Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

Metilfenidato causa alterações neuroquímicas e comportamentais em ratos

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Orientadora: Profa Dra Angela Terezinha de Souza Wyse

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de Mestre em Bioquímica.

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Ao meu marido Lucácio,
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SUMÁRIO

RESUMO

O Transtorno de Déficit de Atenção/Hiperatividade é um transtorno prevalente e debilitante, diagnosticado com base em persistentes níveis de hiperatividade, desatenção e impulsividade. Fármacos estimulantes têm sido eficazes no tratamento desse transtorno, sendo que o metilfenidato é o agente terapêutico mais prescrito. Semelhante às ações celulares e comportamentais dos estimulantes cocaína e anfetamina, o metilfenidato aumenta a liberação e bloqueia a recaptação de dopamina e noradrenalina no cérebro de mamíferos. Milhares de crianças são tratadas com o metilfenidato para o déficit de Atenção/Hiperatividade, mas as consequências neuroquímicas desse tratamento a longo prazo, ainda não estão completamente elucidadas. No presente trabalho nós investigamos os efeitos do metilfenidato sobre alguns parâmetros bioquímicos e comportamentais em ratos. Considerando que os efeitos do metilfenidato sobre o metabolismo do sistema nervoso central são pouco conhecidos e que a Na^+,K^+-ATPase é essencial para o funcionamento normal do cérebro, inicialmente avaliamos o efeito desse psicoestimulante (1,0, 2,0 e 10,0 mg/kg) sobre a atividade dessa enzima em cérebro de ratos jovens (25 dias de idade) e adultos (60 dias). Resultados mostraram que a administração aguda de metilfenidato aumentou a atividade da Na^+,K^+-ATPase em hipocampo, córtex pré-frontal e estriado de ratos jovens e adultos. A administração crônica de metilfenidato a ratos jovens também estimulou a Na^+,K^+-ATPase em hipocampo e córtex pré-frontal, mas não em estriado. Em ratos adultos, o metilfenidato estimulou a Na^+,K^+-ATPase em todas as estruturas cerebrais estudadas, sugerindo que a ativação dessa enzima pode ser resultado dos efeitos do metilfenidato sobre o desenvolvimento do cérebro e excitabilidade neuronal. Considerando que estudos recentes têm indicado que o metilfenidato pode causar alterações no comportamento de animais, também investigamos o efeito da administração crônica de metilfenidato (2,0 mg/Kg) sobre a memória espacial em ratos jovens (15 dias). O imunoconteúdo do fator neurotrófico derivado do encéfalo (BDNF) e a atividade da acetilcolinesterase (AChE) em hipocampo e córtex pré-frontal de ratos também foram avaliados. Os resultados mostraram que os animais tratados com metilfenidato apresentaram prejuízo na memória espacial na tarefa do labirinto aquático de Morris. Observamos também uma redução no imunoconteúdo de BDNF e aumento na atividade da AChE no córtex pré-frontal, mas não em hipocampo de ratos tratados com metilfenidato. Nossos resultados sugerem que o déficit na memória espacial pode estar relacionado com a diminuição nos níveis de BDNF e aumento da AChE no córtex pré-frontal de ratos jovens submetidos à administração de metilfenidato. Os resultados desse trabalho, em conjunto, mostraram que o metilfenidato causa alterações neuroquímicas e comportamentais em animais, que podem ser prejudiciais ao desenvolvimento do cérebro.

ABSTRACT

Attention-deficit hyperactivity disorder is a prevalent and debilitating disorder diagnosed on the basis in persistent levels of overactivity, inattention and impulsivity. Stimulant medications have been effective for the treatment of this disorder, and the methylphenidate is the most prescribed therapeutic agent. Similar to the cellular and behavioral actions of the stimulants cocaine and amphetamine, the methylphenidate enhances the release and blocks the reuptake of dopamine and norepinephrine in mammalian brain. Thousands of children receive methylphenidate for attention deficit/hyperactivity disorder, yet the long-term neurochemical consequences of treatment are unknown. In the present work we investigate the effects of methylphenidate on some biochemical and behavioral parameters in rats. Considering that methylphenidate effects on central nervous system metabolism are poorly known and that Na^+,K^+-ATPase is essential to brain normal function, we initially evaluated the effect of this psychostimulant (1.0, 2.0 or 10.0 mg/Kg) on Na^+,K^+-ATPase activity in the cerebrum of young (25 days old) and adult (60 days old) rats. Our results showed that acute methylphenidate administration increased Na^+,K^+-ATPase activity in hippocampus, prefrontal cortex, and striatum of young and adult rats. Chronic administration of methylphenidate to young rats also increased Na^+,K^+-ATPase activity in hippocampus and prefrontal cortex, but not striatum. In adult rats, methylphenidate increased the Na^+,K^+-ATPase activity in all cerebral structures studied, suggesting that the activation of this enzyme might be the result of the effects of methylphenidate on brain development and neuronal excitability. Considering that recent studies have indicated that methylphenidate causes behavior alterations in animals, also investigated the effect of chronic methylphenidate (2.0 mg/Kg) administration to young rats (15 days old) on spatial memory. Brain-derived neurotrophic factor immunocontent (BDNF) and acetylcholinesterase (AChE) activity in hippocampus and prefrontal cortex were also evaluated. Results showed that methylphenidate-treated rats presented impaired performance on Morris water maze task. We also observed reduction on BDNF immunocontent and increased AChE activity in prefrontal cortex, but not in hippocampus of rats treated with methylphenidate. Our results suggested that the deficit in spatial memory may be related to decreased BDNF immunocontent and increased AChE in prefrontal cortex of young rats subjected to methylphenidate administration. The results of this work, together, showed that the methylphenidate causes neurochemical and behavioral changes in animals, which can be harmful to brain development.

LISTA DE ABREVIATURAS

ACh - acetilcolina

AChE - acetilcolinesterase

ATP - trifosfato de adenosina

BDNF - fator neurotrófico derivado do encéfalo

DSM-IV - Manual de Diagnóstico e Estatística dos Transtornos Mentais, 4ª edição

MAPK - proteína quinase ativada por mitógeno

MFD – metilfenidato

PET - Tomografia por Emissão de Pósitron

PI3K - fosfoinositídeo 3-quinase

SNC - sistema nervoso central

TDA - transportador de dopamina

TDAH - Transtorno de Déficit de Atenção/Hiperatividade

TrkB - receptor de quinase relacionado à tropomiosina

LISTA DE FIGURAS

INTRODUÇÃO

CAPÍTULO I

CAPÍTULO II

LISTA DE TABELAS

CAPÍTULO II

1. INTRODUÇÃO

1.1. Transtorno de Déficit de Atenção/Hiperatividade

O Transtorno de Déficit de Atenção/Hiperatividade (TDAH) está entre as doenças psiquiátricas mais comuns na infância e adolescência, afetando aproximadamente 5,29% de indivíduos menores de 18 anos (POLANCZYK et al., 2007). O TDAH é caracterizado por desatenção, hiperatividade e impulsividade, sendo que os sintomas frequentemente persistem na idade adulta. As consequências desse transtorno incluem prejuízo educacional, profissional e risco aumentado de desenvolvimento de outras desordens psiquiátricas (ROHDE et al.,1999; BIEDERMAN, 2003).

O diagnóstico do TDAH é fundamentalmente clínico, pois atualmente não existe nenhum marcador bioquímico para esse transtorno (TRIPP e WICKENS, 2009). O critério classificatório mais utilizado na literatura é fornecido pela quarta edição do DSM-IV, Manual de Diagnóstico e Estatística dos Transtornos Mentais (AMERICAN PSYCHIATRIC ASSOCIATION, 1994). O DSM-IV classifica os sintomas do TDAH em dois grupos: desatenção e hiperatividade/impulsividade. De acordo com esses critérios, são necessários seis ou mais sintomas em pelo menos um dos grupos para caracterizar o TDAH. Além disso, esses sintomas devem, no mínimo, persistir por seis meses e estarem presentes em dois ou mais ambientes distintos (por exemplo: casa e escola), o que pode causar prejuízo acadêmico e social para os indivíduos. Com base

nesses sintomas são reconhecidos três tipos clínicos de TDAH: predominantemente desatento, hiperativo-impulsivo ou combinado quando apresenta, no mínimo, seis sintomas em ambos os grupos.

É importante destacar que para o diagnóstico do TDAH é necessário correlacionar os sintomas com a história de vida da criança, desde que a desatenção, a hiperatividade e a impulsividade, isoladamente, podem ser resultantes de problemas no relacionamento das crianças com os pais e amigos, sistemas educacionais inadequados ou outros transtornos comumente encontrados na infância e adolescência. Além disso, o diagnóstico realizado antes dos sete anos de idade deve ser cauteloso, pois entre quatro e cinco anos um certo grau de hiperatividade em crianças é aceitável, uma vez que o desenvolvimento neuroevolutivo do encéfalo, com a completa mielinização da área pré-frontal, ocorre nessa faixa etária (MICK et al., 2002).

O TDAH apresenta uma elevada prevalência de comorbidades tais como, transtornos disruptivos do comportamento (transtorno de conduta e transtorno de oposição desafiante), depressão, ansiedade, transtornos na aprendizagem e abuso de drogas (BIEDERMAN et al., 1992; GORDON, 1993; BIEDERMAN et al., 1996).

Estudos demonstram que os sintomas do TDAH podem ser originados de disfunções no funcionamento cerebral, porém os mecanismos envolvidos ainda são pouco conhecidos. Entretanto, a influência de fatores genéticos e ambientais é amplamente aceito na literatura (TANNOCK, 1998). Evidências bioquímicas, farmacológicas e neurobiológicas indicam o envolvimento dos sistemas dopaminérgico,

noradrenérgico e serotoninérgico na fisiopatologia desse transtorno (PLISZKA et al., 1996; CASTELLANOS, 1997).

Os genes para o receptor D_4 de dopamina e para o transportador de dopamina (TDA_1) são os mais estudados, principalmente quanto aos polimorfismos. Pacientes com TDAH exibem uma maior concentração de transportadores de dopamina no cérebro em relação aos indivíduos normais (COOK et al., 1995; KRAUSE et al., 2000; FARAONE et al., 2001). Achados também indicam a participação de genes do sistema noradrenérgico na etiologia dessa doença, como os genes da enzima dopamina-β-hidroxilase e do receptor adrenérgico α2A (ROMAN et al., 2003).

Dados na literatura mostram que possíveis agentes ambientais ou lesões no lobo frontal podem estar relacionados de forma indireta com o TDAH. O fumo e o álcool na gestação, o baixo peso ao nascer e a prematuridade estão sendo considerados fatores de risco para o desenvolvimento desse transtorno. Por outro lado, pacientes com lesões no lobo frontal podem apresentar sintomas de esquecimento, distração, impulsividade e desorganização similares aos encontrados no TDAH (FARAONE et al., 2003; THAPAR et al., 2003).

As primeiras teorias bioquímicas propostas para explicar o TDAH foram baseadas nas catecolaminas, visto que regiões relacionadas à sua fisiopatologia são inervadas por neurônios dopaminérgicos e noradrenérgicos. Os circuitos fronto-subcorticais, possivelmente implicados no TDAH, são ricos tanto em dopamina, quanto em noradrenalina (FARAONE e BIEDERMAN, 1998).

O sistema dopaminérgico além de desempenhar funções essenciais para a seleção, iniciação e manutenção das funções motoras também pode regular funções cognitivas (FARAONE e BIEDERMAN, 1998; SWANSON et al., 1998; KUCZENSKI e SEGAL, 2001). Além das evidências da participação da dopamina na fisiopatologia do TDAH, outros neurotransmissores como a noradrenalina e serotonina parecem estar envolvidos nesse transtorno (PLISZKA et al., 1996; GREVET et al., 2007).

O tratamento do TDAH envolve uma abordagem múltipla. O uso de psicoterapia comportamental e/ou fármacos estimulantes do sistema nervoso central (SNC) são as intervenções mais bem documentadas (ABIKOFF e GITTELMAN, 1985). Tendo em vista que a farmacoterapia tem um papel fundamental no manejo dos sintomas desse transtorno, a literatura apresenta os psicoestimulantes como as medicações de primeira escolha para o TDAH (GREENHILL et al., 1999).

1.2. Metilfenidato (Ritalina®)

O metilfenidato (MFD, metil 2-fenil-2-(2-piperidil) acetato – nomenclatura IUPAC) (Figura 1) tem sido amplamente utilizado em crianças para o tratamento do TDAH desde 1960. Além do TDAH, está indicado no tratamento da narcolepsia (GOODMAN e GILMAN, 2003). Estudos têm mostrado que o MFD melhora a função motora e cognitiva de pacientes com doença de Parkinson (AURIEL et al., 2009) e sintomas

de depressão na população geriátrica (FISCH, 1985). Embora ainda controverso, o MFD também tem sido utilizado com sucesso no tratamento da apatia e perda de motivação em pacientes com doença de Alzheimer (TEIXEIRA e CARAMELLI, 2006).

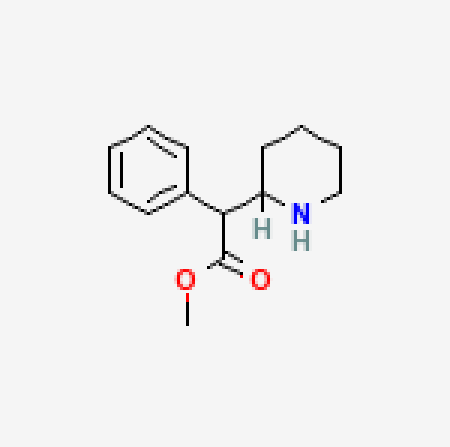

Figura 1 - Estrutura química do metilfenidato

Fonte: www.chemicalregister.com/upload/cr/113-45-1.png

Existe uma considerável quantidade de dados atestando a segurança e eficácia do MFD; entretanto, o seu mecanismo de ação ainda não está completamente elucidado (SOLANTO, 1998; CONNERS, 2002). Estudos mostram que o MFD bloqueia o transportador de dopamina, resultando no aumento da disponibilidade desse neurotransmissor na fenda sináptica, principalmente em estriado e córtex pré-frontal. Embora o MFD tenha uma maior afinidade pelos transportadores de dopamina, ele também atua sobre os sistemas noradrenérgico e serotoninérgico, bloqueando seus transportadores (GATLEY et al., 1996; KUZENSKI e

SEGAL, 1997).

