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EFEITOS DO METILFENIDATO: UMA ABORDAGEM EXPERIMENTAL

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“Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.”

MARK TWAIN

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RESUMO

Embora o uso e o abuso de metilfenidato estejam aumentando na infância e na adolescência, pouco se sabe sobre as consequências de sua utilização em longo prazo sobre o cérebro em desenvolvimento. O objetivo do presente estudo foi investigar os efeitos neuroquímicos hipocampais e comportamentais do tratamento crônico com metilfenidato em ratos jovens. Também avaliamos se o tratamento com metilfenidato (1µM) influencia a via Akt-mTOR em células de feocromocitoma de ratos (PC12), um modelo celular bem caracterizado. Ratos Wistar receberam injeções intraperitoneais de metilfenidato (2,0 mg/kg) ou volume equivalente de solução salina (controles), uma vez por dia, do 15º ao 45º dia de idade. O tratamento com metilfenidato alterou o perfil de aminoácidos, diminuindo os níveis de glutamina, bem como a captação de glutamato e a atividade da Na^+,K^+-ATPase. Não foram observadas alterações no imunoconteúdo dos transportadores de glutamato (GLAST e GLT-1), no imunoconteúdo das subunidades catalíticas da Na^+,K^+-ATPase (α_1, α_2 e α_3) e nos parâmetros de equlíbrio redox. Os níveis de ATP foram diminuídos pelo metilfenidato e as atividades da citrato sintase, succinato desidrogenase e complexos da cadeia respiratória (II, II-III e IV), bem como a massa e o potencial de membrana mitocondrial não foram alterados pelo metilfenidato. Hipofosforilação da GFAP e a redução do seu imunoconteúdo foram observadas em ratos tratados com metilfenidato. Os neurofilamentos de médio e alto peso molecular também foram hipofosforilados, porém seus imunoconteúdos não foram alterados. O imunoconteúdo da proteína fosfatase 1 e 2A foram aumentados. O conteúdo total de gangliosídeos e fosfolipídeos, assim como os principais gangliosídeos (GM1, GD1a e GD1b) e fosfolipídeos cerebrais foram reduzidos pelo tratamento com metilfenidato. O colesterol total também foi reduzido no hipocampo desses animais. Além disso, resultados mostraram que a administração crônica de metilfenidato causou uma perda de astrócitos e neurônios no hipocampo de ratos jovens. O imunoconteúdo de BDNF e pTrkB, bem como os níveis de NGF foram reduzidos, enquanto que os níveis de TNF-α e IL-6 e os imunoconteúdos de Iba-1 e caspase 3 foram aumentados. A razão pERK/ERK e o imunoconteúdo de PKCaMII foram reduzidos, porém a razão pAkt/Akt e pGSK-3β/GSK-3β não foram alterados. O imunoconteúdo de SNAP-25 foi diminuído e GAP-43 e sinaptofisina não foram alterados. A atividade exploratória e a memória de reconhecimento de objetos foram prejudicadas pelo metilfenidato. Em relação às células, o tratamento com metilfenidato de curta duração diminuiu as razões pAkt/Akt, pmTOR/mTOR e pS6K/S6K, bem como o imunoconteúdo de pFoxO1. Por outro lado, o tratamento em longo prazo aumentou a razão de pAkt/Akt, pmTOR/mTOR e pGSK-3β/GSK-3β. Os níveis de fosforilação de 4E-BP1 foram diminuídos aos 15 e 30 minutos e aumentados em 1 e 6 h pelo metilfenidato. A razão pCREB/CREB foi diminuída. Esses resultados fornecem evidências adicionais de que a exposição crônica precoce ao metilfenidato pode ter efeitos complexos, bem como fornece novas bases para a compreensão das consequências neuroquímicas e comportamentais associadas ao tratamento com esse psicoestimulante. Além disso, fornece uma nova base para a compreensão dos mecanismos associados ao tratamento com metilfenidato, que pode conduzir estudos futuros.

Palavras-chave: metilfenidato; excitotoxicidade; inflamação; função mitocondrial; Na^+,K^+-ATPase; lipídeos de membrana; hipocampo; fatores neurotróficos/memória; células de feocromocitoma de ratos; sinalização celular

ABSTRACT

Although the use, and misuse, of methylphenidate is increasing in childhood and adolescence, there is little information about the consequences of this psychostimulant chronic use on brain and behavior during development. The aim of the present study was to investigate neurochemical, histochemical, and behavioral effects of chronic methylphenidate treatment to juvenile rats. We also attempted to determine whether the treatment with methylphenidate (1μM) influences Akt-mTOR signaling pathways in rat pheochromocytoma cells (PC12), a well characterized cellular model. Wistar rats received intraperitoneal injections of methylphenidate (2.0 mg/kg) or an equivalent volume of 0.9 % saline solution (controls), once a day, from the 15th to the 45th day of age. Results showed that chronic methylphenidate altered amino acid profile in hippocampus, decreasing the levels of glutamine. Glutamate uptake and Na^+,K^+-ATPase activity were decreased after treatment in rat hippocampus. No changes were observed in the glutamate transporters (GLAST and GLT-1), immunocontent of catalytic subunits of Na^+,K^+-ATPase (α_1, α_2 and α_3), and redox status. Methylphenidate provoked a decrease in ATP levels, while citrate synthase, succinate dehydrogenase, respiratory chain complexes activities (II, II-III and IV), as well as mitochondrial mass and mitochondrial membrane potential were not altered. Methylphenidate also provoked hypophosphorylation of GFAP and reduced its immunocontent. Middle and high molecular weight neurofilament subunits (NF-M, NF-H) were hypophosphorylated by methylphenidate on KSP repeat tail domains, while their immunocontents were not altered. Methylphenidate increased protein phosphatase 1 and 2A immunocontents. Methylphenidate decreased the total content of ganglioside and phospholipid, as well as the main brain gangliosides (GM1, GD1a, and GD1b) and the major brain phospholipids in rat hippocampus. Total cholesterol content was also reduced in the hippocampi of juvenile rats by methylphenidate. In addition, methylphenidate caused loss of astrocytes and neurons in the hippocampus of juvenile rats. BDNF and pTrkB immunocontents and NGF levels were decreased, while TNF-α and IL-6 levels, as well as Iba-1 and caspase 3 cleaved immunocontents (microglia marker and active apoptosis marker, respectively) were increased. ERK and PKCaMII signaling pathways, but not Akt and GSK-3β, were decreased. SNAP-25 was decreased after methylphenidate treatment, while GAP-43 and synaptophysin were not altered. Both exploratory activity and object recognition memory were impaired. In relation to PC12 cells, short term methylphenidate treatment decreased the pAkt/Akt, pmTOR/mTOR and pS6K/S6K ratios, as well as pFoxO1 immunocontent. On the other hand, long term treatment increased pAkt/Akt, pmTOR/mTOR and pGSK-3β/GSK-3β ratios. Phosphorylation levels of 4E-BP1 were decreased at 15 and 30 minutes and increased at 1 and 6 h by methylphenidate. pCREB/CREB ratio was decreased. These findings provide additional evidence that early-life exposure to methylphenidate can have complex effects during central nervous system development, as well as provide new basis for understanding of the biochemical and behavioral effects associated with methylphenidate treatment.

Keywords: methylphenidate; excitotoxicity; inflammation; mitochondrial function; Na^+,K^+-ATPase; membrane lipids; hippocampus; neurotrophins/memory; PC12 cells; cell signaling

LISTA DE ABREVIATURAS

Akt: proteína-cinase B

AMPA: α-amino-3-hidroxi-5-metil-4-isoxazolpropionato

AMPc: adenosina monofosfato cíclico

ATP: adenosina trifosfato

ADP: adenosina difosfato

BDNF: fator neurotrófico derivado do encéfalo

β-Arr2: β-arrestina 2

CAT: catalase

COX: citocromo *c* oxidase

CREB: proteína de ligação responsiva ao AMPc

DA: dopamina

DAT: transportador de dopamina

D2R: receptor de dopamina 2

DSM-V: Manual Diagnósico e Estatístico de Transtornos Mentais – 5ª edição

4E-BP1: proteína 1 ligante do fator de iniciação eucariótico 4E

ERK: cinase regulada por sinal extracelular ½

$FADH_2$: flavina adenina dinucleotídeo (forma reduzida)

FI: filamento intermediário

FoxO1: fator de transcrição O1

GAP-43: proteína associada ao crescimento 43

GFAP: proteína glial fibrilar ácida

GLAST/EAAT1: transportador de aspartato-glutamato

GLT-1/EAAT2: transportador de glutamato

GPx: glutationa peroxidase

GTP: trifosfato de guanosina

GSK-3β: glicogênio sintase cinase-3β

Iba-1: marcador de microglia

IL-6: interleucina 6

KSP: lisina-serina-prolina

MAP: proteína ativada por mitógeno

MF: microfilamento

MPTP: 1-metil-4-fenil-1,2,3,6-tetraidropiridina

MT: microtúbulo
mTORC1: complexo alvo da rapamicina de mamífero 1
NADH: nicotinamida adenina dinucleotídeo (forma reduzida)
NF: neurofilamento
MFD: metilfenidato
NF-L: neurofilamento de baixo peso molecular
NF-M: neurofilamento de médio peso molecular
NF-H: neurofilamento de alto peso molecular
NGF: fator de crescimento nervoso
NMDA: N-metil-D-aspartato
p70 S6K: proteína cinase ribossomal S6 de 70 kDA
PC12: células de feocromocitoma de ratos
Pi: fosfato inorgânico
PI3K: fosfatidilinositol-3 cinase
PKA: proteína cinase dependente de AMPc
PKC: proteína cinase C
PKCaMII: proteína cinase dependente cálcio/calmodulina II
PP1: proteína fosfatase 1
PP2A: proteína fosfatase 2A
PP2B: calcineurina
pro-BDNF: precursor do BDNF
pTrkB: receptor tirosina cinase B fosforilado
SDH: succinato desidrogenase
SNAP-25: proteína associada à sinaptossoma 25
SNC: sistema nervoso central
SOD: superóxido dismutase
TDAH: Transtorno de Déficit de Atenção/Hiperatividade
TNF-α: fator de necrose tumoral-alfa
Trk: receptor tirosina cinase

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1. INTRODUÇÃO

1.1 Metilfenidato

O metilfenidato (MFD), um psicoestimulante análogo das anfetaminas, tem sido amplamente utilizado em crianças para o tratamento do Transtorno de Déficit de Atenção/Hiperatividade (TDAH). O MFD também é o medicamento de escolha para o tratamento da narcolepsia (Johnston et al., 2011a,b). Além disso, estudos têm mostrado que o MFD melhora a função motora e cognitiva de pacientes com doença de Parkinson (Auriel et al., 2009). Embora ainda controverso, esse fármaco também tem sido utilizado com sucesso no tratamento da apatia e perda de motivação em pacientes com doença de Alzheimer (Ruthirakuhan et al., 2018).

O MFD promove a remissão dos sintomas, bem como a melhora de aspectos funcionais importantes como a interação social e o desempenho acadêmico em grande parte dos portadores de TDAH (Johnston et al., 2011a,b). No entanto, o MFD também é amplamente utilizado por indivíduos não portadores de TDAH com a finalidade de melhorar a atenção e aumentar o desempenho cognitivo (Johnston et al., 2011a,b; Swanson e Volkow, 2003). Apesar de os mecanismos neurais envolvidos na eficácia terapêutica do MFD no tratamento dos sintomas do TDAH ainda não sejam completamente conhecidos, acredita-se que sua atuação esteja, pelo menos em parte, relacionada ao aumento de dopamina (DA), uma vez que, assim como a cocaína e a anfetamina, o MFD aumenta os níveis de DA por meio do bloqueio dos seus transportadores (Johnston et al., 2011a,b; Volkow et al., 2002) (Figura 1). Embora o MFD tenha uma maior afinidade pelo transportador de DA (DAT), ele também atua sobre os sistemas noradrenérgico, serotoninérgico e

glutamatérgico (Gainetdinov, 2001; Gatley et al., 1996; Kuczenski e Segal, 1997).

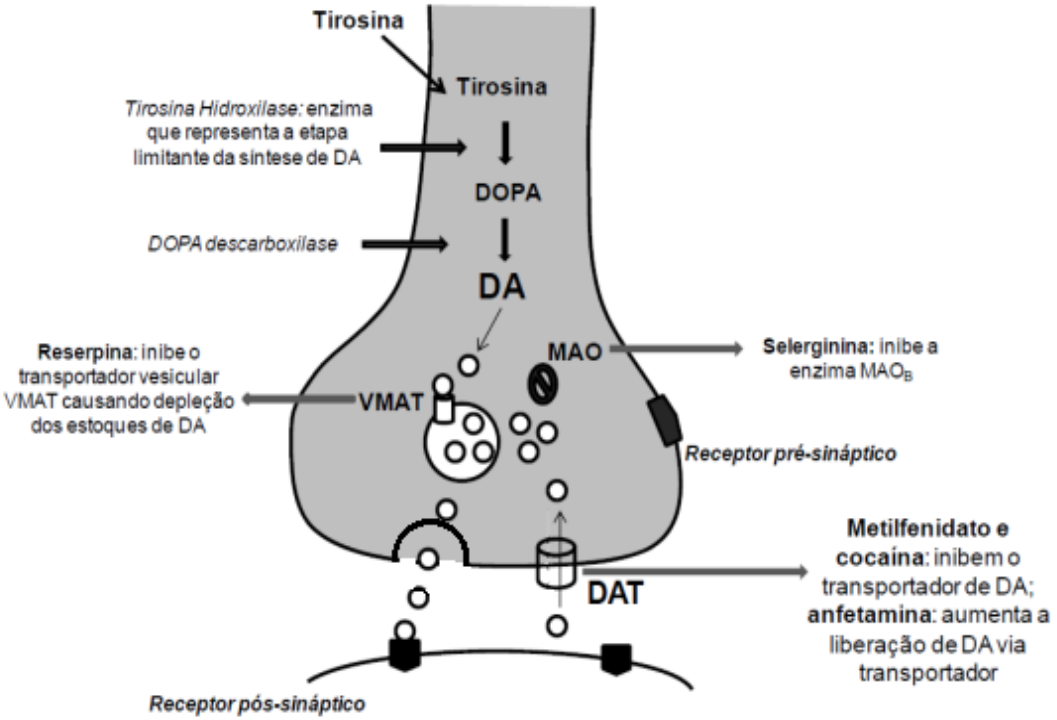

Figura 1. Sinapse dopaminérgica (Nestler et al., 2009, modificado).

Esquema dos eventos envolvidos na síntese e recaptação da DA. A síntese de DA inicia-se pela transformação do aminoácido tirosina, que provém da dieta, em DOPA, pela ação da enzima tirosina hidroxilase. Na sequência, a DOPA é convertida em DA pela ação da enzima DOPA descarboxilase. A DA recém-sintetizada é armazenada em vesículas, onde permanece protegida da degradação enzimática via MAO, até ser liberada por exocitose. Uma vez liberada, a DA atua em receptores pré e pós-sinápticos tendo sua ação interrompida principalmente pela recaptação via DAT. Esses transportadores representam o principal alvo de atuação dos psicoestimulantes, dentre eles o MFD (Nestler et al., 2009).

O MFD apresenta ação curta, com uma meia-vida de 2 a 3 horas (Leonard et al., 2004). Sua absorção é completa e rápida, atravessando a barreira sangue-sangue facilmente, devido à sua lipossolubilidade (Auriel et al., 2009). O MFD é desesterificado pelo sistema microssomal hepático produzindo principalmente o ácido ritalínico. Esse metabólito possui baixa afinidade pelo

DAT e é facilmente eliminado pela urina devido a sua hidrofilia (Johnston et al., 2011a,b).

No cérebro humano, a farmacocinética do MFD foi investigada por meio de Tomografia por Emissão de Pósitron e carbono-11 ([^{11}C] metilfenidato) (Volkow et al., 1995) e os resultados mostraram que os níveis cerebrais de MFD atingem o pico entre 4 a 10 minutos após a administração intravenosa do mesmo. O pico da concentração cerebral após administração oral ocorre em 60 minutos e mais de 50% dos DATs são bloqueados (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 grastrintestinal, permeabilidade da barreira sangue-cérebro e resposta ao tratamento. Doses abaixo de 5 mg/kg/dia são consideradas doses clínicas, acima disso são consideradas doses de abuso ou recreacionais (Johnston et al., 2011a,b). 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 (Johnston et al., 2011a,b).

1.2 O uso abusivo de metilfenidato

O abuso de psicoestimulantes é um sério problema de saúde pública internacional devido ao seu alto potencial de dependência e o risco elevado de comprometimento neurológico quando utilizado por um longo período (Cadet e Krasnova, 2009). Alterações sobre o sistema dopaminérgico, estressse oxidativo, disfunção mitocondrial e excitotoxicidade têm sido alguns dos efeitos envolvidos na neurotoxicidade dos psicoestimulantes. Além disso, tem sido

mostrado que essas substâncias podem promover neuroinflamação, alterações na barreira sangue-cérebro e na neurogênese (Gonçalves et al., 2014; Loureiro-Vieira et al., 2017).

Apesar de o MFD ser amplamente utilizado na clínica, inúmeras questões e incertezas permanecem sobre os efeitos moleculares e suas consequências sobre o sistema nervoso central (SNC) após o seu uso prolongado (Duong et al., 2012; Gonçalves et al., 2014; Loureiro-Vieira et al., 2017; Quansah et al., 2017a). Alguns estudos têm mostrado que o MFD promove efeitos semelhantes aos das anfetaminas (Kollins et al., 2001). Além disso, tem sido mostrado que a alteração catecolaminérgica persistente durante o desenvolvimento pode afetar a sinaptogênese, gliogênese e a mielinização (Rice e Barone, 2000). A preocupação acerca do uso prolongado de MFD é ainda maior pelo fato de que esse psicoestimulante vem sendo extensivamente utilizado por crianças e jovens adultos que não reunem os critérios para serem diagnosticados com TDAH de acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais – 5ª edição (DSM-V), com o objetivo de aumentar o seu desempenho cognitivo (Johnston et al., 2011a,b; Verster e van Niekerk, 2012).

