotor IV de fator neurotrófico derivado do cérebro (BDNF), elevando os níveis dessa neurotrofina no hipocampo (Einat et al, 2003; Frey et al, 2006; Yasuda et al, 2009). O BDNF é importante para a neurogênese, diferenciação e sobrevivência neuronal. Essa neurotrofina é expressa em grande quantidade no cérebro, principalmente, em áreas que estão ligadas a cognição e comportamento emocional como o hipocampo e a amídala (Strakowski et al., 2005). Diversos estudos mostram que o BDNF está envolvido na fisiopatologia do TB, encontrando-se diminuído tanto nas fases maníacas quanto nas depressivas (Cunha et al., 2006; Grande et al., 2010).

Foi demonstrado que, em concentrações terapêuticas Li e VPA são inibidores da enzima glicogênio sintase quinase 3 (GSK-3) (Klein & Melton, 1996). A GSK-3 é uma proteína constantemente ativada, regula múltiplos substratos de serina/ treonina e é regulada por diversas vias de sinalização (ex. Wnt, PI$_3$K, PKA, PKC entre muitas outras) (Doble & Woodgett, 2003). Em mamíferos existem duas isoformas de GSK3: GSK-3α e GSK-3β. Essa enzima regula apoptose e plasticidade celular (Crowder & Freemam, 2000; Franco et al., 2004; Zhao et al., 2007), sendo que a atividade aumentada de GSK-3 estimula processos apoptóticos enquanto que sua inibição atenua ou previne apoptose (Gould et al., 2006).

Outro alvo de Li e VPA é a proteína quinase C (PKC). A inibição da PKC foi sugerida como um dos mecanismos dos estabilizadores do humor de grande importância (Manji & Lenox, 2000; Manji & Chen, 2002). A PKC é encontrada principalmente no cérebro, sendo essencial para os processos de neurotransmissão pré e pós-sináptica, regulando a excitabilidade neuronal, a liberação dos neurotransmissores e a plasticidade celular (Zarate & Manji, 2009). Estudos pré-clinicos mostram que a administração do Li leva a redução de PKC no córtex frontal e hipocampo de ratos (Lenox et al., 1992; Manji et al., 1993; 1999). Chen e colegas (1994) mostram que VPA produz efeitos similares aos do Li, sugerindo que a inibição da PKC tem também um importante papel nos efeitos terapêuticos desse estabilizador do humor.

1.5. Modelo Animal de Mania Induzido por Anfetamina

Apesar do TB ser um transtorno psiquiátrico comum que leva a sérios problemas de saúde, pouco se sabe sobre sua fisiopatologia. Como discutido anteriormente, o TB é um transtorno multifatorial que possui diversos sintomas, incluindo episódios de mania recorrentes, depressão e estados mistos - o que dificulta o desenvolvimento de um modelo

animal adequado (Machado-Vieira et al., 2004). Embora haja uma grande dificuldade em mimetizar o TB em animais, vários modelos animais de mania ou depressão têm sido desenvolvidos com o intuito de mimetizar alguns aspectos comportamentais dessas condições psiquiatricas (Machado-Vieira et al., 2004; Frey et al., 2006a; Herman et al., 2007; Jornada et al., 2010).

Os modelos animais são considerados importantes ferramentas para o estudo do TB, através dos quais, tem-se proposto novos sistemas de neurotransmissão e vias de sinalização intracelular envolvidas na modulação desta psicopatologia (Machado-Vieira et al., 2004). Para ser válido um modelo animal em transtornos psiquiátricos devem ser consideradas três características principais: 1) se o modelo mimetiza os sintomas da doença determinada (validade de face), 2) a habilidade do modelo em reproduzir alguns aspectos fisiopatológicos da doença (validade de constructo) e, finalmente, 3) se os agentes terapêuticos usados no tratamento revertem os sintomas induzidos no modelo animal (validade preditiva) (Ellenbroek & Cools, 1990).

A hiperatividade induzida por psicoestimulantes em ratos é o modelo animal de mania mais aceito na literatura; porque contém os três principais critérios para o desenvolvimento de um modelo animal adequado. A administração de AMPHs em roedores induz hiperlocomoção, comportamento de risco, insônia e aumento da atividade sexual (validade de face); além disso, aumenta os níveis de DA no cérebro – também visto em pacientes bipolares - (validade de constructo) e responde a fármacos antimaníacos como antipsicóticos, Li e VPA (validade preditiva) (Fiorino & Phillips, 1999; Frey et al., 2006 b,c,d).

Como descrito anteriormente, os tipos mais comum de AMPHs são a d-AMPH e a m-AMPH, que são análogas e possuem estruturas químicas semelhantes (Hoffman & Lefkowitz, 1996). Recentemente, nosso grupo de pesquisa mostrou que m-AMPH induz dano oxidativo maior que d-AMPH (da-Rosa et al., 2011). Além disso, Li e VPA revertem e previnem

hiperatividade induzida por m-AMPH somente quando administrada em uma dose muito baixa, de 0,25 mg/kg, (da-Rosa, 2011), enquanto que esses estabilizadores do humor revertem e previnem a hiperatividade induzida por d-AMPH na dose de 2 mg/kg (Frey et al., 2006a,b,c,d).

1.6. O Metabolismo Energético no Transtorno Bipolar

Os seres vivos precisam de energia para realizar várias funções, como, por exemplo, o transporte ativo de íons e moléculas, síntese de macromoléculas e outras biomoléculas. A maior parte da energia necessária para realizar essas funções é obtida com a oxidação de substâncias pela respiração celular. O ATP é o principal combustível da célula na maioria dos processos que precisam de energia. A energia é liberada pela hidrólise de ATP e serve para impulsionar uma série de reações (Nelson & Cox, 2000).

Vários estudos têm sugerido que alterações no metabolismo energético celular têm um papel chave no TB. Anormalidades no metabolismo energético cerebral de pacientes bipolares foram encontradas a partir de testes de neuroimagem funcional e espectroscopia de ressonância magnética (Deicken et al., 1995; Dager et al., 2004; Frey et al., 2007; Regenold et al., 2009).

Após a glicólise, o piruvato é descarboxilado a acetil CoA pela piruvato desidrogenase. A oxidação completa de acetil CoA no ciclo de Krebs resulta na produção de NADH, $FADH_2$ e GTP. O ciclo de Krebs é um sistema bioquímico composto por várias enzimas e etapas. Na primeira etapa, a enzima citrato sintase (CS) catalisa a condensação de oxalaceto com grupamento acetil-coA, visando a formação de ácido cítrico (Shepherd and Garland, 1969). Na ultima etapa do ciclo de Krebs, a enzima malato desidrogenase (MD) catalisa a desidrogenação de malato à oxaloacetato (Kelly et al., 1989). A succinato desidrogenase (SD)

faz parte tanto do ciclo de Krebs quanto da cadeia de transporte de elétrons (complexo II) por isso essa enzima é um dos marcadores mais importantes do ciclo de Krebs (Tyler, 1992).

A disfunção no ciclo de Krebs pode alterar a taxa de metabolismo no cérebro e a produção de radicais livres. O ciclo de Krebs ocorre na matriz mitocondrial e consiste de uma sequência de reações onde, em cada volta do ciclo, são formadas três moléculas de NADH, uma de $FADH_2$, duas de CO_2 e uma de GTP. O NADH e $FADH_2$ produzidos no ciclo de Krebs são carreadores de elétrons e são oxidados na cadeia respiratória para a produção de ATP pela fosforilação oxidativa (Marks et al., 1996; Nelson & Cox, 2000) (Figura 1).
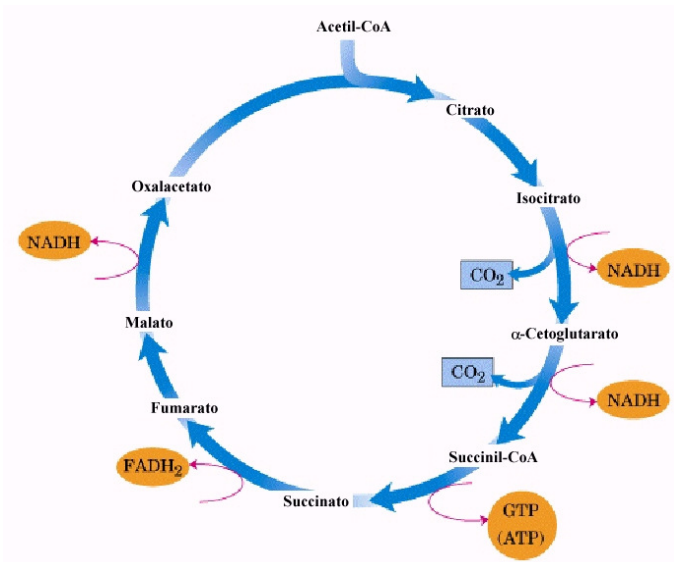

Figura 2. Ciclo de Krebs (Adaptado de NELSON e COX, 2000).

Após o ciclo de Krebs, ocorre outro processo chamado fosforilação oxidativa, no qual os elétrons passam por uma série de complexos enzimáticos (complexo I, II, III, IV) - localizados na membrana interna da mitocôndria – juntamente com essa transferência ocorre o bombeamento de prótons para o espaço intermembrana. O gradiente eletroquímico resultante desse processo permite que o complexo adenosina 5'-trifosfato ATP sintase, ou complexo V, sintetize ATP a partir de ADP mais fosfato inorgânico (Pi) (Horn & Barrientos, 2008) (Figura 3).
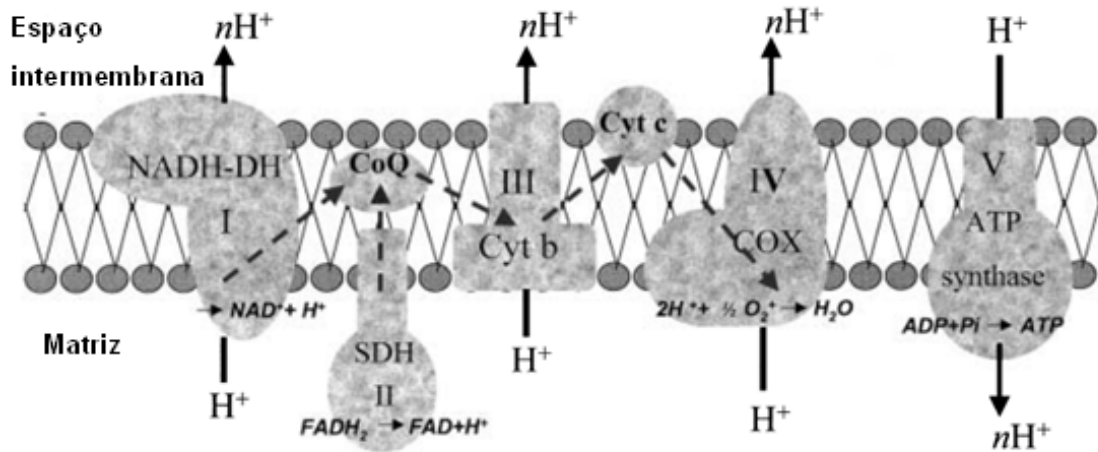
Figura 3. Formação de ATP pela Cadeia de Transporte de elétrons. I = complexo I, II = complexo III = complexo III, IV = complexo IV, V = complexo V Representação esquemática da cadeia respiratória mitocondrial (Adaptado de Ben-Schachar, 2002).

Estudos *postmortem* de neuroimagem e genéticos têm sugerido que a diminuição da função mitocondrial com consequente prejuízo no metabolismo energético celular é uma hipótese atrativa para explicar a fisiopatologia da bipolaridade. Um estado energético celular anormal pode levar à perda da função e da plasticidade neuronal e, consequentemente, a alterações cognitivas e comportamentais características do TB (Steckert et al., 2010).

Estudos clínicos também demonstram alterações na enzima creatina quinase (CK) em pacientes com TB (Vale et al.,1974; Segal et al., 2007). A CK é uma enzima que catalisa a transfosforilação reversível da creatina pelo trifosfato de adenosina e desempenha um papel fundamental na reconstituição e no transporte de energia, principalmente, em células com elevado consumo de energia, incluindo neurônios, miócitos e cardiomiócitos (Andres et al., 2008).

2. Justificativa

O conhecimento bioquímico e enzimático peculiar ao TB é uma ferramenta importante para o desenvolvimento de novos fármacos e para auxiliar na compreensão da fisiopatologia desenvolvida neste transtorno do humor, melhorando assim, a terapêutica utilizada bem como o prognóstico dos pacientes bipolares. Para tanto, faz-se necessário o desenvolvimento de um modelo animal que mimetize o TB, bem como o conhecimento do metabolismo energético envolvido nesta patologia tanto em um modelo animal de TB quanto em pacientes bipolares.

3. Objetivo

Avaliar alterações comportamentais e parâmetros do metabolismo energético no sangue periférico de pacientes bipolares e em cérebro de ratos em um modelo animal de mania induzido por metanfetamina.

3.1. Objetivos Específicos

1) Avaliar os efeitos da administração aguda de dextro-anfetamina e meta-anfetamina sobre a atividade locomotora, exploratória, comportamento de risco e estereotipia em ratos Wistar.

2) Avaliar os efeitos da administração aguda de dextro-anfetamina e meta-anfetamina sobre a atividade das enzimas do ciclo de Krebs (Citrato Sintase, Succinato Desidrogenase e Malato Desidrogenase) em cérebro de ratos Wistar.

3) Avaliar os efeitos da administração aguda de dextro-anfetamina e meta-anfetamina sobre a atividade dos complexos da cadeia respiratória mitocondrial (Complexo I, II, II-III e IV) em cérebro de ratos Wistar.

4) Avaliar os efeitos da administração aguda de dextro-anfetamina e meta-anfetamina sobre a atividade da enzima Creatina Quinase em cérebro de ratos Wistar.

5) Avaliar a atividade locomotora e exploratória em um modelo animal de mania induzido por meta-anfetamina.

6) Avaliar os efeitos da administração crônica de meta-anfetamina na atividade das enzimas do ciclo de Krebs (Citrato Sintase, Succinato Desidrogenase e Malato Desidrogenase) em um modelo animal de mania induzido por meta-anfetamina.

7) Avaliar os efeitos da administração crônica de meta-anfetamina na atividade dos complexos da cadeia respiratória mitocondrial (Complexo I, II, II-III e IV) em um modelo animal de mania induzido por meta-anfetamina.

8) Avaliar os efeitos da administração crônica de meta-anfetamina na atividade da enzima Creatina Quinase em cérebro de ratos Wistar em um modelo animal de mania induzido por meta-anfetamina.

9) Avaliar a atividade da enzima Creatina Quinase em sangue periférico de pacientes bipolares, comparando as fases de mania, depressão e eutimia.

4. Artigo Cientifico

4.1. Artigo Cientifico 1

BEHAVIORAL CHANGES AND BRAIN ENERGY METABOLISM DYSFUNCTION IN RAT TREATED WITH METHAMPHETAMINE OR DEXTROAMPHETAMINE.

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Behavioral changes and brain energy metabolism dysfunction in rats treated with methamphetamine or dextroamphetamine.