O MFD é uma substância quiral cuja farmacologia específica está situada totalmente no enantiômero D. No cérebro humano, o enantiômero D se liga aos transportadores de dopamina, enquanto o enantiômero L não apresenta essa capacidade de ligação (VOLKOW et al., 2005).

O MFD apresenta ação curta, com uma meia-vida de 2 a 3 horas. A concentração plasmática é aproximadamente 10 ng/ml 2 horas após a ingestão oral (CHAN et al., 1983). Sua absorção é completa e rápida, atravessando a barreira hematoencefálica facilmente, devido a sua lipossolubilidade (AURIEL et al., 2009). Após a absorção, em um período de 48 a 96 horas, o MFD é metabolizado através do sistema microssomal hepático. O principal metabólito urinário é um produto desesterificado, o ácido ritalínico, que apresenta pouca afinidade pelos transportadores de dopamina (GOODMAN e GILMAN, 2003).

No cérebro humano, a farmacocinética do MFD foi investigada através de Tomografia por Emissão de Pósitron (PET) e carbono-11 ([^{11}C] metilfenidato) (VOLKOW et al., 1995). Resultados mostram que os níveis cerebrais de MFD atingem o pico entre 4 a 10 minutos após a administração intravenosa de [^{11}C] metilfenidato. Por via oral, esse psicoestimulante atinge o pico da concentração cerebral após 60 minutos, bloqueando mais de 50% dos transportadores de dopamina (VOLKOW et al., 2002).

As doses diárias necessárias para alcançar os benefícios clínicos variam amplamente em crianças e adultos devido à variabilidade na absorção pelo trato grastintestinal, permeabilidade da barreira

hematoencefálica e resposta ao tratamento. A dose clínica pode variar de 0,1 a 1,6 mg/Kg/dia, normalmente dividida em 3 a 4 doses (KIMKO et al., 1999; SWANSON et al., 2003). Para a obtenção de melhor resposta terapêutica, a dose deve ser ajustada individualmente (SWANSON et al., 1991). Os efeitos colaterais mais comuns são agitação, diminuição do apetite, euforia, insônia e nervosismo. A intoxicação é caracterizada por um quadro de hiperatividade simpática, incluindo hipertensão, taquicardia e hipertermia (CORDIOLI et al., 2005).

O uso do MFD aumentou significativamente nos últimos anos, entretanto, as consequências da sua utilização ainda são pouco conhecidas. A maioria dos estudos foi conduzida em adultos, mas pouco é conhecido sobre a ação do MFD no cérebro jovem. Estudos mostram que o córtex pré-frontal, núcleo accumbens e amígdala, regiões cerebrais envolvidas em funções cognitivas, motivacionais, atencionais e emocionais, são vulneráveis aos efeitos agudos e crônicos do MFD. Nesse contexto, Moll e cols. (2001) mostraram que a administração de MFD durante o período pós-natal tem efeitos duradouros sobre o desenvolvimento do sistema dopaminérgico em ratos. O MFD também alterou a expressão de genes imediatos c-fos e zif-268 em ratos adolescentes; e essas alterações persistiram durante a vida adulta dos animais (BRANDON e STEINER, 2003; CHASE et al., 2003).

1.3. Na^+,K^+-ATPase

A Na^+,K^+-ATPase (EC 3.6.3.9) ou bomba de Na^+ é uma proteína integral de membrana responsável pelo co-transporte de três íons Na^+ para o meio extracelular e dois íons K^+ para o meio intracelular, para cada molécula de trifosfato de adenosina (ATP) hidrolisada. Ela consome cerca de 40-60% do ATP cerebral para manter o gradiente eletroquímico necessário à excitabilidade neuronal, regulação do volume celular, balanço osmótico e para o transporte de moléculas ligadas ao co-transporte de Na^+; como glicose, aminoácidos e neurotransmissores (ERECIŃSKA e SILVER, 1994; KAPLAN, 2002; JORGENSEN et al., 2003).

Quanto à estrutura, a Na^+,K^+-ATPase é formada por duas subunidades catalíticas α com dez segmentos transmembrânicos, que contêm os sítios de ligação para Na^+, K^+, ATP e glicosídios cardíacos, duas subunidades β regulatórias glicosiladas, e uma subunidade γ com ação moduladora. Essa enzima apresenta uma distribuição ampla nas células dos mamíferos, sendo encontrada em maior concentração no cérebro (SKOU e ESMANN, 1992; TAGUCHI et al., 2007).

No ciclo catalítico da Na^+,K^+-ATPase, a subunidade α é fosforilada e desfosforilada em um resíduo de ácido aspártico para formar um β-aspartil fosfato durante a translocação de íons, estabilizando sua estrutura em duas formas, E_1 e E_2 (Figura 2). A forma E_1 é estabilizada pela ligação de três íons Na^+. Quando ocorre a fosforilação da enzima há uma perda da afinidade pelos íons Na^+ e liberação dos mesmos no meio extracelular.

A enzima passa a forma E_2, com alta afinidade por íons K^+, ligando assim dois íons, o que provoca sua desfosforilação, seguida pela perda da afinidade pelos íons K^+, que são liberados no meio intracelular. Finalmente, a enzima liga ATP novamente voltando à forma E_1, que tem alta afinidade por Na^+ (VASILETS e SCHWARZ, 1993; KAPLAN, 2002; JORGENSEN et al., 2003).

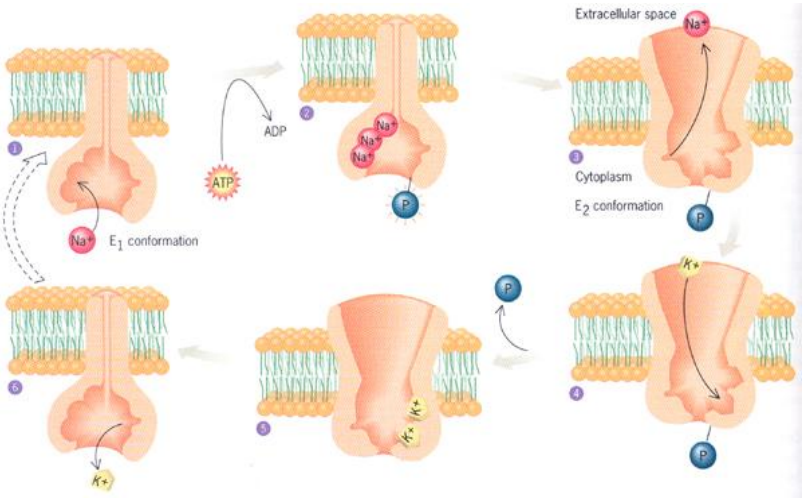

Figura 2. Ciclo catalítico da Na^+,K^+-ATPase (Adaptado de Devlin, 2003)

Independente da sua função como bomba de íons, a Na^+,K^+-ATPase é capaz de regular a expressão de seus genes e crescimento celular através da comunicação com o núcleo. Estudos mostram que essa enzima pode funcionar como receptor na transdução de sinal para a ouabaína, hormônios e neurotransmissores (XIE e ASKARI, 2002; TAGUCHI et al., 2007).

Considerando a importância da Na^+,K^+-ATPase para o funcionamento normal do SNC, a inibição da sua atividade tem sido

associada a diversas neuropatologias, como a isquemia cerebral (WYSE et al., 2000), epilepsia, crises convulsivas e doença de Alzheimer (LEES, 1993; HATTORI et al., 1998). Dados na literatura mostram que essa enzima é inibida por radicais livres, produtos de lipoperoxidação e alterações na fluidez da membrana (DOBROTA et al., 1999; KURELLA et al., 1999; CHAKRABORTY et al., 2003).

A Na^+,K^+-ATPase tem um papel fundamental na excitabilidade neuronal, bem como na captação e liberação de catecolaminas e serotonina (MATA et al., 1980; HERNÁNDEZ, 1987, 1992). Estudos em animais mostram que drogas psicoativas como a anfetamina (ZUGNO et al., 2009), fluoxetina (ZANATTA et al. 2001), selegilina (CARAGEORGIOU et al. 2003), haloperidol, carbamazepina e lítio (WOOD et al. 1989) estimulam a atividade da Na^+,K^+-ATPase.

1.4. Acetilcolinesterase

O sistema colinérgico é uma das mais importantes vias de modulação do SNC, desempenhando um papel fundamental em várias funções vitais, como aprendizado, memória, motivação, recompensa, fluxo sanguíneo cerebral e processamento sensorial e motor (MESULAM et al., 2002; SOFUOGLU e MOONEY, 2009).

A maioria dos neurotransmissores é removida da fenda sináptica por recaptação. Esse é o caso das sinapses dopaminérgicas, noradrenérgicas, glutamatérgicas, gabaérgicas e serotoninérgicas. Em contraste, a transmissão colinérgica é finalizada pela rápida hidrólise do

neurotransmissor acetilcolina (ACh) mediada pela enzima acetilcolinesterase (AChE) nas sinapses colinérgicas e junção neuromuscular (ZIMMERMAN e SOREQ, 2006).

A AChE (EC 3.1.1.7) é uma serina hidrolase, que pertence à família α/β hidrolase (CYGLER et al., 1993; SOREQ e SEIDMAN, 2001). Esta enzima hidrolisa uma ampla variedade de substratos, mostrando alta especificidade para o neurotransmissor ACh. A clivagem do substrato em produto ocorre através de duas etapas: acilação da enzima, seguida de desacilação envolvendo uma molécula de água. Esse processo acontece no sítio ativo localizado no interior da enzima, denominado tríade catalítica, formado pela serina, histidina e glutamato (SOREQ e SEIDMAN, 2001).

Além do seu papel clássico na transmissão colinérgica, a AChE tem sido associada a ações não colinérgicas como crescimento de neuritos (LAYER et al, 1993), diferenciação pós-sináptica (CHÁCON et al., 2003), hematopoiese, osteogênese (GRISARU et al.,1999), adesão celular (SILMAN e SUSSMAN, 2005) e regulação de funções imunes (KAWASHIMA e FUJI, 2000).

A AChE existe nas formas globular e assimétrica. A forma globular é composta por monômeros (G1), dímeros (G2) ou tetrâmeros (G4) da subunidade catalítica. A forma G4 ligada à membrana é a mais abundante no SNC (DAS et al., 2001). A forma assimétrica é encontrada na junção neuromuscular e consiste de um (A4), dois (A8) ou três (A12) tetrâmeros catalíticos ligados covalentemente a uma subunidade de colágeno Q (ALDUNATE et al., 2004).

A hipofunção colinérgica tem sido associada a prejuízos cognitivos característicos de algumas doenças neurodegenerativas. Nesse contexto, o tratamento com inibidores da AChE aumenta os níveis de ACh, e isso pode melhorar o aprendizado e a memória em pacientes com a doença de Alzheimer (BALLARD et al., 2005). Além do seu envolvimento na aprendizagem e memória, a ACh também desempenha um papel relevante na dependência de drogas (WILLIAMS e ADINOFF, 2008).

1.5. Fator Neurotrófico Derivado do Encéfalo

O fator neurotrófico derivado do encéfalo (do inglês - *brain-derived neurotrophic factor* – BDNF), um membro da família de polipeptídeos que inclui o fator de crescimento neural, neurotrofina-3 e neurotrofina 4/5, é a mais abundante neurotrofina expressa no SNC (THOENEN, 1995; LEWIN e BARDE, 1996). Como outros neuropeptídeos, o BDNF é sintetizado como um pró-peptídeo (pró-BDNF) sendo proteoliticamente transformado na forma madura (mBDNF). O pró-BDNF se liga e ativa o receptor pró-apoptótico p75, enquanto o mBDNF se liga e ativa o receptor de quinase relacionado à tropomiosina (TrkB) (BIBEL e BARDE, 2000).

O BDNF se encontra co-localizado pré e pós-sinapticamente com o receptor TrkB. A ligação ao receptor TrkB leva à dimerização e autofosforilação de resíduos de tirosina no domínio intracelular do receptor e ativação subsequente de precursores de sinalização citoplasmática, incluindo a proteína quinase ativada por mitógeno (MAPK), fosfolipase C e fosfoinositídeo 3-quinase (PI3K) (KAPLAN e

MILLER, 2000).

Existe um grande número de estudos indicando o papel do BDNF na modulação da formação, maturação e plasticidade de sinapses glutamatérgicas e gabaérgicas (GOTTMANN et al., 2009). Além disso, o BDNF está envolvido na sobrevivência neuronal, diferenciação fenotípica e manutenção de neurônios dopaminérgicos, colinérgicos e serotoninérgicos (ALDERSON et al., 1990; HYMAN et al., 1991; ALTAR et al., 1994; MARTIN-IVERSON et al., 1994; EATON e WHITTEMORE, 1996).

O BDNF também é essencial na modulação da expressão gênica e cognição (POO, 2001; LU et al., 2005) e está presente em maior quantidade no hipocampo, mas também em córtex, cerebelo e prosencéfalo basal, áreas cerebrais que são vitais para os processos de aprendizado e memória (TIMMUSK et al., 1993; PRUUNSILD et al., 2007).

O BDNF tem sido implicado na fisiopatologia de várias doenças neurodegenerativas e neuropsiquiátricas como doença de Alzheimer (SCHINDOWSKI et al., 2008; TAPIA-ARANCIBIA et al., 2008), Huntington (GAUTHIER et al., 2004), transtorno bipolar, depressão (KAPCZINSKI et al., 2008b; RANTAMÄKI e CASTRÉN, 2008) e TDAH (LANKTREE et al., 2008). O papel central do BDNF no desenvolvimento do sistema dopaminérgico e o envolvimento desse neurotransmissor com o TDAH, sugerem que alterações na expressão do BDNF podem aumentar o risco para o desenvolvimento desse transtorno (TSAI, 2003).