1.3 Geração e diferenciação celular

O MFD aumenta os níveis de DA em várias regiões do cérebro, incluindo o hipocampo. A DA tem um importante papel modulador na neurogênese durante o desenvolvimento do encéfalo (Klein et al., 2018; Reinoso et al., 1996; Todd, 1992). Neste período, o estabelecimento de ligações sinápticas adequadas é essencial para a sobrevivência de células neurais que são

dependentes de fatores tróficos como o fator neurotrófico derivado do encéfalo (BDNF) (Varendi et al., 2015) e o fator de crescimento nervoso (NGF) (Wiesmanna e de Vos, 2001). Em nível molecular, as neurotrofinas promovem a sobrevivência neuronal ao se ligarem a receptores tirosina-cinases (Trk) e ativarem vias intracelulares como a via das proteínas ativadas por mitógeno/cinase regulada por sinal extracelular ½ (MAP/ERK) e/ou a via do fosfatidilinositol-3 cinase/proteína-cinase B (PI3K/Akt) (Teng e Hempstead, 2004). Além das neurotrofinas, proteínas sinápticas, tais como a proteína associada à sinaptossoma 25 (SNAP-25), a proteína associada ao crescimento 43 (GAP-43) e a sinaptofisina também são muito importantes para o processo de sinaptogênese e maturação da conectividade sináptica funcional durante o desenvolvimento cerebral (Davletov et al., 2007; Rice e Barone, 2000).

No que diz respeito à neurogênese, os trabalhos têm sido controversos. Ratos jovens injetados com MFD (2 mg/kg, duas vezes por dia, durante 15 dias) apresentaram uma diminuição na sobrevivência em longo prazo de células jovens no hipocampo, sem alterações na proliferação (Lagace et al., 2006). Schaefers et al. (2009) também relataram que o tratamento crônico com MFD (5 mg/kg, durante 30 dias) não interferiu com o número de células proliferativas no giro dentado. Por outro lado, foi descrito um aumento da proliferação celular e diferenciação de neuroblastos após tratamento com MFD (10 mg/kg, uma vez por dia, durante 28 dias) (Lee et al., 2012).

1.4 Neuroinflamação

Níveis aumentados de DA estão associados à resposta inflamatória no encéfalo, aumentando os níveis de citocinas e quimiocinas (Jang et al., 2012).

Os mediadores inflamatórios podem exercer tanto efeitos benéficos quanto deletérios no SNC. Proteção imunológica e auxílio na remoção de neurônios em processo de morte celular estão entre os papéis benéficos. Por outro lado, quando esses marcadores estão presentes em grandes quantidades em nichos neurogênicos no hipocampo eles podem prejudicar a plasticidade sináptica, o que conduz à disfunção cognitiva em transtornos neuropsiquiátricos e neurodegenerativos, tais como depressão, doença de Alzheimer e doença de Parkinson (Borsini et al., 2015).

A relação entre MFD e neuroinflamação também tem sido pouco investigada. Porém, foi mostrado que ratos submetidos à administração de MFD apresentam ativação microglial (Sadasivan et al., 2012). Esses mesmos autores também observaram um aumento nos níveis de mRNA do fator de necrose tumoral-alfa (TNF-α) e interleucina 6 (IL-6) em estriado após uma única administração de MFD. Hipertrofia e acúmulo de astrócitos em torno das paredes capilares do encéfalo, juntamente com o aumento da espessura da membrana também foram observados após tratamento com MFD (Bahcelioglu et al., 2009).

1.5 Sistema glutamatérgico

O aminoácido glutamato é o principal neurotransmissor excitatório no SNC de mamíferos e está envolvido em diversas funções fisiológicas importantes, tais como aprendizado, memória, desenvolvimento e envelhecimento (Reiner e Levitz, 2018). O glutamato é sintetizado e estocado em vesículas nos neurônios pré-sinápticos a partir da glutamina proveniente

das células gliais, em uma reação catalisada pela enzima glutaminase (Meldrum, 2000).

A liberação de glutamato na fenda sináptica ocorre por exocitose dependente de Ca^{2+}, decorrente da despolarização do terminal pré-sináptico (Attwell, 2000) (Figura 2). No meio extracelular, o glutamato exerce suas funções biológicas ao se ligar a receptores glutamatérgicos localizados na superfície das células neurais, principalmente nos neurônios pré e pós-sinápticos. Esses receptores são classificados como ionotrópicos ou metabotrópicos, conforme suas características moleculares. Os receptores ionotrópicos possuem um canal iônico em sua estrutura e, quando ativos, se tornam permeáveis a íons como Na^{+}, Ca^{2+} e K^{+}; esses receptores são subdivididos em N-metil-D-aspartato (NMDA), α-amino-3-hidroxi-5-metil-4-isoxazolpropionato (AMPA) e cainato. Os receptores metabotrópicos são moléculas acopladas a proteínas G, que atuam modulando a atividade de enzimas como adenilato ciclase e fosfolipase C; esses receptores são subdivididos em receptores do grupo I, II e III (Reiner e Levitz, 2018).

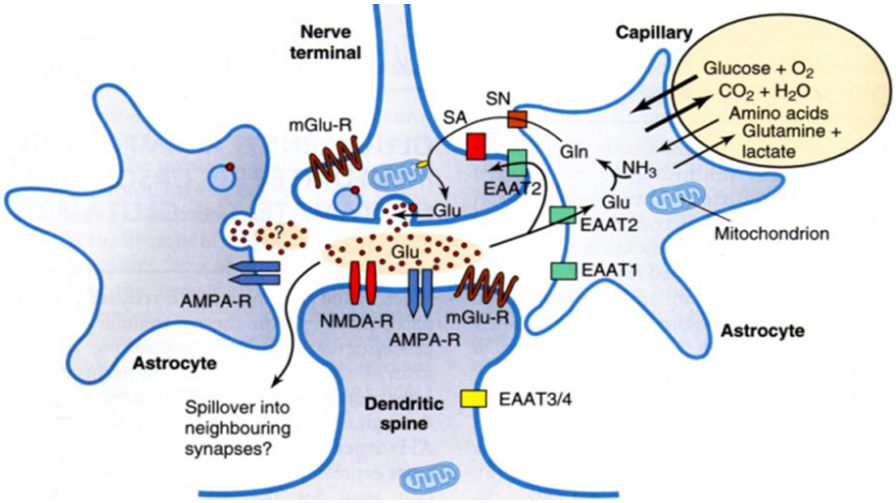

Figura 2. Sinapse glutamatérgica (Siegel et al., 2006).

EAAT1-4: transportador de aminoácidos excitatórios; Gln: glutamina; Glu: glutamato; NMDA-R e AMPA-R: receptores ionotrópicos de glutamato NMDA e AMPA, respecitvamente; mGlu-R:

receptor metabotrópico de glutamato; SA e SN: sistema A e sistema N de transporte de glutamina.

Após o glutamato exercer sua ação em seus receptores, a sinalização glutamatérgica é finalizada por um processo denominado captação de glutamato, onde o glutamato é removido da fenda sináptica por transportadores especializados, dependentes de Na^+, localizados principalmente nas membranas gliais (Anderson e Swanson, 2000). Há cinco diferentes isoformas de transportadores de glutamato, identificadas até o momento: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 e EAAT5, sendo que GLAST e GLT-1 são quantitativamente, os principais transportadores de glutamato em todas as regiões cerebrais, responsáveis por aproximadamente 90% da captação desse aminoácido (Reiner e Levitz, 2018).

O processo de captação de glutamato é realizado contra o gradiente de concentração, uma vez que a concentração desse aminoácido na fenda sináptica é muito menor que a sua concentração intracelular (Nicholls, 2008). Para isso, os transportadores usam o gradiente eletroquímico gerado pela enzima Na^+,K^+-ATPase para o co-transporte de 3 Na^+:1 H^+: 1 glutamato para o meio intracelular, enquanto que 1 íon K^+ é lançado para o meio extracelular, com gasto de uma molécula de adenosina trifosfato (ATP) (Kanner, 2006). Há evidências de que o transporte de glutamato possa ser inibido pela ação de radicais livres que, possivelmente, atuam sobre os grupamentos sulfidrilas dos seus transportadores (Sheldon e Robinson, 2007; Volterra et al., 1994).

A amplitude da sinalização glutamatérgica depende basicamente da concentração e do tempo de permanência do glutamato na fenda sináptica (Reiner e Levitz, 2018). Desse modo, cabe ressaltar a importância do

funcionamento adequado dos transportadores para uma efetiva captação de glutamato, uma vez que a superestimulação dos receptores glutamatérgicos pode levar a excitotoxicidade. Este processo envolve influxo de íons Na^+, Ca^{2+}, estresse oxidativo, déficit energético, e que pode culminar em morte neuronal (Maragakis e Rothstein, 2001; 2004; Nicholls, 2008). Nesse contexto, a excitotoxicidade parece estar implicada na fisiopatologia de várias doenças agudas e crônicas do SNC incluindo epilepsia, isquemia e doença de Alzheimer (Maragakis e Rothstein, 2004). Estudos recentes têm mostrado o possível envolvimento do sistema glutamatérgico nos efeitos promovidos pelo MFD (Urban et al., 2013; Cheng et al., 2014). Nesse contexto, nós temos mostrado que a administração crônica de MFD em ratos jovens aumentou os níveis de glutamato no líquido cefalorraquidiano e diminuiu a captação de glutamato em córtex pré-frontal de ratos jovens (Schmitz et al., 2015).

1.6 Na^+,K^+-ATPase e lipídeos de membrana

A Na^+,K^+-ATPase é uma proteína integral de membrana que usa a energia da hidrólise de uma molécula de ATP para transportar 3 íons Na^+ para o compartimento extracelular e 2 íons K^+ para o compartimento intracelular, simultaneamente. A Na^+,K^+-ATPase funcional é composta por duas subunidades α e duas subunidades β. As subunidades α são responsáveis pela atividade catalítica da enzima e sofrem fosforilação e mudança conformacional acoplada à hidrólise de ATP e ao transporte dos íons Na^+ e K^+. A subunidade β é uma proteína glicosilada de adesão intercelular necessária para direcionar a subunidade α para a membrana plasmática. Outra subunidade ainda menor (γ)

tem sido implicada na regulação da atividade dessa enzima (Geering, 2008) (Figura 3).

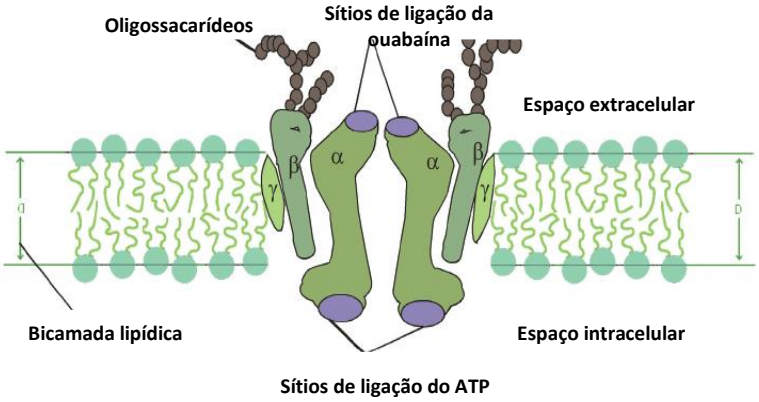

Figura 3. Estrutura da Na^+,K^+-ATPase (Modificado de Suhail, 2010)

A isoforma α_1 é ubiquamente expressa enquanto a α_2 é expressa em pequenos subconjuntos de neurônios, porém em muitos astrócitos. As isoformas α_3 são expressas exclusivamente em neurônios de várias estruturas encefálicas (Bottger et al., 2011; McGrail et al., 1991).

O gradiente de íons Na^+ e K^+ gerado pela Na^+,K^+-ATPase é importante para uma série de funções celulares tais como regulação do volume celular, pH, manutenção do potencial de membrana em repouso e transporte de moléculas ligadas ao co-transporte de Na^+, como aminoácidos, neurotransmissores e glicose (Blanco, 2005; Jorgensen et al., 2003). Nos neurônios, a restauração dos gradientes de Na^+ e K^+ pela isoforma α_3 é muito importante para a manutenção da excitabilidade neuronal, condução do potencial de ação em axônios mielinizados e para o transporte secundário de neurotransmissores acoplados ao Na^+ (Benarroch, 2011). Em astrócitos, a α_2 é co-localizada com diferentes transportadores de glutamato, gerando o

gradiente eletroquímico necessário para a captação desse neurotransmissor e, consequentemente, a finalização da sinalização glutamatérgica (Rose et al., 2009).

A regulação da Na^+,K^+-ATPase inclui diversos fatores como disponibilidade de substrato, componentes da membrana celular, hormônios e fosforilação (Lopina, 2000; Wang e Yu, 2005). Além disso, essa enzima é sensível ao ataque de radicais livres (Wang et al., 2003), sendo inibida por metabólitos formados durante a peroxidação lipídica e por alterações na membrana plasmática (Chakraborty et al., 2003; Cornelius et al., 2015; Dencher et al., 2007).

A atividade da Na^+,K^+-ATPase é importante para a remoção de glutamato da fenda sináptica e alterações na sua atividade parecem estar associadas a diversas patologias que afetam o SNC, tais como enxaqueca (Suhail, 2010), doença de Alzheimer (Vitvitsky et al., 2012), depressão (Goldstein et al., 2006), doença de Parkinson e epilepsia (Aperia, 2007; Benarroch, 2011).

Estudos em animais mostram que substâncias psicoativas como anfetamina (Zugno et al., 2009), fluoxetina (Zanatta et al., 2001), selegilina (Carageorgiou et al., 2003), haloperidol, carbamazepina e lítio (Wood et al,. 1989), bem como MFD (Scherer et al., 2009) alteram a atividade da Na^+,K^+-ATPase. No entanto, ainda faltam estudos para compreender os efeitos da administração crônica de MFD sobre a função da Na^+,K^+-ATPase, especialmente durante o desenvolvimento encefálico.

Cornelius et al. (2015) mostraram também que a atividade da Na^+,K^+-ATPase é sensível a alterações na composição dos lipídeos de membrana.

Nesse contexto, as membranas celulares são compostas por três principais classes de lipídeos: esfingolipídeos, fosfolipídeos e colesterol, os quais desempenham uma série de funções fisiológicas importantes (Cornelius et al., 2015) (Figura 4).

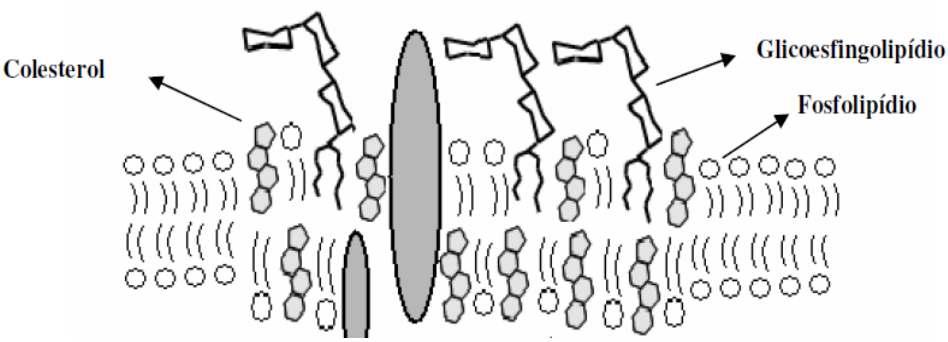

Figura 4. Representação esquemática dos lipídeos de membara. Adaptado de Alchorn, 2007.

Os gangliosídeos são glicoesfingolipídeos complexos, com um ou mais resíduos de ácido siálico na molécula, altamente concentrados nas membranas celulares neurais (Cornelius et al., 2015; Sonnino et al., 2007). Os principais gangliosídeos presentes no encéfalo são GM1, GD1a, GD1b e GT1b, os quais são sintetizados a partir de um precursor comum derivado da lactosilceramida (Kolter et al., 2002). Esses lipídeos apresentam uma variedade de funções importantes nos neurônios, como a participação na proliferação e diferenciação neuronal, na adesão celular, na mielinização e na transmissão sináptica (Cornelius et al., 2015; Kolter et al., 2002; Mocchetti et al., 2005). Adicionalmente, She e colaboradores (2005) demonstraram que os gangliosídeos afetam a plasticidade sináptica em hipocampo, sendo efetivos para atenuar déficits cognitivos em ratos.

Os fosfolipídeos compreendem um grupo heterogêneo de compostos que geralmente apresentam as seguintes regiões: grupo cabeça polar unido ao glicerol por uma ponte fosfodiéster e cadeias hidrocarbonadas longas. Esses

lipídeos constituem o esqueleto das membranas neurais e conferem a essas, fluidez e permeabilidade iônica; além disso, são requeridos para a função de proteínas transmembrana, como receptores e canais iônicos (Cornelius et al., 2015).

Os fosfolipídeos mais abundantes são: fosfatidilcolina, fosfatidiletanolamina, fosfatidilinositol e fosfatidilserina (Cornelius et al., 2015; Farooqui et al., 2004). A esfingomielina, também considerada um fosfolipídeo por apresentar a fosfocolina como grupo cabeça polar, está presente em altas concentrações na mielina (Cornelius et al., 2015).

O colesterol, molécula derivada do ciclopentanoperidrofenantreno, também é um constituinte integral das membranas plasmáticas, localizando-se especialmente no tecido neural (Cornelius et al., 2015; Ohvo-Rekilä et al., 2002). Apresenta diversas funções como a modulação de propriedades físico-químicas das membranas, a formação da mielina, a sinaptogênese e a liberação de neurotransmissores (Mauch et al., 2001; Valenza e Cattaneo, 2006). O colesterol, juntamente com os glicoesfingolipídeos, está concentrado em microdomínios de membrana resistentes à solubilização com detergentes, denominados *lipid rafts*, os quais parecem ser plataformas para os eventos de transdução de sinal (Paratcha e Ibanez, 2002).