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Abstract

Previous studies have demonstrated that amphetamines (AMPHs), such as dextroamphetamine (d-AMPH) and methamphetamine (m-AMPH) differentially induce oxidative damage in the rat brain. However, the mechanism to explain this difference is unknown. It is well described in literature that AMPHs inhibit the mitochondrial respiratory chain and cause changes to behaviour when administered to experimental animals. Besides this, mitochondria play a key role in energy metabolism and are also the main producers of reactive oxygen species as well as being the source of pro- and anti-apoptotic key factors. The goal of the present study was thus to investigate the potency of the two drugs on the behaviour and energetic dysfunction in the brain of rats. Male adult Wistar rats were given single intraperitoneal injections of saline (0.9% NaCl), d-AMPH (2 mg/kg) or m-AMPH (0.25, 0.5, 1 or 2 mg/kg). The animals were submitted to the open field task for behavioral assessment. The energy metabolism parameters were measured in the prefrontal cortex, amygdala, hippocampus and striatum. D-AMPH and m-AMPH (all doses administered) increased the crossing and rearing behaviours of animals, but no significant difference between d-AMPH and m-AMPH was observed. The numbers of visits to the centre were increased by d-AMPH and m-AMPH only at 2 mg/kg. Likewise, at a high dose (2mg/kg), the injection of m-AMPH increased the amount of sniffing, but this was not seen with d-AMPH. The AMPHs significantly decreased the activities of Krebs cycle enzymes (citrate synthase and succinate dehydrogenase), mitochondrial respiratory chain complexes (I, II, III, IV) and creatine kinase; nevertheless, this effect varied depending on the brain region evaluated. However, only in the prefrontal did m-AMPH (at 0.5 and 1 mg/kg) induce complex IV inhibition greater than d-AMPH. In summary, this study demonstrated that at high doses, m-AMPH, increased stereotyped (sniffing) behaviour in rats, but d-AMPH did not. However, this study shows that d-AMPH and m-AMPH seem to have similar effects on the brains energetic metabolism.

Introduction

Amphetamines (AMPHs) are central nervous system stimulants and act on multiple sites of pre-synaptic dopaminergic neurotransmission, however, the three primary targets are considered to be competitive blockade of the dopamine transport (DAT), depletion of vesicular dopamine (DA) stores, and reversal of DAT function driving a non-exocytotic form of DA release called efflux (Kuczenski and Segal 1994; Fleckenstein et al. 2007).

The abuse of AMPHs has become a major public health problem worldwide and the use of these stimulants has significant psychiatric and medical consequences, including psychosis, dependence, overdose and death (Chen et al., 2010) plus there are concerns about potential neurotoxicity (Volkow et al., 2001). Clinical studies have shown that the chronic use of AMPHs leads to functional alterations and neurodegerative changes in various brain regions (Wilson et al., 1996; Ernst et al., 2000).

AMPHs are synthetic chemicals with a structure similar to amphetamine, comprising a broad range of psychoactive derivatives such as methamphetamine (m-AMPH) and dextroamphetamine (d-AMPH). Previous studies demonstrated that amphetamines (AMPHs), such as dextroamphetamine (d-AMPH) and methamphetamine (m-AMPH) differentially induce oxidative damage in the rat brain. However, the mechanism to explain this difference is unknown (da-Rosa et al., 2011).

Mitochondria are membrane-enclosed organelles which generate most of the cell's supply of adenosine triphosphate (ATP) by a process called oxidative phosphorylation (Calabrese et al., 2001). Electrons are passed along a series of respiratory enzyme complexes located in the inner mitochondrial membrane, and the energy released by this electron transfer is used to pump protons across the membrane. The resultant electrochemical gradient enables another complex, adenosine 5′-triphosphate (ATP) synthase, to synthesize the energy carrier ATP (Horn and Barrientos, 2008).

Citrate synthase (CS) is localized in the mitochondrial matrix and catalyzes the condensation of oxaloacetate and the acetyl group of acetyl coenzyme-A, the first step of Krebs cycle (Shepherd and Garland, 1969). On the other hand, malate dehydrogenase (MD) catalyzes the dehydrogenation of l-malate to oxaloacetate in the final step of TCA cycle (Kelly et al., 1989). Succinate dehydrogenase (SD) is still one of the most reliable markers of the mitochondrial capability to supply an adequate amount of ATP, as it is part of both the Krebs cycle and the respiratory chain (complex II) (Tyler, 1992).

The increase in extracellular DA concentration induced by AMPHs (Sulzer and Rayport 1990) causes overproduction of the toxic metabolite of DA oxidation (Wrona et al. 1997), leading to oxidative damage to proteins, lipids and DNA in the neurons (Gluck et al. 2001, Eyerman and Yamamoto 2007; Yamamoto & Zhu 1998). Valvassori and colleagues (2010) showed that chronic amphetamine administration resulted in a marked inhibition of complexes I, II, III and IV of the mitochondrial respiratory chain in the rat brain. It is well described in literature that mitochondria are the major source of reactive oxygen species (ROS), which are produced in the complexes of the electron transport chain (Mattiasson et al., 2003). Moreover, a shift in the antioxidant/pro-oxidant balance toward oxidative stress may inhibit electron transport chain complexes, leading to decreases in ATP production and cellular dysfunction (Calabrese et al., 2001).

Besides that, creatine kinase (CK) is an enzyme that catalyses the reversible transphosphorylatin of creatine by ATP and plays a key role in cellular energy buffering and energy transport, particularly in cells with high and fluctuating energy requirements, including neurons. The CK system plays a significant role in the brain, and a functional impairment of this system leads to a deterioration in energy metabolism (Andres et al., 2008).

In the present study m-AMPH and d-AMPH were compared to determine the potency of the two drugs on behavior, activities of Krebs cycle enzymes (citrate synthase and

succinate dehydrogenase), mitochondrial respiratory chain complexes (I, II, III, IV) and creatine kinase energetic metabolism in the brain of rats.

Experimental methods

Animals

The subjects were adult male Wistar rats (weighting 250–350 g) obtained from our breeding colony. The animals were housed five to a cage, with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) at a temperature of 22 ± 1°C. All experimental procedures were performed in accordance with, and with the approval of the local Ethics Committee in the use of animals at the Universidade do Extremo Sul Catarinense. All experiments were performed at the same time during the day to avoid circadian variations.

Drugs and pharmacological procedures

The drugs, d-AMPH and m-AMPH (Sigma, St Louis, Missouri, USA), were dissolved in saline (0.9% NaCl). The solutions were prepared immediately before use and were protected from the light during the experimental session. The total number of rats used in this experiment were 72 (n = 12 animals per group). Animals received a single injection of m-AMPH (0.25, 0.5, 1 or 2 mg/kg body weight) or d-AMPH (2 mg/kg body weight) in a volume of 1 mL/kg, administered intraperitoneally (i.p.). The control group received an injection of saline (0.9% NaCl) in a volume of 1 mL/kg. Locomotor activity was measured 2 h after the injection and the rats were killed by decapitation right after the open-field task.

Behavior patterns of rat in the open field test

The task was performed in a 40×60 cm open field surrounded by 50 cm high walls. The floor of apparatus was constructed from varnished wood and divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle and left to explore the arena for 5 min. The following behavioural parameters were assessed in the open field test:

Crossings: Total number of square crossings during the entire test period (Ericson et al., 1991; Prut and Belzung, 2003; Wultz et al., 1990).

Rearings: Total number of erect postures during the entire test period (Ericson et al., 1991; Prut and Belzung, 2003).

Visits to center: Total number of visits to the centre of open-field. A center square of 30x30cm was defined as the "center" area of the field.

Grooming: Total time (in seconds) of grooming behaviour during the entire test period. Include rat paw licking, nose/face grooming, head washing, body grooming/scratching, leg licking and tail/genitals grooming (Kalueff and Tobimaa, 2004, 2005; Kalueff et al., 2007).

Sniffing: Total time (in seconds) of sniffing behaviour during the entire test period. Rat sniffs the environment in moving (walking + rearing) (Casarrubea et al., 2008, 2009a, 2009b, Meyerson and Höglund, 1981).

Tissue and homogenate preparation

The prefrontal cortex, amygdala, hippocampus and striatum were removed and homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at $800 \times g$ for 10 min at 4°C and the supernatants kept at −70oC until being used for enzymes activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days.

The protein content was determined by the method described by Lowry and colleagues (Lowry et al., 1951) using bovine serum albumin as standard.

Activities of enzymes of Krebs cycle

Citrate synthase activity: Citrate synthase activity was assayed according to the method described by Shepherd and Garland (1969). The reaction mixture contained 100 mM Tris, pH 8.0, 100 mM acetyl CoA, 100 mM 5,5′-di-thiobis-(2- nitrobenzoic acid), 0.1% triton X-100, and 2–4 μg supernatant protein and was initiated with 100 μM oxaloacetate and monitored at 412 nm for 3 min at 25 °C.

Malate dehydrogenase activity: Malate dehydrogenase was measured as described by Kitto (1969). Aliquots (20 mg protein) were transferred into a medium containing 10 mM rotenone, 0.2% Triton X-100, 0.15 mM NADH, and 100 mM potassium phosphate buffer, pH 7.4, at 37°C. The reaction was started by the addition of 0.33 mM oxaloacetate. Absorbance was monitored as described above.

Succinate dehydrogenase activity: Succinate dehydrogenase activity was determined according to the method of Fischer and colleagues (1985), and measured by following the decrease in absorbance due to the reduction of 2,6-di-chloro-indophenol (2,6-DCIP) at 600nm with 700nm as a reference wavelength ($\varepsilon = 19.1$ mM-1 cm-1) in the presence of phenazine methasulphate (PMS). The reaction mixture consisting of 40mM potassium phosphate, pH 7.4, 16mM succinate and 8μM 2,6-DCIP was pre-incubated with 40–80μg homogenate protein at 30oC for 20 min. Subsequently, 4mM sodium azide, 7μM rotenone and 40μM 2,6-DCIP were added and the reaction was initiated by the addition of 1mM PMS and was monitored for 5 min.

Activities of mitochondrial respiratory chain enzymes

Complex I activity: NADH dehydrogenase (complex I) was evaluated according to Cassina and Radi (1996) by the determination of the rate of NADH-dependent ferricyanide reduction at $\lambda = 420$ nm.

Complex II activity: The activities of succinate-2,6- dichloroindophenol (DCIP)-oxidoreductase (complex II) was determined by the method described by Fischer and colleagues (1985). Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at $\lambda = 600$ nm.

Complex II-III activity: The activity of succinate:cytochrome c oxidoreductase (complex III) was determined by the method described by Fischer and colleagues (1985). Complex II-III activity was measured by cytochrome c reduction using succinate as substrate at $\lambda = 550$ nm.

Complex IV activity: The activity of cytochrome c oxidase (complex IV) was assayed according to the method described by Rustin and colleagues (1994), measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c (prepared by reduction of cytochrome with $NaBH_4$ and HCl) at $\lambda = 550$ nm with 580 nm as the reference wavelength ($\varepsilon = 19.1$ mM-1 cm-1). The activities of the mitochondrial respiratory chain complexes were calculated as nmol. min-1. mg protein-1.

Activity of creatine kinase enzyme

Creatine kinase activity was measured in brain homogenates pretreated with 0.625 mM lauryl maltoside. The reaction mixture consisted of 60mM Tris–HCl, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$ and approximately 0.4–1.2 µg protein in a final volume of 100 µL. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of 3.2 mmol of ADP plus 0.8 mmol of reduced glutathione. The reaction was stopped after 10

min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 100 μL 2%α-naphtol and 100 μL 0.05% diacetyl in a final volume of 1 mL and read spectrophotometrically after 20 min at 540 nm. Results were expressed as units/min×mg protein.

Statistical analysis

Data were analyzed by one-way analysis of variance followed by the Tukey test when F was significant and are expressed as mean ± standard deviation. All analyses were performed using the Statistical Package for the Social Science (SPSS; version 16.0) software.

Results

The effects of d-AMPH and m-AMPH administration on open-field behaviors:

The results for locomotor activity are shown in Fig. 1A. We replicated here previous data from our group (da-Rosa et al., 2011), the administration of d-AMPH (2 mg/kg) and m-AMPH at 0.25, 0.5, 1 and 2 mg/kg increased rat spontaneous locomotion (crossing) and exploration (rearing), when compared with the control group. No significant difference was observed between any dose of m-AMPH and d-AMPH, indicating that all tested doses of m-AMPH are equivalent to 2 mg/kg of d-AMPH in activating locomotor and exploratory activity. The administration of d-AMPH and m-AMPH at 2 mg/kg increased visits to center of open-field, suggesting that the two drugs at a dose of 2mg/kg increased risk behavior in animals. In addition, m-AMPH at 2mg/kg displayed an increase in sniffing (Fig. 1B), indicating stereotyped behavior.

The effects of d-AMPH and m-AMPH administration on activities of enzymes of Krebs cycle. Citrate synthase (CS), malate dehydrogenase (MD) and succinate dehydrogenase (SD) activities were measured in prefrontal cortex, hippocampus and striatum of rats.

m-AMPH administration at 1 mg/kg inhibited CS activity in the prefrontal and hippocampus of rats. Administration of d-AMPH (2 mg/kg) and m-AMPH at 0.5, 1 and 2 mg/kg also inhibited CS in the amygdala and striatum of rats (Fig. 2A). We also observed decreased activity of SD (Fig. 2B) in the striatum after d-AMPH (2mg/kg) and m-AMPH at 0.5 and 1 mg/kg. No change was observed in MD activity after d-AMPH or m-AMPH injection in any brain structure evaluated (Fig. 2C).

The effects of d-AMPH and m-AMPH administration on activities of mitochondrial respiratory chain enzymes. Complex I, II, III and IV activities were measured in the prefrontal cortex, hippocampus and striatum of rats.

Administration of d-AMPH (2mg/kg) and m-AMPH at all doses (0.25, 0.5, 1 and 2 mg/kg) administered significantly inhibited complex I activity in the rat's amygdala (Fig. 3A). m-AMPH at 0.5 and 1 mg/kg induced a decrease of complex II activity in the amygdala of rats (Fig. 3B). The activity of complex II–III was decreased in prefrontal, amygdala and hippocampus after d-AMPH or m-AMPH at 0.5, 1 and 2 mg/kg administration (Fig. 3C). In the striatum the complex II activity was decreased after d-AMPH and m-AMPH at all doses (0.25, 0.5, 1 and 2 mg/kg) was administered (Fig. 3C). Complex IV activity was also decreased in prefrontal after d-AMPH and m-AMPH at 0.5 and 1 mg/kg; in the amygdala following administration of d-AMPH and m-AMPH at 1mg/kg; in the hippocampus after d-AMPH and m-AMPH at all doses administered and finally in the striatum after d-AMPH and m-AMPH at 0.5, 1 and 2 mg/kg.

The effects of d-AMPH and m-AMPH administration on creatine kinase activity (CK) in the prefrontal cortex, hippocampus and striatum of rats.

d-AMPH and m-AMPH at 0.5, 1 and 2 mg/kg significantly inhibited CK activity in the prefrontal. In the amygdala only the highest dose of m-AMPH (2 mg/kg) decreased activity of CK. Finally, in the striatum d-AMPH decreased and m-AMPH increase de activity of this enzyme.