Dados na literatura apontam para a participação dos sistemas

glutamatérgico e gabaérgico, bem como proteínas cinases e mecanismos hormonais nos processos de memória (IZQUIERDO e MEDINA, 1997; CAMMAROTA et al., 2005). Além disso, tem sido proposto o envolvimento da Na^+,K^+-ATPase, AChE e BDNF na modulação da cognição (SATO et al., 2004; WYSE et al., 2004; BALLARD et al., 2005; LU et al., 2005).

1.6. Memória

A memória não é estática, isolada ou uma única função cerebral; memória pode ser definida como uma complexa rede de diferentes funções inter-relacionadas trabalhando juntas para gerenciar as informações (PAUL et al., 2009). Suas fases compreendem a aquisição, a consolidação e a evocação de uma grande diversidade de informações (IZQUIERDO, 2002; SQUIRE e KANDEL, 2003). A aquisição é obtida através de experiências; a consolidação compreende a fase em que a informação obtida é processada e a evocação é a recordação, a lembrança daquilo que foi previamente aprendido (SQUIRE, 2004).

De uma maneira geral, a memória pode se classificada em dois grandes grupos: declarativa, referente à informação que é transmitida ou expressa e não-declarativa, representada por informações sobre habilidades motoras ou sensoriais que não podem ser transmitidas oralmente. A memória também pode ser classificada quanto ao tempo de duração, curta ou longa duração (IZQUIERDO, 2002).

A memória espacial é responsável pelo conhecimento, codificação, armazenamento e recuperação de informações sobre o arranjo espacial

dos objetos ou rotas específicas (KESSELS et al., 2001). Ela não pode ser classificada em um dos grupos mencionados anteriormente, pois envolve aspectos da memória declarativa e não-declarativa, bem como de curta e longa duração (MOSCOVITCH et al., 2006). Esse tipo de comportamento pode ser considerado uma expressão de curiosidade natural ou representar uma necessidade de adquirir informações sobre um estímulo ou ambiente novo (THINUS-BLANC, 1996).

A memória espacial pode ser dividida em memória espacial de trabalho ou memória espacial de referência. A memória espacial de trabalho é transitória e precede as memórias de curta e longa duração. No momento em que a informação é recebida ela determina se a mesma é nova ou já consta nos arquivos; e quanta informação será armazenada nos sistemas de curta e longa duração. Ela serve para manter por alguns segundos, no máximo poucos minutos, a informação que está sendo processada no momento. Seu processamento depende da atividade elétrica de neurônios do córtex pré-frontal. A memória espacial de referência exibe uma maior capacidade de armazenamento, duração e resistência aos interferentes do que a memória espacial de trabalho, sendo processada pelo circuito hipocampal e suas conexões (IZQUIERDO, 2002).

Um dos métodos mais utilizados para a avaliação da memória espacial em roedores é o labirinto aquático de Morris. Esse teste comportamental foi desenvolvido por Morris, em 1982, para avaliar o papel dos estímulos visuais como referências proximais e distais para a memória espacial em ratos. Dados na literatura mostram que a memória

espacial depende da ação de neurotransmissores em diversas áreas cerebrais. Lesões no hipocampo, córtex pré-frontal e estriado prejudicam o aprendizado de animais nessa tarefa (D`HOOGE e DE DEYN, 2001).

O uso prolongado de psicoestimulantes como a anfetamina e cocaína têm sido associado à disfunção cognitiva envolvendo aprendizado e memória. Estruturas cerebrais como o hipocampo e córtex pré-frontal, que são essenciais a funções cognitivas, são altamente sensíveis a derivados anfetamínicos (CAMARASA, 2008; SANTUCCI, 2008).

1.7. Objetivos

1.7.1 Objetivo geral

Considerando que: (1) os mecanismos de ação do MFD ainda não estão bem elucidados e (2) a Na^+,K^+-ATPase, AChE e BDNF são importantes para a função cerebral e estão relacionados aos mecanismos de memória, o objetivo geral do nosso estudo foi investigar o efeito do MFD sobre alguns parâmetros neuroquímicos (Na^+,K^+-ATPase, AChE e BDNF) e comportamentais em ratos.

Este trabalho será dividido em dois capítulos como segue:

- **Capítulo I**

Objetivos específicos:

- Investigar o efeito da administração aguda de MFD (1, 2 e 10 mg/Kg) sobre a atividade de Na^+,K^+-ATPase em hipocampo, córtex pré-frontal e estriado de ratos jovens e adultos;
- Verificar o efeito da administração crônica de diferentes doses de MFD sobre a atividade de Na^+,K^+-ATPase em cérebro de ratos jovens e adultos.

- **Capítulo II**

Objetivos específicos:

- Investigar o efeito da administração crônica de MFD sobre a memória espacial de referência e memória espacial de trabalho em ratos jovens na tarefa do labirinto aquático de Morris;
- Verificar a influência do tratamento crônico com MFD sobre o imunoconteúdo de BDNF em hipocampo e córtex pré-frontal de ratos jovens;
- Avaliar a atividade da AChE em hipocampo e córtex pré-frontal de ratos jovens submetidos à administração crônica de MFD.

2. ARTIGOS CIENTÍFICOS

CAPÍTULO I – ARTIGO 01

Methylphenidate treatment increases Na^+, K^+-ATPase activity in the cerebrum of young and adult rats

Emilene B. S. Scherer, Cristiane Matté, Andréa G. K. Ferreira, Karin M. Gomes, Clarissa M. Comim, Cristiane Mattos, João Quevedo, Emilio L. Streck and Angela T. S. Wyse

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Methylphenidate treatment increases Na^+, K^+-ATPase activity in the cerebrum of young and adult rats

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Abstract Methylphenidate is a central nervous system stimulant used for the treatment of attention-deficit hyperactivity disorder. Na^+, K^+-ATPase is a membrane-bound enzyme necessary to maintain neuronal excitability. Considering that methylphenidate effects on central nervous system metabolism are poorly known and that Na^+, K^+-ATPase is essential to normal brain function, the purpose of this study was to evaluate the effect of this drug on Na^+, K^+-ATPase activity in the cerebrum of young and adult rats. For acute administration, a single injection of methylphenidate (1.0, 2.0, or 10.0 mg/Kg) or saline was given to rats on postnatal day 25 or postnatal day 60, in the young and adult groups, respectively. For chronic administration, methylphenidate (1.0, 2.0, or 10.0 mg/Kg) or saline injections were given to young rats starting at postnatal day 25 once daily for 28 days. In adult rats, the same regimen was performed starting at postnatal day 60. Our results showed that acute methylphenidate administration increased Na^+, K^+-ATPase activity in hippocampus, prefrontal cortex, and striatum of young and adult rats. In young rats, chronic administration of methylphenidate also enhanced Na^+, K^+-ATPase activity in hippocampus and prefrontal cortex, but not in striatum. When tested in adult rats, Na^+, K^+-ATPase activity was increased in all cerebral structures studied. The present findings suggest that increased Na^+, K^+-ATPase activity may be associated with neuronal excitability caused by methylphenidate.

Keywords Methylphenidate · Na^+, K^+-ATPase · Hippocampus · Prefrontal cortex · Striatum

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Introduction

The psychostimulant methylphenidate (MPH, Ritalin®) is used in the treatment of hyperkinetic and other forms of attention-deficit/hyperactivity disorders (Chase et al. 2003; Banaschewski et al. 2006). It has been known that attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric disease of prevalence heterogeneous that can affect 3–9% of school-aged children and 4% of adults (Heiligenstein et al. 1998; Faraone et al. 2003; Polanczyk et al. 2007). This disorder, commonly diagnosed during childhood, is characterized by inattention, impulsivity, and hyperactivity (Goldman et al. 1998; Miller and Castellanos 1998). The long-term consequences include poor school performance, delinquency, difficulties with peers and family, and other antisocial behavior (Taylor et al. 2004).

Patients with ADHD exhibit dysfunction of dopaminergic and noradrenergic circuits, including prefrontal, subcortical regions (e.g., striatum), and limbic regions (e.g., hippocampus) (Arnsten et al. 1996; Dinn et al. 2001; Castellanos and Tannock 2002; Bush et al. 2005).

Biochemical studies have shown that MPH, like amphetamine, enhances the release and blocks the reuptake of noradrenaline and dopamine in mammalian brain (Ferris et al. 1972; Biederman and Faraone 2005; Volkow et al. 2005). The distribution of MPH in brain is heterogeneous, and the maximum concentration occurs in the striatum, cortex, and cerebellum (Volkow et al. 2005). The effects of MPH treatment on central nervous system (CNS) development have been investigated since other psychoactive drugs such as fluoxetine and reboxetine may cause changes in the system of neurotransmitters during brain maturation (Moll et al. 2001; Bock et al. 2005).

Na^+, K^+-ATPase (E.C 3.6.1.37), an enzyme concentrated at nerve ending membranes, is responsible for generating and maintaining of ionic gradient necessary for neuronal excitability, consuming about 40–50% of the ATP produced in brain (Erecinska and Silver 1994). This enzyme has been related to various aspects of neural function, where it might modulate directly or indirectly the signaling transmission, neurotransmitter release and uptake, and neurogenesis (Choi 1988; Xie and Askari 2002; Deisseroth et al. 2004). Studies show that Na^+, K^+-ATPase activity is altered in various disorders affecting the brain, such as ischemia (Wyse et al. 2000a), neurodegenerative diseases (Hattori et al. 1998), neuropsychiatric disorders (Kurup and Kurup 2002; Goldstein et al. 2006), and animal models of depression and mania (Gamaro et al. 2003; Zugno et al. 2009). The relationship between Na^+, K^+-ATPase activity and neurotransmitter release has been demonstrated, suggesting that this enzyme could play a role in the neurotransmission modulation (Hernandez 1992a, b; Yang et al. 2007). Recent studies showed that amphetamine administration increased Na^+, K^+-ATPase activity in hippocampus of rats (Zugno et al. 2009).

Therefore, considering that Na^+, K^+-ATPase activity is critical for normal brain function, and that little is known about the neurochemical and behavioral consequences of MPH treatment on the developing brain, in the present study we investigated the effect of acute and chronic administration of MPH on Na^+, K^+-ATPase activity in hippocampus, prefrontal cortex, and striatum of young and adult rats. We used these cerebral structures because there are data showing that after administration of MPH an increase of this drug in hippocampus, cerebral cortex, and striatum occurs (Volkow et al. 2005).

Materials and methods

Animals

Male young (25 days old, $n = 40$) and adult (60 days old, $n = 40$) Wistar rats were obtained from Central Animal House of Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil. They were caged in groups of five with free access to food and water, and were maintained on a 12-h light–dark cycle (lights on at 7:00 am), at a temperature of 23 ± 1°C. This study was performed in accordance with the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care and the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996); with the approval of Ethics Committee from Universidade do Extremo Sul Catarinense.

Acute administration of methylphenidate

A single intraperitoneal (i.p.) injection of MPH (1.0, 2.0, or 10.0 mg/kg of body weight) or saline (0.9% NaCl) was given to rats on postnatal day (PD) 25 ($n = 20$) or PD 60 ($n = 20$) in the young and adult groups, respectively ($n = 5$ per group totaling 8 groups). The doses chosen were based on previous reports suggesting that MPH doses lower than 5.0 mg/kg might better reflect clinical use (Gerasimov et al. 2000), while the dose of 10.0 mg/kg would mimic *recreational* use (Valvassori et al. 2007). Two hours after the injection, the animals were killed by decapitation without anesthesia, the brain was removed and hippocampus, prefrontal cortex, and striatum were obtained.

Chronic administration of methylphenidate

In young rats, MPH (1.0, 2.0, or 10.0 mg/kg, i.p.) or saline (0.9% NaCl) injections were given starting at PD 25 once a day, for 28 days (last injection at PD 53; $n = 20$). In adult rats, the same regimen was performed starting at PD 60 (last injection at PD 88; $n = 20$) totaling 8 groups ($n = 5$). Two hours after the last injection, the animals were killed by decapitation, the brain was removed and hippocampus, prefrontal cortex, and striatum were obtained (Chase et al. 2003).

Tissue preparation

The hippocampus, prefrontal cortex, and striatum were homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at $1000 \times g$ for 10 min; the supernatants were removed for Na^+, K^+-ATPase activity determination.

Na^+, K^+-ATPase activity assay

The reaction mixture for Na^+, K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 µl.

After 10 min of pre-incubation at 37°C, the reaction was initiated by addition of ATP to a final concentration of 3.0 mM, and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays according to the method of Wyse et al. (2000b). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

Protein determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan multiple range test when the F test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Values of $P < 0.05$ were considered significant.

Results

We initially investigated the effect of acute MPH administration on Na^+, K^+-ATPase activity in cerebrum of rats. Results showed that MPH (2.0 and 10.0 mg/kg) significantly increased Na^+, K^+-ATPase in hippocampus (Fig. 1a; $F(3,12) = 20.59$; $P < 0.001$), prefrontal cortex (1.0, 2.0, and 10.0 mg/kg) (Fig. 1b; $F(3,12) = 25.10$; $P < 0.001$), and striatum (2.0 mg/kg) (Fig. 1c; $F(3,14) = 3.36$; $P < 0.05$) of young rats. Figure 2 shows that Na^+, K^+-ATPase was significantly increased in hippocampus in all tested doses (Fig. 2a; $F(3,12) = 13.34$; $P < 0.001$), prefrontal cortex in 2.0 and 10.0 mg/kg (Fig. 2b; $F(3,12) = 8.50$; $P < 0.01$), and striatum in all tested doses (Fig. 2c; $F(3,12) = 8.13$; $P < 0.01$) of adult Wistar rats subjected to acute MPH administration.