Sabe-se que alterações na composição lipídica podem acarretar mudanças nas propriedades físicas das membranas e, consequentemente, levar a disfunção neuronal (Cornelius et al., 2015; Farooqui et al., 2004; Swapna et al., 2006; Valenza e Cattaneo, 2006). Nesse contexto, variações no conteúdo e na composição dos gangliosídeos têm sido observadas em condições como hipóxia (Yin et al., 2006), isquemia (Kwak et al., 2005) e

doença de Alzheimer (Barrier et al., 2007). Adicionalmente, há estudos demonstrando que o conteúdo encefálico de colesterol e fosfolipídeo diminui com o avanço da idade (Svennerholm et al., 1994), bem como em pacientes com desordens neurodegenerativas (Svennerholm e Gottfries, 1994; Farooqui et al., 2004).

1.7 Radicais livres e estresse oxidativo

O radical livre pode ser definido como qualquer espécie química que contém um ou mais elétrons desemparelhados no seu orbital mais externo. A molécula com um elétron desemparelhado se torna mais instável e reativa (Gutteridge e Halliwell, 2018). Eles são gerados nos sistemas vivos quando aproximadamente 5% do oxigênio molecular não são completamente reduzidos até a formação de água na cadeia transportadora de elétrons (Halliwell e Whiteman, 2004; Valko et al., 2004).

As espécies reativas podem exercer tanto papel benéfico quanto deletério para os sistemas vivos. Elas têm papel benéfico quando atuam, por exemplo, no combate a agentes infecciosos e nos processos de sinalização celular. Por outro lado, elevadas concentrações de espécies reativas causam danos às biomoléculas como lipídeos, proteínas e DNA, que podem levar a perda de função celular. Enzimas, transportadores e receptores podem ter sua estrutura alterada quando oxidadas por espécies reativas com consequente prejuízo das suas funções (Halliwell, 2012; Valko et al., 2007).

Em condições fisiológicas as espécies reativas são mantidas em equilíbrio devido, em grande parte, a capacidade de neutralização do sistema de defesa antioxidante enzimático e não enzimático. Entretanto, quando esse

equilíbrio é rompido ocorre um processo denominado de estresse oxidativo e que pode estar associado a diversas patologias. Todas as células aeróbias sofrem danos oxidativos, no entanto, o encéfalo dos mamíferos é altamente sensível. Alguns fatores que tornam o SNC mais suscetível às espécies reativas incluem: alto consumo de oxigênio, presença de neurotransmissores que sofrem auto-oxidação como DA, serotonina e noradrenalina, alta concentração de ferro, lipídeos de membrana ricos em ácidos graxos insaturados, bem como modesto sistema de defesa antioxidante (Halliwell, 2012). Evidências têm sugerido o envolvimento do estresse oxidativo na patogênese de doenças neurodegenerativas como Huntington, Alzheimer e Parkinson (Mancuso et al., 2010; Petrozzi et al., 2007).

Vários estudos demonstram que o estresse oxidativo está provavelmente envolvido nos mecanismos neurotóxicos induzidos pelo MFD. O tratamento agudo com MFD aumenta a atividade da superóxido dismutase (SOD) e catalase (CAT), bem como a peroxidação de lipídeos em encéfalo de ratos jovens e adultos (Gomes et al., 2008; Martins et al., 2006). Aumento do índice de dano ao DNA também foi verificado em estriado e hipocampo de ratos jovens submetidos ao tratamento com MFD (Andreazza et al., 2007). No entanto, apesar desses dados, mais estudos são necessários para compreender a causa e as consequências da alteração no equilíbrio redox promovido pelo MFD sobre o SNC.

1.8 Bioenergética cerebral

O encéfalo possui pouca reserva energética comparada à sua elevada atividade metabólica; portanto, necessita de um suprimento contínuo de

glicose, seu principal substrato energético. A glicólise em conjunto com o ciclo de Krebs e a fosforilação oxidativa são as rotas essenciais para a produção de energia cerebral (Zhou et al., 2018).

A glicólise é composta por uma sequência de reações que ocorrem no citosol e que formam o piruvato como produto final. O ciclo de Krebs ocorre na matriz mitocondrial e consiste em uma sequência de reações onde, em cada volta do ciclo, são formadas três moléculas de nicotinamida adenina dinucleotídeo reduzida (NADH), uma de flavina adenina dinucleotídeo reduzida ($FADH_2$), duas de dióxido de carbono e uma de trifosfato de guanosina (GTP). O NADH e $FADH_2$ produzidos no ciclo de Krebs são carreadores de elétrons e são utilizados na cadeia respiratória para a produção de ATP na fosforilação oxidativa (Zhou et al., 2018).

A cadeia respiratória é composta por quatro complexos enzimáticos e dois transportadores móveis de elétrons (coenzima Q e citocromo *c*) que realizam a transferência dos elétrons desde as coenzimas reduzidas até o aceptor final, o oxigênio. Os elétrons provenientes do NADH são entregues ao complexo I (ou NADH desidrogenase ou NADH: ubiquinona oxirredutase) que transfere esses elétrons para a ubiquinona, formando ubiquinol. O complexo II (ou succinato: ubiquinona oxirredutase) é formado pela enzima succinato desidrogenase (SDH) e três subunidades hidrofóbicas. Esse complexo reduz a ubiquinona a ubiquinol com elétrons do $FADH_2$, provenientes da oxidação do succinato a fumarato no ciclo de Krebs. O complexo III (citocromo bc_1 ou ubiquinona-citocromo *c* oxirredutase) transfere elétrons do ubiquinol para o citocromo *c*. O complexo IV, também denominado citocromo *c* oxidase (COX), catalisa a transferência dos elétrons do citocromo *c* reduzido para o oxigênio, reduzindo-o

a água. O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas, pelos complexos I, III e IV. Com isso, cria-se um gradiente eletroquímico transmembrana que é utilizado como força próton-motriz pelo complexo V (ATP sintase) para síntese de ATP a partir de adenosina difosfato (ADP) e fosfato inorgânico (Pi), processo denominado de fosforilação oxidativa (Zhou et al., 2018) (Figura 5).

Problemas no funcionamento normal da cadeia respiratória mitocondrial levam à diminuição da síntese de ATP. Nesse contexto, dados da literatura sugerem que uma diminuição do metabolismo energético pode estar implicada na fisiopatologia de alguns transtornos neurológicos, como demência, isquemia cerebral e as doenças de Alzheimer e Parkinson (Beal, 2007; Gibson et al., 2009; Kowaltowski et al., 2009; Schurr, 2002; Zhou et al., 2018).

Em relação à função mitocondrial, os efeitos do MFD parecem ser dependentes da idade do animal e do protocolo de administração. MFD (1, 2 ou 10 mg/kg, uma vez por dia, durante 28 dias) aumentou a atividade da Na^+,K^+-ATPase (Scherer et al., 2009), bem como a atividade de enzimas envolvidas no metabolismo energético em estruturas cerebrais de ratos adultos (Fagundes et al., 2007). Por outro lado, a administração aguda de MFD (1, 2 ou 10 mg/kg) reduziu a atividade dos complexos da cadeia respiratória mitocondrial no encéfalo (Fagundes et al., 2010a; Fagundes et al., 2010b). No entanto, mais estudos são necessários para avaliar os efeitos do MFD sobre a bioenegética encefálica.

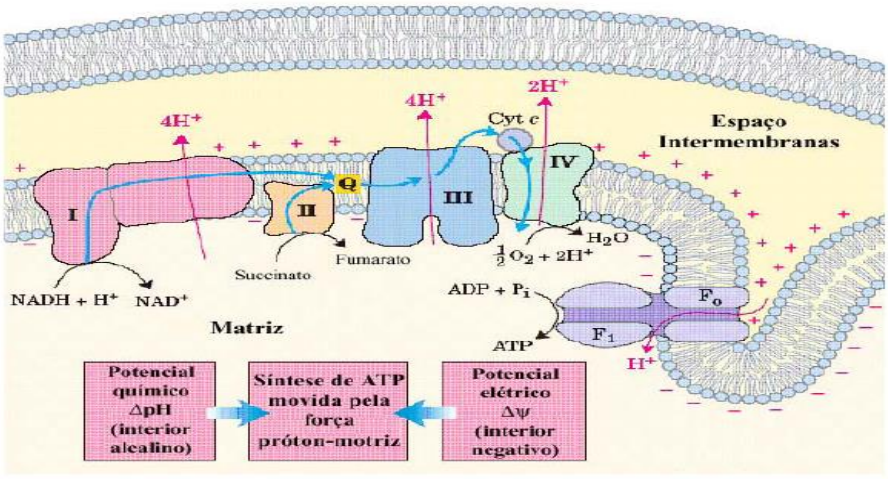

Figura 5. Fosforilação oxidativa (Nelson e Cox, 2014).

1.9 Citoesqueleto

Todas as funções realizadas na célula viva estão de alguma forma associadas a uma sofisticada rede de filamentos de proteínas com diferentes composições, distribuições e funções na célula, formando uma malha integrada conhecida como citoesqueleto (Huber et al., 2015). No entanto, a característica mais marcante do citoesqueleto diz respeito à sua capacidade de responder a sinais e condições às quais as células são submetidas, rearranjando e participando da resposta celular adaptativa a estímulos de sobrevivência (Bolin et al., 2016), proliferação (Yi et al., 2015), diferenciação (Compagnucci et al., 2016), migração (Le Clainche e Carlier, 2008) ou morte (Xiong et al., 2015). O citoesqueleto é um ponto final das vias de sinalização, adaptando as células a respostas imediatas ou duradouras em organismos saudáveis e doentes.

O citoesqueleto da maioria das células animais é formado por uma malha de três subsistemas de filamentos interligados: microfilamentos (MFs), microtúbulos (MTs) e os filamentos intermediários (FIs) (Figura 6). Estes sistemas proteicos atuam em conjunto com proteínas acessórias e/ou vias de

sinalização que permitem que o citoesqueleto participe nas mais variadas respostas celulares.

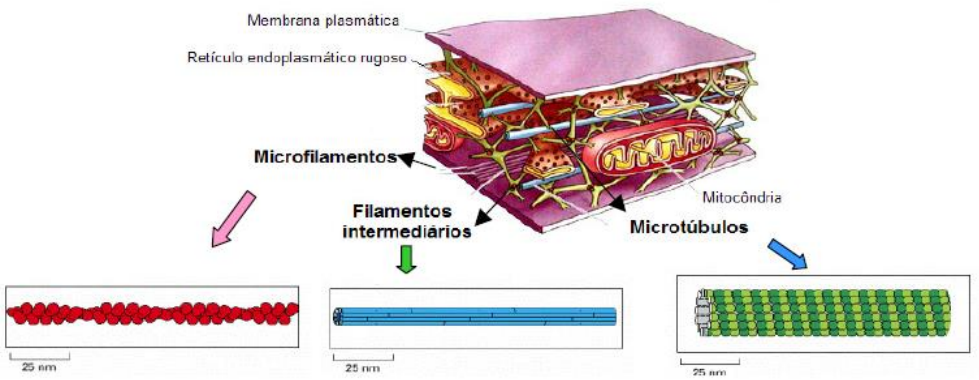

Figura 6. Representação esquemática dos constituintes do citoesqueleto. Adaptado de Albert et al., 2002 e www.mie.utoronto.ca/labs/lcdlab/biopic, acessado em 07.11.2016

Os neurônios são células extremamente polarizadas nas quais o citoesqueleto [constituído de MTs, MFs e neurofilamentos (NFs)] desempenha um papel crucial na manutenção da estrutura e função dessas células (Gentil et al., 2015). Os NFs são os componentes mais abundantes do citoesqueleto de grandes axônios mielinizados do SNC e periférico (Perrot e Eyer, 2009). Eles são constituídos da associação de três subunidades: NF de baixo, médio e alto peso molecular (NF-L, NF-M e NF-H) que são distribuídos de diferentes maneiras em um NF individual (Figura 7).

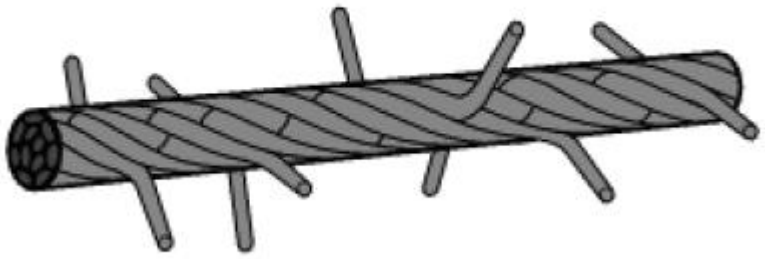

Figura 7. Representação esquemática de um NF, onde a região central é formada pelas três subunidades (NF-L, NF-M e NF-H) e as projeções laterais são constituídas pelas regiões carboxi-terminais das subunidades NF-M e NF-H. Adaptado de Kirkpatrick & Brady, 1999.

A subunidade NF-L pode se auto montar, formando o núcleo do filamento. As subunidades NF-M e NF-H são dispostas perifericamente no filamento, com suas longas e flexíveis caudas ricas em domínios altamente carregados de múltiplos locais de fosforilação, projetando-se radialmente a partir do esqueleto do filamento (Beck et al., 2010).

NFs podem interagir com organelas, tais como núcleo (Mellad et al., 2011), mitocôndrias e retículo endoplasmático (Rao et al., 2002; 2011), bem como outros componentes do citoesqueleto e da membrana plasmática (Macioce et al., 1999; Wiche e Winter, 2011; Koutras e Levesque, 2011). Além disso, Ehlers e colaboradores (1998) observaram uma interação do NF-L com os receptores NMDA, o que poderia ter implicações no papel do citoesqueleto na sinalização do glutamato. Nos axônios de neurônios maduros, um grande número de NFs orientados longitudinalmente e fosforilados desempenham um papel fundamental, aumentando o diâmetro dos axônios mielinizados. Uma das funções dos NFs nos neurônios é controlar o calibre axonal e, consequentemente, a velocidade da condutividade nervosa. Os NFs são transportados do corpo celular onde são sintetizados, para serem entregues ao longo do axônio por um mecanismo chamado de transporte axonal. Esse mecanismo é dependente do nível de fosforilação do NF-M e NF-H, dessa forma, uma inadequada fosforilação do terminal carboxila poderia comprometer o transporte axonal do NF (Yabe et al., 2001).

Entre as células da glia, os astrócitos são componentes-chave do SNC. Eles são considerados importantes elementos para a comunicação do encéfalo

(Volterra e Meldolesi, 2005), bem como são importantes para a eficácia da sinapse, produção de gliotransmissores e fatores tróficos que modulam a função neuronal (Volterra e Meldolesi, 2005; Pirttimaki e Parri, 2013). A proteína glial fibrilar ácida (GFAP) é o principal FI expresso em astrócitos maduros, contribuindo para a manutenção da força mecânica e a forma das células. Além disso, evidências mostram que a GFAP desempenha também um papel crítico sobre a motilidade/migração, proliferação, homeostase glutamatérgica, crescimento de neuritos e lesão/proteção celular (Middeldorp e Hol, 2011). Astrócitos lesionados responderam com hipertrofia, regulação positiva e rearranjo da rede de GFAP, tornando-se astrócitos reativos (Hol e Pekny, 2015). A superexpressão da GFAP altera a expressão de genes críticos na comunicação neuronal (Orre et al., 2014), que pode ser uma resposta precoce em um processo de doença crônica, como a doença de Alzheimer (Kamphuis et al., 2012; 2014).

1.10 Fosforilação de proteínas do sistema nervoso central

A fosforilação é um importante mecanismo regulador da função de proteínas em que grupos volumosos e carregados negativamente são cataliticamente adicionados ou removidos para a cadeia lateral dos aminoácidos serina, treonina e tirosina, alterando a função biológica do substrato. A fosforilação é um processo altamente específico e hierárquico catalisado por proteínas cinases, uma das maiores famílias de enzimas em eucariotos, enquanto a defosforilação é catalisada por proteínas fosfatases, revertendo os efeitos da fosforilação (Ubersax e Ferrel, 2007) (Figura 8).

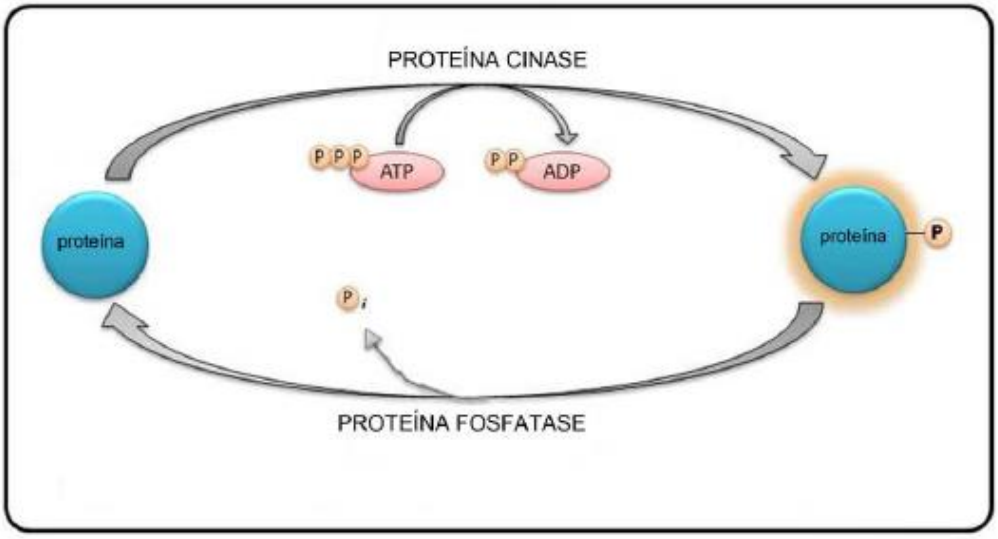

Figura 8. Regulação das proteínas celulares por fosforilação. Proteínas cinases transferem grupos fosforil do ATP para resíduos de serina, treonina ou tirosina em um substrato proteico. A remoção desses grupos é catalisada por proteínas fosfatases. Adaptado de Purves et al., 2005.