Discussion

The present study was designed to compare the doses of 0.25, 0.5, 1 or 2 mg/kg of m-AMPH with 2 mg/kg of d-AMPH - known to cause hyperactivity and inhibition of mitochondrial respiratory chain complexes in the brain of rats (Valvassori et al., 2010) - on behavior and energetic metabolism parameters in the prefrontal cortex, amygdala, hippocampus and striatum of rats.

We replicated here previous data from our group (da-Rosa et al., 2011); the administration of d-AMPH (2 mg/kg) and m-AMPH at 0.25, 0.5, 1 and 2 mg/kg were equipotent at increased rat spontaneous locomotion (crossing) and exploration (rearing). An additional element observed in this study was that m-AMPH and d-AMPH both at 2 mg/kg, increased the number of visits to the center of the open-field, showing the equivalence of the two drugs to induce risk-taking behavior in rats. According to Einat (2006), risk-taking behaviour may represent the mirror image of anxiety in many tests. Administration of AMPHs in rodents increases risk-taking behavior by reducing anxiety–like measures such as increasing the time spent in the centre of an open-field (Einat et al., 2003).

In our study, we found that the administration of m-AMPH (but not d-AMPH) in high doses (2 mg/kg), induces sniffing behaviour. AMPHs and nicotine increased stereotypy until 80 min after the administration of these psychotropic drugs in rats (Izawa et al., 2006).

Commonly abused drugs, such as cocaine and methamphetamine stimulate dopamine transmission in the nucleus accumbens, inducing behavioural activation in rodents and other laboratory animals (Di Chiara, 2002).

In the present work, we observed that the activities of enzymes of the Krebs cycle - CS, SD - mitochondrial respiratory chain – complex I, II, II-II, IV - and CK were inhibited in the brain of rats submitted to the administration of d-AMPH and m-AMPH; however, these changes varied according to the brain structure and biochemical analysis. m-AMPH and d-AMPH decreased complex II-III, complex IV and CK activities in the prefrontal; nevertheless, m-AMPH also inhibits the CS activity in this brain structure. In the amygdala, both m-AMPH and d-AMPH reduced CS, complex I, II-III and IV and CK activities; in addition, m-AMPH administration also decreased complex II. In the hippocampus, m-AMPH and d-AMPH administration decreased complex II-III and complex IV activities; although m-AMPH injection also decreased CS activity. Finally, in the striatum, both amphetamines inhibited the activities of CS, SD, complex II-III, complex IV and CK. As can be seen, the effects of m-AMPH and d-AMPH on cerebral metabolism is heterogeneous between the structures.

It is well known that the amygdala modulates the limbic system, controlling an iterative circuit, prefrontal–striatal–thalamic, which controls complex socioemotional behaviours (Strakowski et al., 2000; Strakowski et al., 2005). Amphetamines act on the mesocorticolimbic dopamine system which projects from the ventral tegmental area to the nucleus accumbens, olfactory tubercle, frontal cortex and amygdala (Kalivas and Stewart 1991; Volkow et al. 2003). Above the nucleus accumbens (or ventral striatum) is the dorsal striatum that has been implicated in the locomotor response to amphetamines (Hamamura et al. 1991; Nestler 2001). The frontal cortex is an important cerebral area involved in working memory, in decision making, inhibitory control and in selecting and retaining information to

produce executive control (Royall 2002; Huang et al., 2004; Rinaldi et al., 2007). Glutamatergic projections from the prefrontal cortex to the nucleus accumbens also play an important role in the action of amphetamines (Kalivas et al. 2005). In addition, dopaminergic innervation is critical for long term changes in synaptic efficacy in hippocampus and the interactions between dopamine and glutamate receptors are essential for prefrontal and hippocampal cognitive functions (Gurden et al., 2000; Li et al., 2003; Huang et al., 2004; Granado et al., 2008; Yang et al., 2000; Chen et al., 2004; Tseng and O'Donnell, 2004; Nowak and Corces, 2000).

According to the data presented in this article, previous studies from our and other laboratories have demonstrated that administration of d-AMPH and m-AMPH in rats also inhibited enzymes of energetic metabolism (Valenzuela and Villanueva, 1987; Valvassori et al., 2010; Corrêa et al., 2007; Streck et al., 2008; Moretti et al., 2011; Bachmann et al., 2009). However, there are no studies comparing the two drugs on energy metabolism.

The amphetamines have been associated to long-term deficits in dopaminergic and serotonergic systems in the brain, resulting from dopamine- and glutamate-generated reactive oxygen species (LaVoie et al., 1999; Page et al., 2001). Several studies suggest that dopamine autoxidation can form reactive quinones that attack and potentially inhibit the function of intracellular proteins. In addition to dopamine autoxidation, metabolism of dopamine by monoamine oxidase can increase H_2O_2 production and iron-dependent ROS production (Spina and Cohen, 1989). Monoamine oxidase is located in the outer membrane of mitochondria and could be a significant source of ROS production mainly in dopaminergic neurons (Adam-Vizi, 2005). Mitochondria are the main source of reactive oxygen species (ROS), which are produced in the complexes of the electron transport chain (Mattiasson et al., 2003). Moreover, a shift in the antioxidant/pro-oxidant balance toward oxidative stress may inhibit electron

transport chain complexes, leading to decreases in ATP production and cellular dysfunction (Calabrese et al., 2001).

Oxidative stress induced by the amphetamines (da-Rosa et al., 2011) with consequent impairment in mitochondrial complex activity can be a possible explanation for the results of this study. We note here that in general, even lower doses of m-AMPH (most of the doses administered) were equivalent to 2mg/kg of d-AMPH to inhibit enzymes of energy metabolism. In contrast, a recent study from our laboratory showed that d-AMPH and m-AMPH increased oxidative lipid and protein damage, but m-AMPH even at lower doses, was more potent than d-AMPH (da-Rosa et al., 2011). This discrepancy can be explained at least in part by the fact that mitochondria play a key role in ROS formation but also in the antioxidant response (Starkov, 2008).

Superoxide produced in the mitochondria is rapidly dismutated by enzyme manganese superoxide dismutase in the mitochondrial matrix (Forman and Azzi, 1997) or by copper/zinc superoxide dismutase in the intermembrane space and the cytosol reducing the superoxide radical into hydrogen peroxide (H_2O_2) (Fridovich, 1995; Loschen et al., 1973). H2O2 is also a ROS, which is moderately membrane-permeable and able to leave the mitochondrial matrix and, when produced in excess, is released from the cell. In the presence of Fe^{3+}, hydroxyl radical (•OH), the more reactive radical, is also formed from H_2O_2 and superoxide. On the whole, H2O2 is eliminated by glutathione or it is converted to H_2O in the enzyme reactions by catalase or glutathione peroxidase/glutathione reductase present in both the mitochondrial matrix and the cytosol (Halliwell and Gutteridge, 1999).

In summary, this study demonstrated that at high doses, m-AMPH, (but not d-AMPH) increased stereotyped behaviour (sniffing) in rats. However, this study shows that d-AMPH and m-AMPH seem to have similar effects on brain energetic metabolism.

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Legends to Figures

Figure 1: Open field test. **A)** Free movement in the open field: crossings (dF=5; F=54.842; p<0.0001), rearings (dF=5; F=30.766; p<0.0001) and visits to center (dF=5; 51.101; p<0.0001). **B)** Stereotypy: grooming (dF=5; F=5.869; p<0.0001) and sniffing (dF=5; F=34.515; p<0.0001). *Different from Saline group.

Figure 2: Activities of enzymes of Krebs cycle. **A)** Citrate synthase activity: In the prefrontal (dF=5; F=10.261; p<0.0001), amygdala (dF=5; F=14.819; p<0.0001), hippocampus (dF=5; F=2.809; p=0.034) and striatum (dF=5; F=6.189; p=0.001). **B)** Succinate dehydrogenase activity: In the prefrontal, amygdala, hippocampus and striatum (dF=5; F=8.714; p<0.0001). **C)** Malate dehydrogenase activity: In the prefrontal, amygdala, hippocampus and striatum. *Different from Saline group.

Figure 3: Activities of mitochondrial respiratory chain enzymes. **A)** Complex I activity: In the prefrontal, amygdala (dF=5; F=10.533; p<0.0001), hippocampus and striatum. **B)** Complex II activity: In the prefrontal, amygdala (dF=5; F=3.819; p=0.018), hippocampus and striatum. **C)** Complex II-III activity: In the prefrontal (dF=5; F=18.526; p<0.0001), amygdala (dF=5; F=9.714; p<0.0001), hippocampus (dF=5; F=6.967; p<0.0001) and striatum (dF=5; F= 34.235; p<0.0001). **D)** Complex IV activity: In the prefrontal (dF=5; F=23.848; p<0.0001), amygdala (dF=5; F=6.283; p=0.002), hippocampus (dF=5; F=7.522; p=0.001) and striatum (dF=5; F=8.655; p<0.0001). *Different from Saline group. #Different from d-AMPH 2mg/kg group.

Figure 4: Activity of creatine kinase: In the prefrontal (dF=5; F=12.053; p<0.0001), amygdala (dF=5; F=9.633; p<0.0001), hippocampus and striatum (dF=5; F=13.712; p<0.0001). *Different from Saline group. #Different from d-AMPH 2mg/kg group.
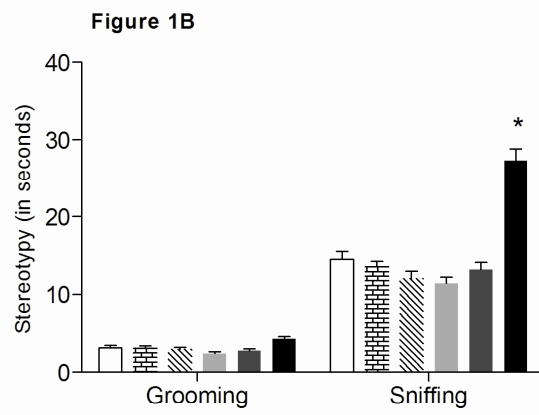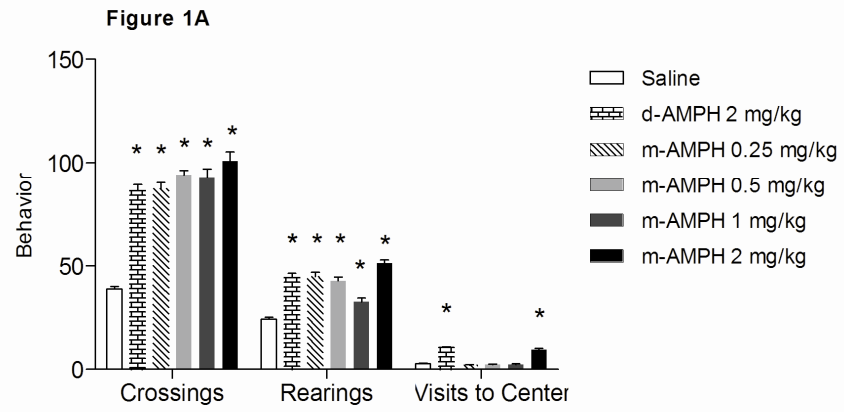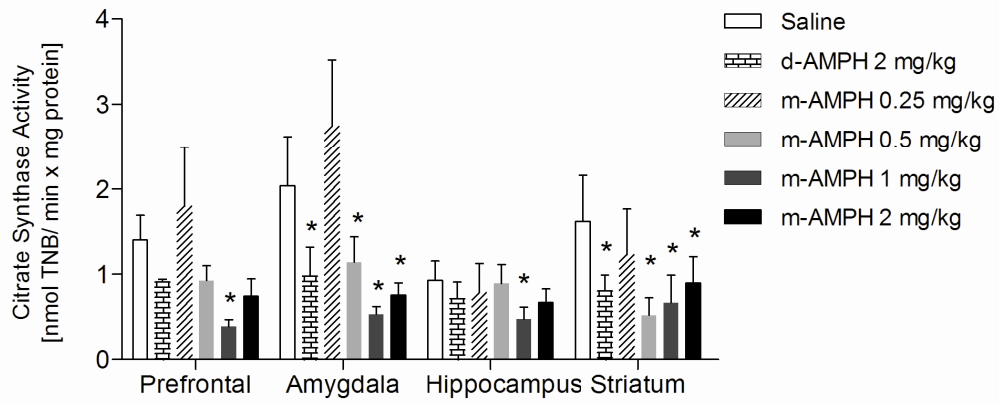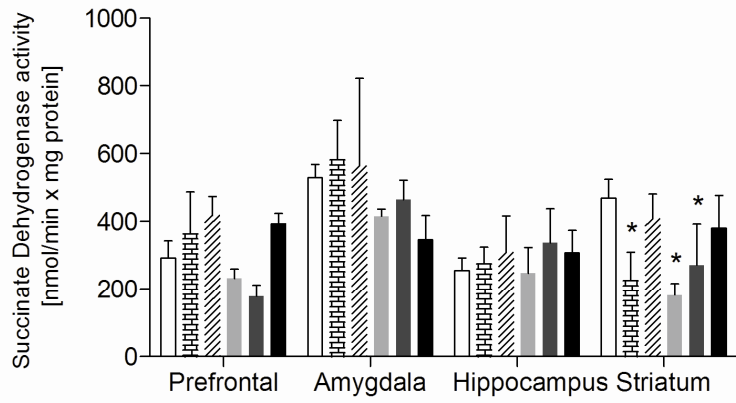
Figure 1A