Next, the effect of chronic MPH administration on Na^+, K^+-ATPase activity in cerebrum of rats was evaluated. Figure 3 shows that MPH, at the dose of 2.0 and 10.0 mg/kg, significantly increased Na^+, K^+-ATPase in hippocampus (Fig. 3a; $F(3,14) = 3.93$; $P < 0.05$), as well in prefrontal cortex in all tested doses (Fig. 3b; $F(3,13) = 6.13$; $P < 0.01$). Besides, Na^+, K^+-ATPase activity was not affected in striatum (Fig. 3c; $F(3,13) = 0.904$; $P > 0.05$) of young rats. Finally, MPH significantly increased Na^+, K^+-ATPase in hippocampus (only at 10.0 mg/kg) (Fig. 4a; $F(3,15) = 15.18$; $P < 0.001$), prefrontal cortex (2.0 and 10.0 mg/kg) (Fig. 4b; $F(3,16) = 11.54$; $P < 0.001$), and striatum, in all tested doses, (Fig. 4c; $F(3,15) = 14.37$; $P < 0.001$) of adult rats treated chronically.

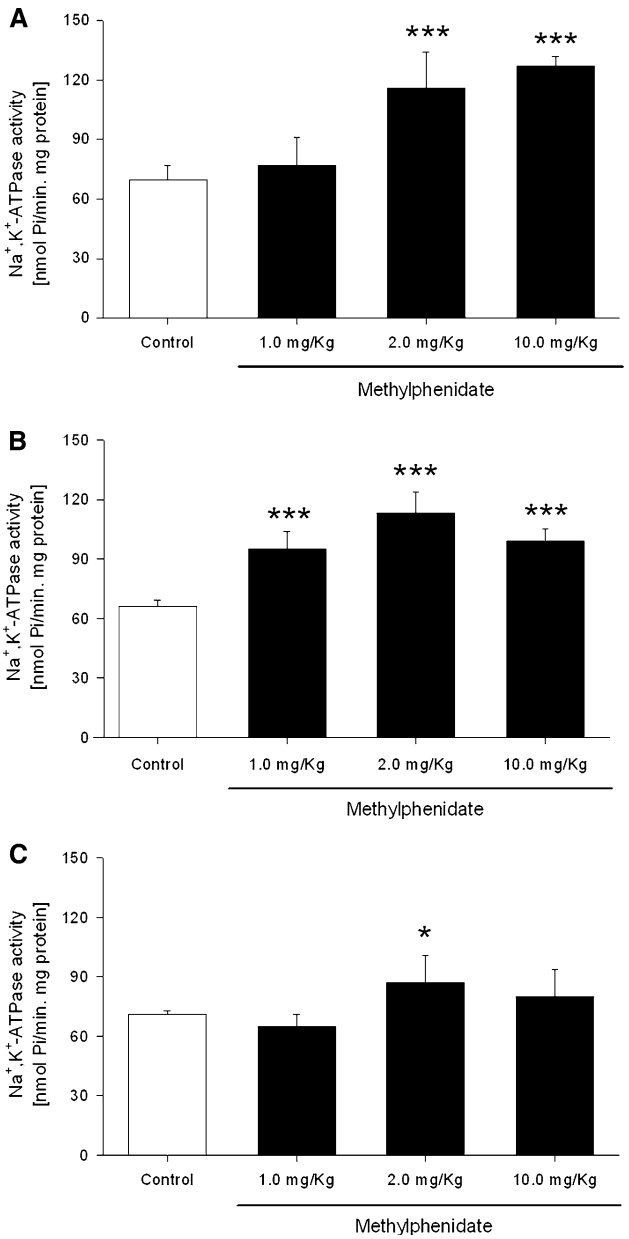

Fig. 1 Effect of acute administration of methylphenidate on Na^+, K^+-ATPase activity in hippocampus (**a**), prefrontal cortex (**b**), and striatum (**c**) of young rats. Results are expressed as mean ± SD for four to five independent experiments performed in duplicate. * $P < 0.05$, *** $P < 0.001$ compared to control (Duncan's multiple range test)

Discussion

In the present study we evaluated the effect of a single or chronic administration of MPH on Na^+, K^+-ATPase activity in different cerebral structures from young and

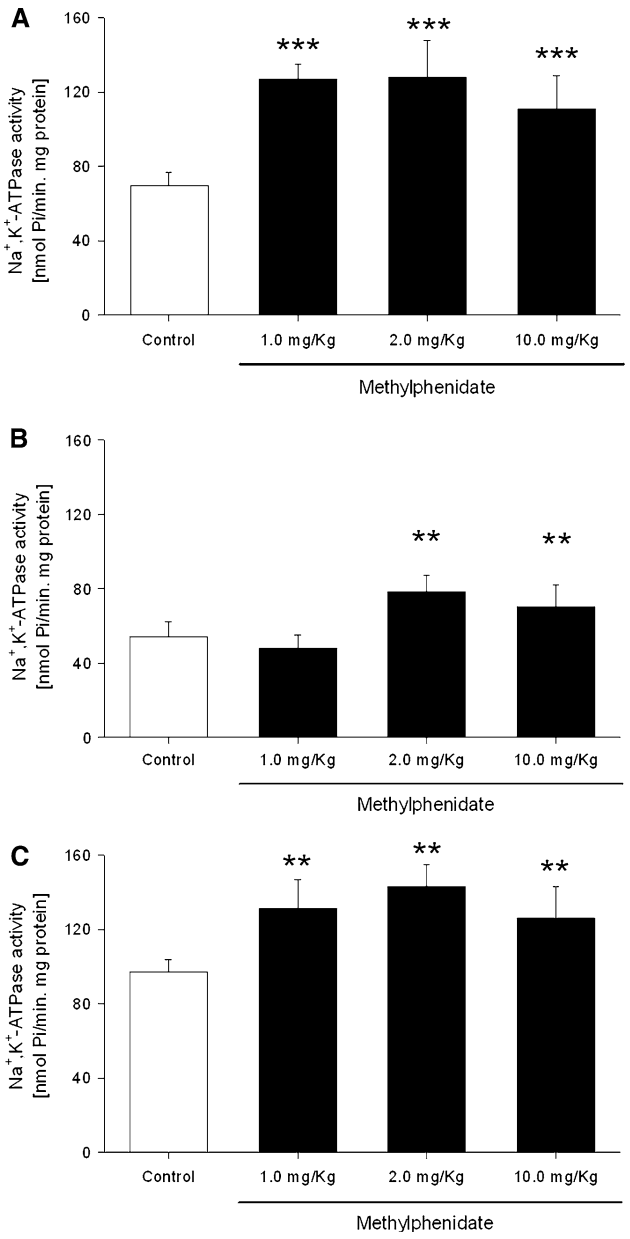

Fig. 2 Effect of acute administration of methylphenidate on Na^+, K^+-ATPase activity in hippocampus (**a**), prefrontal cortex (**b**), and striatum (**c**) of adult rats. Results are expressed as mean ± SD for four to five independent experiments performed in duplicate. ** $P < 0.01$, *** $P < 0.001$ compared to control (Duncan's multiple range test)

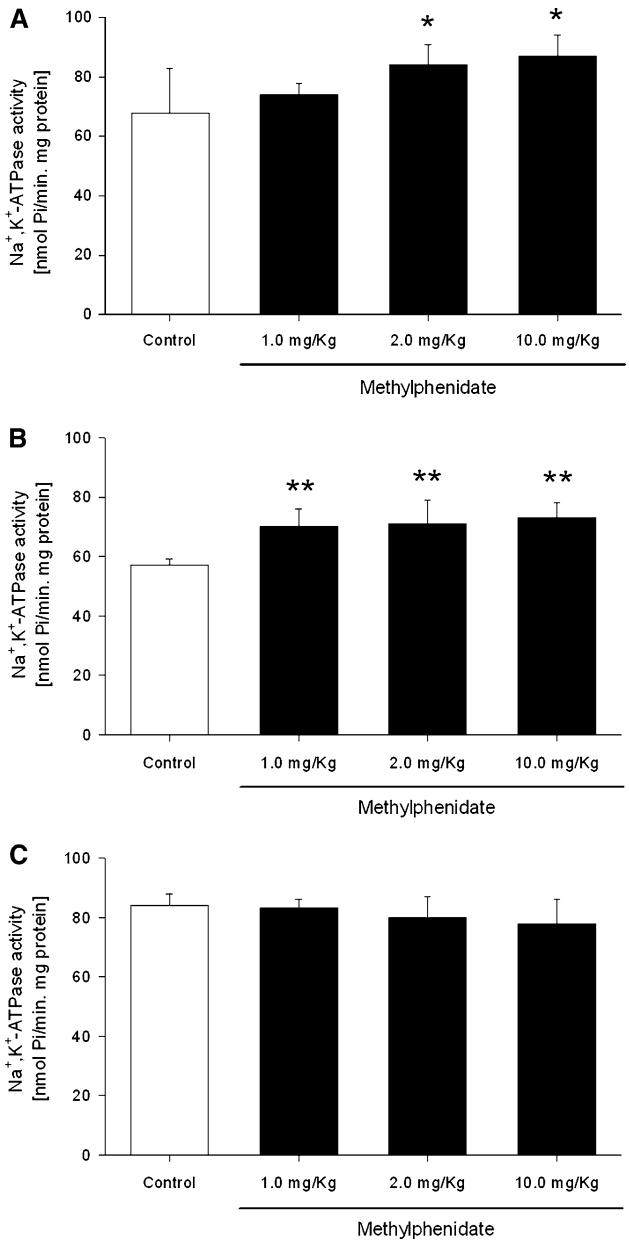

Fig. 3 Effect of chronic administration of methylphenidate on Na^+, K^+-ATPase activity in hippocampus (**a**), prefrontal cortex (**b**), and striatum (**c**) of young rats**.** Results are expressed as mean ± SD for four to five independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$ compared to control (Duncan's multiple range test)

adult rats. Our findings showed that MPH acute administration enhanced Na^+, K^+-ATPase activity in hippocampus, prefrontal cortex, and striatum of young and adult rats. Chronic administration of MPH to young rats also increased Na^+, K^+-ATPase activity in hippocampus and prefrontal cortex, but not in striatum. In adult rats, MPH enhanced Na^+, K^+-ATPase activity in all brain structures evaluated. Differences between young and adult rats' response after MPH treatment have been reported, and some researchers suggest that these differences depend on the brain area studied, of the dose, as well as of the age of exposure to the drug (Volkow et al. 1998; Yano and Steiner 2005; Chase et al. 2007).

Methylphenidate increases dopamine and noradrenaline in the synaptic cleft (Kuczenski and Segal 1997; Overtoom et al. 2003). Although the efficacy of MPH for ADHD is well recognized, clinical and animal studies showed that MPH influences the brain development (Moll et al. 2001;

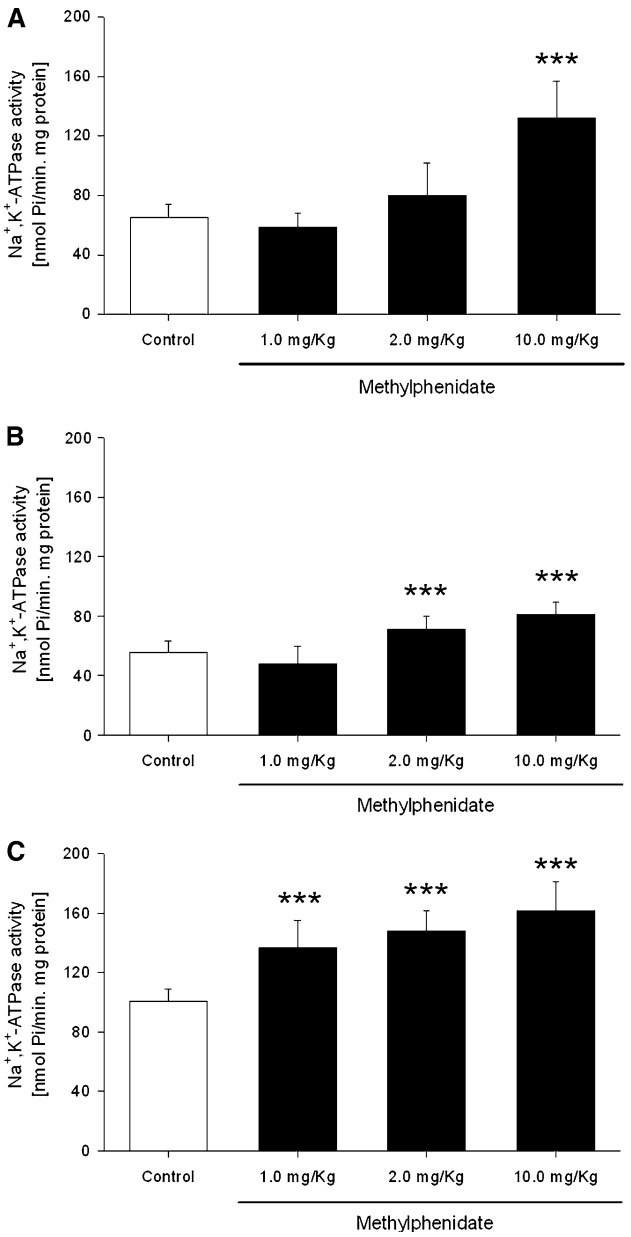

Fig. 4 Effect of chronic administration of methylphenidate on Na^+, K^+-ATPase activity in hippocampus (**a**), prefrontal cortex (**b**), and striatum (**c**) of adult rats. Results are expressed as mean ± SD for four to five independent experiments performed in duplicate. *** $P < 0.001$ compared to control (Duncan's multiple range test)

Shaw et al. 2009). Exposure of the immature brain to drugs such as amphetamine and MPH may alter gene expression and could lead to permanent changes in cellular responsiveness, synaptic connectivity (Robinson and Kolb 2004; Stanwood and Levitt 2004; Andersen 2005), and interfere with normally developing neurotransmitter systems (Moll et al. 2001). Studies showed that the administration of MPH to young rodents produces notable changes in behavior, neurophysiology, and biochemistry in adulthood. These effects may be associated with dopamine receptor overstimulation (Achat-Mendes et al. 2003; Bolanos et al. 2003; Carlezon et al. 2003; Adriani et al. 2006). In addition, MPH stimulates energy metabolism enzymes in the brain of young and adult rats (Fagundes et al. 2007; Scaini et al. 2008).

Na^+, K^+-ATPase is involved in several physiological functions such as regulation of the cell volume, cell differentiation, and maintenance of sodium and potassium equilibrium through biological membranes. Since this enzyme is involved in cellular excitability and cell energy metabolism (Erecinska and Silver 1994), modifications in the modulatory action of Na^+, K^+-ATPase can be involved in behavioral sensitization induced by psychostimulants (Munhoz et al. 2003).