A fosforilação desempenha um papel importante regulando a organização estrutural e função dos FIs (Sihag et al., 2007). NFs estão entre as proteínas mais fosforiladas no SNC. Eles são sintetizados no corpo celular, mas são extensivamente fosforilados depois que eles são transportados para o axônio. A fosforilação aminoterminal desempenha um papel importante na regulação do equilíbrio de montagem/desmontagem das subunidades NF-L e NF-M dos NFs (Omary et al., 2006; Shea e Chan, 2008). Os grupos fosfatos do grupamento aminoterminal presentes na GFAP e NF-L são adicionados por cinases proteicas dependentes de segundos mensageiros, tais como proteína cinase dependente de adenosina monofosfato cíclico (AMPc) (PKA), proteína cinase dependente de Ca^{2+}/calmodulina II (PKCaMII) e proteína cinase C (PKC) (Sihag et al., 2007).

Os locais de fosforilação de NF-M e NFH estão localizados em regiões de repetição Lys-Ser-Pro (KSP) no domínio caudal dessas subunidades.

Fosforilação das repetições KSP tornam o NF-H a molécula mais extensivamente fosforilada do SNC. As repetições de KSP são fosforiladas por cinases, como Cdk5 (Zhou et al., 2010), MAPK, tais como ERK½, JNK e p38MAPK, bem como glicogênio sintase cinase-3 (GSK-3) (Grant e Pant, 2000; Lee et al., 2014).

As Ser-Thr fosfatases mais frequentes envolvidas na modulação nos níveis de fosforilação dos FIs são: proteína fosfatase 1 (PP1), proteína fosfatase 2A (PP2A) e calcineurina (PP2B) (Pierozan e Pessoa-Pureur, 2018).

O desequilíbrio entre fosforilação e defosforilação das proteínas do citoesqueleto estão associadas à neurodegenaração (Perrot et al., 2008; Perrot e Eyer, 2009). Nesse contexto, tem sido mostrado que NFs hipofosforilados são mais suscetíveis à quebra proteolítica (Goldstein et al., 1987; Pant, 1988; Pierozan e Pessoa-Pureur, 2018).

1.11 Comportamento e metilfenidato

A memória pode ser definida como uma complexa rede de diferentes funções inter-relacionadas trabalhando juntas para gerenciar as informações (Asok et al., 2018). Suas fases compreendem a aquisição, a consolidação e a evocação de uma grande diversidade de informações e pode ser classificada quanto ao tempo de duração em curta ou longa duração (Asok et al., 2018; Izquierdo, 2002; Squire e Kandel, 2003).

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). 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

(Asok et al., 2018).

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 e seu processamento depende da atividade elétrica de neurônios do córtex pré-frontal. Por outro lado, 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 (Asok et al., 2018).

O uso prolongado de psicoestimulantes como a anfetamina e cocaína tem sido associado à disfunção cognitiva envolvendo aprendizado e memória. Estruturas encefálicas como o hipocampo e córtex pré-frontal, que são essenciais para as funções cognitivas, são altamente sensíveis a derivados anfetamínicos (Camarasa et al., 2008; Loureiro-Vieira et al., 2017; Quansah et al., 2017a,b; Santucci, 2008).

Nesse contexto, 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), bem como aumentou os efeitos depressivos e reduziu a habituação a um ambiente familiar (Carlezon, 2003). Animais adultos tratados com MFD (2 mg/kg) na adolescência foram menos sensíveis aos estímulos de recompensa naturais como a sacarose e mostraram maior sensibilidade a situações aversivas (Bolaños et al, 2003). Nesse contexto, nosso grupo mostrou que ratos tratados com MFD apresentaram desempenho inferior aos animais controles no teste do labirinto aquático de Morris depois de terem

recebido MFD (2 mg/kg) do 15º ao 45º dia de idade (Scherer et al., 2010).

Heyser e colaboradores (2004) observaram alteração no teste de reconhecimento de objetos em ratos jovens tratados por sete dias com MFD (5 mg/kg). Além disso, 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 mostrou que a exposição ao MFD, desde a adolescência até a idade adulta, em 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, idade dos animais, padrão de administração do fármaco e a complexidade da tarefa utilizada (Bethancourt et al., 2009).

1.12 Células de feocromocitoma de ratos (PC12)

A linhagem celular PC12, derivada de feocromocitoma de rato, um tumor decorrente das células cromafins da medula suprarrenal, é um modelo muito importante e bem conhecido para o estudo de eventos neurobiológicos e neuroquímicos (Jaeger, 1985). Essas células têm sido amplamente utilizadas como modelo experimental *in vitro* para estudar os efeitos de vários compostos neuroativos, uma vez que apresentam muitas macromoléculas citosólicas e ligadas à membrana correspondentes a neurônios (Shafer e Atchison, 1991). Nesse contexto, foi relatado que as células PC12 diferenciadas expressam o receptor D2 de DA (D2R) (Zhu et al., 1997) e DAT (Kadota et al., 1996), o que

torna essa linhagem celular um modelo bastante adequado para estudar os efeitos do MFD.

Funcionalmente, o MFD é um inibidor de alta afinidade do DAT (Johnston et al., 2011a,b; Kuczenski e Segal, 2001; Leonard et al., 2004; Volkow et al., 2001). O DAT desempenha um papel essencial na terminação da sinalização dopaminérgica, uma vez que os níveis de DA são principalmente regulados pela remoção de DA extracelular e sua reciclagem de volta ao neurônio (Klein et al., 2018).

A DA é um importante neurotransmissor modulador que controla várias atividades fisiológicas e comportamentais no encéfalo de mamíferos, incluindo controle motor, motivação e humor (Klein et al., 2018). A perda de função dos neurônios dopaminérgicos tem sido implicada na patogênese da doença de Parkinson, esquizofrenia e dependência química (Klein et al., 2018; Sotinikova et al., 2006). Aproximadamente 80% da DA encontrada no SNC está localizada no estriado onde se liga aos seus receptores (Carlsson, 1959), em neurônios que expressam especificamente os receptores de DA, D1R e D2R (Gingrich e Caron, 1993). Nesse contexto, Sadasivan e colaboradores (2012) mostraram que ratos pré-tratados com MFD apresentam uma maior perda neuronal no estriado quando submetidos à neurotoxina 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP), utilizada para induzir a doença experimental de Parkinson.

Existem vários estudos que sugerem um papel específico do D2R na regulação da função do DAT (Meiergerd et al., 1993; Batchelor e Schenk, 1998; Dickinson et al., 1999), bem como uma interação física entre eles (Dickinson et al., 1999). O bloqueio do DAT pelo MFD está bem estabelecido, mas os efeitos

do MFD por meio de sua ligação ao D2R não têm sido investigados. Nesse contexto, estudos recentes *in vivo* revelaram que o D2R exerce sua ação de maneira independente do AMPc, promovendo a formação de um complexo de sinalização composto pela Akt, PP2A e β-arrestina 2 (β-Arr2) (Beaulieu et al., 2004; 2005). A formação desse complexo leva à inativação de Akt após a defosforilação de seu resíduo de treonina 308 (Thr-308) por PP2A (Beaulieu et al., 2005). A inativação de Akt em resposta a DA resulta na ativação da GSK-3β, que por sua vez contribui para a expressão de comportamentos associados à DA (Beaulieu et al., 2004). Nesse contexto, respostas diminuídas aos estímulos de recompensa, assim como aumento do comportamento depressivo e ansioso, foram observadas em ratos tratados com MFD (Achat-Mendes et al., 2003; Adriani et al., 2006; Andersen et al., 2002; Bolanos et al., 2003; Carlezon et al., 2003; Mague et al., 2005) .

A via mTORC1 (complexo alvo de rapamicina em mamíferos 1) é um importante regulador da síntese proteica e sua atividade pode ser regulada pela Akt. A síntese de importantes proteínas implicadas na plasticidade sináptica e na consolidação da memória têm sido associada com a tradução dependente de mTORC1 (Antion et al., 2008; Hoeffer et al., 2011; Bhattacharya et al., 2012; Santini et al., 2011). A Akt quando fosforilada, fosforila e ativa mTORC1, que por sua vez, promove a tradução, principalmente por meio da fosforilação da proteína 1 ligante do fator de iniciação eucariótico 4E (4E-BP1) e da proteína cinase ribossomal S6 de 70 kDA (p70 S6K) (Antion et al., 2008; Hoeffer et al., 2011; Bhattacharya et al., 2012; Santini et al., 2011).

No entanto, outros importantes fatores que podem regular a síntese proteica, inclusive de proteínas sinápticas, estão sendo associados ao

tratamento com psicoestimulantes (Bartl et al., 2010; Zheng et al., 2013). Nesse contexto, Bartl e colaboradores (Bartl et al., 2010) mostraram que os níveis da proteína de ligação responsiva ao AMPc (CREB) aumentaram após o tratamento com MFD. Por outro lado, os níveis do fator de transcrição O1 fosforilado (pFoxO1) foram reduzidos no estriado de ratos Sprague-Dawley após a administração de d-anfetamina (Zheng et al., 2013).

Para nosso conhecimento, há pouca informação sobre os efeitos do MFD sobre a função de proteínas de sinalização celular. No entanto, alterações na função dessas proteínas podem estar envolvidas nos efeitos celulares do MFD, uma vez que suas atividades podem ser reguladas pelo D2R, que é amplamente expresso no SNC, bem como finamente regulado pelo DAT, o principal alvo do MFD.

2. OBJETIVOS

2.1 Objetivo geral

A fim de melhor compreender os efeitos do uso do MFD, o objetivo **geral** do presente trabalho foi avaliar parâmetros neuroquímicos e comportamentais associados a esse psicoestimulante.

2.2 Objetivos específicos

Os objetivos específicos do presente trabalho serão subdivididos em quatro capítulos, os quais correspondem a artigos científicos, como seguem:

2.2.1 CAPÍTULO I

Avaliar o efeito da administração crônica de MFD em hipocampo de ratos jovens sobre:

- as concentrações de diferentes aminoácidos (aspartato, glutamato, serina, histidina, glutamina, glicina, alanina, tirosina, triptofano, metionina, valina, fenilalanina, isoleucina, leucina, ornitina e lisina);
- a captação de glutamato, bem como o imunoconteúdo dos transportadores de glutamato, GLAST e GLT-1;
- a atividade da Na^+,K^+-ATPase, bem como o imunoconteúdo das subunidades catalíticas (α_1, α_2 e α_3);

- alguns parâmetros de estresse oxidativo, a saber: os níveis de espécies reativas, dano a proteínas e lipídeos, bem como a atividade de enzimas antioxidantes [SOD, CAT e glutationa peroxidase (GPx)];
- alguns parâmetros de metabolismo energético, a saber: nível de ATP, citrato sintase, SDH, atividade dos complexos da cadeia respiratória (II, II-III e IV), bem como a massa mitocondrial e o potencial de membrana mitocondrial.

2.2.2 CAPÍTULO II

Avaliar o efeito da administração crônica de MFD em hipocampo de ratos jovens sobre:

- a fosforilação e imunoconteúdo dos NFs (NF-L, NF-M e NF-H) e GFAP;
- o imunoconteúdo de fosfatases (PP1, PP2A e PP2B);
- o conteúdo de gangliosídeos (GM1, GD1a, GD1b and GT1b);
- o conteúdo de fosfolipídeos (esfingomileina, fosfatidilcolina, fosfatidilserina, fosfatidiletanolamina e fosfatidilinositol);
- a concentração de colesterol.

2.2.3 CAPÍTULO III

Avaliar o efeito da administração crônica de MFD em hipocampo de ratos jovens sobre:

- o número de neurônios e astrócitos;

- os níveis ou imunoconteúdo das neurotrofinas (NGF e BDNF), do pro-BDNF (precursor do BDNF) e do pTrkB (receptor tirosina cinase B ativado/fosforilado);
- o imunoconteúdo das proteínas sinápticas (SNAP-25, GAP-43 e sinaptofisina);
- os níveis de TNF-α e IL-6, bem como o imunoconteúdo de Iba-1 (marcador de microglia);
- o imunoconteúdo da caspase 3 clivada (marcador de apoptose);
- as vias de sinalização celular (MAP/ERK, Akt, GSK-3β);
- o desempenho dos animais nas tarefas de labirinto em cruz elevado, campo aberto e memória de reconhecimento de objetos.

2.2.4 CAPÍTULO IV

Avaliar o efeito a curto e/ou longo prazo do tratamento com MFD (1μM) em células PC12 sobre:

- a razão pAkt/Akt e pmTOR/mTOR;
- a razão pp70 S6K/p70 S6K;
- os níveis de fosforilação da 4E-BP1;
- a atividade de CREB, FoxO1 e GSK-3β.

3. PROCEDIMENTOS EXPERIMENTAIS E RESULTADOS

3.1 Administração crônica de metilfenidato

Os capítulos I, II e III correspondem a artigos científicos de pesquisa experimental realizados em ratos Wistar. Os ratos foram pesados e injetados por via intraperitoneal com solução salina (grupo controle) ou 2,0 mg/kg de metilfenidato (grupo MFD), uma vez por dia, durante trinta dias consecutivos a partir do 15º dia de vida. O MFD foi dissolvido em solução salina e injetado num volume de 1 mL/100 g de peso corporal. O grupo controle recebeu volume equivalente de solução salina. Essa dose e via de administração foram selecionadas porque mimetizam as doses terapêuticas em termos de magnitude dos efeitos neuroquímicos e comportamentais (Gerasimov et al., 2000).

Vinte e quatro horas após a última injeção (45º dia de vida), os animais foram submetidos às tarefas comportamentais ou decapitados sem anestesia. O hipocampo foi dissecado e processado de acordo com cada análise.

Optamos por começar a administrar o MFD em ratos com 15 dias de vida, pois as primeiras três semanas de vida em ratos são caracterizadas por intensa sinaptogênese, mielinização e gliogênese, comparável à primeira infância em seres humanos (Rice and Barone, 2000). Administramos por 30 dias, pois esse período mimetiza a utilização crônica em seres humanos (Andreazza et al., 2007).

No presente estudo, decidimos investigar os efeitos sobre o hipocampo porque pouco se sabe como a exposição precoce ao MFD pode afetar o

funcionamento dessa estrutura na vida adulta (Andersen, 2005; Carias et al., 2018; Oakes et al., 2018). O hipocampo desempenha um papel importante em comportamentos afetivos, dependência química e recaída (Carlezon et al., 2005; Duman et al., 1997; Nestler et al., 2002). Além disso, as ações do MFD sobre o hipocampo podem ser centrais nos efeitos em longo prazo uma vez que o tratamento com esse psicoestimulante em jovens roedores aumenta os níveis de noradrenalina num padrão dose-dependente (Kuczenski e Segal, 2002). Portanto, identificar as alterações induzidas pelo MFD sobre o hipocampo pode ser crucial na interpretação dos mecanismos que contribuem para as alterações comportamentais e neuroquímicas associadas a esse psicoestimulante.

3.2 Cultura celular e tratamento

O capítulo IV corresponde ao artigo científico de pesquisa experimental realizado em células PC12. Esse modelo celular foi escolhido porque tem sido amplamente usado para estudar os efeitos de vários compostos neuroativos como cocaína (Shafer e Atchison, 1991; Iman et al., 2005), bromocriptina e quinpirole (agonistas D2R) (Chiasson et al., 2006), himantano (fármaco antiparkinsoniano) (Abaimov et al., 2008), 6-hidroxidopamina (Xu et al., 2013), e MFD (Grünblatt et al., 2013; Bartl et al., 2010), uma vez que é uma célula neuronal dopaminérgica e pode sintetizar, armazenar, secretar e captar DA (Pan et al., 2012). Além disso, foi mostrado que células PC12 diferenciadas expressam D2R (Zhu et al., 1997) e DAT (Kadota et al., 1996), o que faz dessa linhagem um modelo apropriado para estudar os efeitos do MFD.

Nós escolhemos tratar as células com 1 μM de MFD porque tem sido demonstrado que concentrações micromolares produzem níveis extracelulares de DA superiores aos níveis intracelulares (Kuczenski e Segal, 1997; Easton et al., 2007; Volz et al., 2008).

A solução de MFD foi protegida da luz e as diluições foram sempre preparadas para cada tratamento. As células foram incubadas com MFD sob condições escuras, a fim de reduzir a sua degradação.

3.3 Capítulo I

Methylphenidate decreases ATP levels and impairs glutamate uptake and Na^+,K^+-ATPase activity in juvenile rat hippocampus

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Methylphenidate Decreases ATP Levels and Impairs Glutamate Uptake and Na^+,K^+-ATPase Activity in Juvenile Rat Hippocampus

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Abstract The study of the long-term neurological consequences of early exposure with methylphenidate (MPH) is very important since this psychostimulant has been widely misused by children and adolescents who do not meet full diagnostic criteria for ADHD. The aim of this study was to examine the effect of early chronic exposure with MPH on amino acids profile, glutamatergic and Na^+,K^+-ATPase homeostasis, as well as redox and energy status in the hippocampus of juvenile rats. Wistar male rats received intraperitoneal injections of MPH (2.0 mg/kg) or saline solution (controls), once a day, from the 15th to the 45th day of age. Results showed that MPH altered amino acid profile in the hippocampus, decreasing glutamine levels. Glutamate uptake and Na^+,K^+-ATPase activity were decreased after chronic MPH exposure in the hippocampus of rats. No changes were observed in the immunocontents of glutamate transporters (GLAST and GLT-1), and catalytic subunits of Na^+,K^+-ATPase (α_1, α_2, and α_3), as well as redox status. Moreover, MPH provoked a decrease in ATP levels in the hippocampus of chronically exposed rats, while citrate synthase, succinate dehydrogenase, respiratory chain complexes activities (II, II–III, and IV), as well as mitochondrial mass and mitochondrial membrane potential were not altered. Taken together, our results suggest that chronic MPH exposure at early age impairs glutamate uptake and Na^+,K^+-ATPase activity probably by decreasing in ATP levels observed in rat hippocampus.