Figure 1B

Figure 2A

Figure 2B

Figure 2C
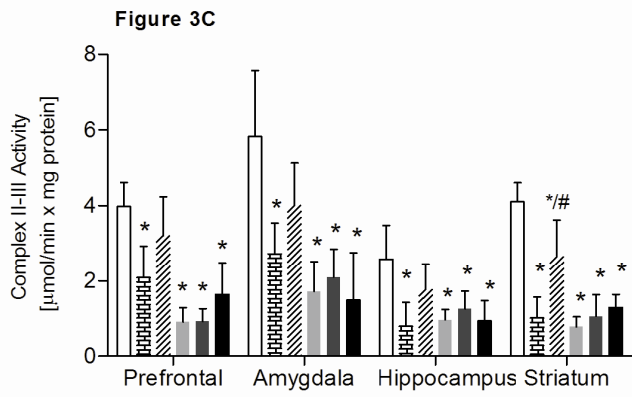
Figure 3A
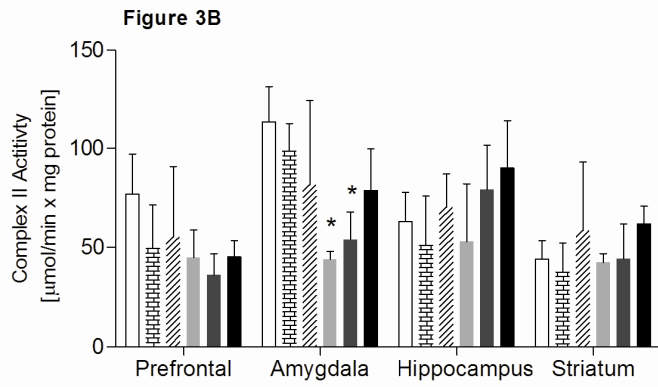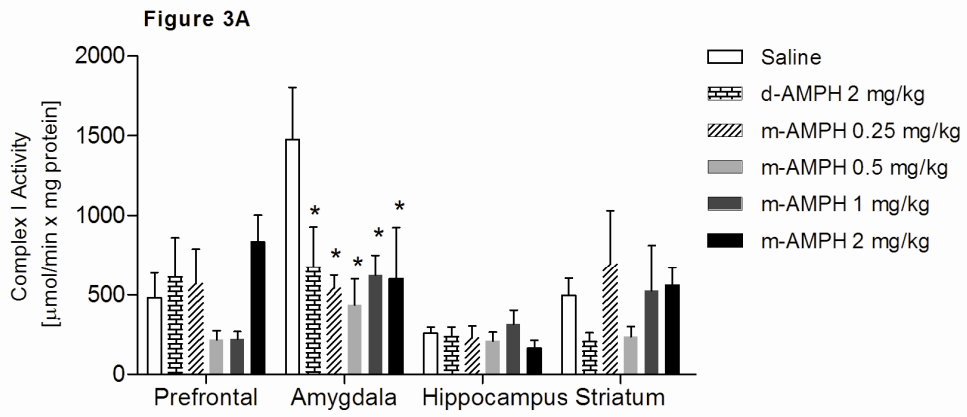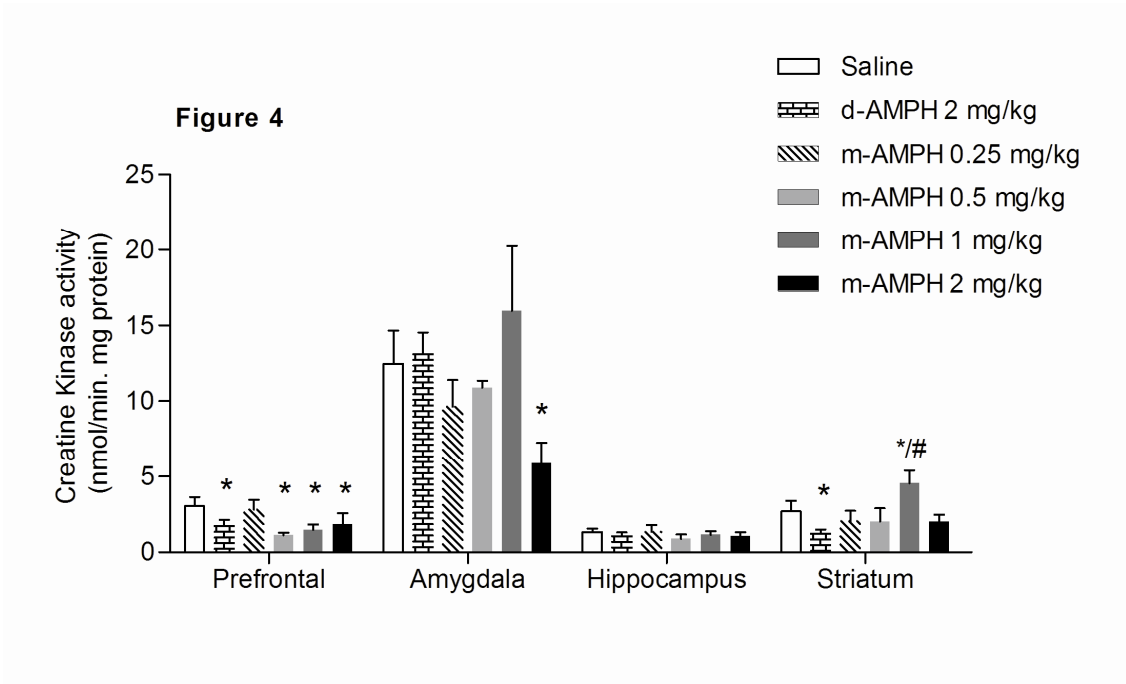
Figure 3B

Figure 3C

Figure 3D

Figure 4
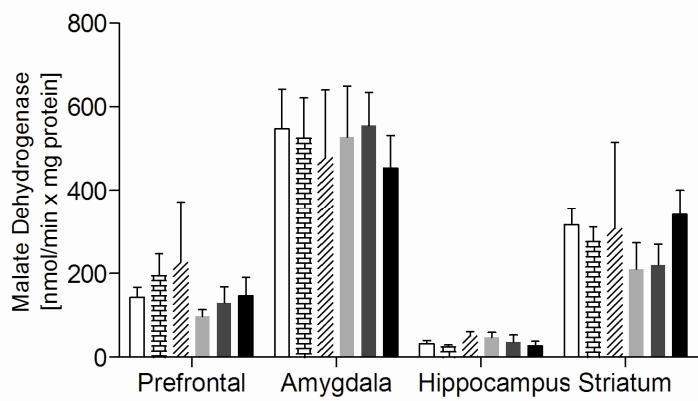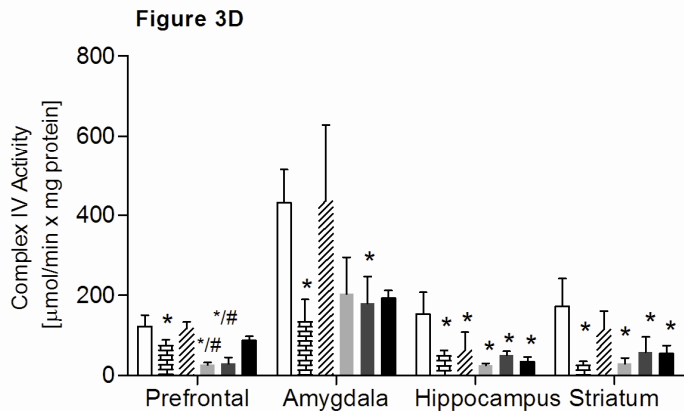
4.2 Artigo Cientifico 2

LITHIUM AND VALPROATE MODULATE ENERGY METABOLISM IN AN ANIMAL MODEL OF MANIA INDUCED BY METHAMPHETAMINE.

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Lithium and Valproate Modulate Energy Metabolism in an Animal Model of Mania Induced by Methamphetamine

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Abstract

Animal models have been important to study intracellular systems involved in bipolar disorder (BD). The animal model of mania induced by amphetamine has been considered a good model for the study of BD. However, some studies have shown differences between amphetamines to induce both behavioral and biochemical changes. In recent years our research laboratory has validated animal model of mania induced by d-AMPH and has found behavioral and biochemical changes induced by d-AMPH as well as in BD. The aim of this

study is to assess behavioral and energy metabolism changes in an animal model of mania induced by metamphetamine (m-AMPH). Wistar rats were first given m-AMPH or saline for 14 days, and then, between days 8 and 14, rats were treated with lithium (Li), valproate (VPA) or saline (Sal). Locomotor behavior was assessed using the open-field task and activities of Krebs cycle enzymes (citrate synthase and succinate dehydrogenase), mitochondrial respiratory chain complexes (I, II, III and IV) and creatine kinase were measured in brain structures (prefrontal, amygdala, hippocampus and striatum). Li and VPA reversed m-AMPH-induced hyperactivity. The administration of m-AMPH inhibited activities of Krebs cycle enzymes and complexes of mitochondrial respiratory chain in all analyzed structures. Li and VPA reversed m-AMPH-induced energetic metabolism dysfunction; however, the effects of Li and VPA were dependent on the brain region analyzed. These findings suggested that m-AMPH also is able to induced hyperactivity and energetic metabolism dysfunction, both seen in BD. Besides, the results suggest that the animal model of mania induced by m-AMPH fulfills adequate face, construct and predictive validity as an animal model of mania.

Key words: m-AMPH; animal model of mania; Krebs cycle enzymes; complexes of mitochondrial respiratory chain

Introduction

Although bipolar disorder (BD) is a common psychiatric disorder that leads to serious health problems, little is known about its pathophysiology. BD is a multifactorial illness and has diversity symptoms including recurrences of mania, depression, and mixed states; this hampers the development of a suitable animal model (Machado-Vieira et al., 2004). Despite the difficulties inherent in modeling BD in animals, several behavioral animal models of mania or depression have been developed to try to mimic some aspect of behavioral changes

found in this psychiatric condition (Frey et al., 2006a; Jornada et al., 2010; Herman et al., 2007; Machado-Vieira et al., 2004).

The psychostimulant-induced hyperactivity is the best established animal model of mania; because it contains the three major criteria for the development of an adequate animal model: face validity, construct validity and predictive validity. The amphetamines AMPHs administration in rodents causes hyperlocomotion, insomnia and increased sexual drive (face validity) (Fiorino and Phillips, 1999); besides, reproduces pathophysiological characteristics of the bipolar condition (construct validity) (Frey et al., 2006d) and responses to antimanic agents, such as antipsychotics and mood stabilizers (predictive validity) (Frey et al., 2006b; Frey et al., 2006c).

The most common types of AMPHs are dextroamphetamine (d-AMPH) and methamphetamine (m-AMPH). m-AMPH and d-AMPH have similar chemical structures, since, m-AMPH is the N-methylated analog of d-AMPH (Hoffman and Lefkowitz, 1996). Despite their structural similarities, some studies have reported differences between two drugs, particularly in the induction of behavioral and neurochemical changes. Epidemiological evidence indicates that m-AMPH abuse rates are greater than those of d-AMPH (SAMHSA, 2007). Recently, our research group showed that m-AMPH induces oxidative damage greater than d-AMPH (da-Rosa et al., 2011). Besides, Li and VPA reversed and prevented m-AMPH-induced hyperactivity when m-AMPH was administered at 0.25 mg/kg (da-Rosa et al., *in press*), while the mood stabilizers reversed and prevented hyperactivity induced by d-AMPH at 2mg/kg (Frey et al., 2006a,b,c,d).

Several studies have suggested that dysfunctional cellular energy metabolism have a central role in BD. Abnormalities in energy metabolism were found in functional assays and in magnetic resonance spectroscopy studies (Regenold et al., 2009; Dager et al., 2004; Deicken et al., 1995; Frey et al., 2007). In an animal model of mania, Li and VPA were able

to reverse and prevent d-AMPH-induced mitochondrial dysfunction, suggesting that one of the mechanisms of action of mood stabilizers may be decreasing the amount of dopamine available, and stabilizing mitochondrial function in the pathophysiology of BD (Valvassori et al., 2010).

Dysfunctions in the Krebs cycle can be capable of alter the rate of brain metabolism and the production of free radicals. After glycolisis, pyruvate is decarboxylated to acetyl CoA by the pyruvate dehydrogenase. The conversion of acetyl CoA to CO_2 in the Krebs cycle results in production of NADH for the electron transport chain and subsequent production of ATP. Krebs cycle is a chemical system made up of several enzymes and steps. In the first step, citrate synthase (CS) catalyzes the condensation of oxaloacetate and the acetyl group of acetyl coenzyme-A (Shepherd and Garland, 1969). In the final step of Krebs cycle, malate dehydrogenase (MD) catalyzes the dehydrogenation of l-malate to oxaloacetate (Kelly et al., 1989). Succinate dehydrogenase (SD) is part of both the Krebs cycle and the respiratory chain (complex II); therefore, this enzyme is one of the most important markers of the mitochondrial ability to supply an adequate amount of ATP (Tyler, 1992).

After the Krebs cycle, there is another process called oxidative phosphorylation, which electrons are passed along a series of respiratory enzyme complexes (complex I, II, III and IV) located in the inner mitochondrial membrane, and the energy released by this electron transfer is used to pump protons across the membrane. The resultant electrochemical gradient enables another complex, adenosine 5′-triphosphate (ATP) synthase, to synthesize ATP from ADP plus Pi (Horn and Barrientos, 2008).

The main symptoms of BD are neurovegetative abnormalities, impulsivity, and psychosis, suggesting that anterior limbic brain networks controlling these behaviors are dysfunctional. It is well known that the amygdala modulates the limbic system, controlling an

iterative circuit, prefrontal–striatal–thalamic, which control complex socioemotional behaviors (Strakowski et al., 2000; Strakowski et al., 2005).

Thus, we examined the activities of mitochondrial enzymes of the Krebs cycle (CS, MD and SD) and respiratory enzyme complexes (complex I, II, III and IV) in amygdala, prefrontal, striatum and hippocampus of rats submitted to an animal model of mania induced by m-AMPH.

Experimental methods

Animals

The subjects were adult male Wistar rats (weighting 250–350 g) obtained from our breeding colony. The animals were housed five to a cage, with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) at a temperature of 22 ± 1°C. All experimental procedures were performed in accordance with, and with the approval of the local Ethics Committee in the use of animals at the Universidade do Extremo Sul Catarinense. All experiments were performed at the same time during the day to avoid circadian variations.

Drugs and pharmacological procedures

The animals received one daily intraperitoneal injection (i.p.) of m-AMPH 0.25mg/kg or saline (Sal) for 14days (45 animals per group). On the 8th day of treatment, the animals in the saline and d-AMPH group were divided in 3 groups (15 animals per group): 1) treatment with Li (47.5 mg/kg i.p.); 2) treatment with VPA (200 mg/kg i.p.) and 3) treatment with Sal for 7 days twice a day for all drugs. On the 15th day of treatment, the animals received a single injection of m-AMPH or Sal and locomotor activity was assessed 2 h after the last injection. The rats were killed by decapitation immediately after the open-field task and amygdala,

prefrontal, striatum and hippocampus were dissected, rapidly frozen and stored -70º C until assayed.

Locomotor Activity

Locomotor activity was assessed using the open-field task as previously described (Barros et al., 2002; Frey et al., 2006a; b; c). This task was performed in a 40x60 cm open field surrounded by 50 cm high walls, made of brown plywood, with the floor divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle, and left free to explore the arena for 5 min. Crossings of the black lines (locomotor activity/ horizontal activity) and rearings (exploratory activity/ vertical activity) were counted.

Tissue and homogenate preparation

The prefrontal cortex, amygdala, hippocampus and striatum were removed and homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at $800 \times g$ for 10 min at 4°C and the supernatants kept at -70oC until being used for enzymes activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days. The protein content was determined by the method described by Lowry and colleagues (Lowry et al., 1951) using bovine serum albumin as standard.

Activities of enzymes of Krebs cycle

Citrate synthase activity: Citrate synthase activity was assayed according to the method described by Shepherd and Garland (1969). The reaction mixture contained 100 mM Tris, pH 8.0, 100 mM acetyl CoA, 100 mM 5,5′-di-thiobis-(2- nitrobenzoic acid), 0.1% triton X-100,

and 2–4 μg supernatant protein and was initiated with 100 μM oxaloacetate and monitored at 412 nm for 3 min at 25 °C.

Malate dehydrogenase activity: Malate dehydrogenase was measured as described by Kitto (1969). Aliquots (20 mg protein) were transferred into a medium containing 10 mM rotenone, 0.2% Triton X-100, 0.15 mM NADH, and 100 mM potassium phosphate buffer, pH 7.4, at 37°C. The reaction was started by the addition of 0.33 mM oxaloacetate. Absorbance was monitored as described above.