For our knowledge, our results provide the first experimental demonstration that MPH stimulates Na^+, K^+-ATPase in cerebrum of young and adult rats. Interestingly, it is important to note that other psychoactive drugs such as amphetamine (Zugno et al. 2009), fluoxetine (Zanatta et al. 2001), selegiline (Carageorgiou et al. 2003), haloperidol, carbamazepine, and lithium (Wood et al. 1989) also increase Na^+, K^+-ATPase activity in the rat brain.

In summary, in the present study we demonstrated that MPH increased significantly the activity of Na^+, K^+-ATPase in cerebrum of young and adult rats. The mechanisms by which MPH administration stimulated Na^+, K^+-ATPase were not clear; however, we suggested that the activation of this enzyme might be the result of the effects of methylphenidate on brain development and neuronal excitability since Na^+, K^+-ATPase is very important for the normal functioning of CNS. Therefore, further studies are necessary to elucidate the mechanisms involved in the activation of Na^+, K^+-ATPase caused by MPH.

Study limitations

The main limitation of the present study is the fact that we did not evaluate methylphenidate effects in an animal model of ADHD, such as spontaneous hypertensive rats. Moreover, we used i.p. administration of methylphenidate, and patients usually take this drug orally.

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CAPÍTULO II – ARTIGO 02

Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in rat

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Status: A ser submetido

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Abstract

Methylphenidate, a psychostimulant that affects both dopaminergic and noradrenergic systems, is one of the most frequently prescribed treatments for attention-deficit hyperactivity disorder. The present study investigated the effects of chronic administration of methylphenidate to juvenile rats on spatial memory, brain-derived neurotrophic factor immunocontent and acetylcholinesterase activity in hippocampus and prefrontal cortex. Rats received intraperitoneal injections of methylphenidate (2.0 mg/Kg) once a day, from the 15th to the 45th day of age or an equivalent volume of 0.9% saline solution (controls). Twenty-four hours after the last injection, animals were subjected to testing in the Morris water maze. After that, animals were killed and hippocampus and prefrontal cortex were obtained for determination of brain-derived neurotrophic factor immunocontent and acetylcholinesterase activity. Chronic administration of methylphenidate provoked impairment of spatial learning in reference and working memory tasks. A reduction on brain-derived neurotrophic factor immunocontent and an increased acetylcholinesterase activity in prefrontal cortex, but not in hippocampus of rats treated with methylphenidate were also observed. These results suggest that the deficit in spatial memory may be related to decreased brain-derived neurotrophic factor immunocontent and increased acetylcholinesterase in prefrontal cortex of juvenile rats subjected to methylphenidate administration.

Keywords: Methylphenidate; spatial memory; BDNF; acetylcholinesterase.

Introduction

Methylphenidate (MPH, Ritalin®) is a psychostimulant commonly used for the treatment of attention-deficit/hyperactivity disorder (ADHD) and for narcolepsy (Swanson & Volkow, 2002; Thomas, Francescutti-Verbeem, & Kuhn, 2008). ADHD is a complex neurobehavioral disorder affecting school-age children and often persists into adulthood (Chase, Carrey, Soo, & Wilkinson, 2007). It is characterized by varying degrees of inattention, hyperactivity and impulsivity (Biederman & Faraone, 2005; Miller & Castellanos, 1998), with comorbidities such as learning disabilities (Sunohara, Malone, Rovet, Humphries, Roberts, & Taylor, 1999) and mood disorders (Wilens, Biederman, & Spencer, 2002).

MPH blocks both dopamine and noradrenaline transporters, inhibiting the presynaptic reuptake, so increasing the amount of both neurotransmitters in several brain regions including the hippocampus and the prefrontal cortex (Kuczenski & Segal, 2001). These cerebral structures are involved in memory mechanisms (Alonso, Vianna, Depino, Mello e Souza, Pereira, Szapiro, Viola, Pitossi, Izquierdo, & Medina, 2002; Izquierdo, Quillfeldt, Zanatta, Quevedo, Schaeffer, Schimitz, & Medina, 1997) and present their peak of synaptogenesis during childhood and adolescence in humans and rats (Bayer, Altman, Russo, & Zhang, 1993; Klintsova & Greenough, 1999; Rice & Barone, 2000). MPH is safe and effective in treating ADHD symptoms, but the long-term consequences of drug exposure on brain development and behavior remain unknown (Bethancourt, Camarena, & Britton, 2009). Recent investigations have shown that MPH promotes pronounced changes in expression of genes

(Yano & Steiner, 2007), maturation and signalling of the dopaminergic system (Fukui, Svenningsson, Matsuishi, Higashi, Nairn, Greengard, & Nishi, 2003; Moll, Hause, Rüther, Rothenberger, & Huether, 2001).

Brain-derived neurotrophic factor (BDNF) is a member of the family of neurotrophins that modulate different aspects of neuronal function during development and in the mature nervous system (Gottmann, Mittmann, & Lessmann, 2009). This neurotrophin acts through tropomyosin-related kinase B (TrkB) receptors either pre- and postsynaptically to modulate long-term potentiation (LTP), which serves as a molecular model for the synaptic events underlying memory formation (Farmer, Zhao, van Praag, Wodtke, Gage, & Christie, 2004; van Praag, Kempermann, & Gage, 1999). Furthermore, BDNF enhances glutamatergic synaptic transmission (Gottmann et al., 2009) and has been strongly implicated in spatial learning (Hall, Thomas, & Everitt, 2000; Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998; Yamada, Mizuno, & Nabeshima, 2002).

Acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine (ACh) at the synaptic cleft of cholinergic synapses and neuromuscular junctions (Soreq & Seidman, 2001). In addition to its role in cholinergic transmission, AChE is associated with brain development, learning and memory, and neuronal damage (Ballard, Greig, Guillozet-Bongaarts, Enz, & Darvesh, 2005; Metz & Tracey, 2005; Zimmerman & Soreq, 2006). Several “nonclassical” AChE activities have been described as neurite growth (Layer, Weikert, & Alber, 1993), haematopoiesis, osteogenesis (Grisaru, Sternfeld, Eldor, Glick, & Soreq, 1999), as well as

an action as adhesion protein in synaptic development and maintenance (Silman & Sussman, 2005).

The aim of the present study is to investigate the effects of chronic administration of MPH to juvenile rats on spatial memory, as well as on BDNF immunocontent and AChE activity in hippocampus and prefrontal cortex of rats, cerebral structures known to be involved both in the action of MPH in the brain and in memory mechanisms.

Materials and methods

Animals and reagents

Male Wistar rats (15 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22±1ºC) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the ‘‘Principles of Laboratory Animal Care’’ (NIH publication 85-23, revised 1985) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

Chronic administration of methylphenidate

Rats were treated for 30 days (postnatal day 15-45) with an intraperitoneal (i.p.) injection of saline (0.9% NaCl) or MPH (2.0 mg/kg) once a day, resulting in plasma and brain levels of MPH within the range achieved under clinical conditions in humans (Gerasimov, Franceschi, Volkow, Gifford, Gatley, Marsteller, Molina, & Dewey, 2000; Volkow,

Wang, Fowler, Gatley, Logan, Ding, Hitzemann, & Pappas, 1998). Twenty-four hours after the last injections, animals were subjected to behavioral testing. Twenty-four hours after behavioral testing, animals were killed by decapitation without anesthesia, the brain was removed and hippocampus and prefrontal cortex were obtained for analysis of BDNF immunocontent and AChE activity determination.

Behavioral testing

On the 45th day of life, animals were subjected to behavioral testing. The Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks (D'Hooge & De Deyn, 2001; Mc Namara & Skelton, 1993; Netto, Hodges, Sinden, Le Peilet, Kershaw, Sowinski, Meldrum, & Gray, 1993) was used. The behavioral experiments were conducted between 7h and 12h a.m. The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water maintained at constant temperature of 23◦C. The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video camera mounted above the center of the tank.

Reference memory task

The task consisted of 7 training and one test sessions. In the acquisition phase, rats had daily sessions of 4 trials per day for 7 days to find the platform, submerged 2 cm under the water surface, placed on the

center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the 4 starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failure, they were gently guided to it; all animals were allowed to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was of 15–20 min (Netto et al., 1993). One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross over the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measure for spatial memory (Netto et al., 1993). In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial.

Working memory task

After 1 week, the working memory version of Morris water maze was performed. The task consisted of 4 consecutive trials per day, with a 20 min inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every

subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance (Netto et al., 1993).

Open field task

The task was run in a wooden box measuring 60 cm × 40 cm × 50 cm with a frontal glass wall, whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 2 min. The number of squares crossed with the four paws from one square to another and rearings were measured as indicative of motor activity (Netto, Dias, & Izquierdo, 1986).

Analysis of BDNF immunocontent

Mature BDNF protein was assessed using the E-Max ELISA kit (Promega) according to the manufacturer's recommendations. Briefly, hippocampus and prefrontal cortex were individually homogenized in lysis buffer containing: 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and centrifuged for 3 min at 14,000 rpm at 4 °C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated on a 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody and blocked with Block and Sample buffer. After sample incubation, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Then color

reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/mL, was performed in each plate.

Acetylcholinesterase assay

For the AChE assay, the hippocampus and prefrontal cortex were homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10min at 1000 x g. The supernatants were used for the enzymatic AChE analyses. AChE activity was determined according to Ellman et al. (1961), with some modifications. Hydrolysis rates were measured at acetylthiocholine concentration of 0.8 mM in 300 µL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25 ºC. About 15 µL of hippocampus and prefrontal cortex supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30s). All samples were run in triplicate.

Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

Differences between groups in the Morris Water Maze performance were analyzed by one-way (ANOVA), repeated measures analysis of variance (ANOVA) or Student's T test; data from the probe trial

parameters, open field test, BDNF immunocontent and AChE activity were analyzed by Student's T test. A $p < 0.05$ was considered significant. Descriptive statistics data were expressed as mean ± SEM or mean ± SD. All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC- compatible computer.

Results

Experiment 1: Effect of MPH on reference memory task in the Morris water maze

Figure 1 shows that chronic MPH administration affects the acquisition phase of reference memory. Repeated measures ANOVA (days versus groups) revealed a significant groups [$F(1,22) = 5.739$; $p < 0.05$] and days effect [$F(6,22) = 46.307$; $p < 0.001$]. Student's T test showed differences in days 2 and 4 between the two groups (both $p < 0.05$). Four parameters were evaluated in the test session, latency to cross over the location of the platform, the number of target crossings and the time spent in the target and opposite quadrants (Fig. 2A–D). The latency to cross over the platform location for the first time was significantly increased by MPH treatment when compared to saline group (A) [$t(22) = 2.822$; $p < 0.05$]. However, MPH administration did not affect the number of crossings over platform location area (B) [$t(22) = 1.669$; $p > 0.05$], time spent in the target quadrant (C) [$t(22) = 1.804$; $p > 0.05$], nor time in the opposite quadrant (D) [$t(22) = 1.580$; $p > 0.05$].

Experiment 2: Effect of MPH on working memory task in the Morris water maze

It was also shown that chronic MPH administration affected working memory in the Morris water maze. One-way ANOVA showed that MPH alters performance of rats on trials 3 [$F(1,22) = 10.702$; $p < 0.01$] and 4 [$F(1,22) = 69.544$; $p < 0.001$] (Fig.3). Figure 4 demonstrates that MPH-treated rats exhibit significant difference between T1-T3 [$t(22) = 2.856$; $p < 0.01$].

Experiment 3: Effect of chronic administration of MPH on open field task

In order to verify whether MPH would affect motor activity, we submitted both groups (with and without MPH treatment) to the open field arena. MPH did not alter the number of crossings [$t(22) = 1.153$; $p > 0.05$] nor of rearings [$t(22) = 0.700$; $p > 0.05$] (Table 1).

Experiment 4: Effect of chronic administration of MPH on BDNF immunocontent

Mature BDNF levels in hippocampus and prefrontal cortex of rats were measured after behavioral testing. Figure 5 shows that BDNF immunocontent was not affected in hippocampus [Fig. 5A; $t(8) = 0.172$; $p > 0.05$] when compared to control group. However, its administration significantly reduced BDNF levels in prefrontal cortex [Fig. 5B; $t(8) = 2.745$; $p < 0.05$] of experimental animals.

Experiment 5: Effect of chronic administration of MPH on AChE activity

Next, the effect of chronic MPH administration on AChE activity in hippocampus and prefrontal cortex were evaluated. In hippocampus, AChE activity was not affected by MPH [Fig. 6A; $t(9) = 1.350$; $p > 0.05$]; however its activity was significantly increased in prefrontal cortex [Fig. 6B; $t(9) = 4.253$; $p < 0.05$] of rats after behavioral testing.

Discussion

Present study demonstrates that chronic exposure to therapeutic dose of MPH (2.0 mg/kg) in juvenile rats significantly impairs learning/memory of a reference spatial task in the Morris water maze. MPH-treated animals also showed a reduced efficiency to find the platform position in the working memory task, suggesting a deficit of spatial navigation (Netto et al., 1993). Open-field task performance and the swim speeds (~34.70 cm/s) were used as a measure for spontaneous motor activity and the results indicate that none of the tested groups present any deficit.

Our results are in agreement with previous studies showing that exposure to MPH during early developmental periods causes behavioral changes in rats (Andersen, Arvanitogiannis, Pliakas, LeBlanc, & Carlezon, 2002) such as depressive-like effects in the forced-swin, reduced habituation to a familiar environment (Carlezon, Mague, & Andersen, 2003), increased anxiety-like behaviors, decreased sensitivity to rewarding stimuli (Bolaños, Barrot, Berton, Wallace-Black, & Nestler, 2003) and promotes transient effects on object recognition memory and contextual fear memory (Bethancourt et al., 2009). MPH administered chronically to

juvenile rats (7-35 days of life) affected brain areas involved in cognition, motivated behaviors, appetite, and stress (Gray, Punsoni, Tabori, Melton, Fanslow, Ward, Zupan, Menzer, Rice, Drake, Romeo, Brake, Torres-Reveron, & Milner, 2007) and induced post-treatment impairment in recognition and spatial memory in adult rats (LeBlanc-Duchin & Taukulis, 2009). Other psychostimulants such as cocaine and methamphetamine, administered early in postnatal development, have resulted in long-term alterations in behavior (Dow-Edwards & Hughes, 1995; Vorhees, Ahrens, Acuff-Smith, Schilling, & Fisher, 1994).