Keywords Methylphenidate · Amino acid levels · Glutamatergic homeostasis · Na^+,K^+-ATPase · ATP levels · Mitochondrial function

Introduction

Methylphenidate (MPH), an amphetamine-like stimulant, increases dopamine and norepinephrine levels in the synaptic cleft by blocking its carriers. It is widely used to treat the symptoms of attention deficit hyperactivity disorder (ADHD), which is a complex neuropsychiatric disease worldwide, highly prevalent among children and often persisting into adulthood [1–4]. However, recent studies have reported a growing incidence of MPH misuse among young adults and students who do not meet the criteria for ADHD in search of a cognitive enhancement [5, 6].

The increase of prescribing frequency of the drug creates the need to investigate further the consequences of long-term use of this psychostimulant on the developing normal brain. In line with this, studies show that adult rats submitted to chronic treatment with psychostimulants during childhood and adolescence present changes in neurochemical, behavioral, and molecular parameters [7–11]. In this context, alteration in dopaminergic system, redox status, mitochondrial function, glutamatergic/GABAergic systems, neuroinflammation, blood-brain barrier, and neurogenesis have been associated

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with MPH treatment [12–16]. We have recently shown that chronic treatment at early age with MPH increases glutamate levels in cerebrospinal fluid and impairs glutamate uptake and Na^+,K^+-ATPase activity, as well as causing protein damage in the prefrontal cortex [17]. However, the effects of chronic MPH exposure on glutamatergic homeostasis in juvenile rat hippocampus, a structure of the brain that is crucial to memory process, are still unknown.

Amino acids, including glutamate, can quickly enter the cerebrospinal fluid through the single amino acid transporter and act as neurotransmitters or neurotransmitter precursors or even as an energy source for the brain [18]. Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and plays important roles in processes that depend on brain plasticity such as learning/memory processes [19, 20]. On the other hand, overstimulation of the glutamatergic system may lead to a process known as excitotoxicity [21], which has been implicated in the pathogenesis of various CNS disorders including epilepsy, ischemia, and Alzheimer's disease [22, 23]. Astrocytes play a central role in maintaining glutamate homeostasis due to its ability to remove glutamate from the synaptic cleft via a mechanism known as glutamate uptake, which is accomplished by the glutamate transporters GLT-1 and GLAST [24], and their ability to synthesize glutamine from glutamate [25]. Glutamate transports through its carriers may be affected by the activity of Na^+,K^+-ATPase since they are dependent on the gradient of Na^+ generated by this enzyme [26].

Na^+,K^+-ATPase is an integral membrane protein that regulates neuronal signaling, ion homeostasis, and substrate transportation of most animal cells; it transports 3 Na^+ from within the cell in exchange for 2 K^+ from outside the cell, using the energy derived from hydrolysis of one molecule of ATP [27]. Disturbance in Na^+,K^+-ATPase is observed in various diseases that affect the CNS, including schizophrenia, bipolar mood disorder, major depressive disorder [28], and cerebral ischemia [29]. It has been reported that Na^+,K^+-ATPase activity is particularly susceptible to free radicals attack and/or induction of oxidative stress, and its activity [27, 30]. Reactive oxygen species have been shown to play a role in the behavioral changes after psychostimulant use [31]. In addition, clinical [32] and preclinical [33–37] studies show that MPH may alter brain energy metabolism.

The investigation of the long-term consequences of early exposure with MPH on CNS is very important since the misuse of this psychostimulant is increasing, particularly during childhood and adolescence. In the present study, we initially investigate the effect of early chronic exposure with MPH on amino acid levels in rat hippocampus. We also investigated the effect of MPH on glutamate homeostasis, Na^+,K^+-ATPase, and redox status in the hippocampus of rats. Energy metabolism parameters and mitochondrial function also were tested.

Experimental Procedures

Animals and Reagents

The rats were obtained from the Central Animal House of the Department of Biochemistry of the University Federal of Rio Grande do Sul, Porto Alegre, Brazil. Litters were culled to eight pups on postnatal day (PD) 3 (day of parturition = PD 0) and were kept with the dam until weaning on PD 21. After weaning, the rats were re-housed in boxes containing up to four male rats. Animals were maintained on a 12:12 light/dark cycle at a constant temperature of 22 ± 1 °C, with free access to water and commercial protein chow. We determined that seven per group were sufficient for biochemical studies since it was considered a power estimated at 0.80, an alpha 0.05, and standard deviation values of previous studies. A total of 134 male Wistar rats were used.

The care with animals followed the official governmental guidelines issued by the Brazilian Federation of Societies for Experimental Biology, following the Guide for the Care and Use of Laboratory Animals (No. 80-23, revised 1996) and Arouca Law (Law no. 11.794/2008). In addition, all experimental protocols of this study were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS, RS, Brazil) under license #29651. We further attest that all efforts were made to minimize the number of animals used and their suffering.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Chronic Early Treatment with Methylphenidate

Beginning on PD 15, rats were weighed and injected intraperitoneally with saline solution (control group) or 2.0 mg/kg of methylphenidate (MPH group), once a day, for 30 consecutive days, during the diurnal cycle at 12 ± 1 h [38]. MPH was dissolved in saline and injected at a volume of 1 mL/100 g of body weight. The control group received an equivalent volume of saline solution. This dose and route of administration were selected because they mimic the therapeutic doses in terms of magnitude of neurochemical and behavioral effects [39]. Twenty-four hours after the last injection (PD 45), the animals were decapitated and the hippocampi were removed and processed according to each analysis.

We chose to start administering MPH to rats at 15 days of age because in the first 3 weeks during the postnatal period, rats are characterized by intense synaptogenesis, myelination, and gliogenesis, comparable to early childhood in humans [40]. Treatment lasted 30 days (from 15 to 45 days of life of rats) because this period mimics chronic use in humans [41]. We did not observe any changes in weight gain, behavior, and mortality rate among the groups throughout the treatment.

Determination of Amino Acid Concentrations

Free amino acids in the hippocampus supernatant were determined by HPLC method according to Joseph and Marsden [42]. Briefly, the hippocampus was previously homogenized in 5 vol (1:5, *w*/*v*) of 0.9% saline solution, centrifuged at 800×*g* for 10 min at 4 °C, and the supernatant was taken to amino acid determination. Amino acids were quantitatively determined by relating their chromatographic peak area to those obtained from a known standard mixture and to that of internal standard peak area (homocysteic acid). Fourteen rats were needed for this assay (seven per group).

Glutamate Uptake Assay

It was performed according to Frizzo et al. [43]. Fourteen rats were needed for this assay (seven per group). The hippocampus was cut into 400-μm-thick slices with a McIlwain chopper. Briefly, slices were pre-incubated in Hank's balanced salt solution (HBSS) at 37 °C for 15 min, followed by adding a solution containing 0.33 mCi/mL L-[2,3-3 H] glutamate (Amersham International, UK) with 100 μM unlabeled glutamate at 37 °C. Incubation was stopped after 5 min with two ice-cold washes of 1 mL HBSS, immediately followed by the addition of 0.5 N NaOH. Sodium-independent uptake was determined on ice (4 °C), using HBSS containing *N*-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake and were calculated as nanomoles of glutamate per minute per milligram of protein. Both the specific and non-specific uptakes were performed in triplicate.

Western Blot Analyses

The hippocampus was homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (*v*/*v*) of a solution containing 40% glycerol, 5% mercaptoethanol, and 50 mM Tris–HCl, pH 6.8. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE. The gels were transferred (Trans-blot SD semidry transfer cell; BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5% bovine serum albumin, fraction V). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS). After blocking with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 3% bovine serum albumin for 1 h, the membranes were incubated with primary antibody overnight: 1:1000 rabbit anti-GLAST and rabbit anti-GLT-1 glutamate transporters; and 1:5000 anti-Na^+,K^+-ATPase (α_1 subunit), anti-Na^+,K^+-ATPase (α_2 subunit), anti-Na^+,K^+-ATPase (α_3 subunit), and 1:2000 mouse anti-β-actin. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:10,000. The blot was washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). Membranes were re-probed for anti-β-actin immunoreactivity. The membranes were developed in a photo documenter and band intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the website http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-actin as a loading control to assess protein levels. The data used in statistical analysis were obtained from the ratio of the protein studied and β-actin density unit lines. Fourteen rats (seven per group) were necessary for the determination of western blot analyses.

Na^+,K^+-ATPase Activity Assay

The hippocampus was homogenized in 10 vol (1:10, *w*/*v*) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. Homogenates were centrifuged at 1000×*g* for 10 min at 4 °C. Supernatants were taken for Na^+,K^+-ATPase activity assay. Fourteen rats were needed for this assay (seven per group). Reaction mixture for Na^+,K^+-ATPase activity 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 started by the 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 addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays [29]. Inorganic phosphate (Pi) released was measured by the method of Chan et al. [44] and enzyme-specific activity was expressed as nanomoles Pi per minute per milligram of protein.

Assay of Redox Homeostasis Parameters

Twenty-four rats (six per group) were necessary for the determination of oxidative stress parameters. The hippocampus was homogenized in 10 vol (1:10, *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenates were centrifuged at 800×*g* for 10 min at 4 °C. The pellet was discarded, and the supernatant was immediately separated and used for the measurements of oxidative stress.

2′7′-Dichlorofluorescein Fluorescence Assay Reactive oxygen species production was measured according to the method

of LeBel et al. [45] and based on the oxidation of 2′7′-dichlorofluorescein (H_2DCF). The samples were incubated in a medium containing 100 μM 2′7′-dichlorofluorescein diacetate (H_2DCF-DA) solution. Reaction produces the fluorescent compound dichlorofluorescein (DCF) which is measured at λ_{em} = 488 nm and λ_{ex} = 525 nm. Results were represented as nanomoles DCF per milligram of protein.

Thiobarbituric Acid Reactive Substances (TBARS) TBARS, an index of lipid peroxidation, were measured according to Ohkawa et al. [46]. The samples were incubated in a medium containing 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, and 0.8% thiobarbituric acid. This mixture was vortexed, and the reaction was carried out in a boiling water bath for 1 h. The resulting pink-stained TBARS were determined spectrophotometrically at 535 nm. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard, and the results were represented as nanomoles of TBARS per milligram of protein.

Sulfhydryl Content This assay was performed as described by Aksenov and Markesbery [47], which is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Briefly, 15 μL of homogenate was added to 275 μL of phosphate buffer saline pH 7.4 containing 1 mM EDTA. The reaction was started by the addition of 10 μL of 10 mM DTNB and incubated for 30 min at room temperature in a dark room. The sulfhydryl content is inversely correlated to oxidative damage to the protein. Results were reported as nanomoles of TNB per milligram of protein.

Superoxide Dismutase Assay (SOD) SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is a substrate for SOD. Inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity was then indirectly assayed at 420 nm [48]. A calibration curve was performed with purified SOD as a standard to calculate the activity of SOD present in the samples. The results were represented as units per milligram of protein.

Catalase Assay (CAT) CAT activity was assayed using a SpectraMax M5/M5 Microplate Reader (Molecular Devices; MDS Analytical Technologies, Sunnyvale, CA, USA). The method used is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer pH 7.0 [49]. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute, and the results were represented as units per milligram of protein.

Glutathione Peroxidase Assay (GPx) GPx activity was measured using *tert*-butyl-hydroperoxide as a substrate [50]. NADPH disappearance was monitored at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide, and 0.1 mM NADPH. One GPx unit is defined as 1 μmol of NADPH consumed per minute; the specific activity is represented as units per milligram of protein.

Assay of Energy Homeostasis Parameters

ATP Levels Assay Fourteen rats' (seven per group) hippocampi were immediately dissected and frozen in liquid nitrogen. Each hippocampus was weighed and homogenized in 1 mL of 0.1 M NaOH (to inactivate cellular ATPases activity); centrifugation is not necessary. Samples were assayed using the ATPlite Luminescence ATP detection assay system (Perkin-Elmer, Waltham, MA, USA) according to Witt et al. [51]. The measurement of chemiluminescence was performed using a Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet tissue weights in grams and expressed as micromoles per gram of tissue.

For the determination of the activities of citrate synthase and the respiratory chain complexes, hippocampi of 28 rats (seven per group) were homogenized (1:20, *w*/*v*) in SETH buffer, pH 7.4. For the preparation of mitochondrial fraction, the homogenates were centrifuged at 800×*g* for 10 min at 4 °C and the pellet discarded [52]. The supernatant was then centrifuged at 27,000×*g* for 30 min at 4 °C. The pellet containing the mitochondria was washed three times with saline solution. We used samples containing approximately 0.01–1 mg protein in the assays.

Citrate Synthase Activity It was measured according to Srere [53], by determining DTNB reduction at 412 nm, and calculated as nanomoles of TNB per minute per milligram of protein.Respiratory Chain Complex Activities

The activities of succinate dehydrogenase, succinate: DCIP-oxidoreductase (complex II), and succinate: cytochrome *c* oxidoreductase (complex II–III) were determined according to the method of Fischer et al. [54], and the activity of cytochrome *c* oxidase (complex IV) was determined according to Rustin et al. [55]. The activities of the respiratory chain complexes were calculated as nanomoles per minute per milligram of protein.

Mitochondrial Mass and Mitochondrial Membrane Potential Measurements Hippocampi of 12 rats (6 per group) were mechanically dissociated in PBS containing collagenase to yield digestion to a density of about 200,000 cells/mL. The dissociated contents were then filtered into sterile 50-mL Falcon tubes through a 40-μm nylon cell strainer and

kept on ice until mitochondrial staining. Dissociated cells were stained with 100 nM MitoTracker Green and 100 nM MitoTracker Red (diluted from 1 mM stock solutions in dimethylsulfoxide) for 45 min at 37 °C (and in the dark), according to the method described by Keij et al. [56] and Pendergrass et al. [57], with some modifications, to determine mitochondrial mass and mitochondrial membrane potential, respectively. Immediately after staining, cell suspensions were analyzed on a FACSCalibur flow cytometer, using red (670 nm long pass) and green (530 nm/30) filters. Controls stained with a single dye were used to set compensation. For each sample, 10,000 events corresponding to intact cells (as gated in FSC versus SSC plots) were analyzed. All flow cytometric acquisition and analyses were performed using CELLQuest Pro data acquisition and FlowJo software.

Protein Determination

The protein content of samples was determined using bovine serum albumin as standard, according to Peterson [58] for glutamate uptake, Bradford [59] for Na^+,K^+-ATPase parameters, or Lowry et al. [60] for the other parameters.

Statistical Analysis

Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Student's *t* test was used to evaluate the different parameters after the dates presented a normal distribution in Shapiro–Wilk test. Results are expressed as means ± standard deviation, and differences were considered statistically significant when $p < 0.05$. In all cases, litter effects were controlled by assigning not more than two subjects from a litter to a particular group.

Results

Initially, we evaluated the effect of early chronic treatment with MPH on amino acid profile in the hippocampus of rats. Table 1 shows that MPH caused a significant increase in histidine levels [$t(9) = -5.986$; $p < 0.001$], as well as a decrease in glutamine [$t(11) = 2.989$; $p < 0.05$], leucine [$t(10) = 4.932$; $p < 0.01$], and isoleucine levels [$t(11) = 2.469$; $p < 0.05$], when compared to the control group. The levels of other amino acids examined (aspartate, glutamate, serine, glycine, alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, ornithine, and lysine) were not different from control ($p > 0.05$). Although the levels of tryptophan were not significantly different from controls, the results showed a tendency of increase [$t(11) = -2.118$; $p = 0.058$] in the hippocampus of juvenile rats treated with MPH.

Table 1. Effect of chronic treatment with methylphenidate on amino acid concentrations in hippocampus (μmol/L) of juvenile rats

Amino acid	Control	MPH
Aspartate	717.42 ± 79.87	739.08 ± 51.13
Glutamate	1533.10 ± 125.10	1565.70 ± 99.47
Serine	212.00 ± 24.21	205.51 ± 16.20
Histidine	22.83 ± 1.78	33.06 ± 3.73***
Glutamine	816.27 ± 109.81	647.91 ± 89.88**
Glycine	460.64 ± 43.88	477.62 ± 48.14
Alanine	278.14 ± 28.30	290.21 ± 31.14
Tyrosine	16.64 ± 2.56	16.24 ± 1.14
Tryptophan	12.10 ± 2.11	14.63 ± 3.31
Methionine	21.54 ± 2.88	16.83 ± 1.32
Valine	22.94 ± 4.95	24.75 ± 5.70
Phenylalanine	21.07 ± 1.99	19.38 ± 1.74
Isoleucine	13.14 ± 1.54	11.28 ± 1.08*
Leucine	33.20 ± 2.15	27.88 ± 1.53**
Ornithine	6.84 ± 3.10	6.43 ± 5.62
Lysine	64.46 ± 7.45	61.90 ± 7.57

Results are expressed as mean ± standard deviation for 5–7 animals in each group

Different from control, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (Student's *t* test)

MPH methylphenidate

In order to investigate the change in the levels of glutamine observed, our next step was to evaluate the effects of early MPH treatment on glutamate uptake in the hippocampus of juvenile rats. Figure 1a shows that MPH treatment decreased glutamate uptake in the hippocampus [$t(9) = 3.940$; $p < 0.05$] when compared to the control group. Immunocontent of GLAST and GLT-1 were also evaluated in the hippocampus of juvenile rats, and the results showed that MPH did not alter the immunocontent of these transporters ($p > 0.05$) (Fig. 1b).

We also tested the effect of chronic MPH treatment on Na^+,K^+-ATPase (activity and immunocontent of α_1, α_2, and α_3 subunits), and the results showed that MPH decreased the specific activity of the Na^+,K^+-ATPase [$t(10) = 5.109$; $p < 0.01$] in the hippocampus of juvenile rats but did not cause any alteration on the immunocontent of α_1, α_2, and α_3 subunits ($p > 0.05$) (Fig. 2) when compared to the control group.