Succinate dehydrogenase activity: Succinate dehydrogenase activity was determined according to the method of Fischer and colleagues (1985), and measured by following the decrease in absorbance due to the reduction of 2,6-di-chloro-indophenol (2,6-DCIP) at 600nm with 700nm as a reference wavelength ($\varepsilon = 19.1$ mM-1 cm-1) in the presence of phenazine methasulphate (PMS). The reaction mixture consisting of 40mM potassium phosphate, pH 7.4, 16mM succinate and 8μM 2,6-DCIP was pre-incubated with 40–80μg homogenate protein at 30oC for 20 min. Subsequently, 4mM sodium azide, 7μM rotenone and 40μM 2,6-DCIP were added and the reaction was initiated by the addition of 1mM PMS and was monitored for 5 min.

Activities of mitochondrial respiratory chain enzymes

Complex I activity: NADH dehydrogenase (complex I) was evaluated according to Cassina and Radi (1996) by the determination of the rate of NADH-dependent ferricyanide reduction at $\lambda = 420$ nm.

Complex II activity: The activities of succinate-2,6- dichloroindophenol (DCIP)-oxidoreductase (complex II) was determined by the method described by Fischer and colleagues (1985). Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at $\lambda = 600$ nm.

Complex II-III activity: The activity of succinate:cytochrome c oxidoreductase (complex III) was determined by the method described by Fischer and colleagues (1985). Complex II-III activity was measured by cytochrome c reduction using succinate as substrate at $\lambda = 550$ nm.

Complex IV activity: The activity of cytochrome c oxidase (complex IV) was assayed according to the method described by Rustin and colleagues (1994), measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c (prepared by reduction of cytochrome with NaBH4 and HCl) at $\lambda = 550$ nm with 580 nm as the reference wavelength ($\varepsilon = 19.1$ mM−1 cm−1). The activities of the mitochondrial respiratory chain complexes were calculated as nmol. min-1. mg protein-1.

Activity of creatine kinase enzyme

Creatine kinase activity was measured in brain homogenates pretreated with 0.625 mM lauryl maltoside. The reaction mixture consisted of 60mM Tris–HCl, pH 7.5, containing 7 mM phosphocreatine, 9 mM MgSO4 and approximately 0.4–1.2 μg protein in a final volume of 100 μL. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of 3.2 mmol of ADP plus 0.8 mmol of reduced glutathione. The reaction was stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 100 μL 2%α-naphtol and 100 μL 0.05% diacetyl in a final volume of 1 mL and read spectrophotometrically after 20 min at 540 nm. Results were expressed as units/min×mg protein.

Statistical analysis

Data were analyzed by one-way analysis of variance followed by the Tukey test when F was significant and are expressed as mean ± standard deviation. All analyses were performed using the Statistical Package for the Social Science (SPSS; version 16.0) software.

Results

Li and VPA reversed hyperlocomotion induced by m-AMPH in rats

Results for locomotor activity are shown in Figure 1. There was a significant effect of m-AMPH and mood stabilizers in the number of both crossings [df=5, F=27.57, p<0.001] and rearings [df=5, F=7.43, p<0.001]. Further analysis with Tukey post-hoc tests showed that administration of m-AMPH increased locomotion and rearing behavior in rats treated with Sal (both Ps < 0.001 compared to the group treated with SAL followed by SAL). Treatment with Li and VPA reversed m-AMPH-related hyperlocomotion. Animals given Li or VPA followed by m-AMPH did not differ from the control group given Sal followed by Sal.

Li and VPA treatment attenuate m-AMPH-induced decreases in activities of enzymes of Krebs cycle in rat brain

Results for citrate sinthase activity are shown in Figure 2A. The m-AMPH administration caused a marked decreased of the citrate synthase activity of rat prefrontal, amygdala, hippocampus and striatum. Seven days of treatment with Li and VPA reversed m-AMPH's effects on citrate synthase activity in the amygdala, hippocampus and striatum. In the prefrontal, Li reversed and VPA partially reversed m-AMPH's effects on citrate synthase activity. Chronic use of Li and VPT in saline-treated animals had no influence in citrate

synthase activity in the amygdala hippocampus and striatum. In the prefrontal, VPA alone, but not Li, decreased the citrate synthase activity.

Results for succinato dehydrogenase activity are shown in Figure 2B. The m-AMPH administration decreased succinato dehydrogenase activity in all brain structures evaluated. Treatment with Li reversed the reduction of succinato dehydrogenase activity induced by m-AMPH in the amygdala and hippocampus, but not in the striatum. In the prefrontal, the Li administration in the m-AMPH pretreated rats increased succinate dehydrogenase activity when compared to control group. Besides, in the hippocampus, succinate dehydrogenase activity was significantly decreased in the Sal+Li group. Seven days of treatment with VPA reversed m-AMPH's effects on succinate dehydrogenase in the striatum, but not in the prefrontal and amygdala. In the hippocampus, succinate dehydrogenase activity was significantly increased in the VPA+m-AMPH group. We found also that treatment with VPA alone, succinate dehydrogenase activity was significantly enhanced in the prefrontal when compared to control group.

Results for malate dehydrogenase activity are shown in Figure 2C. After m-AMPH administration, malate dehydrogenase activity also decreased in the amygdala, hippocampus and striatum. The Li and VPA treatment reversed m-AMPH's effects on the malate dehydrogenase activity in the hippocampus and striatum. In the amygdala, VPA, but not Li, partially reversed m-AMPH's effects on malate dehydrogenase activity.

Li and VPA treatment attenuate m-AMPH-induced decreases in activities of respiratory chain complexes in rat brain

m-AMPH administration inhibited the respiratory chain complexes (I, II, II-III and IV) in all brain structure evaluated.

Results for complex I activity are shown in Figure 3A. The Li and VPA administration reversed m-AMPH's effects on complex I activity in the prefrontal and amygdala of rats. The VPA treatment, but not Li, reversed the m-AMPH-induced the decreased in complex I activity in the hippocampus and striatum.

Results for complex II activity are shown in Figure 3B. The Li and VPA treatment reversed m-AMPH's effects on complex II activity in the hippocampus and striatum. However, in the prefrontal and amygdala VPA, but not Li, significantly increased the complex II activity when compared to control group.

Results for complex II-III activity are shown in Figure 3C. The Li and VPA treatment attenuate the m-AMPH-induced the decreased in complex II-III activity in the hippocampus and striatum. Seven days of treatment with VPA attenuate m-AMPH's effects on complex II-III activity in the prefrontal and amygdala; unlike, Li administration potentiates the m-AMPH's effects in these cerebral structures.

Results for complex IV activity are shown in Figure 3D. Treatment with Li and VPA reversed m-AMPH-related complex IV dysfunction in the amygdala and striatum. In the prefrontal, VPA, but not Li, reversed m-AMPH's effects on complex IV activity. In the amygdala, Li and VPA partially reversed the m-AMPH's effects on complex IV activity.

Effects of Li and VPA on creatine kinase activity in the rats submitted to animal model of mania induced by m-AMPH

The m-AMPH administration decreased CK activity in the prefrontal of rats and the mood stabilizers, Li and VPA, were not able to reverse this damage. In the amygdala and hippocampus, CK activity was decreased in the Sal+VPA and m-AMPH+Li groups. In the striatum CK activity was decreased in the Sal+VPA group.

Discussion

Initially, we reproduced data from previous studies from our laboratory, which Li and VPA reversed the hyperactivity induced by m-AMPH at 0.25mg/kg (da-Rosa et al., *in press*). It is know that mood stabilizers reversed and prevented hyperactivity induced by d-AMPH at 2mg/kg (Frey et al., 2006a; b; c; d).

Accumulating evidence suggests that energetic metabolism dysfunction contribute to the pathogenesis of BD. Impairment of complex I was found in the prefrontal cortex of bipolar patients (Andreazza et al., 2010). In a large-scale DNA microarray analysis in *postmortem* brains was showed a global down-regulation of mitochondrial genes, such as those encoding respiratory chain components, in BD (Iwamoto et al., 2005). In an elegant study, Cataldo and colleagues (2010) have demonstrated that mitochondria from patients with BD have size and distributional abnormalities compared with control group. In the brain, individual mitochondria profiles had significantly smaller areas, on average, in BD samples. In peripheral cells, mitochondria in BD samples were concentrated proportionately more within the perinuclear region than in distal processes. These changes in mitochondrial morphology and distribution appear to be linked to diverse cellular events, such as differentiation, aging, and apoptosis. In addition, abnormalities in energy metabolism of BD patients were also found in functional assays and in magnetic resonance spectroscopy studies (Regenold et al., 2009; Dager et al., 2004; Deicken et al., 1995; Frey et al., 2007).

In previous studies, we have demonstrated also that d-AMPH at 2mg/kg inhibited citrate synthase (Correa et al., 2007) and mitochondrial respiratory chain complexes I, II, II-III and IV (Valvassori et al., 2010) activities in brain of rats, which was reversed by Li and VPA. As described above, changes in energy metabolism are strongly linked to BD; therefore, it is interesting the development of animal models with this feature. In order to study the action mechanisms of classical drugs on this system and still test new drugs.

Here, we demonstrated that chronic administration of m-AMPH at 0.25mg/kg was able to inhibit the cycle Krebs enzymes activities: citrate synthase, succinate dehydrogenase and malate dehydrogenase. Moreover, we demonstrated also that m-AMPH at 0.25mg/kg inhibit mitochondrial respiratory chain complexes (I, II, II-III and IV) in all brain structure evaluated, as well as d-AMPH at 2mg/kg as demonstrated in previous studies (Valvassori et al., 2010). From the results obtained in this study, we suggested that the decreased Krebs cycle enzymes activity induced by m-AMPH inhibit energy production by mitochondrial respiratory chain via Krebs cycle. Recently, was showed that m-AMPH administration decreased Krebs cycle intermediates in the urine and increased glucose in the plasma of rats (Shima et al., 2011). Kim and colleagues (2009) have demonstrated dose-dependent frontal hypometabolism on fluorodeoxy-D-glucose-positron emission tomography (FDG-PET) in methamphetamine abusers. Impaired energy metabolism results in depletion of ATP in cells, and ATP depletion as a consequence of mitochondrial toxicity may be related to a number of m-AMPH-induced toxicities.

Another significant finding in this study is that mood stabilizers, Li and VPA, attenuated the AMPH's effects on Krebs cycle enzymes activity and, consequently, reduced the impairment on respiratory chain complexes activity. Our results are in agreement with a previous study, which showed that that long-term treatment with Li or VPA protected against m-AMPH-induced toxicity at the mitochondrial level (Bachmann et al., 2009). Additionally, the mood stabilizers prevented the m-AMPH-induced reduction of mitochondrial cytochrome c, the mitochondrial anti-apoptotic Bcl-2/Bax ratio, and mitochondrial cytochrome oxidase activity (Bachmann et al., 2009).

Kazuno (2008) and colleagues have showed that VPA may stabilize intracellular calcium in cells with high mitochondrial calcium levels. The excessive release of glutamate by m-AMPH may induce neuronal damage through receptor-mediated intracellular Ca^{2+}

overload and increased reactive oxygen species (ROS) production (Arundine and Tymianski, 2004; Choi, 1995; Lu et al., 2008). The Li and VPA treatment exert neuroprotective effects against cytotoxicity by inhibiting the glutamate-induced increase of intracellular free Ca^{2+} concentration (Shao et al., 2005). Besides, it is well known that m-AMPH-induced increased DA oxidized metabolites inhibit the mitochondrial respiratory system, both in vivo and in vitro (Przedborsk et al., 1993). The Li and VPA treatment inhibit the dopaminergic system by a mechanism of second messenger activation, which are activated by D2 receptors (Yatham et al., 2002). Together with our results, these studies suggest that mood stabilizers, Li e VPA, attenuate the glutamate and DA systems, protecting the mitochondria against m-AMPH-induced damage.

Finally, we can observe that m-AMPH inhibit the CK activity only in the prefrontal; however, Li and VPA were not able to reverse this enzyme alteration. In a previous study of our laboratory we have showed that d-AMPH at 2mg/kg inhibited CK activity in hippocampus, striatum and cortex, but not in prefrontal cortex, and administration of Li or VPA did not reverse the enzyme inhibition (Streck et al., 2008). This discrepancy can be explain by the difference, in the structure and in the dose administered, between the two drugs. Here we demonstrated also that chronic VPA administration, in the saline pretreated rats, decreased CK activity in all brain structures evaluated. Additionally, despite m-AMPH alone did not alter CK activity in the amygdala and hippocampus, the Li treatment (Li+m-AMPH) decreased CK activity in this brain structures. Couthon and colleagues (1997) have demonstrated that incubation of dimeric MM-creatine kinase (MM-CK) with LiCl results in dissociation of the subunits and complete enzyme inactivation.

These findings suggested that m-AMPH also is able to induced hyperactivity and energetic metabolism dysfunction, both seen in BD. Besides, the results suggest that the

animal model of mania induced by m-AMPH fulfills adequate face, construct and predictive validity as an animal model of mania.

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Legends to Figures

Figure 1: Open field test. Free movement in the open field: crossings (dF=5; F=27.568; p<0.0001), rearings (dF=5; F=7.430; p<0.0001). *Different from Sal+Sal group. #Different from m-AMPH+Sal group.

Figure 2: Activities of enzymes of Krebs cycle. **A)** Citrate synthase activity: In the prefrontal (dF=5; F=27.845; p<0.0001), amygdala (dF=5; F=16.176; p<0.0001), hippocampus (dF=5; F=8.799; p<0.0001) and striatum (dF=5; F=6.796; p<0.0001). **B)** Succinate dehydrogenase activity: In the prefrontal (dF=5; F=20.922; p<0.0001), amygdale (dF=5; F=22.592; p<0.0001), hippocampus (dF=5; F=25.361; p<0.0001) and striatum (dF=5; F=14.491; p<0.0001). **C)** Malate dehydrogenase activity: In the prefrontal (dF=5; F=4.332; p<0.0001), amygdala (dF=5; F=38.111; p<0.0001), hippocampus (dF=5; F=8.945; p<0.0001) and striatum (dF=5; F=15.033; p<0.0001). *Different from Sal+Sal group. #Different from m-AMPH+Sal group.