It has been previously reported that MPH treatment increases Na^+,K^+-ATPase activity in the cerebrum of young and adult rats (Scherer, Matté, Ferreira, Gomes, Comim, Mattos, Quevedo, Streck, & Wyse, 2009) and there is evidence showing that this enzyme might play a relevant role in the molecular mechanisms of learning (Sato, Tanaka, Ohnishi, Teramoto, Irifune, & Nishikawa, 2004; Wyse, Bavaresco, Reis, Zugno, Tagliari, Calcagnotto, & Netto, 2004).

Some neurochemical parameters that could to be related to memory impairment were also investigated. Our results show that MPH reduced BDNF immunocontent in prefrontal cortex, but not in the hippocampus of rats. Prefrontal cortex, whose proper functioning depends on maintaining a delicate balance of dopamine and norepinephrine activities, mediates executive abilities functions, such as working memory, attention regulation, behavioral inhibition, planning and organization (Arnsten & Li, 2005; Hastings, Parsey, Oquendo, Arango, & Mann, 2004). The lack of effect of MPH on this parameter in hippocampus is puzzling,

however, we suggest that the prefrontal cortex is more sensitive to drug action, since recent biochemical studies showed that MPH have more potent effects in this structure than in other brain areas (Arnsten, 2009; Drouin, Page, & Waterhouse, 2006; Kuczenski & Segal, 2002). Furthermore, lower levels of cortical BDNF have been associated with impaired working memory in dopamine transporter knockout mice (Li, Arime, Hall, Uhl, Cui, & Sora, 2009).

BDNF is a neurotrophin implicated in almost all aspects of central nervous system (CNS) development, including neuronal survival and proliferation, differentiation and maintenance of dopaminergic, cholinergic and serotonergic neurons (Alderson, Alterman, Barde, & Lindsay, 1990; Altar, Whitehead, Chen, Wortwein, & Madsen, 2003; Eaton & Whittemore, 1996) and synaptic plasticity (Gottmann et al., 2009). Memory acquisition and consolidation are associated with an increase in BDNF levels and the activation of its TrkB receptor, since this neurotrophin is one of the mediators of LTP at glutamatergic synapse in the CNS (Gottmann et al., 2009; Yamada & Nabeshima, 2003).

Moreover, effects of BDNF vary substantially depending on specific experimental conditions, e.g., the exact time point during development, the brain area studied, the location of BDNF release and the pattern of synaptic stimulation (Gottmann et al., 2009). MPH presented little effect on BDNF expression in the striatum of juvenile rats (25-38 days of life) (Chase et al., 2007). Moreover, Banerjee and colleagues (2009) showed that a single injection of MPH (2.0 mg/kg, i.p.) induced a reduction of BDNF mRNA in hippocampal and cortical brain regions of juveniles,

whereas effects in adults rats were significantly lower. It has been shown that the administration of others psychoactive drugs such as haloperidol and risperidone decreased BDNF levels in hippocampus, frontal and occipital cortices of rats (Angelucci, Mathé, & Aloe, 2000).

AChE is one of the most important enzymes in many living organisms, including humans and vertebrates, and is located greater quantity in the nervous system and in muscles, playing very important role in nerve signal transmission (Soreq & Seidman, 2001). The reduction in brain levels of ACh has been associated with cognitive alterations of some dementias. Treatment with AChE inhibitors increases ACh levels, and this can improve learning and memory in patients with Alzheimer's disease (Ballard et al., 2005).

Present reports show also that MPH increased AChE activity in prefrontal cortex, but not in the hippocampus of rats. The stimulation of this enzyme by MPH might decrease ACh levels, what could be associated with the memory impairment observed. In this context, there is evidence that ACh is involved in the behavioral effects of stimulant drugs such as amphetamine, cocaine and MPH. Repeated exposure to amphetamine induced disruption of prefrontal cholinergic activity and attentional performance in an animal model of cognitive symptoms for schizophrenia (Kozak, Martinez, Young, Brown, Bruno, & Sarter, 2007). Tzavara and colleagues (2006) showed that acute administration of MPH (1.0 and 3.0 mg/kg) increased cortical and hippocampal ACh release in rats.

The administration of galantamine, a drug inhibitor of AChE, reversed symptoms such as agitation, excitement and stereotyped behavior induced by amphetamine in monkeys (Andersen, Werge, & Fink-Jensen, 2007). Another study in rats showed that AChE inhibitors block the locomotor sensitization induced by cocaine (Hikida, Kitabatake, Pastan, & Nakanishi, 2003).

In conclusion, our results demonstrate that MPH impairs spatial learning/memory, reduces BDNF immunocontent and increases AChE in prefrontal cortex of rats. Although the mechanism(s) involved are not clearly defined, these findings are consistent with other studies reporting that this psychostimulant causes neurochemical and behavioral alterations in animals. Taken together, these observations are, at least in part, consistent with a hypothesis that impaired working memory may be associated with the decreased prefrontal cortex BDNF levels and increased of AChE activity found in juvenile rats subjected to chronic administration of MPH.

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Legends to Figures

Figure 1. Effect of chronic methylphenidate administration on spatial memory acquisition phase. Data show latencies to find the platform across blocks of four trials on each day and are expressed as mean ± SEM for 12 animals in each group. Different from control, *p <0.05 (repeated measures ANOVA). MPH – methylphenidate.

Figure 2. Effect of chronic methylphenidate administration on performance of spatial memory test session parameters namely: time spend to cross the platform (a), number of crossings on the platform (b), time spend in the target quadrant (c) and the time spend in the opposite quadrant (d). Data are expressed as mean ± SEM for 12 independent animals in each group. Different from control, *p <0.05 (Student's t test). MPH – methylphenidate.

Figure 3. Effect of chronic methylphenidate administration on performance in working memory version of Morris water maze. Data are latencies to find the platform on each trial during the 4 days and are expressed as means ± SEM for 12 animals in each group. Different from control, **p <0.01, ***p <0.001 (one-way ANOVA). MPH – methylphenidate.

Figure 4. Effect of chronic methylphenidate administration on performance in working memory. Data are expressed as mean ± SEM for 12 animals in

each group. Significant difference between T1-T3, **p <0.01 MPH – methylphenidate.

Figure 5. Effect of chronic methylphenidate administration on BDNF concentration in hippocampus (A) and prefrontal cortex (B) of rats. Results are expressed as mean ±SD for 5 animals in each group. Different from control, *p <0.05 (Student's t test). MPH – methylphenidate.

Figure 6. Effect of chronic methylphenidate administration on acetylcholinesterase (AChE) activity in hippocampus (A) and prefrontal cortex (B) of rats. Data are expressed as mean ± SD for 5-6 animals in each group. Different from control, *p <0.05 (Student's t test). MPH – methylphenidate.

FIGURA 1

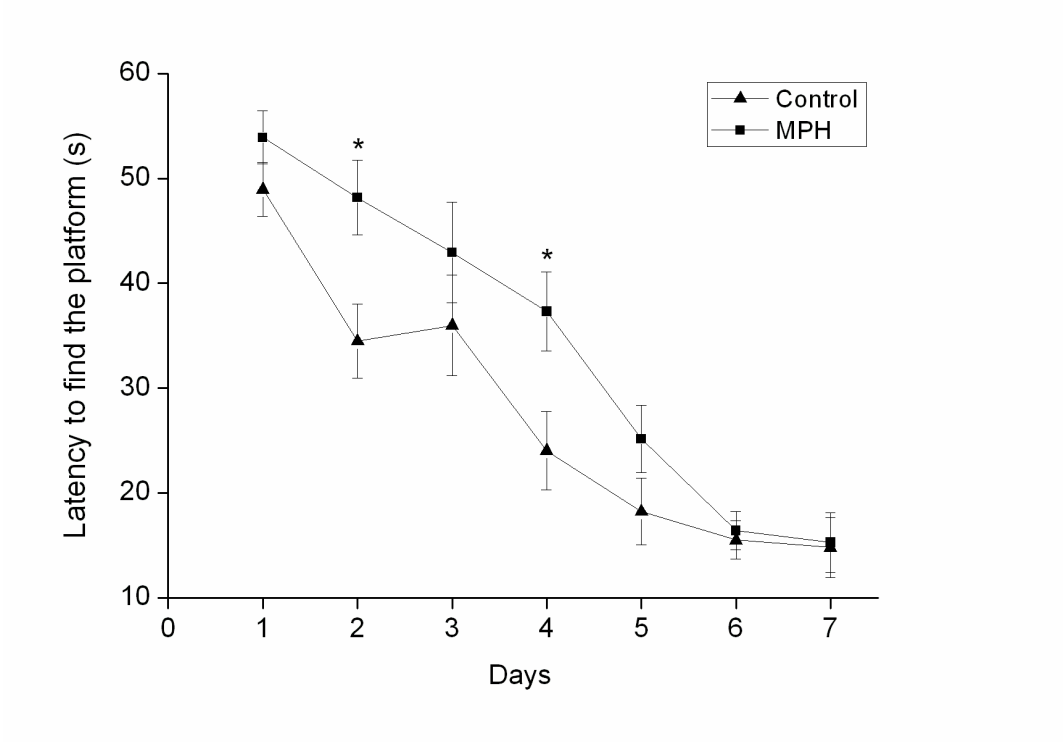
Latency to find the platform (s)
Days
Control
MPH
*
*
60
50
40
30
20
10
0
1
2
3
4
5
6
7

FIGURA 2

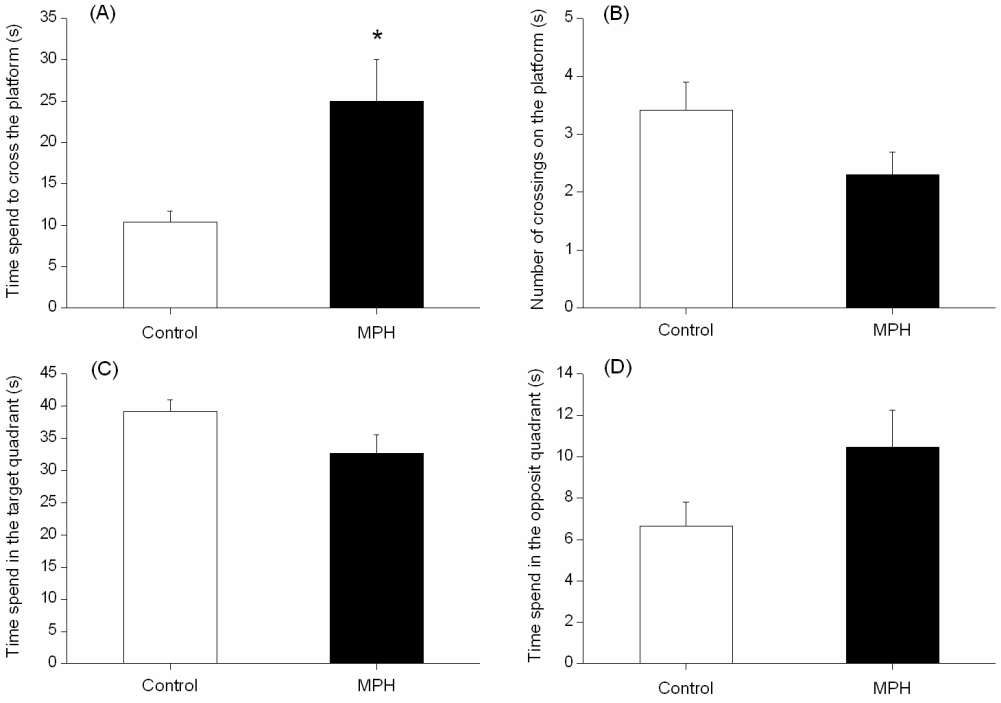
(A)
Time spend to cross the platform (s)
35
30
25
20
15
10
5
0
*
Control
MPH
(B)
Number of crossings on the platform (s)
5
4
3
2
1
0
Control
MPH
(C)
Time spend in the target quadrant (s)
45
40
35
30
25
20
15
10
5
0
Control
MPH
(D)
Time spend in the opposit quadrant (s)
14
12
10
8
6
4
2
0
Control
MPH

FIGURA 3

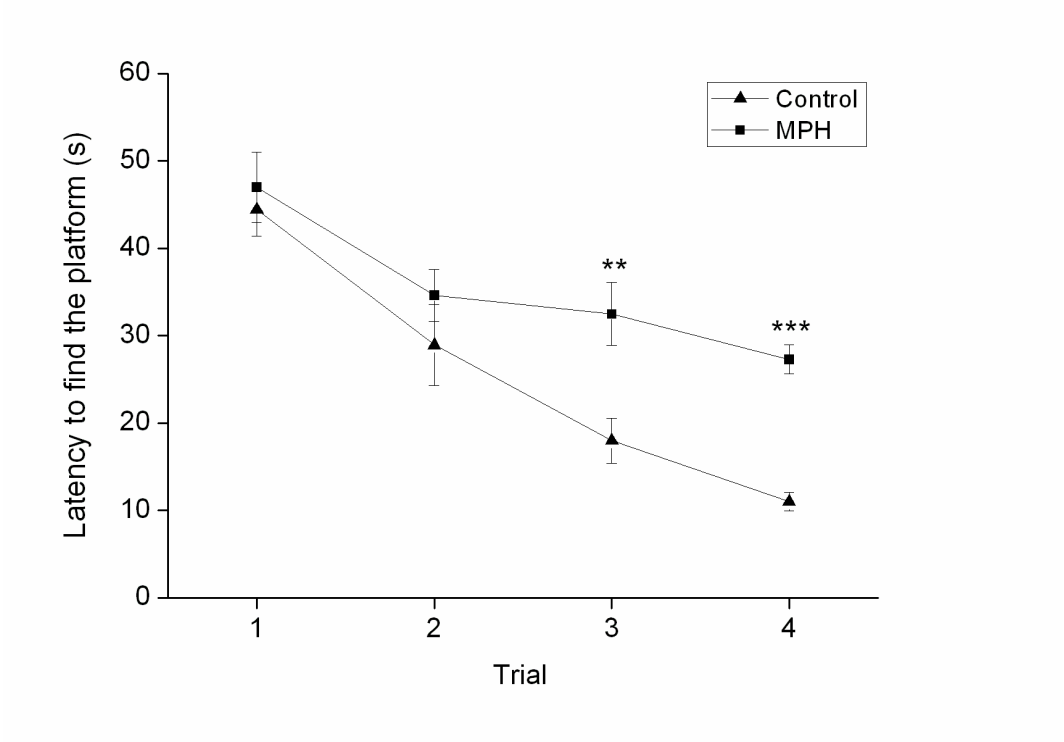
Latency to find the platform (s)
60
50
40
30
20
10
0
1
2
3
4
Trial
Control
MPH
**
***