Given our results showing that MPH decreased Na^+,K^+-ATPase activity and considering that oxidative stress and energy availability could affect the enzyme, we investigated some redox status and energy parameters in the hippocampus of juvenile rats. Table 2 shows that animals treated chronically with MPH did not show significant changes in any parameters tested: reactive species levels and damage to lipids and proteins and superoxide dismutase, catalase, and glutathione peroxidase activities ($p > 0.05$) in rat hippocampus. However, MPH caused a reduction in ATP intracellular levels in the

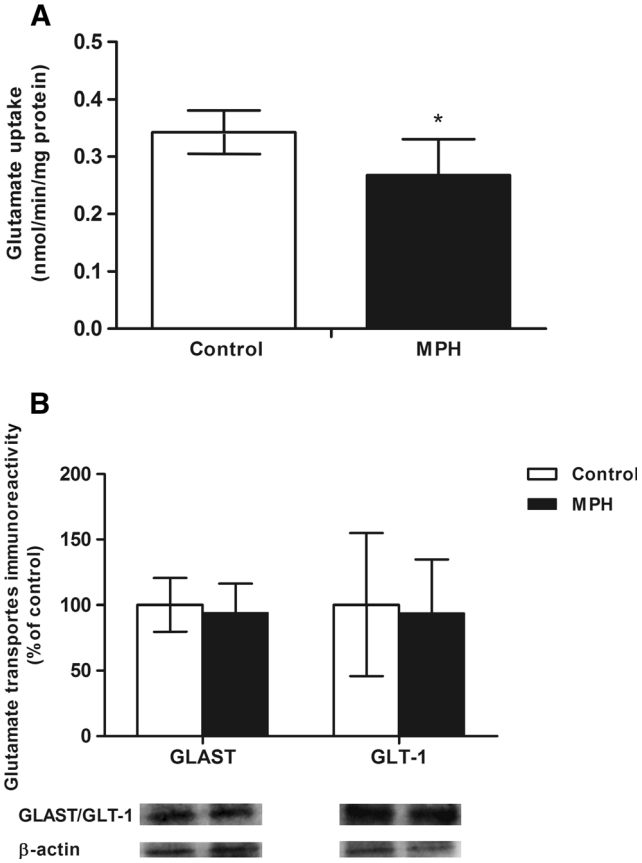

Fig. 1. Effect of chronic methylphenidate exposure on glutamate uptake in slices (**a**) and on glutamate transporters' immunocontent of GLAST and GLT-1 (**b**) in homogenates of the hippocampus of juvenile rats. All lanes received equivalent amounts (30 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for five to six animals in each group. Different from control, $^{*}p < 0.05$ (Student's *t* test). *MPH* methylphenidate

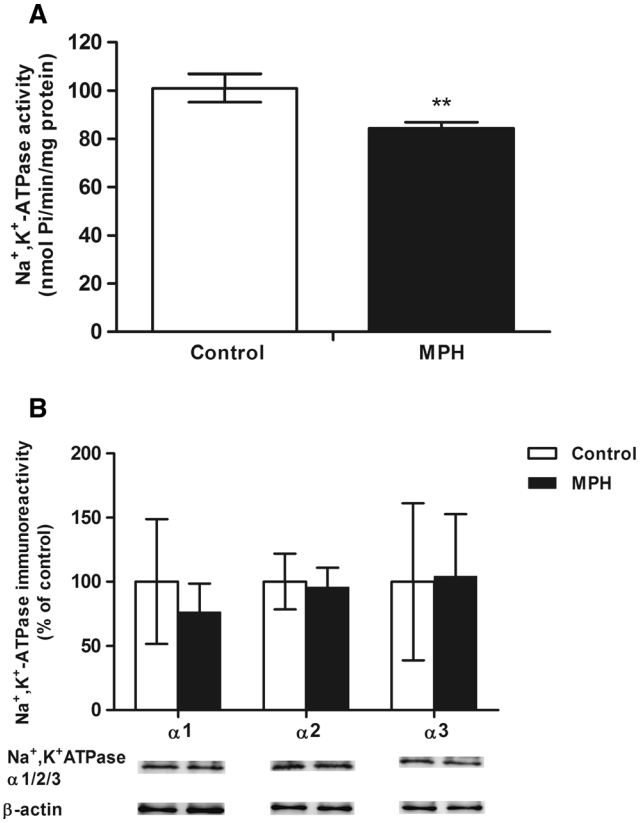

Fig. 2. Effect of chronic methylphenidate exposure on Na^+,K^+-ATPase specific activity (**a**) and on immunocontent (**b**) of the α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase in homogenates of the hippocampus of juvenile rats. All lanes received equivalent amounts (30 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for six to seven animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t* test). *MPH* methylphenidate

hippocampus of juvenile rats [$t(12) = -2.923$; $p < 0.05$], but citrate synthase, succinate dehydrogenase, and respiratory chain complexes activities (II, II–III, and IV) were not altered by MPH exposure ($p > 0.05$) (Fig. 3).

Finally, mitochondrial mass and mitochondrial membrane potential also were tested in the hippocampus of juvenile after chronic MPH exposure, and we did not observe any changes in these parameters when compared to the control group ($p > 0.05$) (data not shown).

Discussion

Since we have demonstrated that MPH alters the amino acid concentrations in cerebrospinal fluid and increases glutamate levels probably by inhibition of glutamate uptake in prefrontal cortex [17], we extended our study investigating the effects of chronic MPH exposure on amino acid profile and glutamate uptake in the hippocampus of juvenile rats. Results showed that chronic exposure to MPH decreased glutamine levels and impaired glutamate uptake in the hippocampus of juvenile rats, suggesting that MPH provokes an imbalance in

Table 2. Effect of chronic treatment with methylphenidate on parameters of oxidative stress in hippocampus of juvenile rats

	Control	MPH
DCF (nmol/mg protein)	54.56 ± 6.07	55.05 ± 3.46
TBARS levels (nmol/mg protein)	0.68 ± 0.13	0.73 ± 0.16
SH content (nmol TNB/mg protein)	31.93 ± 2.26	33.69 ± 1.79
SOD activity (units/mg protein)	5.70 ± 1.23	6.65 ± 0.47
CAT activity (units/mg protein)	3.54 ± 0.48	3.52 ± 0.82
GPx activity (units/mg protein)	25.73 ± 2.58	25.75 ± 2.01
SOD/CAT ratio	1.61 ± 0.24	1.99 ± 0.53

Results are expressed as mean ± standard deviation for 5–6 animals in each group

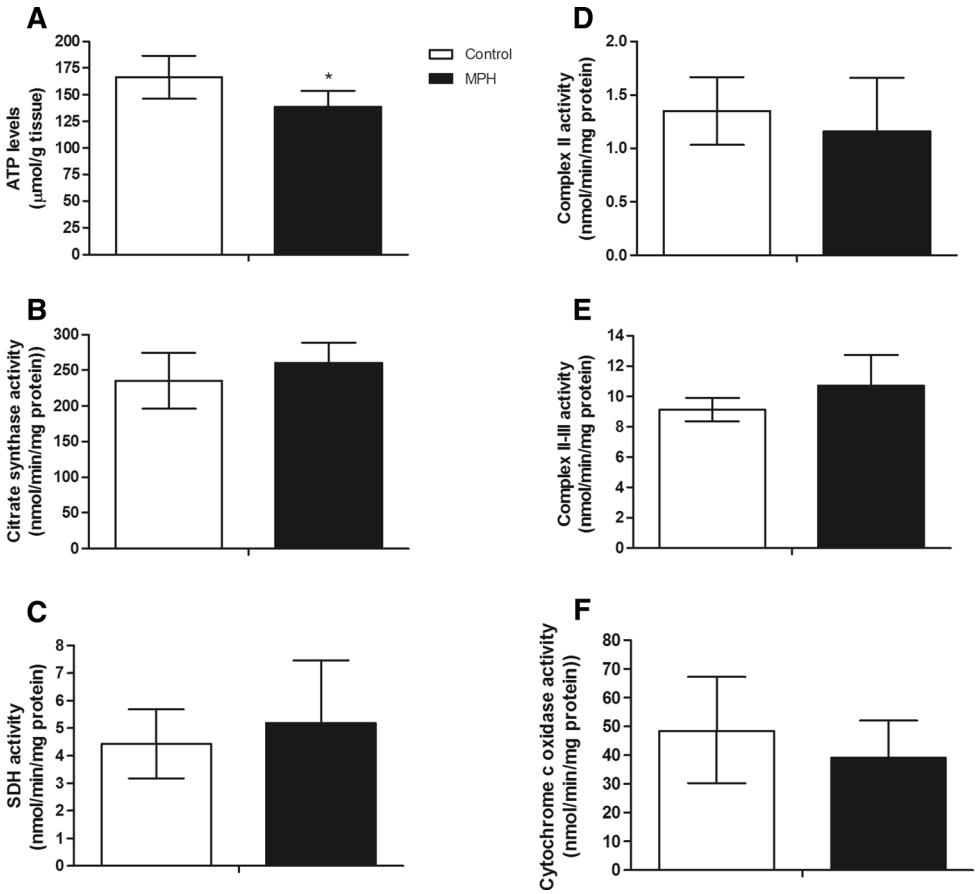

Fig. 3. Effect of chronic methylphenidate exposure on ATP levels (**a**), citrate synthase activity (**b**), succinate dehydrogenase activity (**c**), complex II activity (**d**), complex II–III activity (**e**), and complex IV activity (**f**) in the hippocampus of juvenile rats. Results are expressed as mean ± standard deviation for five to six animals in each group. Different from control, $*p < 0.05$ (Student's *t* test). *MPH* methylphenidate, *SDH* succinate dehydrogenase

glutamatergic system that can lead to the glutamatergic excitotoxicity.

It has been shown that MPH increases norepinephrine levels in the hippocampus of adolescent rodents in a dose-dependent pattern [61]. The hippocampus plays a prominent role in affective behaviors, drug taking, and relapse [62, 63]. Moreover, glutamatergic and dopaminergic systems (main systems affected by MPH exposure) are intricately interlinked via intracellular signaling pathways as well as via physical interactions [64]. However, it is still unknown how chronic exposure to MPH during development can compromise the glutamatergic homeostasis in the hippocampus. To investigate the impairment in the glutamate uptake caused by chronic MPH exposure, we evaluated the immunocontent of glutamate transporters responsible to account for the majority of extracellular glutamate, GLAST, and GLT-1 [65] in the hippocampus of juvenile rats subjected to the MPH. The results suggest that the decrease in glutamate uptake induced by MPH does not involve a quantitative change in the glutamate transporters, but it could be due to a possible alteration of its proper function since we did not observe any significant change in their immunocontents.

The proper functionality of the glutamate uptake depends on the gradient of Na^+ generated by the Na^+,K^+-ATPase [26]. Based on this, we evaluated the activity of Na^+,K^+-ATPase and observe that the activity of this enzyme is decreased in the hippocampus of juvenile rats chronically exposed to the MPH. This decrease in Na^+,K^+-ATPase activity in rat hippocampus may be associated with the decrease in glutamate uptake observed in this study. These changes could contribute, at least in part, with the increase in glutamate levels observed previously in cerebrospinal fluid [17]. It is also important to mention that our results disagree with those of Scherer and colleagues [66] who found an increase in Na^+,K^+-ATPase activity in the hippocampus of rats subject to chronic MPH treatment. However, in this study, the treatment started from the 25th day of age and the animals were decapitated 2 h after the last administration, suggesting that the effects of MPH on this enzyme depend on the experimental model.

Na^+,K^+-ATPase is a membrane-embedded protein highly sensible to free radical insult [67], and the glutamate transport can also be impaired by oxidative stress [68]. In order to better understand the alterations observed in Na^+,K^+-ATPase and glutamate uptake caused by chronic MPH exposure, we also investigated the effects of MPH on some redox homeostasis parameters at 24 h after the last injection of this psychostimulant. Results showed that MPH did not alter any of the redox parameters evaluated. Interestingly, we have previously shown a decrease in reactive species formation and lipid peroxidation in the hippocampus of juvenile rats at 2 h

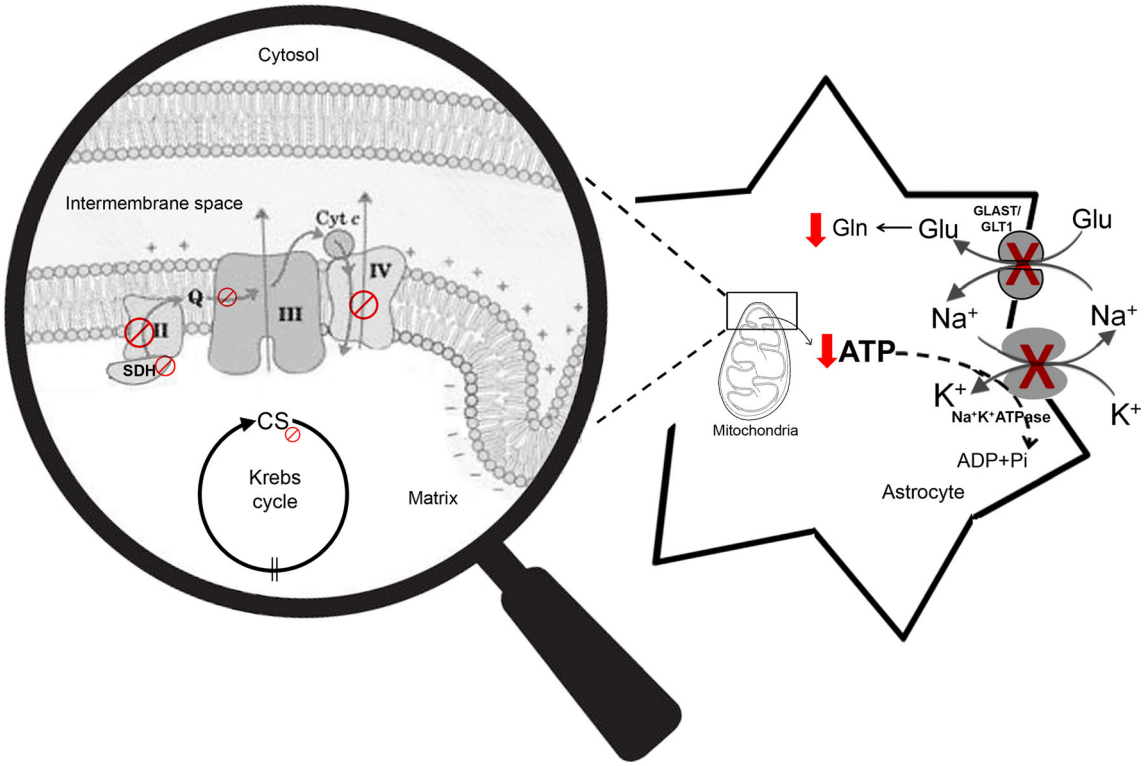

Fig. 4. Graphical summary of the processes that were quantified throughout the investigations, highlighting the main effects of chronic methylphenidate exposure on crucial parameters for the proper functioning of central nervous system. In summary, we demonstrated that chronic MPH exposure at an early age impairs glutamate uptake and Na^+,K^+-ATPase activity in the hippocampus of juvenile rats probably by decreasing ATP levels. *Gln* glutamine, *Glu* glutamate, *CS* citrate synthase, *SDH* succinate dehydrogenase, *CII* complex II, *CIII* complex III, *CIV* complex IV

after the last injection of MPH [69]. Martins et al. [70] showed that acute MPH (2 mg/kg) administration to juvenile rats 25 days of age and chronic MPH administration to adult rats (from 60 to 88 days of age) decrease lipid peroxidation while chronic MPH administration to young rats (from 25 to 53 days of age) increases this parameter in the hippocampus at 2 h after the last injection [70]. Carbonyl levels (indicative of protein damage) [70], superoxide dismutase and catalase activities [71], and superoxide production [72] were not changed in the hippocampus by MPH treatment. Moreover, the striatum has shown to be more susceptive to DNA damage than the hippocampus [41]. Thus, we could suggest that the hippocampus appears to be a brain structure less susceptible to changes in redox homeostasis when juvenile rats are subjected to chronic MPH exposure.

Na^+,K^+-ATPase consumes about 40–50% of the ATP produced in brain to pump Na^+ and K^+ across the cell membrane [27, 30]. In addition, it has been shown that the activation of glutamate receptors increases energy consumption and rapidly decreases the neuronal ATP levels [73, 74]. Based on these considerations and in order to better understand the alterations observed on glutamate uptake and Na^+,K^+-ATPase activity caused by MPH, in the present study, we investigated the effect of chronic MPH exposure on ATP levels in the hippocampus of juvenile rats. Results showed a significant decrease in ATP levels. So far, we may presume that the decrease in glutamate uptake observed in the hippocampus of juvenile rats chronically exposed to MPH may be caused by a decrease in the ATP levels since this process is dependent on the Na^+ gradient generated by Na^+,K^+-ATPase and the functionality of this enzyme is highly dependent on energy.

In order to clarify the decrease in ATP levels provoked by chronic MPH exposure in the hippocampus of juvenile rats, we also investigated the activity of citrate synthase (this enzyme catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate and controls the first step of the Krebs cycle) and succinate dehydrogenase, as well as activities of complexes of mitochondrial respiratory chain (II, II–III, and IV). Results show that chronic exposure to MPH did not change any of these parameters in the hippocampus of juvenile rats. In addition, mitochondrial mass and mitochondrial membrane potential were not altered in this study either. It has been shown that chronic MPH treatment of young and adult rats (from the 25th to the 53rd and from the 60th to the 88th day of age, respectively; 2 mg/kg) decreased citrate synthase and isocitrate dehydrogenase activities but did not alter malate dehydrogenase in the hippocampus [36]. Complex IV activity was increased in the hippocampus of juvenile rats [33] and

decreased in adult rats chronically exposed to MPH [34]. Glucose uptake and lactate release were not also altered by MPH in the hippocampus [37]. On the other hand, Fagundes et al. [35] showed that MPH inhibited mitochondrial respiratory chain (complex I, II, III, and IV) in the hippocampus of adult rats (60 days of age) subjected to a single injection of MPH. Scaini et al. [75] showed that chronic MPH treatment (2 mg/kg, from the 25th to the 53rd day of age) promoted an increase in creatine kinase activity in the hippocampus. This enzyme is a secondary source of ATP and is activated when the demand of energy is large and the production of ATP from oxidative phosphorylation is impaired or not enough [75]. Taking all this into account, we suggest that chronic treatment with MPH affects brain bioenergetics, and we believe that the apparent conflicts observed between our results and the results found in the literature regarding oxidative phosphorylation, oxidative stress, and Na^+,K^+-ATPase are due to differences in age of the animals at the start of treatment, duration of treatment, and the time after the last injection.