Figure 3: Activities of mitochondrial respiratory chain enzymes. **A)** Complex I activity: In the prefrontal (dF=5; F=17.512; p<0.0001), amygdala (dF=5; F=16.018; p<0.0001), hippocampus (dF=5; F=44.667; p<0.0001) and striatum(dF=5; F=32.298; p<0.0001). **B)** Complex II activity: In the prefrontal (dF=5; F=23.680; p<0.0001), amygdala (dF=5; F=19.278; p<0.0001), hippocampus (dF=5; F=6.466; p<0.001) and striatum (dF=5; F=10.529; p<0.0001). **C)** Complex II-III activity: In the prefrontal (dF=5; F=58.859; p<0.0001), amygdala (dF=5; F=111.150; p<0.0001), hippocampus (dF=5; F=26.548; p<0.0001) and striatum (dF=5; F= 33.750; p<0.0001). **D)** Complex IV activity: In the prefrontal (dF=5; F=46.454; p<0.0001), amygdala (dF=5; F=18.474; p=0.002), hippocampus (dF=5; F=11.713; p=0.001) and striatum (dF=5; F=18.617; p<0.0001). *Different from Sal+Sal group. #Different from m-AMPH+Sal group.
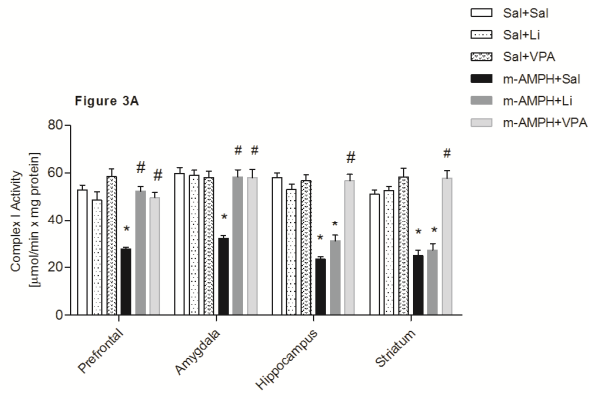
Figure 4: Activity of creatine kinase: In the prefrontal (dF=5; F=25.298; p<0.0001), amygdala (dF=5; F=9.96; p<0.0001), hippocampus (dF=5; F=3.08; p<0.03) and striatum (dF=5; F=6.103; p<0.002). *Different from Sal+Sal group. #Different from m-AMPH+Sal group.

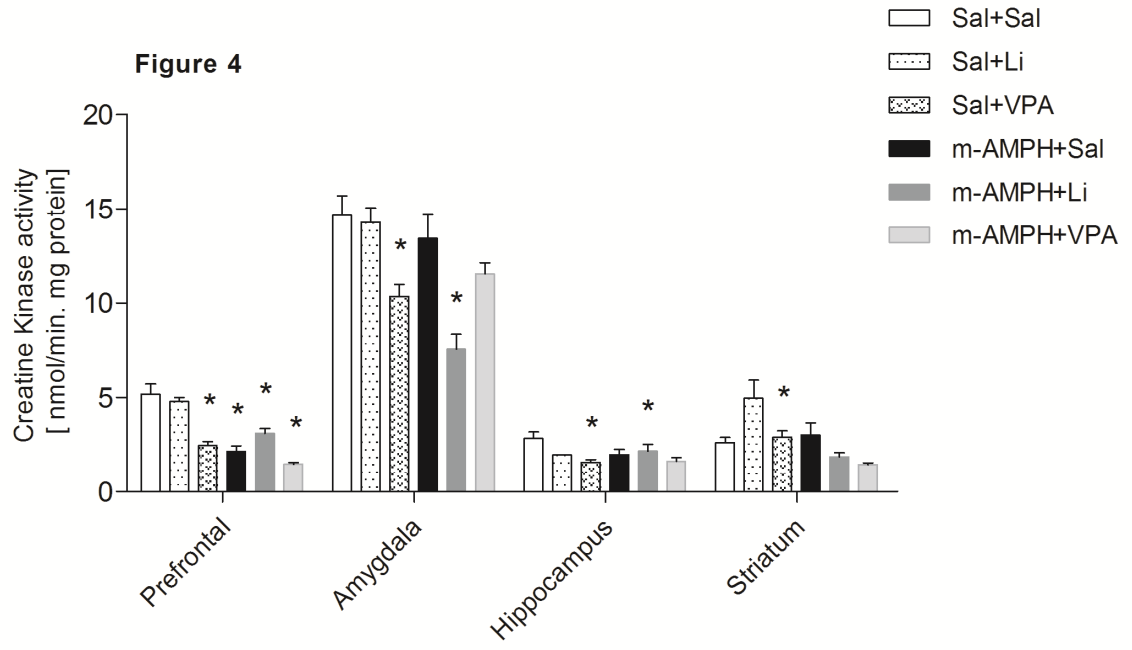Figure 1

Figure 2A

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Figure 3A

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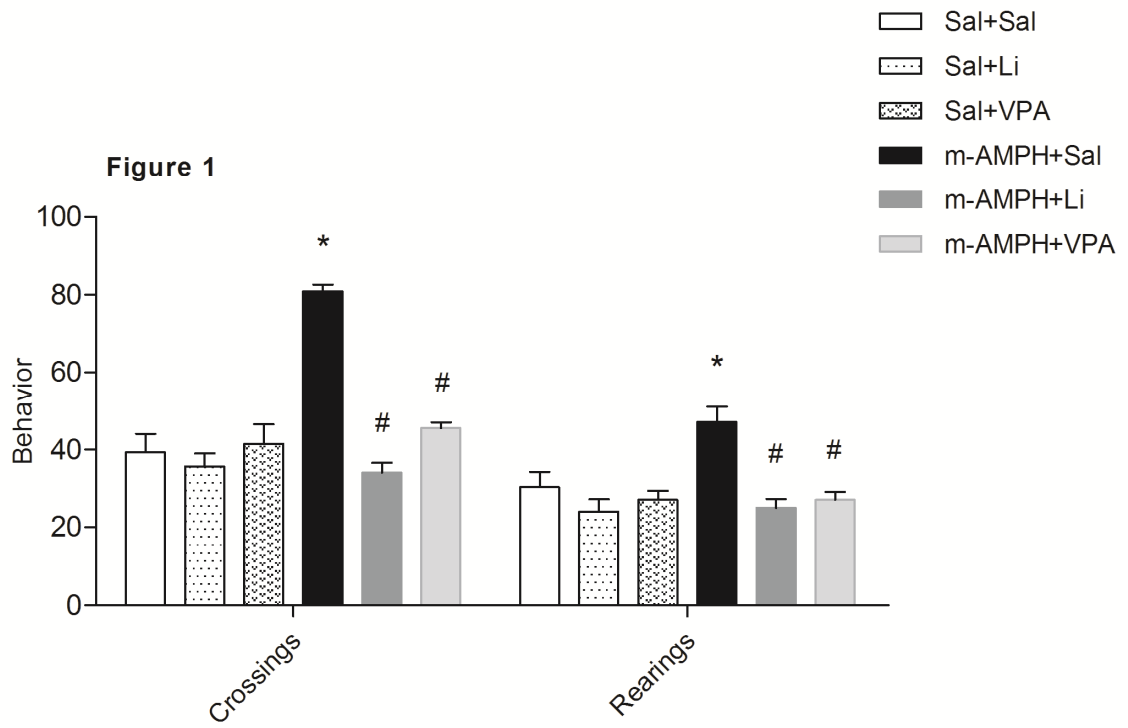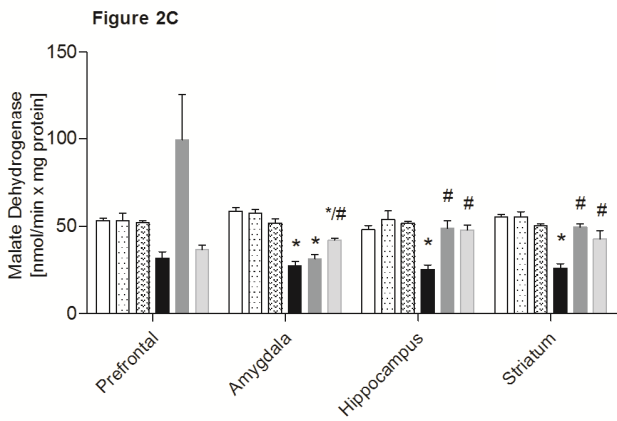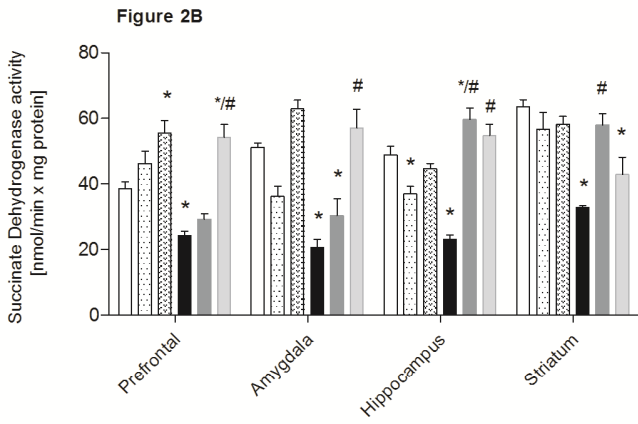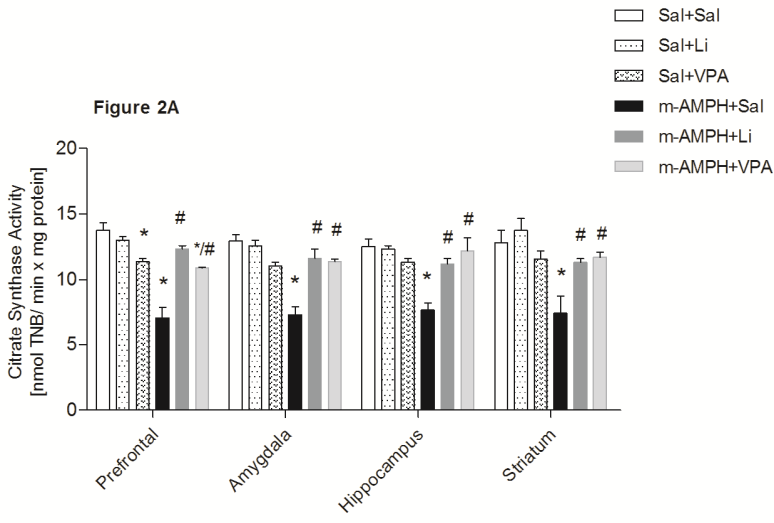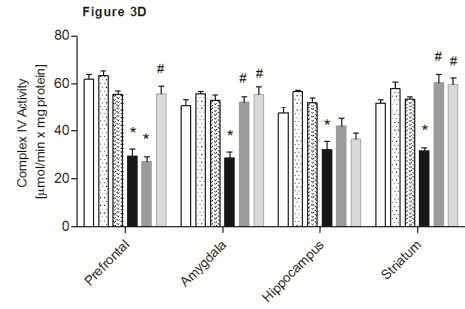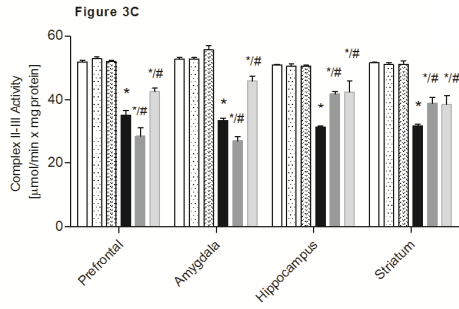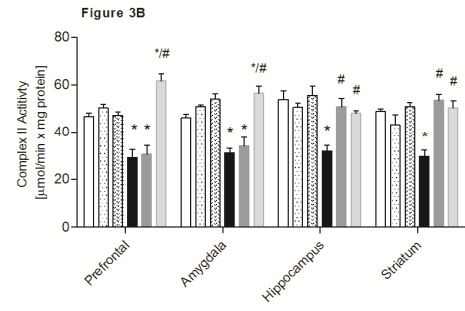
4.3 Artigo Cientifico 3

**CREATINE KINASE LEVELS IN PATIENTS WITH BIPOLAR DISORDER:
DEPRESSIVE, MANIC, AND EUTHYMIC PHASES**
*COMPARAÇÃO DAS FASES DE DEPRESSÃO, MANIA E EUTIMIA SOBRE OS NÍVEIS DE
CREATINA QUINASE EM PACIENTES BIPOLARES*

Gustavo Feier, Samira S. Valvassori, Gislaine T. Rezin, Márcio Búrigo, Emilio L. Streck,
Flávio Kapczinski, João Quevedo

Creatine kinase levels in patients with bipolar disorder: depressive, manic, and euthymic phases

Comparação das fases de depressão, mania e eutimia sobre os níveis de creatina quinase em pacientes bipolares

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Abstract

Objective: Bipolar disorder is a severe, recurrent, and often chronic psychiatric illness associated with significant functional impairment, morbidity, and mortality. Creatine kinase is an important enzyme, particularly for cells with high and fluctuating energy requirements, such as neurons, and is a potential marker of brain injury. The aim of the present study was to compare serum creatine kinase levels between bipolar disorder patients, in the various phases (depressive, manic, and euthymic), and healthy volunteers. **Method:** Forty-eight bipolar patients were recruited: 18 in the euthymic phase; 17 in the manic phase; and 13 in the depressive phase. The control group comprised 41 healthy volunteers. The phases of bipolar disorder were defined as follows: euthymic—not meeting the DSM-IV criteria for a mood episode and scoring < 8 on the Hamilton Depression Rating Scale (HDRS) and Young Mania Rating Scale (YMRS); manic—scoring < 7 on the HDRS and > 7 on the YMRS; depressive—scoring > 7 on the HDRS and < 7 on the YMRS. Patients in mixed phases were excluded. Blood samples were collected from all participants. **Results:** Creatine kinase levels were higher in the manic patients than in the controls. However, we observed no significant difference between euthymic and depressive patients in terms of the creatine kinase level. **Conclusion:** Our results suggest that the clinical differences among the depressive, manic, and euthymic phases of bipolar disorder are paralleled by contrasting levels of creatine kinase. However, further studies are needed in order to understand the state-dependent differences observed in serum creatine kinase activity.

Descriptors: Case-control studies; Bipolar disorder; Water level measurement/adverse effects; Creatine kinase; Depression

Resumo

Objetivo: O transtorno do humor bipolar é uma doença psiquiátrica grave, recorrente e crônica associada a significativo prejuízo funcional, morbidade e mortalidade. A creatina quinase tem sido proposta como um marcador de dano cerebral. A creatina quinase é uma enzima importante principalmente para células que necessitam de uma grande quantidade de energia, como os neurônios. O objetivo do presente estudo foi comparar os níveis de creatina quinase entre as fases depressiva, maníaca e eutímica de pacientes com transtorno do humor bipolar. **Método:** Para avaliação dos níveis de creatina quinase no soro, 48 pacientes bipolares foram recrutados; 18 estavam eutímicos, 17 estavam em mania e 13 em episódio depressivo. Foi feita também uma comparação com um grupo controle que incluiu 41 voluntários saudáveis. Grupo eutimia: foram incluídos os pacientes que não cumpriam os critérios do DSM-IV para episódios de humor e deveriam ter a pontuação inferior a oito nas escalas de avaliação de mania (YMRS) e depressão (HDRS); grupo mania: foram incluídos os pacientes que apresentavam YMRS > 7 e HDRS < 7; grupo depressão: foram incluídos os pacientes que apresentavam HDRS > 7 e YMRS < 7. Os pacientes em episódios mistos não foram incluídos no estudo. Amostras de sangue foram coletadas de todos os participantes. **Resultados:** Durante a mania, os níveis de creatina quinase foram aumentados em comparação com voluntários saudáveis. Entretanto, não houve diferença significativa nos níveis de creatina quinase em pacientes eutímicos e depressivos, quando comparados com o grupo controle. **Conclusão:** Nossos resultados sugerem que as fases maníaca, depressiva e eutímica do transtorno do humor bipolar, além de apresentarem sintomatologia distinta, também podem ser diferenciadas pelo nível de creatina quinase presente no sangue do paciente. Entretanto, mais estudos são necessários para entender as diferenças observadas na atividade da creatina quinase durante as fases do transtorno do humor bipolar.