FIGURA 4

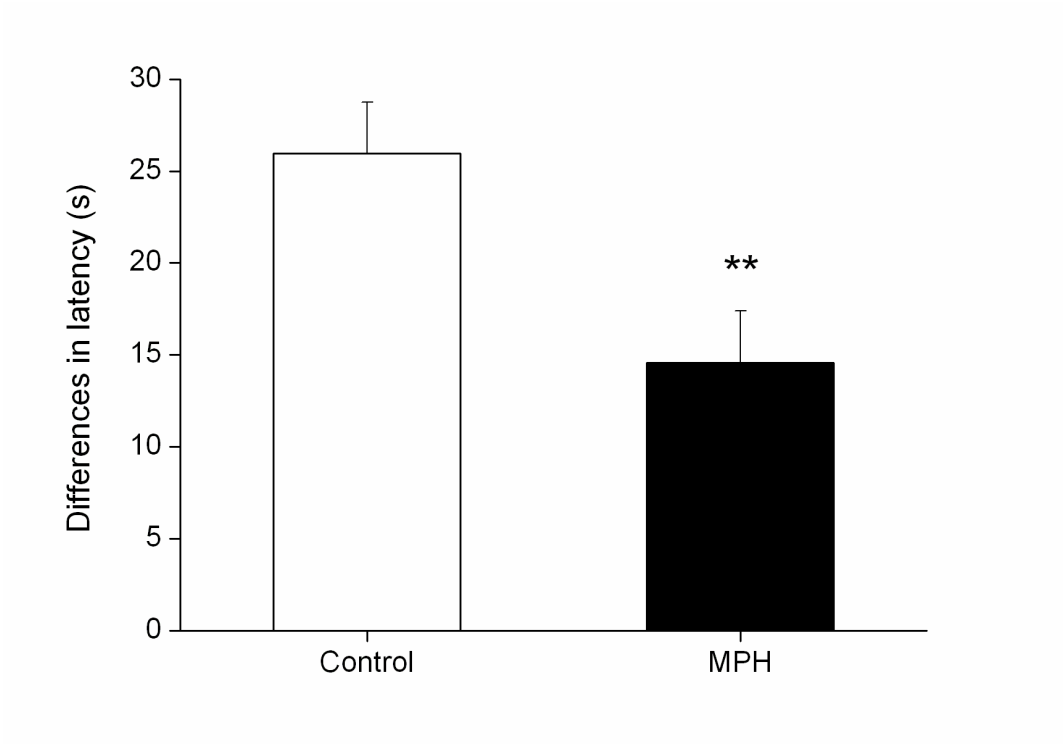

FIGURA 5

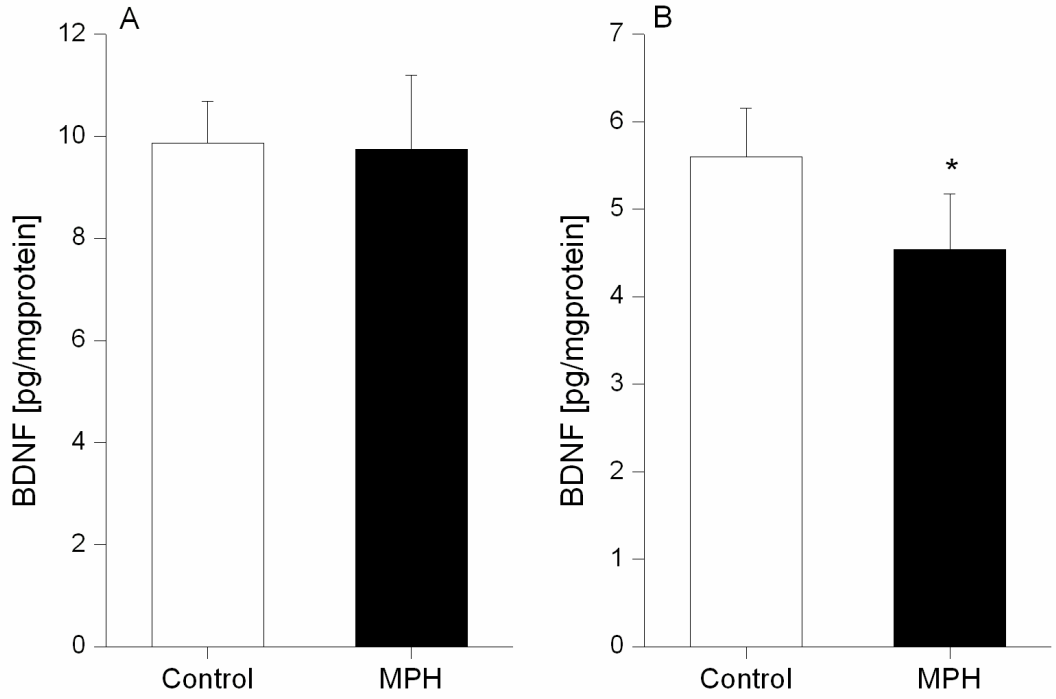
A
B
BDNF [pg/mgprotein]
BDNF [pg/mgprotein]
12
10
8
6
4
2
0
7
6
5
4
3
2
1
0
*
Control
MPH
Control
MPH

FIGURA 6

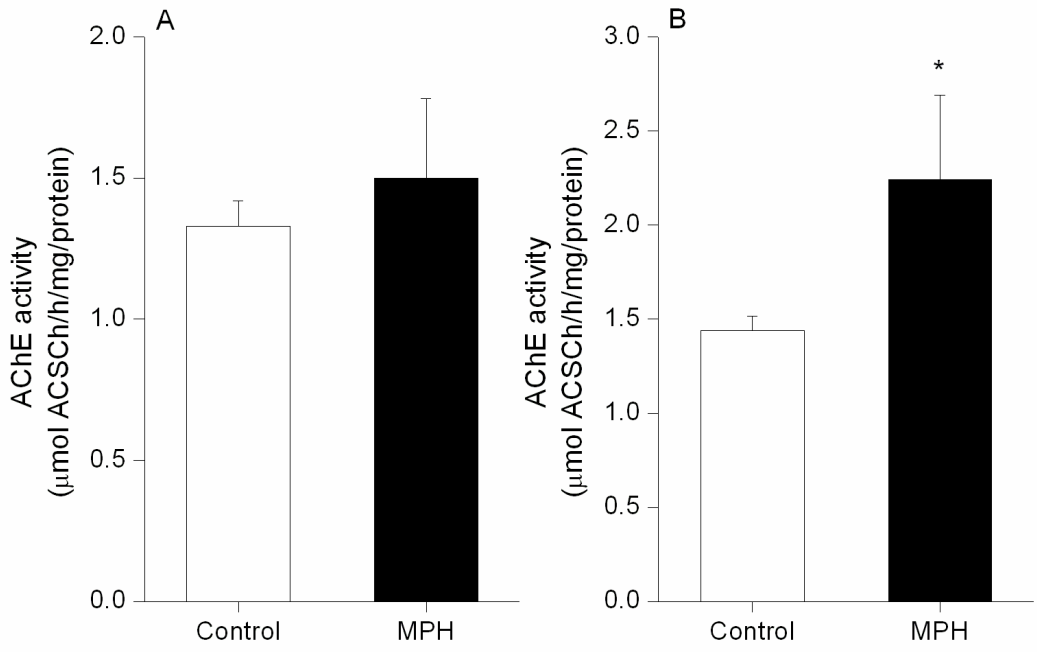
A
AChE activity
(µmol ACSCh/h/mg/protein)
2.0
1.5
1.0
0.5
0.0
Control
MPH
B
AChE activity
(µmol ACSCh/h/mg/protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
MPH

TABELA 1

Effect of chronic methylphenidate administration on performance (number of crossings and rearings) in the open field task.

Groups	Number of crossings	Number of rearings
Control	30.66 ± 2.62	10.83 ± 0.89
Methylphenidate	33.23 ± 4.29	9.92 ± 0.93

Data are expressed as mean ± SEM for 12 animals in each group. There were no significant differences between groups, $p > 0.05$ (Student's T test).

3. DISCUSSÃO

O psicoestimulante MFD tem um perfil neurofarmacológico semelhante à anfetamina e cocaína, sendo um dos mais prescritos tratamentos para o TDAH, um transtorno comportamental associado à disfunção no sistema catecolaminérgico e anormalidades cerebrais (WILENS, 2008).

O MFD diminui o comportamento impulsivo e melhora a atenção em pacientes com TDAH. Estudos sugerem que o MFD atenua os sintomas desse transtorno através do bloqueio dos transportadores de dopamina, inibindo a sua recaptação. Os receptores dopaminérgicos cerebrais são abundantes e se distribuem principalmente no córtex frontal, estriado, sistema límbico e hipotálamo (BERRIDGE e ROBINSON, 1998).

Dados na literatura mostram que o MFD inibe a recaptação de noradrenalina e estimula receptores alfa e beta-adrenérgicos no neurônio pós-sináptico (BENNETT et al., 1999). Evidências também sugerem que o MFD pode atuar sobre o sistema serotoninérgico (GATLEY et al., 1996; KUZENSKI e SEGAL, 1997).

Os possíveis efeitos adversos produzidos pela intervenção psicofarmacológica e a falta de informações sobre os efeitos do MFD no neurodesenvolvimento têm gerado preocupações quanto às consequências da sua utilização (ACCARDO e BLONDIS, 2001; KLEIN-SCHWARTZ, 2003). Dessa forma, os estudos em animais são muito importantes para elucidar os mecanismos pelos quais o MFD altera o

funcionamento do SNC.

Recentes estudos mostraram que a exposição do cérebro imaturo ao MFD causa alterações bioquímicas e comportamentais em ratos, como alteração na expressão dos genes imediatos c-fos e fos-B (CHASE et al., 2005), ativação da via de sinalização da proteína cinase A no córtex pré-frontal (PASCOLI et al., 2005) e ansiedade (BOLAÑOS et al., 2003). Além disso, a persistente alteração catecolaminérgica causada pela administração crônica de MFD durante o desenvolvimento pode afetar a sinaptogênese, gliogênese e mielinização (LEVITT et al., 1997; BARONE et al., 2000; RICE e BARONE, 2000).

Considerando que pouco é conhecido sobre as consequências da utilização do MFD durante períodos prolongados, e que a Na^+,K^+-ATPase é uma enzima fundamental para o funcionamento normal do SNC, no presente estudo, inicialmente investigamos o efeito da administração aguda e crônica de 1, 2 e 10 mg/Kg de MFD sobre a atividade da Na^+,K^+-ATPase em hipocampo, córtex pré-frontal e estriado de ratos jovens e adultos. As doses escolhidas foram baseadas em dados da literatura, sugerindo que doses inferiores a 5 mg/Kg refletem o uso clínico (GERASIMOV et al., 2000) e a dose de 10 mg/Kg mimetiza o uso recreacional (VALVASSORI et al., 2007).

Nossos resultados mostraram que a administração aguda de MFD estimulou a atividade da Na^+,K^+-ATPase em hipocampo, córtex pré-frontal e estriado de ratos jovens (25 dias) e adultos (60 dias). A administração crônica de MFD a ratos jovens também aumentou a atividade dessa enzima em hipocampo e córtex pré-frontal e não alterou a atividade da

Na^+,K^+-ATPase em estriado. Em ratos adultos, o MFD estimulou a atividade dessa enzima em todas as estruturas cerebrais avaliadas.

Nossos achados estão de acordo com vários estudos mostrando que a resposta ao tratamento com MFD varia de acordo com a área cerebral analisada, a dose e a idade de exposição ao fármaco. Neste contexto, Scaini e cols. (2008) mostraram que a administração aguda de MFD estimulou a atividade da enzima creatina cinase em córtex pré-frontal, hipocampo, estriado e córtex cerebral, mas não em cerebelo de ratos jovens e adultos. Os autores observaram que a administração crônica de MFD estimulou a atividade da enzima em todas as estruturas cerebrais avaliadas; a dose mais elevada (10 mg/Kg) apresentou efeitos mais pronunciados. Outra investigação demonstrou que ratos jovens submetidos à administração aguda de MFD apresentaram aumento na produção de superóxido no cerebelo (1, 2 e 10 mg/Kg) e hipocampo (somente 10 mg/Kg). O tratamento crônico com MFD não causou alteração nesse parâmetro em nenhuma estrutura cerebral analisada. Entretanto, em ratos adultos, a administração aguda desse psicoestimulante não teve efeito sobre a produção de superóxido e o tratamento crônico diminuiu esse parâmetro em cerebelo (GOMES et al., 2009).

A Na^+,K^+-ATPase está envolvida em várias funções fisiológicas, como a regulação do volume celular, diferenciação e manutenção do equilíbrio de sódio e potássio através das membranas biológicas. Desde que essa enzima está envolvida na excitabilidade neuronal e no metabolismo energético (ERECIŃSKA e SILVER, 1994), modificações na

sua atividade podem estar envolvidas na sensibilização comportamental induzida por psicoestimulantes (MUNHOZ et al., 2003).