Although glucose is a major source of energy for the brain, amino acid oxidation may also serve as an energy source. In this context, we showed a reduction in leucine and isoleucine concentrations in the hippocampus of juvenile rats chronically treated with MPH. In accordance with this result, we have recently shown that chronic MPH exposure decreased the levels of leucine and serine in prefrontal cortex and increased the levels of ornithine in cerebrospinal fluid [17]. These results suggest a bigger degradation of amino acids in brains of rats subjected to chronic MPH exposure as an alternative source of energy in an attempt to supply the ATP levels, but without success.

Histamine is a neurotransmitter that controls a wide variety of neurobiological and behavioral functions, and it is synthesized in neurons from the essential amino acid histidine [76]. Histamine facilitates directly the activation of NMDA receptor containing NR2B subunit through its binding to the modulatory site of polyamines present in this receptor [77]. In addition, the increase in histamine release has been associated with improvement of cognitive function in experimental models [78]. In this study, we found an increase in histidine concentration in the hippocampus of juvenile rats subjected to chronic MPH exposure. Recently, we have also shown an increase in the levels of this amino acid in cerebrospinal fluid and prefrontal cortex of rats subjected to the same experimental model [17]. These results suggest that chronic MPH exposure decreases the conversion of histidine to histamine to prevent an overstimulation of the glutamatergic system. Thus, the beneficial role of histamine on cognition, attention, and alert could be impaired.

It has been shown that psychostimulant drugs, such as MPH, are associated with dopamine- and glutamate-generated reactive oxygen species [79, 80]. These reactive oxygen species attack and potentially inhibit the function of intracellular proteins [81], including complexes of the electron transport chain [82, 83]. Defects in the mitochondrial oxidative phosphorylation function limit ATP production [84, 85], and energy impairment is linked to neuronal death and neurodegeneration [86–91]. In this context, we have recently showed that Wistar rats treated with MPH (2.0 mg/kg), once a day, from the 15th to the 45th day of age showed an astrocyte and neuron loss and inhibition of cell survival and activation of cell death pathways regulated by cytokines and neurotrophins in the hippocampus [92]. For all these, the study of the consequences associated with chronic MPH exposure on early stages of brain development is fundamental since this psychostimulant has been widely misused by children and adolescents who do not meet full diagnostic criteria for ADHD [12, 93, 94]. Although other brain regions such as the prefrontal cortex and striatum are involved in MPH mechanisms, the hippocampus plays an important role in cognition as well as in depression- and anxiety-related behaviors [95, 96]. It has been shown that MPH actions in the hippocampus may be central to MPH long-term effects since the treatment with this psychostimulant in adolescent rodents increases norepinephrine levels in the hippocampus in a dose-dependent pattern [61].

In summary, in the present study, we demonstrated that chronic MPH exposure at an early age impairs glutamate uptake and Na^+,K^+-ATPase activity in the hippocampus of juvenile rats, and these findings can be explained, at least in part, by the decrease in ATP levels observed, as shown in Fig. 4. These results provide a new basis for understanding the neurochemical actions of the MPH after chronic exposure during early stages of brain development and suggest that the misuse of this psychostimulant should be strictly monitored in youth.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest with the contents of this article.

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3.4 Capítulo II

Methylphenidate disrupts cytoskeletal homeostasis and reduces membrane-associated lipid content in juvenile rat hippocampus

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ORIGINAL ARTICLE

Methylphenidate disrupts cytoskeletal homeostasis and reduces membrane-associated lipid content in juvenile rat hippocampus

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Abstract

Although methylphenidate (MPH) is ubiquitously prescribed to children and adolescents, the consequences of chronic utilization of this psychostimulant are poorly understood. In this study, we investigated the effects of MPH on cytoskeletal homeostasis and lipid content in rat hippocampus. Wistar rats received intraperitoneal injections of MPH (2.0 mg/kg) or saline solution (controls), once a day, from the 15th to the 44th day of age. Results showed that MPH provoked hypophosphorylation of glial fibrillary acidic protein (GFAP) and reduced its immunocontent. Middle and high molecular weight neurofilament subunits (NF-M, NF-H) were hypophosphorylated by MPH on KSP repeat tail domains, while NFL, NFM and NFH immunocontents were not altered. MPH increased protein phosphatase 1 (PP1) and 2A (PP2A) immunocontents. MPH also decreased the total content of ganglioside and phospholipid, as well as the main brain gangliosides (GM1, GD1a, and GD1b) and the major brain phospholipids (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine). Total cholesterol content was also reduced in the hippocampi of juvenile rats treated with MPH. These results provide evidence that disruptions of cytoskeletal and lipid homeostasis in hippocampus of juvenile rats are triggers by chronic MPH treatment and present a new basis for understanding the effects and consequences associated with chronic use of this psychostimulant during the development of the central nervous system.

Keywords Methylphenidate · Cytoskeleton · Intermediate filaments · Cholesterol · Gangliosides · Phospholipids

Abbreviations

ADHD	Attention deficit hyperactivity disorder
CNS	Central nervous system
GFAP	Glial fibrillary acidic protein
IFs	Intermediate filaments
KSP	Lysine-serine-proline
MPH	Methylphenidate
NF	Neurofilament
NFH	High molecular weight neurofilament subunit
NFL	Light molecular weight neurofilament subunit
NFM	Middle molecular weight neurofilament subunit
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Calcineurin

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Introduction

Methylphenidate (MPH) is a psychostimulant drug widely used to treat attention deficit hyperactivity disorder (ADHD) (Johnston et al. 2011a, b). However, the exponential increase in MPH prescription in recent years suggests its misuse among individuals who do not meet the full diagnostic criteria for ADHD as young children (2–4-year-olds) (Zito et al. 2000),

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and among students in search of cognitive improvement (Johnston et al. 2011a, b). This widespread use raises concerns about the long-term consequences and lasting effects of MPH on the developing central nervous system (CNS), which continues to grow and mature well in the second to third decade of life (Sowell et al. 2003).

MPH is described to block monoamine reuptake, and increase extracellular brain concentrations of both noradrenaline and dopamine (Kuczenski and Segal 2001; Volkow et al. 2001) which has been associated with neurotoxicity (Volkow et al. 2001; Cyr et al. 2003). Previous experiments conducted in young rodents have provided evidence that exposure to MPH in early life may disrupt brain maturation (Andersen 2005). In this context, MPH treatment has been associated with the reduction of cell density in the adult rat hippocampus (Motaghinejad et al. 2016), neuronal loss in the striatum (Sadasivan et al. 2012) and attenuation of adult hippocampal neurogenesis in rats (Lagace et al. 2006). In addition, exposure of the immature brain to MPH may alter gene expression and could lead to permanent changes in cellular responsiveness and synaptic connectivity (Andersen et al. 2008). It has been shown that MPH treatment alters Arc (activity-regulated cytoskeletal associated protein), a pivotal factor in activity-dependent neuronal plasticity in the brain in response to stimulation (Chase et al. 2007; Gronier et al. 2010; Banerjee et al. 2009; Quansah et al. 2017a).

It has been described that the interruption of neuronal connectivity causes cell death in the immature brain more rapidly and with higher frequency than in the mature brain (Nyakas et al. 1996; Kudryashov et al. 2001). There is also a report showing that the hippocampus is the most vulnerable cerebral structure to these effects (Vannucci 1990). The use and misuse of MPH are increasing, particularly during childhood and adolescence, periods characterized by rapid development of CNS and intense cellular proliferation and growth (Johnston et al. 2011a, b; Zito et al. 2000). There are few studies concerning the consequences of early exposure of this psychostimulant on the CNS. Previous studies have shown that MPH causes loss of astrocytes and neurons in the hippocampus of juvenile rats likely due to an inflammatory pathway activation and caspase-3 activation which are associated with memory impairment (Schmitz et al. 2016a). Recently, we showed that rats subjected to chronic MPH exposure decreased hippocampal glutamate uptake, ATP levels, and Na^+,K^+-ATPase activity, a membrane-embedded enzyme (Schmitz et al. 2016b). It is therefore important to emphasize that membrane proteins like Na^+,K^+-ATPase are regulated by both general lipid–protein interactions, where the physical properties of the bilayer such as hydrophobic thickness, curvature stress, and elastic moduli affect the membrane protein conformational mobility, and by specific lipid–protein interactions, where lipids interact chemically at lipid-binding sites located on the protein (Cornelius et al. 2015).

Lipids are important components of all mammalian cells and have a variety of biological functions. For example, they play critical roles in structural integrity maintenance, lipid bilayer formation, energy reservoir formation, and as precursors for second messengers in various signaling pathways. The importance of lipids in cell signaling and physiology has been demonstrated by many CNS disorders (Adibhatla and Hatcher 2007). In addition, it was demonstrated that membrane/lipid rafts and the cytoskeleton interact dynamically and regulate many facets of eukaryotic cell function and adaptation to changing environments (Head et al. 2014).

In this study, we investigated the effects of early chronic exposure with MPH on cytoskeletal homeostasis [phosphorylation and immunocontents of intermediate filaments (IFs) such as glial fibrillary acidic protein (GFAP) and light, middle, and high molecular weight neurofilament subunits (NFL, NFM and NFH), as well as phosphatase protein immunocontents such as phosphatase 1 (PP1), 2A (PP2A) and 2B (PP2B)] in hippocampus of juvenile rats. We also evaluated hippocampal lipid contents [(total contents and main ganglioside and phospholipid species) and total cholesterol]. We hypothesized that the disruption of the IF cytoskeletal-associated phosphorylation system and altered membrane-associated lipid content may be involved in the neural changes induced by MPH in hippocampus of juvenile rats.

Experimental procedures

Animals and reagents

Male rats were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Litters were culled to eight pups on postnatal day (PD) 3 (day of parturition = PD 0) and were kept with the dam until weaning on PD 21. After weaning, the rats were re-housed in boxes containing up to four male rats. Animals were maintained on a 12–12 light-dark cycle at a constant temperature of 22 ± 1 °C, with free access to water and commercial protein chow. We determined that 7 per group were sufficient for biochemical studies. Since it was considered a power estimated at 0.80, an alpha = 0.05 and standard deviation values of previous studies. A total of 70 male Wistar rats were used.

The care with animals followed the official governmental guidelines in issued by the Brazilian Federation of Societies for Experimental Biology, following the NIHGuide for the Care and Use of Laboratory Animals (8th edition, 2011) and Arouca Law (Law n° 11.794/2008). According to Ethics Committee of Federal University of Rio Grande do Sul

(UFRGS, RS, Brazil), the degree of severity of this project is moderate and all experimental protocols of this study were approved under license #29651. We further attest that all efforts were made to minimize the number of animals used and their suffering.

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil. Platinum Taq DNA polymerase and SuperScript-II RT pre-amplication system were from Invitrogen. All other chemicals were of analytical grade and were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Chronic early treatment with methylphenidate

Beginning on PD 15, rats were weighed and injected intraperitoneally with saline solution (control group) or 2.0 mg/kg of methylphenidate (MPH group), once a day, for thirty consecutive days, during the diurnal cycle at 12 h ± 1 h (Schmitz et al. 2012a, b, c). MPH was dissolved in saline and injected at a volume of 1 mL/100 g of body weight. Control group received the equivalent volume of saline solution. The dose and route of administration were selected because they mimic the therapeutic doses in terms of the magnitude of neurochemical and behavioral effects (Gerasimov et al. 2000). Twenty-four hours after the last injection (PD 45), the animals were decapitated and the hippocampi were removed and processed according to each analysis.

We chose to start administering MPH to rats at postnatal day 15 since the first three weeks during the postnatal period in rats are characterized by intense synaptogenesis, myelination, and gliogenesis, comparable to early childhood in humans (Rice and Barone Jr 2000). Treatment lasted thirty days (from 15 to 44 days of life of rats) since this period mimics chronic use in humans (Andreazza et al. 2007).

Cytoskeletal analysis

Rats were killed by decapitation and the hippocampi were dissected onto Petri dishes placed on ice and cut into 400 mm thick slices with a McIlwain chopper. Forty two rats were needed for this assay (three independent experiments were performed with 14 animals each, 7 per group).

Preincubation

Before phosphorylation, tissue slices were stabilized for 20 min at 30 °C in a Krebs-Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 mM leupeptin, 0.7 mM antipain, 0.7 mM pepstatin and 0.7 mM chymostatin.

In vitro ^{32}P incorporation experiments

After stabilization, the preincubation medium was changed and incubation was carried out at 30 °C with the addition of 100 μL of the basic medium containing 80 μCi of [^{32}P]orthophosphate. Labeling reaction was normally allowed to proceed for 30 min at 30 °C and stopped with 1 mL of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, 50 mM Tris–HCl, pH 6.5), and the protease inhibitors described above. Excess of radioactivity was removed from the slices by two washings with stop buffer.

Preparation of the high salt-triton insoluble cytoskeletal fraction from tissue slices

After ^{32}P–orthophosphate labeling reaction, the IF-enriched cytoskeletal fraction was extracted, from the hippocampal slices, as described by Funchal et al. (2003). Briefly, after incubation, slices were homogenized in 400 μL of ice-cold high salt buffer containing 5 mM KH_2PO_4, pH 7.1, 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors described above. Homogenate was centrifuged at 15,800 x g for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet suspended with the same volume of the high salt medium, centrifuged as described, and the supernatant was discarded. The Triton-insoluble pellet, containing NF subunits and GFAP constituting the IF-enriched cytoskeletal fraction was dissolved in 1% SDS and protein concentration was determined.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Equal protein concentrations of the IF-enriched cytoskeletal fraction from controls and MPH treated animals were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE (Laemmli 1970). After drying, gels were exposed to T-MAT films at −70 °C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Preparation of total protein homogenate

Tissue slices were homogenized in 100 μL of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% (*w*/*v*) SDS. For electrophoretic analysis, samples were dissolved in 25% (*v*/v) solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min.

Western blot analysis

Tissue homogenate (80 μg) or cytoskeletal fraction (60 μg) was analyzed by SDS-PAGE. Fourteen rats were needed for this assay ($n = 7$ per group). Gels were transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 mM NaCl, 20 mM Trizma, pH 7.5), followed by a 2 h incubation in blocking solution (TBS plus 5% bovine serum albumin, fraction V). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS). After blocking with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 3% bovine serum albumin for 1 h, the membranes were incubated for 24 h at 4 °C with the primary antibodies listed in Table 1. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:10,000. The blot was washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). Membranes were re-probed for anti-β-actin immunoreactivity. The membranes were developed in a photodocumenter and band intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the web site (http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-actin as a loading control to assess protein levels. The data used in the statistical analysis were obtained from the ratio of the protein studied and β-actin density unit lines. For determination of Western blot analyses we used fourteen rats (seven per group).

Table 1 Primary antibodies used for western blot analyzes

Primary antibody	Dilution	Purchased from
anti-GFAP	1:500	Sigma
anti-NFL	1:1000	Sigma
anti-NFM	1:400	Sigma
anti-NFH	1:1000	Sigma
anti-pKSP repeats	1:1000	Cell signaling
anti-PP1	1:1000	Sigma
anti-PP2A	1:1000	Sigma
anti-PP2B	1:1000	Sigma
anti-β-actin	1:1000	Cell signaling

GFAP, glial fibrillary acidic protein; *NFL*, neurofilament of light molecular weight; *NFM*, neurofilament of middle molecular weight; *NFH*, neurofilament of high molecular weight; *PP1*, protein phosphatase 1 α; *PP2A*, protein phosphatase 2 A (c-subunit); *PP2B*, calcineurin (α-subunit)

Lipid content analysis

Lipid extraction

Hippocampi were weighed and homogenized in a 2:1 mixture of chloroform:methanol (C:M, 2:1, *v*/v) to a 20-fold dilution of tissue mass and centrifuged at 800 x g for 10 min. The pellet was re-homogenized in C:M (1:2) to a 10-fold dilution of original sample mass (Folch et al. 1957). The C:M extracts were combined and this pool was used for the following determinations. Fourteen rats were needed for this assay ($n = 7$ per group).

Total gangliosides, phospholipids and cholesterol determinations

Aliquots from the total lipid extracts were used for ganglioside determination by the N-acetyl-neuraminic acid (NeuAc) quantification with the resorcinol-hydrochloric acid method described by Svennerholm (Svennerholm 1957) with an adaptation to aqueous medium using dimethyl sulphoxide (Fragoso and Trindade 2015). Phospholipid and cholesterol were quantified in aliquots from total lipid extracts according to the method of Bartlett (1959) and to the Trinder-enzymatic technique (Röschlau et al. 1974), respectively.

Thin layer chromatography (TLC) analysis

Ganglioside species were analyzed by HPTLC and this technique was performed on 10 cm × 10 cm Merck sheets of silica gel 60 using a developing tank described by Nores et al. (1994). Aliquots of the total lipid extracts containing 30 nmol of NeuAc suspended in 10 μL C:M (1:1) were spotted on 8 mm lanes. HPTLC was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, *v*/v) and secondly C:M:0.25% $CaCl_2$ (60:36:8, v/v/v). Ganglioside profile was visualized with resorcinol reagent (Svennerholm 1957; Lake and Goodwin 1976). The chromatographic bands were quantified by scanning densitometry at 580 nm with a CS 9301 PC SHIMADZU densitometer. Individual ganglioside values are expressed as percent of control. The terminology used herein for gangliosides is that recommended by Svennerholm (1963).

Phospholipid species were analyzed by HPTLC using chloroform:methanol: acetic acid:water (C:M:Aac:W, 86:14:4:1, v/v/v/v) as the solvent system which is a modification of the theoretical under phase (Folch et al. 1957). Aliquots of total lipid extracts containing a quantity equivalent to 15 μmol of inorganic phosphorus (Pi) suspended in 10 μL of C:M (2:1) were spotted on the same plate size described above. Phospholipid bands were visualized with Comassie-Blue R250 (Nakamura and Handa 1984). The chromatographic bands were quantified by scanning densitometry at 500 nm

with a CS 9301 PC SHIMADZU densitometer. Individual phospholipid values are expressed as percent of control.