Descritores: Estudos de casos e controles; Transtorno bipolar; Medição de níveis/efeitos adversos; Creatina quinase; Depressão

Introduction

Bipolar disorder (BD) is a severe, recurrent, and chronic psychiatric disorder that is associated with suicide, as well as with significant functional impairment and morbidity. Patients with BD typically experience recurrent

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changes in mood, including manic, depressive, and mixed episodes.[1-3]

Creatine kinase (CK), an enzyme that catalyses the reversible transphosphorylation of creatine by adenosine triphosphate, plays a key role in energy buffering and energy transport, particularly in cells with high and fluctuating energy requirements, including neurons.[4] CK is found mainly in the skeletal muscle, heart, and brain; substantially elevated serum levels of CK usually indicate damage or stress in one or more of these. Enhanced serum CK activity is found also in a substantial proportion of patients hospitalized with acute schizophrenia or affective psychosis.[5-10] A recent study of BD patients demonstrated that serum CK levels were higher in manic patients than in depressive patients. Likewise, when tested within the same patient, serum CK levels were found to be higher during the manic phase than in the depressive phase. In agreement with this, it has been demonstrated that CK levels increase in the cerebrospinal fluid and serum of BD patients after an acute episode.[11,12]

Despite recent data indicating that serum CK levels are elevated in BD, we found no studies evaluating CK levels in manic, depressive, and euthymic BD patients. Therefore, this study was designed to investigate whether changes in serum CK levels are associated with mood episodes.

Method
1. Subjects
The present study was approved by the *Ethics Committee* in Human Research of *Universidade do Extremo Sul de Santa Catarina* (UNESC), protocol number: 42/2008. We evaluated 48 patients with BD type I, recruited from among those enrolled in the Bipolar Disorders Program of the *Hospital de Clínicas de Porto Alegre*, located in the city of Porto Alegre, Brazil. Of those 48 patients, 18 were in the euthymic state, 17 were in the manic state, and 13 were in the depressive state. We also recruited a comparison group of 41 healthy volunteers. Psychiatric diagnoses were based on clinical interviews and were confirmed with the structured clinical interview for DSM-IV Axis I personality disorders (SCID-I). Manic and depressive symptoms were assessed using the Young Mania Rating Scale (YMRS) and the Hamilton Depression Rating Scale (HDRS), respectively. Acute manic or depressive episodes were defined by DSM-IV-TR criteria. Patients were divided into three groups as follows: euthymic—patients who did not meet the DSM-IV criteria for a mood episode and who scored < 8 on the HDRS and YMRS; manic—patients who scored < 7 on the HDRS and > 7 on the YMRS; and depressive—patients who scored > 7 on the HDRS and < 7 on the YMRS. Patients in mixed phases were excluded. The healthy volunteers were screened for psychiatric disorders using the SCID-I, non-patient version. The healthy subjects were not on medication and had no history of major psychiatric disorders, dementia, or mental retardation. Any individual (patient or control) who had a medical condition that could result in elevated CK levels—including acute kidney injury, heart disease and musculoskeletal disorders—was excluded, as were those who had received intramuscular injections.

2. Determination of serum CK levels
From each subject, 5 mL of blood were drawn by venipuncture into an anticoagulant-free vacuum tube. The blood was immediately centrifuged at 3000 × g for 5min, and the serum was stored at –80°C for subsequent assay. The reaction mixture for CK assay contained 100mmol/L of Tris-HCl buffer, pH 7.5, 30mmol/L of phosphocreatine, 20mmol/L of glucose, 12mmol/L of magnesium acetate, 10 μmol/L of diadenosine pentaphosphate, 15mmol/L of sodium azide, 20mmol/L of N-acetylcysteine, 2mmol/L of adenosine diphosphate, 5mmol/L of adenosine monophosphate, 2mmol/L of nicotinamide adenine dinucleotide, 3500U/L of hexokinase, 2000U/L of glucose-6-phosphate dehydrogenase, and approximately 1.5μg of protein, in a final volume of 1200μL. The CK activity was calculated based on the appearance (formation) of reduced nicotinamide adenine dinucleotide, monitored with a spectrophotometer at 340nm at 37°C. The upper limit of normal for CK is 150U/L for women and 175U/L for men.[13]

3. Statistics
Descriptive analyses are presented as number and percentage. Demographic and clinical characteristics were analyzed using the chi-square test and ANOVA, as necessary. Levels of CK were analyzed by one-way ANOVA, followed by Tukey's post hoc test when the F value was significant, and are expressed as mean and standard deviation. All analyses were performed with the Statistical Package for the Social Sciences 17.0 (SPSS Inc., Chicago, IL, USA). The level of significance was set at p < 0.05.

4. Ethics
The study design was approved by the local research ethics committee, and all participants gave written informed consent.

Results
Demographic and clinical characteristics of the patients and controls are presented in Table 1. All groups were homogeneous regarding age and sex. No statistical difference was found between the BD phases in terms of years since diagnosis or number of manic or depressive episodes. Serum CK levels were significantly higher in the manic patients than in the controls (2.12 ± 0.42 vs. 1.84 ± 0.26U/μL, p = 0.013; Figure 1). In addition, CK levels were significantly lower in the depressed patients (1.72 ± 0.16) than in the manic patients (p = 0.005). Furthermore, CK levels in the depressive and euthymic patients (1.89 ± 0.33 for the latter) did not differ significantly from those observed for the controls.

Discussion
Our results show that serum levels of CK are higher in manic BD patients than in healthy control subjects. Although CK levels were higher in the manic patients than in the depressed patients, the levels observed in the latter group did not differ significantly from those obtained for the controls. One of the most important findings of the study is that there was no significant difference between the euthymic patients and the controls in terms of the serum CK levels. In clinical practice, serum CK is measured as a marker of myocardial infarction (heart attack), rhabdomyolysis (severe muscle breakdown), muscular dystrophy, and acute kidney injury. In view of this, it should be borne in mind that the elevated serum CK levels seen in the manic patients evaluated here might simply represent increased motor activity.

In agreement with our findings, Segal et al. showed that CK levels are higher in manic patients than in those who are depressed.[11] In addition, cerebrospinal fluid and serum levels of CK have been shown to increase in BD patients after an acute episode.[12,14,15] Furthermore, there is strong evidence that

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Table 1 - Characteristics of the patients and controls

| Characteristic | Control group (n = 41) | Bipolar disorder patients | | | p-value |
		Euthymic (n = 18)	Manic (n = 17)	Depressed (n = 13)	
Female, n (%)	28 (68.3)	15 (83.3)	14 (82.4)	10 (76.9)	0.53*
Age, mean ± SD	42.5 ± 11.39	39.71 ± 3.37	40 ± 11.94	50.33 ± 10.99	0.09**
HDRS score, mean ± SD		3.59 ± 1.42	6.75 ± 2.05	18.23 ± 9.28	0.001**
YMRS score, mean ± SD		5.94 ± 1.09	35.19 ± 9.74	2.38 ± 1.71	0.001**
Years since diagnosis, mean ± SD		17.57 ± 9.19	17.12 ± 8.16	21.6 ± 10.10	0.724**
Number of manic episodes, mean ± SD		13.5 ± 8.48	14.5 ± 8.22	10 ± 7.5	0.543**
Number of depressive episodes, mean ± SD		15.66 ± 12.82	12 ± 3.39	13.57 ± 5.96	0.691**
Creatine kinase levels (U/µL), mean ± SD	1.84 ± 0.26	1.89 ± 0.38	2.12 ± 0.41	1.72 ± 0.15	0.005**

HDRS = Hamilton Depression Rating Scale. YMRS = Young Mania Rating Scale. *Chi-square test. **One-way ANOVA and Tukey's post hoc test.

metabolic impairment and mitochondrial dysfunction are involved in the pathophysiology of BD.[16-20] The phosphocreatine/ CK energy circuit, which is important for maintaining normal energy homeostasis,[21,22] has a number of integrated functions, such as temporary energy buffering and energy transfer, as well as regulating metabolic capacity.[23] In view of these data, we can suggest that the increased CK activity seen in manic BD patients is a compensatory mechanism related to the mitochondrial damage that occurs in BD.

Soni et al. evaluated patients suffering from a variety of psychoses and graded those patients on the basis of the degree of psychomotor activity.[24] Serum creatine phosphokinase (CPK) levels were found to be related to the degree of psychomotor activity, irrespective of the diagnostic category. Retarded patients and withdrawn patients had normal serum CPK, but transitory increases in CPK were observed as those patients returned to normal psychomotor activity, suggesting that nonphysiological motor activity is more directly related to the rise of serum CPK than is motor activity *per se*. However, a postmortem study[25] showed that expression of CK

Figure 1 - Serum creatine kinase levels in the depressive, manic, and euthymic phases of bipolar disorder and in controls. Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test when F was significant.
*p = 0.013, control vs. manic; **p = 0.005, manic vs. depressed.

mRNA is decreased in the hippocampus and dorsolateral prefrontal cortex of BD patients. In addition, decreased CK activity has been reported in a rat model of d-amphetamine-induced mania.[20] That finding could be explained by the fact that increased dopamine activity inhibits CK. In a clinical study of patients with bipolar I or II disorder (BP I or BP II),[26] brain phosphorus metabolism was measured by phosphorus-31 magnetic resonance spectroscopy. The authors found that phosphocreatine levels were significantly lower in the BP II patients, regardless of the BD phase, than in a group of normal controls, suggesting that brain high-energy phosphate metabolism is impaired in BP II and that there are pathophysiological differences between BP I and BP II.

To our knowledge, this is the first study of its type to include euthymic patients. The inclusion of such a group could provide data about the persistence of altered serum CK levels during remission. However, because our sample was small, the present study did not have sufficient power to detect possible differences between the groups. Another limitation of our study is that we did not evaluate the effects that mood stabilizers or other drugs have on CK activity. However, it is of note that we compared the BD (phase) subgroups with the control group. The fact that the levels of the CK in the euthymic BD patients were comparable to those observed for the controls might indicate that alterations in CK levels are associated with acute mood episodes, especially acute mania. Although CK activity does not explain the entire psychiatric profile, it does shed light on certain topics including the potential development of alternative treatments and the possibility that treatment response could be monitored more specifically by measuring the levels of biological markers in plasma.

Conclusion

In conclusion, our results suggest that the clinical differences among the depressive, manic, and euthymic phases of BD are paralleled by contrasting levels of CK. However, further studies are needed in order to understand the state-dependent differences observed in serum CK activity.

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Disclosures

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Gustavo Feier	UNESC	-	-	-	-	-	-
Samira S. Valvassori	UNESC	-	CAPES	-	-	-	-
Gislaine T. Rezin	UNESC	-	CNPq	-	-	-	-
Márcio Búrigo	UFRGS	-	-	-	-	-	-
Emilio L. Streck	UNESC	CNPq*** FAPESC** Instituto Cérebro e Mente* UNESC*	-	-	-	-	-
Flávio Kapczinski	UFRGS	CNPq***	-	-	-	-	-
João Quevedo	UNESC	CNPq*** FAPESC** Instituto Cérebro e Mente*	-	-	-	-	-

*Modest
**Significant
*** Significant. Amounts given to the author's institution or to a colleague for research in which the author has participation, not directly to the author.
Note: UNESC = Universidade do Extremo Sul Catarinense, UFRGS = Universidade Federal do Rio Grande do Sul; CNPq = Conselho Nacional de Desenvolvimento Científico e Tecnológico; FAPESC = Fundação de Amparo á pesquisa do Estado de Santa Catarina; CAPES = Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.
For more information, see Instructions for Authors.

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5. Discussão

O modelo animal de mania induzido por psicoestimulantes é descrito na literatura como um bom modelo animal de TB (Machado-Vieira et al., 2004). Nos ultimos seis anos nosso grupo de pesquisa tem validado o modelo animal de mania induzido por d-AMPH, com o qual temos sugerido que a dopamina tem um papel fundamental tanto nos sintomas maníacos quanto nos danos neuronais observados em pacientes bipolares (Frey et al., 2006a, b, c, d). Entretanto, relata-se diferenças entre os tipos de anfetaminas em induzir alterações comportamentais e neuroquímicas (Samhsa, 2007; da-Rosa et al., 2011). Tendo em vista o fato, torna-se importante avaliar se existem diferenças entre tipos de AMPHs em mimetizar comportamentos do tipo maníacos em animais experimentais. Além disso, cada vez mais estudos sugerem que o TB está associado à disfunções no metabolismo energético (Deicken et al., 1995; Dager et al., 2004; Frey et al., 2007; Regenold et al., 2009).

No presente estudo foi avaliada a diferença entre d-AMPH e m-AMPH sobre o comportamento e disfunções no metabolismo energético no cérebro de ratos. Foram avaliados também os efeitos de Li e VPA sobre as alterações comportamentais e do metabolismo energético induzidos por m-AMPH. Em adição, foram comparados os níveis de CK no soro de pacientes bipolares nas fases depressiva, maníaca e eutímica.

Na primeira fase do estudo foram comparadas as doses de 0,25, 0,5, 1 ou 2mg/kg de m-AMPH com 2mg/kg de d-AMPH – dose já estabelecida pelo grupo de pesquisa, a qual induz hiperatividade e inibição dos complexos da cadeia respiratória mitocondrial no cérebro de ratos (Frey et al., 2006 a, b, c, d; Valvassori et al., 2010) – sobre tarefas comportamentais e sobre parâmetros do metabolismo energético em córtex pré-frontal, amígdala, hipocampo e estriado de ratos.

No presente estudo reproduzimos dados prévios do nosso grupo (da-Rosa et al, 2011), onde a administração de d-AMPH (2 mg/kg) e m-AMPH nas doses de 0,25, 0,5, 1 e 2mg/kg foram equivalentes em aumentar a atividade locomotora e exploratória nos ratos. Adicionalmente, foi observado no presente estudo que m-AMPH e d-AMPH, ambos na dose de 2mg/kg, aumentaram o número de visitas ao centro do campo aberto, mostrando a equivalência das duas anfetaminas em induzir comportamento de risco. De acordo com Einat (2006), comportamento de risco pode representar a ansiedade em muitos testes. Naturalmente quando colocado em um campo iluminado aberto, pela primeira vez, ratos e camundongos tendem a permanecer na periferia do aparelho ou contra as paredes, pois espaços abertos constituem situações de risco predador. A administração de AMPHs em roedores aumenta comportamentos de risco, pois reduzem a ansiedade e, consequentemente, os animais aumentam o tempo gasto no centro do campo aberto (Einat et al., 2003).

No presente estudo encontramos também que a administração de m-AMPH (mas não d-AMPH) em altas doses (2mg/kg) induz comportamento estereotípico (sniffing e grooming). Em um estudo prévio foi demonstrado que AMPHs e nicotina aumentam estereotipia em ratos - em até 80 min após a administração dessas drogas (Izawa et al., 2006). É bem descrito na literatura que drogas de abuso, como a cocaína e a m-AMPH, que estimulam a transmissão de dopaminérgica no núcleo accumbens, induzem ativação comportamental em roedores e outros animais de laboratório (Di Chiara, 2002).