O MFD aumenta a quantidade de dopamina e noradrenalina na fenda sináptica (KUCZENSKI e SEGAL, 1997; OVERTOOM et al., 2003). A dopamina pode regular a função da Na^+,K^+-ATPase através da ativação de segundos mensageiros que promovem a inserção ou remoção dessa enzima da membrana plasmática (BARNARD et al., 1997; BRISMAR et al., 1998; RIDGE et al., 2002). Sabe-se que o metabolismo cerebral é ativado por elevadas concentrações de K^+ extracelular, que parece ser dependente da atividade da Na^+,K^+-ATPase para a manutenção dos seus níveis basais (MCDOUGAL, 1997; HASSEL e SONNEWALD, 2002). Receptores D_2 de dopamina afetam canais de K^+ e podem levar à estimulação da Na^+,K^+-ATPase (YAMAGUCHI et al., 1996).

A estimulação da Na^+,K^+-ATPase pela noradrenalina também tem sido relatada. Segundo Mallick e cols. (2000), a noradrenalina aumentou a atividade da Na^+,K^+-ATPase em cérebro de ratos através do receptor α1A, possivelmente por desfoforilação da enzima. Nesse contexto, estudos sugerem que a fenilalanina estimulou a atividade da Na^+,K^+-ATPase em cérebro de ratos devido à sua conversão em noradrenalina (TSAKIRIS et al., 1998). Em adição, estudos mostram que a administração aguda de desipramina, um inibidor da recaptação de noradrenalina utilizado no tratamento da depressão, aumentou a atividade da Na^+,K^+-ATPase em hipotálamo e mesencéfalo de ratos (VIOLA e RODRIGUEZ, 2007). Andrews e Lavin (2006) mostraram que o MFD aumentou a excitabilidade no córtex pré-frontal de ratos pela ativação de receptores α_2

noradrenérgicos.

Tem sido demonstrado que o MFD altera o metabolismo energético cerebral. A administração crônica de MFD estimulou a atividade de enzimas da cadeia respiratória no cerebelo, hipocampo, córtex pré-frontal e estriado de ratos, sugerindo um aumento na produção de ATP (FAGUNDES et al., 2007). Propõe-se, então, que esses efeitos no metabolismo energético poderiam estar associados à estimulação da Na^+,K^+-ATPase demonstrada em nosso estudo, desde que uma parte considerável do ATP cerebral é utilizado para sustentar a atividade desta enzima (ERECIŃSKA e SILVER, 1994).

Em neurociência tem sido reconhecido que as sinapses são modificáveis; as mudanças a curto e a longo prazo podem resultar de fatores intrínsecos e extrínsecos do ambiente. Essa plasticidade sináptica dos neurônios desempenha um papel importante em fenômenos como organização neuronal durante o desenvolvimento, o aprendizado e a formação da memória (ROBINSON e KOLB, 2004).

Anormalidades na plasticidade sináptica podem resultar de lesões cerebrais, influências ambientais negativas e crônica exposição a drogas psicotrópicas (ROBINSON e BERRIDGE, 2003; ROBINSON e KOLB, 2004). A exposição prolongada aos psicoestimulantes durante o desenvolvimento do SNC pode ocasionar mudanças neuronais permanentes, tais como alterações nos sistemas de neurotransmissores (ANDERSEN, 2003; STANWOOD e LEVITT, 2004). Neste contexto, dados na literatura sugerem que a persistente estimulação dos sistemas dopaminérgico e noradrenérgico pode contribuir para o déficit cognitivo

(ARNSTEN e LI, 2005).

Nesse trabalho, também investigamos o efeito da administração crônica de MFD a ratos jovens sobre a memória espacial de referência e a memória espacial de trabalho. Os ratos foram tratados por 30 dias, do 15º ao 45º dia de vida, com 2 mg/Kg de MFD. Ao término do tratamento, os animais foram submetidos à tarefa do labirinto aquático de Morris. Nessa tarefa comportamental, a memória avaliada é dependente principalmente do hipocampo, entretanto, outras estruturas cerebrais como os córtices parietal e pré-frontal também estão envolvidas na formação da memória espacial (D`HOOGE e DE DEYN, 2001).

Os resultados mostraram que a administração crônica de MFD prejudicou a aprendizagem/memória na fase de aquisição do labirinto aquático de Morris e na latência para cruzar, pela primeira vez, o local da plataforma. Os ratos tratados com MFD também apresentaram um prejuízo na memória espacial de trabalho. A tarefa do campo aberto e a velocidade de natação foram utilizadas como controle da atividade motora e os resultados obtidos demonstraram que os animais tratados não apresentavam alterações nesses parâmetros quando comparados aos controles.

Em concordância com nossos resultados, estudos mostram que a exposição ao MFD durante o desenvolvimento causa alterações comportamentais em ratos. A administração de MFD a ratos do 20º ao 35º dia de vida modificou a sensibilidade à cocaína na vida adulta (60 dias), aumentou os efeitos depressivos e reduziu a habituação a um ambiente familiar (CARLEZON et al., 2003). Animais adultos tratados com MFD (2

mg/Kg) na adolescência foram menos sensíveis aos estímulos de recompensa naturais como a sacarose, e apresentaram prejuízo no comportamento sexual. Além disso, esses animais mostraram maior sensibilidade a situações aversivas (BOLAÑOS et al., 2003).

Heyser e cols. (2004) observaram prejuízo na tarefa de reconhecimento de objetos em ratos jovens tratados por sete dias com MFD (5 mg/Kg). Em adição, doses similares de MFD administradas a ratos jovens durante 21 dias causaram prejuízo no mesmo teste comportamental, e esse efeito persistiu até 42 dias após a administração desse psicoestimulante (LeBLANC-DUCHIN e TAUKULIS, 2007).

Um estudo recente mostrou que a exposição ao MFD, desde a adolescência até a idade adulta de ratos, produz efeitos transitórios sobre tarefas comportamentais dependentes do hipocampo. Esses resultados sugerem que os efeitos do MFD sobre os processos cognitivos variam de acordo com o tempo de tratamento, o padrão de administração do fármaco e a complexidade da tarefa utilizada (BETHANCOURT et al., 2009).

Também foram investigados no presente estudo alguns parâmetros neuroquímicos que poderiam estar relacionados ao prejuízo na memória. Após os testes comportamentais foram avaliados o imunoconteúdo de BDNF e a atividade da enzima AChE em hipocampo e córtex pré-frontal de ratos. Essas estruturas cerebrais foram escolhidas porque elas estão envolvidas tanto na ação do MFD no SNC (VOLKOW et al. 2005) quanto nos mecanismos de memória (IZQUIERDO, 2002).

Os resultados mostram que o MFD reduziu o imunoconteúdo de

BDNF e estimulou a atividade da AChE em córtex pré-frontal, mas não em hipocampo de ratos. A ausência do efeito do MFD sobre esses parâmetros no hipocampo é intrigante, entretanto, podemos sugerir que o córtex pré-frontal é mais sensível à ação do fármaco, uma vez que recentes estudos bioquímicos mostraram que o MFD tem efeitos mais potentes no córtex pré-frontal em relação às outras estruturas cerebrais (KUCZENSKI e SEGAL, 2002; DROUIN et al., 2006; ARNSTEN, 2009).

O córtex pré-frontal, cujo funcionamento depende da manutenção de um delicado equilíbrio de dopamina e noradrenalina, medeia habilidades executivas como memória de trabalho, regulação da atenção, inibição comportamental, planejamento e organização (HASTINGS et al., 2004; ARNSTEN e LI, 2005).

Durante o desenvolvimento, a expressão de BDNF é mais abundante no cérebro do que em outros tecidos e aumenta constantemente durante o período pós-natal (HOFER et al., 1990; WETMORE et al.,1990; FRIEDMAN et al., 1991). O BDNF é a neurotrofina que se encontra mais distribuída no encéfalo, principalmente em hipocampo e córtex, sendo indispensável para a consolidação da memória (ALONSO et al., 2002).

Há evidências na literatura sugerindo que a dopamina regula os níveis de BDNF corticais e alguns desses efeitos podem envolver os receptores D_1. A infusão de antagonistas de receptores D_1 no córtex pré-frontal de primatas prejudicou a memória de trabalho (SAWAGUCHI e GOLDMAN-RAKIC, 1991). Além disso, em camundongos “knockout” para o transportador de dopamina, os baixos níveis de BDNF no córtex foram

associados a um prejuízo na memória de trabalho (LI et al., 2009).

Vários estudos têm mostrado que as mudanças na expressão do BDNF podem variar de acordo com diversos fatores tais como: o tipo de droga, dose, via de administração, duração do tratamento (TARDITO et al., 2006), fase do desenvolvimento, área cerebral analisada e padrão de estimulação sináptica (GOTTMAN et al., 2009). Além disso, o estresse oxidativo tem sido associado a baixos níveis de BDNF (KAPCZINSKI et al., 2008a).

A administração aguda e crônica de MFD a ratos jovens (25 dias) não alterou a expressão de BDNF no estriado ou córtex (CHASE et al., 2007). Entretanto, Banerjee e cols. (2009) mostraram que uma única dose de MFD (2 mg/Kg) administrada a ratos jovens reduziu a expressão do mRNA do BDNF no hipocampo e córtex. Em ratos adultos, os efeitos foram significativamente mais baixos quando comparados aos animais jovens.

O sistema colinérgico desempenha um papel fundamental nos mecanismos de memória. A enzima AChE contribui para a manutenção da integridade desse sistema através da finalização da ação sináptica do neurotransmissor ACh (ZIMMERMAN e SOREQ, 2006).

A redução nos níveis de ACh cerebral tem sido associada a alterações cognitivas características de algumas demências. Nesse contexto, inibidores reversíveis da AChE têm sido utilizados para melhorar o déficit cognitivo apresentado por pacientes com doença de Alzheimer e outras desordens neurodegenerativas (BALLARD et al., 2005).

Estudos demonstram que o sistema colinérgico está envolvido na

formação da memória espacial. Um grande aumento na ACh cortical foi detectado em ratos durante a execução de tarefas que envolvem a atividade exploratória espontânea e memória de trabalho (GIOVANNINI et al., 1998).

O reconhecimento de objetos parece ser dependente dos níveis de ACh, desde que estudos em animais mostraram que esse comportamento foi prejudicado pela hipofunção colinérgica induzida por fármacos (ENNACEUR e MELIANI, 1992), lesões no sistema colinérgico cortical e idade (BARTOLINI et al., 1996).

Nesse estudo também demonstramos que a administração crônica de MFD aumentou significativamente a atividade da AChE em córtex pré-frontal de ratos. Sugere-se, então que a estimulação dessa enzima diminui os níveis de ACh e isto poderia estar relacionado, pelo menos em parte, com o déficit cognitivo observado nos ratos tratados com MFD.

Nossos achados corroboram com dados da literatura, sugerindo que o sistema colinérgico pode ser modulado por psicoestimulantes. A repetida exposição à anfetamina induziu detrimento na atividade colinérgica pré-frontal em modelo animal de esquizofrenia (KOZAK et al., 2007). Outro estudo mostra que a administração aguda de 1 e 3 mg/Kg de MFD aumentou a liberação de ACh no hipocampo e córtex de ratos (TZAVARA et al., 2006).

A administração de galantamina, fármaco inibidor da AChE, reverteu sintomas como agitação, excitação e comportamento estereotipado induzidos pela anfetamina em macacos (ANDERSEN et al., 2007). Outro estudo conduzido em ratos mostrou que os inibidores da

AChE bloqueiam a sensibilização locomotora induzida pelo consumo de cocaína (HIKIDA et al., 2003).

Apesar dos processos de aprendizagem e memória envolverem múltiplos mecanismos e os efeitos exercidos pelo MFD sobre a cognição não estarem completamente elucidados, sugerimos que o déficit na memória espacial observado nesse estudo pode estar associado, pelo menos em parte, com a redução nos níveis de BDNF e a estimulação da AChE no córtex pré-frontal de ratos jovens.

Em suma, os resultados obtidos nesse estudo demonstraram que o MFD estimulou a atividade das enzimas Na^+,K^+-ATPase e AChE, prejudicou a memória espacial e diminuiu os níveis de BDNF em ratos. Esses achados corroboram com pesquisas mostrando que a administração de MFD a animais promove alterações cerebrais que são importantes para a maturação e o desenvolvimento do SNC.

4. CONCLUSÕES

1. A administração aguda de MFD aumentou significativamente a atividade da Na^+,K^+-ATPase em hipocampo, córtex pré-frontal e estriado de ratos jovens e adultos.
2. O tratamento crônico com MFD estimulou a atividade da Na^+,K^+-ATPase em hipocampo, córtex pré-frontal e estriado de ratos adultos. Por outro lado, em ratos jovens, a atividade da enzima foi aumentada somente em hipocampo e córtex pré-frontal.
3. A administração crônica de MFD a ratos jovens estimulou a atividade da enzima AChE em córtex pré-frontal.
4. Ratos jovens tratados cronicamente com MFD apresentaram redução no imunoconteúdo de BDNF no córtex pré-frontal.
5. A administração crônica de MFD a ratos jovens prejudicou as memórias espaciais de referência e de trabalho.

Sabe-se que o MFD é amplamente utilizado para o tratamento do TDAH durante a infância e adolescência, mas os possíveis efeitos desse tratamento no desenvolvimento cerebral ainda não foram extensamente investigados. Para esclarecer estas questões, a utilização de estudos em animais tem se mostrado um excelente método. Portanto, os resultados do presente trabalho em conjunto, mostrando que o MFD causa alterações neuroquímicas e comportamentais em animais, podem ser úteis, pelo menos em parte, para o esclarecimento de possíveis efeitos desse psicoestimulante sobre o desenvolvimento cerebral.

5. PERSPECTIVAS

1. Avaliar a memória na tarefa aversiva de esquiva inibitória em ratos jovens após o tratamento crônico com MFD.

2. Estudar o efeito do MFD sobre a ansiedade através das tarefas de labirinto em cruz elevado (plus maze) e caixa claro-escuro em ratos jovens.

3. Avaliar alguns parâmetros de estresse oxidativo e excitotoxicidade em animais submetidos ao tratamento crônico com MFD.

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