Foi observado, ainda, que a atividade das enzimas do ciclo de Krebs, citrato sintase e succinato desidrogenase, da cadeia respiratória mitocondrial (complexos I, II, II-III e IV) e CK foram inibidas no cérebro de ratos submetidos à administração de d-AMPH e m-AMPH; no entanto, essas alterações no metabolismo energético variaram de acordo com a estrutura cerebral analisada. m-AMPH e d-AMPH diminuiram a atividade dos complexos II-III e IV e a atividaden da CK no pré-frontal dos ratos; além disso, m-AMPH inibiu a atividade da citrato

sintase nesta estrutura cerebral. Na amígdala, tanto d-AMPH quanto m-AMPH diminuiu a atividade da citrato sintase, dos complexos I, II-III e IV e da CK; adicionalmente, m-AMPH também diminuiu a atividade do complexo II nessa estrutura cerebral. No hipocampo, a administração de ambas as AMPHs diminuiu a atividade dos complexos I e II-III; porém, uma única injeção de m-AMPH também inibiu a atividade da citrato sintase. Finalmente, no estriado, ambas as AMPHs inibiram as atividades da citrato sintase, da succinato desidrogenase, dos complexos I, II, III e IV e da CK. Como podemos observar os efeitos de d-AMPH e m-AMPH são heterogênios entre as estruturas cerebrais.

Quanto às estruturas cerebrais estudadas no presente trabalho podemos citar que a amídala modula o sistema límbico, controlando um sistema interativo, pré-frontal-estriado-tálamo, que controla comportamentos sócio-emocionais complexos (Strakowski et al., 2000; Strakowski et al., 2005). As AMPHs agem sobre o sistema dopaminérgico mesocorticolimbico, o qual projeta prolongamentos neuronais da área tegmental ventral para o núcleo accumbens, tubérculo olfatório, córtex pré-frontal e amígdala (Kalivas & Stewart, 1991; Volkow et al., 2003). O estriado também está envolvido nos efeitos dos psicoestimulantes, principalmente, na resposta locomotora e exploratória (Hamamura et al., 1991;. Nestler, 2001). O córtex pré-frontal é uma importante área cerebral envolvida na memória de trabalho, na tomada de decisões, controle inibitório, na seleção e retenção de informações e controle executivo (Royall, 2002; Huang et al., 2004; Rinaldi et al., 2007). Projeções glutamatérgica do córtex pré-frontal para o estriado também desempenham um papel importante na ação das AMPHs (Kalivas et al., 2005). Além disso, o sistema dopaminérgico é fundamental na eficácia sináptica e, consequentemente, para mudanças de longo prazo no hipocampo. Vale citar também que a interação entre DA e receptores glutamatérgicos são essenciais para funções cognitivas no pré-frontal e hipocampo (Gurden et

al., 2000; Nowak & Corces, 2000; Yang et al., 2000; Li et al., 2003; Chen et al., 2004; Huang et al., 2004; Tseng & O'Donnell, 2004; Granado et al, 2008).

Estudos anteriores do nosso e de outros laboratórios têm demonstrado que a administração de d-AMPH e m-AMPH em ratos inibem as enzimas do metabolismo energético (Valenzuela & Villanueva, 1987; Corrêa et al., 2007; Streck et al, 2008; Bachmann et al., 2009; Valvassori et al., 2010; Moretti et al., 2011). No entanto, não existem estudos comparando as duas drogas sobre o metabolismo energético.

Na segunda parte do trabalho, reproduzimos dados de estudos anteriores de nosso laboratório, no qual Li e VPA reverteram a hiperatividade induzida por m-AMPH na dose de 0.25mg/kg (da-Rosa et al., 2011b). É bem descrito na literatura que os estabilizadores do humor revertem e previnem a hiperatividade induzida por d-AMPH na dose de 2mg/kg (Frey et al, 2006; a, b, c, d).

Evidências sugerem que a disfunção no metabolismo energético está fortemente ligada a fisiopatologia do TB. Em um estudo *postmortem* foi encontrado diminuição da expressão de subunidades do complexo I no córtex pré-frontal de pacientes bipolares (Andreazza et al., 2010). Em uma análise, de *DNA microarray* em larga escala feito em cérebros de pacientes bipolares *postmortem*, foi demonstrado diminuição da expressão de genes mitocondriais, tais como os que codificam componentes da cadeia respiratória (Iwamoto et al., 2005). Em um elegante estudo, Cataldo e colegas (2010) demonstraram que as mitocôndrias de pacientes com TB possuem tamanho menor e anormalidades na sua distribuição celular, quando comparado com grupo controle. Essas alterações na morfologia e distribuição mitocondrial parecem estar ligadas a diversos eventos celulares, como diferenciação, envelhecimento e apoptose. Além disso, anormalidades no metabolismo energético de pacientes com TB também foram encontrados em ensaios funcionais e em estudos de espectroscopia de ressonância magnética (Deicken et al., 1995; Dager et al., 2004; Frey et al., 2007; Regenold et

al., 2009).

Em estudos anteriores, foi demonstrado que a administração de d-AMPH na dose 2mg/kg inibe a atividade da enzima citrato sintase (Correa et al., 2007) e dos complexos da cadeia respiratória mitocondrial I, II, II-III e IV (Valvassori et al., 2010) no cérebro de ratos, sendo que essas alterações induzidas por d-AMPH foram revertidas por Li e VPA. Como descrito anteriormente, as alterações no metabolismo energético estão fortemente ligados à TB, por isso, é interessante o desenvolvimento de modelos animais que mimetizem essa característica para que posteriormente possamos estudar os mecanismos de ação dos fármacos clássicos (Li e VPA) ou ainda testar novos fármacos sobre esse sistema.

Aqui, nós demonstramos que a administração crônica de m-AMPH na dose de 0,25mg/kg foi capaz de inibir a atividade das enzimas do ciclo de Krebs: citrato sintase, succinato desidrogenase e malato desidrogenase. Além disso, demonstramos também que a m-AMPH na dose de 0,25mg/kg inibiu os complexos da cadeia respiratória mitocondrial (I, II, II-III e IV), em todas as estruturas cérebrais avaliadas, assim como a d-AMPH na dose de 2mg/kg como demonstrado em estudo anterior (Valvassori et al., 2010). A partir dos resultados obtidos neste estudo, sugerimos que a diminuição da atividade das enzimas do ciclo de Krebs induzida por m-AMPH pode inibir os complexos da cadeia respiratória mitocondrial via ciclo de Krebs. Kim e colegas (2009) demonstraram hipometabolismo frontal em usuários de m-AMPH em um estudo utilizando tomografia por emissão de pósitrons. O prejuízo no metabolismo energético resulta na diminuição de ATP para a célula e a diminuição de ATP como conseqüência da toxicidade mitocondrial pode estar relacionada a uma série de toxicidade induzida pela m-AMPH.

Outro achado significativo do presente estudo é que os estabilizadores do humor, Li e VPA, atenuam os efeitos da AMPH sobre atividade das enzimas do ciclo de Krebs e, conseqüentemente, reduzem a atividade dos complexos da cadeia respiratória mitocondrial.

Nossos resultados estão de acordo com um estudo anterior, que mostrou que o tratamento crônico com Li ou VPA protege a mitocondria contra a toxicidade induzida por m-AMPH (Bachmann et al., 2009).

Kazuno e colegas (2008) mostraram que VPA pode estabilizar o cálcio intracelular em células com altos níveis de cálcio mitocondrial. A liberação excessiva de glutamato por m-AMPH pode induzir dano neuronal através do aumento intracelular Ca^{2+} mediado por receptores e do aumento da produção de EROs (Choi, 1995; Arundine & Tymianski, 2004; Lu et al., 2008). O tratamento com Li e VPA exerce efeitos neuroprotetores contra a citotoxicidade por inibir o sistema glutamatérgico e, consequentemente, diminuir a concentração de Ca^{2+} livre (Shao et al., 2005). Além disso, é bem descrito na literatura que a m-AMPH aumenta os níveis de DA oxidada, inibindo o sistema respiratório mitocondrial, tanto in vivo quanto in vitro (Przedborsk et al., 1993). O tratamento com Li e VPA inibe indiretamente o sistema dopaminérgico por um mecanismo de ativação de segundos mensageiros, que são ativadas por receptores D2 (Yatham et al., 2002). Juntamente com nossos resultados, estes estudos sugerem que os estabilizadores do humor, Li e VPA, diminuem a sinalização glutamatérgico e a DA, protegendo as mitocôndrias contra os danos induzidos por m-AMPH.

Finalmente, podemos observar que m-AMPH inibiu a atividade de CK somente no pré-frontal e que Li e VPA não foram capazes de reverter essa alteração. Em um estudo anterior de nosso laboratório, nós demostramos que a d-AMPH na dose de 2mg/kg inibiu a atividade de CK no estriado, hipocampo e no córtex, mas não no córtex pré-frontal, e que a administração de Li ou VPA não reverteu a inibição desta enzima induzida por d-AMPH (Streck et al., 2008). Esta discrepância pode ser explicada pela diferença, na estrutura molecular e na dose administrada das duas anfetaminas. No presente estudo, foi demonstrado também que a administração crônica de VPA, nos ratos pré-tratados com salina, diminuiu a

atividade de CK em todas as estruturas do cérebro avaliadas. Além disso, apesar de m-AMPH por si só não alterar a atividade de CK na amígdala e no hipocampo, o tratamento Li (Li + m-AMPH) diminui a atividade de CK nestas estruturas cerebrais. Couthon e colegas (1997) demonstraram que a incubação de quinase dimérica MM-creatina (MM-CK) com LiCl causou dissociação das subunidades e inativação total das enzimas.

Na terceira parte do nosso trabalho, foi encontrado que os níveis séricos de CK são maiores em pacientes bipolares na fase de mania, quando comparados com voluntários saudáveis refletindo possivelmente o aumento da atividade motora. Nós podemos observar também que nível de CK foi maior nos pacientes em mania do que nos pacientes em depressão. Entretanto, não houve diferença significativa nos níveis de CK quando os pacientes em depressão foram comparados ao grupo controle. Outro dado interessante encontrado no presente estudo é que não houve diferença significativa nos níveis de CK entre os pacientes bipolares eutímicos e o grupo controle.

Na prática clínica, os níveis de CK sérica são mensurados como marcador de infarto do miocárdio (ataque cardíaco), rabdomiólise (ruptura muscular grave), distrofia muscular e lesão renal aguda. Em vista disso, deve-se ter em mente que elevados níveis séricos de CK visto nos pacientes bipolares, durante a fase de mania, pode simplesmente representar aumento da atividade motora (Manor et al., 1998).

De acordo com nossos resultados, Segal e colegas (2007) mostraram que os níveis de CK nos pacientes bipolares são maiores na mania do que na depressão. Além disso, foi demonstrado que os níveis de CK no líquido cefalorraquidiano e no soro estavam aumentados em pacientes bipolares após um episódio de mania aguda (Vale et al., 1974; Taylor & Abichandani, 1980; Manor et al., 1998). No entanto, há fortes evidências de que alterações metabólicas e disfunções mitocondriais estão fortemente envolvidas na fisiopatologia da TB (Hough & Chuang, 2000; Streck et al., 2008). O circuito energético fosfocreatina/CK é

importante para manter a homeostase energética (Khuchua et al., 1998; Schlattner & Wallimann, 2000). Essa enzima possui várias funções integradas, como depósito de energia temporária, transferência de energia, bem como a regulação da capacidade metabólica (Saks et al., 1985).

Soni e colegas (1976) avaliaram pacientes com os mais variados tipos de psicoses e os classificou com base no grau de atividade psicomotora. Os níveis de CK foram relacionados com o grau de atividade psicomotora, independentemente da categoria de diagnóstico. Pacientes com retardo mental e em abstinência apresentaram níveis séricos de CK normais. Entretanto, foi observado aumento transitório nos níveis séricos de CK em pacientes que retornavam ao estado psicomotor normal, sugerindo que a atividade motora não fisiológica é mais diretamente relacionada ao aumento dos níveis séricos de CK do que a atividade motora *per se*. Em um estudo *postmortem* foi demonstrado diminuição na expressão de RNAm de CK no hipocampo e no córtex pré-frontal dorsolateral de pacientes com TB (Hermesch et al., 2002). Em um estudo clínico, foi avaliado o metabolismo do fósforo cerebral de pacientes bipolares do tipo I e do tipo II (PBI e PBII), através de espectroscopia de resonancia magnética (Kato et al., 1994). Os autores encontraram que os níveis de fosfocreatina cerebral dos PBII eram significativamente menores, quando comparado com voluntários saudáveis, sugerindo que existem diferenças fisiopatológicas entre PBI e PBII.

Este é o primeiro estudo, que avalia os níveis de CK no soro de pacientes bipolares, que incluiu pacientes eutímicos. A inclusão desse grupo nos estudos é de extrema importância, pois, pode fornecer dados sobre a persistência das alterações bioquímicas durante a remissão. Entretanto, pelo fato de a nossa amostra ser pequena, o presente estudo não teve poder suficiente para detectar possíveis diferenças entre os grupos. Outra limitação do nosso estudo é que nós não avaliamos os efeitos que os estabilizadores do humor ou outros fármacos poderiam ter sobre a atividade da CK. Contudo, o fato de os níveis de CK nos

pacientes eutímicos serem equivalentes aos observados no grupo controle, pode indicar que as alterações na CK estão associadas com episódios de humor agudo, especialmente mania aguda. Embora a atividade da CK não explique todo o perfil psiquiátrico, nos faz pensar em importantes tópicos, incluindo o desenvolvimento de tratamentos alternativos e a possibilidade de que a resposta ao tratamento possa ser monitorada mais especificamente através dos níveis de marcadores biológicos no plasma dos pacientes.

Em resumo os resultados do presente estudo demonstraram que: 1) altas doses de m-AMPH, mas não de d-AMPH, aumentam o comportamento estereotipado em ratos. 2) d-AMPH e m-AMPH tem efeitos similares sobre o metabolismo energético. 3) O tratamento crônico com m-AMPH na dose de 0,25mg/kg é capaz de induzir hiperatividade e disfunção do metabolismo energético, ambos vistos no TB. 4) O modelo animal de mania induzido por m-AMPH cumpre adequadamente os critérios de validade (face, constructo e preditiva) para o desenvolvimento de um modelo animal de mania. 5) As fases maníaca, depressiva e eutímica do TB, além de apresentarem sintomatologia distinta, também podem ser diferenciadas pelo nível de CK presente no soro dos pacientes. Entretanto, mais estudos são necessários para entender as diferenças observadas na atividade da CK durante as fases do TB. Juntos o corpo de resultados mostram que a disfunção do metabolismo energético está fortemente ligada ao TB sendo uma ferramenta importante para auxiliar na compreensão da fisiopatologia desenvolvida neste transtorno do humor, melhorando assim, a terapêutica utilizada bem como o prognóstico destes pacientes bipolares.

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