Ganglioside and phospholipid standards were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Protein determination

The protein content of samples was determined using bovine serum albumin as standard, according to Lowry et al. (1951).

Statistical analysis

Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer. Student's *t* test was used to evaluate the different parameters for data presenting a normal distribution in Shapiro–Wilk test. Results are expressed as means ± standard deviation, and differences were considered statistically significant when $p < 0.05$. In all cases, litter effects were controlled by assigning not more than two subjects from a litter to a particular group.

Results

MPH treatment provokes IF hypophosphorylation

Initially, we evaluated the effects of early chronic treatment with MPH on the phosphorylation level of the IF-enriched cytoskeletal fraction in the hippocampus of juvenile rats. Figure 1a shows that MPH significantly decreased the phosphorylation level in GFAP, NFM and NFH ($p < 0.001$), but did not alter NFL phosphorylation ($p > 0.05$) when compared with the control group. Representative Coomassie blue stained gel and autoradiograph of the proteins studied are shown in Fig. 1b.

GFAP immunocontent was decreased, while the immunocontent of NF subunits were not affected by MPH treatment

In order to investigate the mechanisms involved in the hypophosphorylation, the next step was to evaluate the effects of early MPH treatment on the immunocontent of the IF proteins in hippocampus of rats subjected to the MPH treatment. Figure 2a shows that MPH treatment reduced GFAP ($p < 0.01$), without altering NFL, NFM and NFH immunocontents ($p > 0.05$). In addition, MPH decreased the phosphorylation level of pKSP repeats ($p < 0.01$) (Fig. 2b), corroborating the NFM and NFH hypophosphorylation observed.

PP1 and PP2A immunocontent were increased by MPH treatment

Since dephosphorylation/phosphorylation balance of the IFs may be affected by phosphatase activities, we investigated the protein phosphatases involved in the effects of MPH treatment, analyzing the immunocontents of PP1, PP2A and PP2B. We observed an increase in the immunocontents of PP1 ($p < 0.01$) and PP2A ($p < 0.05$), but not PP2B ($p > 0.05$) in hippocampus of juvenile rats (Fig. 3a, b and c, respectively).

Ganglioside, phospholipid and cholesterol levels are reduced by MPH treatment

The effect of chronic MPH treatment on total lipid content in hippocampus of juvenile rats was evaluated. Table 2 shows that chronic MPH administration significantly decreased the total ganglioside ($p < 0.05$), phospholipid ($p < 0.05$) and cholesterol ($p < 0.01$) contents.

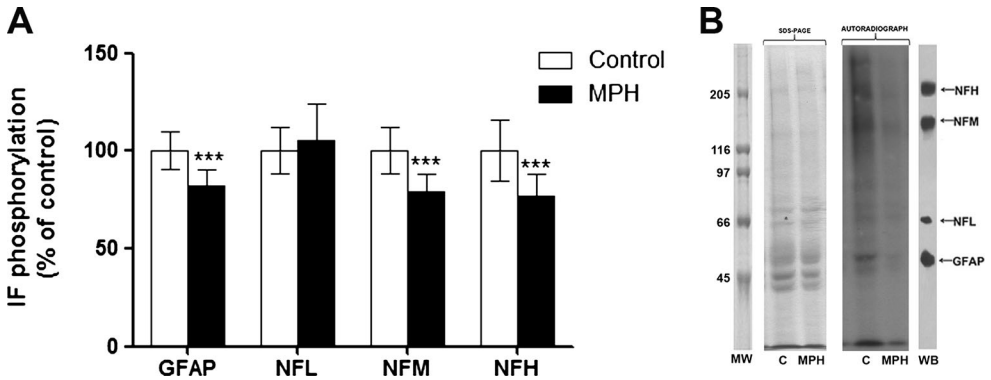

Fig. 1 The effects of chronic methylphenidate treatment on the phosphorylation of intermediate filament proteins in hippocampus of juvenile rats are shown (**a**). Representative stained gel and autoradiograph of the proteins studied are shown (**b**). Results are expressed with the mean ± standard deviation for 5–6 animals in each group and are expressed as percent of the control. Different from control, *** $p < 0.001$ (Student's *t* test). GFAP, glial fibrillary acidic protein; NFH, high molecular weight neurofilament subunit; NFM, middle molecular weight neurofilament subunit; NFL, light molecular weight neurofilament subunit; MPH, methylphenidate

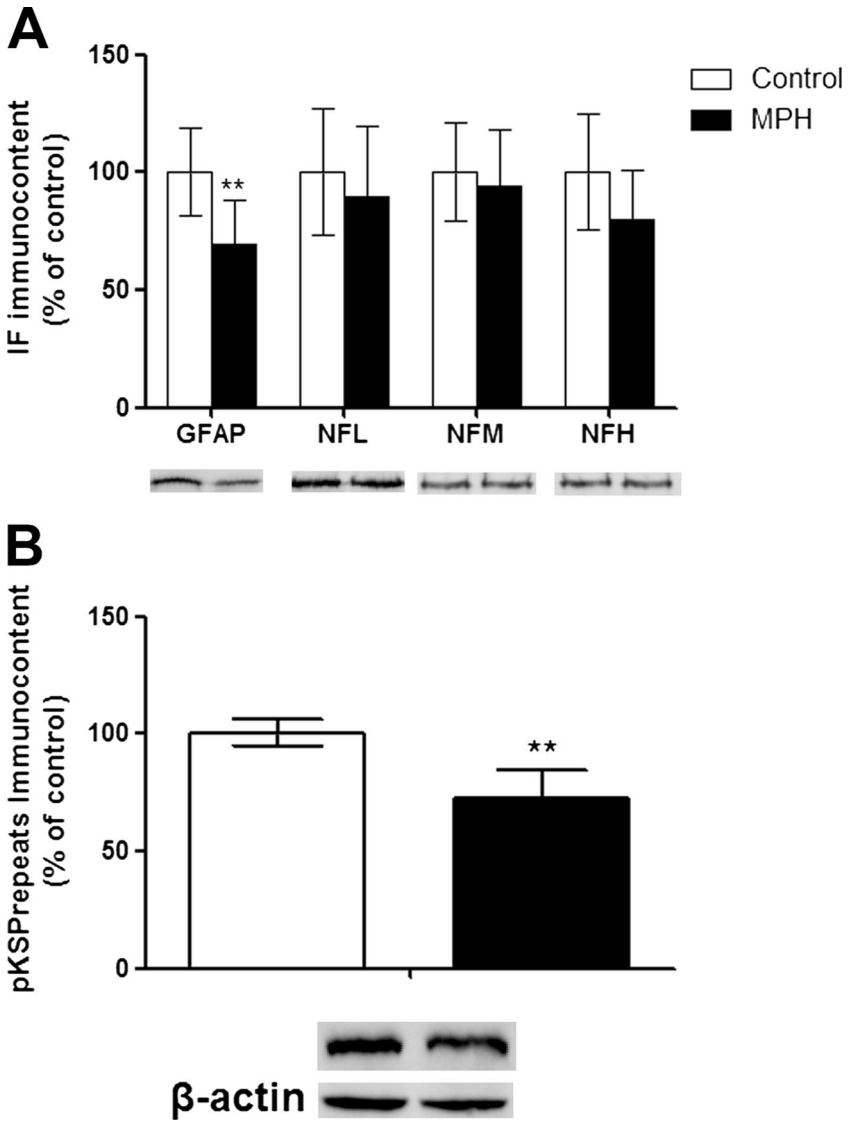

Fig. 2 The effects of chronic methylphenidate treatment on the immunocontents of intermediate filament proteins (GFAP, NFL, NFM, and NFH) are shown (**a**). The effects of the same treatment on phosphoNFH/NFM KSP repeats (**b**) as well as in hippocampus of juvenile rats. Representative Western blots of the proteins studied are shown. Western blot of β-actin was used as loading control. Results are expressed with the mean ± standard deviation for 5–6 animals in each group and expressed as percent of control. Different from control, ** $p < 0.01$ (Student's t test). GFAP, glial fibrillary acidic protein; NFH, high molecular weight neurofilament subunit; NFM, middle molecular weight neurofilament subunit; NFL, light molecular weight neurofilament subunit; MPH, methylphenidate

Next, we evaluated the content of ganglioside species in hippocampus of juvenile rats subjected to chronic MPH treatment. Figure 4a shows that MPH significantly decreased GD1a ($p < 0.05$), GD1b ($p < 0.01$) and GM1 ($p < 0.05$), but did not alter GT1b ($p > 0.05$) content in the hippocampus of juvenile rats treated with MPH. Representative HPTLC migrations are shown in Fig. 4b.

Finally, the effect of MPH on the content of individual phospholipids was investigated. It was observed a reduction of all classes of phospholipids studied such as sphingomyelin ($p < 0.05$), phosphatidylcholine ($p < 0.01$), phosphatidylethanolamine ($p < 0.05$), and phosphatidylserine + phosphatidylinositol ($p < 0.05$) in the hippocampus of juvenile rats subjected to chronic MPH administration (Fig. 5a). Representative TLC migrations are shown in Fig. 5b.

Discussion

In the present study, we initially investigated the effect of chronic MPH treatment on the phosphorylating system associated with cytoskeleton IF in hippocampus of juvenile rats. Neurons express three specific IFs named according to their molecular mass: NFH, NFM and NFL (Friede and Samorajski 1970; Zhu et al. 1997; Zanatta et al. 2012). GFAP is the main IF of mature astrocytes (Middeldorp and Hol 2011). We found that MPH triggers hypophosphorylation of NFM and NFH in juvenile rat hippocampus without altering protein levels. Our data also suggests that PP1 and PP2A participate in these effects as their immunocontents were increased by MPH. Interestingly, altered phosphorylation levels of GFAP were accompanied by diminished immunoreactivity.

Phosphorylation/dephosphorylation balance plays a major role in regulating the structural organization and function of

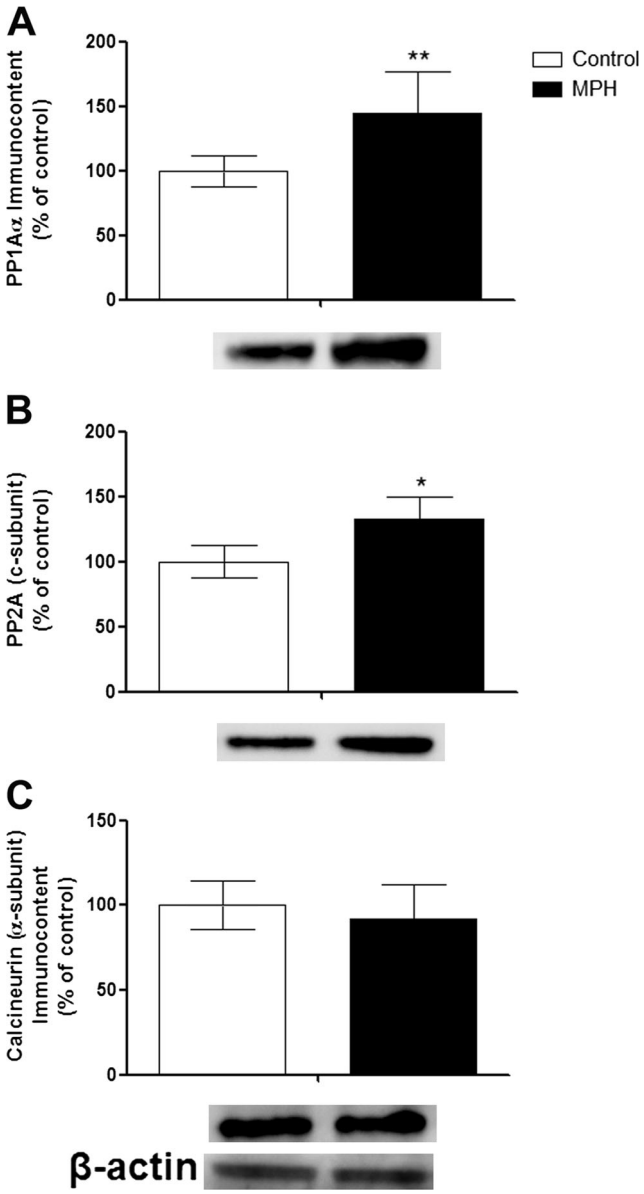

Fig. 3 The effects of chronic methylphenidate treatment on PP1 (**a**), PP2A (**b**) and PP2B (**c**) immuncontents in juvenile rat hippocampus are displayed. Representative Western blots of the proteins studied are shown. Western blot of β-actin was used as loading control. Results are expressed with the mean ± standard deviation for 5–6 animals in each group and expressed as percent of control. Different from control, ** $p < 0.01$ and * $p < 0.05$ (Student's t test). PP1, protein phosphatase 1 α; PP2A, protein phosphatase 2 A (c-subunit); PP2B, calcineurin (α-subunit); MPH, methylphenidate

IFs (Brownlees et al. 2000; Guidato et al. 1996; Sihag et al. 2007; Strack et al. 1997; Veeranna et al. 1998) and hypophosphorylated NF are more susceptible to proteolytic breakdown (Goldstein et al. 1987; Pant 1988). Hypophosphorylation of Lys-Ser-Pro (KSP) repeats of NFM and NFH tail domain was observed in the present work, and this result is in accordance with increased PP1 and PP2A immunocontent. Considering that these phosphorylation sites

Table 2 The effects of chronic treatment with methylphenidate on lipid content in hippocampus of juvenile rats

	Control	MPH
Hippocampus		
Gangliosides	177.44 ± 27.67	141.21 ± 19.77*
Phospholipids	306.82 ± 24.65	251.42 ± 40.85*
Cholesterol	119.85 ± 14.16	95.68 ± 7.17**

Ganglioside, phospholipid and cholesterol content are expressed in nmol NeuAc/mg protein, nmol Pi/mg protein and μg cholesterol/mg protein respectively. Results are expressed as means ± standard deviation for 5–6 animals in each group. Different from control, *$p < 0.05$ and **$p < 0.01$ (Student's t test). MPH, methylphenidate

have been implicated in the regulation of axonal caliber and transport (Veeranna et al. 1998), it is possible that MPH may disrupt axonal cytoskeleton and downregulate axonal transport, interfering with highly regulated processes in the immature brain.

The most frequent Ser-Thr phosphatases involved in the modulation of the phosphorylation levels of IF cytoskeletal proteins are PP1, PP2A and PP2B (Friede and Samorajski 1970). PP1, could in this case, be regulated by DARPP-32, an important endogenous regulator of PP1 activity, whose biochemical effects are dependent on its phosphorylation level at specific sites (Heimfarth et al. 2012; Hakansson et al. 2004). Studies have shown that the DARPP-32/PP1 cascade is a major target for psychostimulant drugs, and dopamine may alter the phosphorylation of DARPP-32 (Svenningsson et al. 2003). Corroborating our findings, Souza et al. (2009) showed that juvenile rats subject to chronic MPH treatment (2 mg/kg, from 25th to 58th day of age) present a decrease in hippocampal DARP-32 immunocontent. Since PP2A could be directly or indirectly activated by intracellular calcium levels leading to the IF hypophosphorylation induced by MPH, we suggest that NFH and NFM hypophosphorylation can be explained, at least in part, by signaling mechanisms downstream of MPH. This would up-regulate PP1 and PP2A catalytic activities in the hippocampus of juvenile rats. However, further investigation will be necessary to verify this proposal.

Concerning the downregulation of GFAP immunocontent, previous immunohistochemical analyses have shown that MPH decreases GFAP in rat hippocampus (Schmitz et al. 2016a). We cannot rule out the fact that GFAP hypophosphorylation could be a consequence of diminished protein levels, not simply a result of a misregulated phosphorylation system associated with the astrocyte cytoskeleton. GFAP is necessary for cell shape maintenance, motility/migration, proliferation, glutamate homeostasis, and protection against CNS injury (Middeldorp and Hol 2011). It has been described that astrocytes of $GFAP^{-/-}$ knockout mice are less efficient in dealing with acute states of injury in the CNS (Pekny and Pekna 2004). Astrocytes are particularly adapted

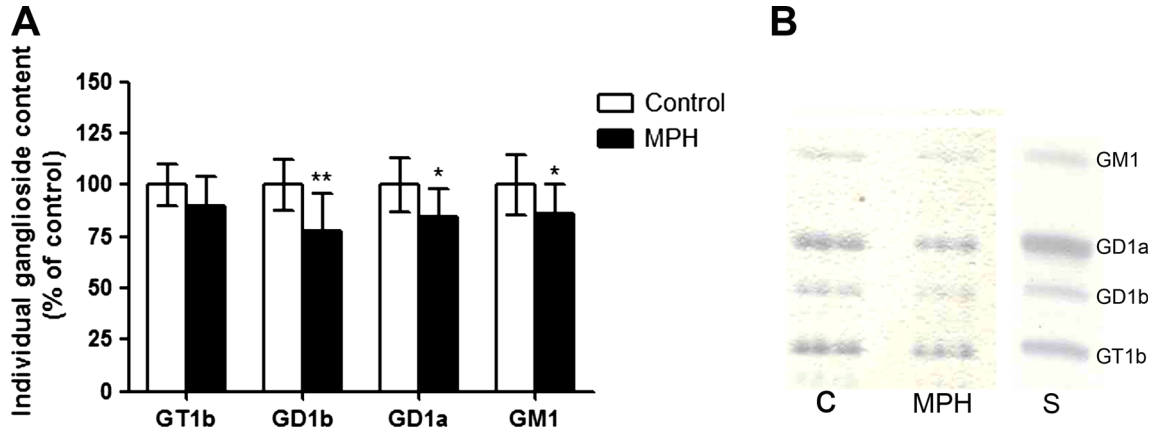

Fig. 4 The effects of chronic methylphenidate treatment on GM1, GD1a, GD1b and GT1b (**a**) levels in the hippocampus of juvenile rats are shown. Representative bands of the gangliosides studied are shown (**b**). Results are expressed as mean ± standard deviation for 5–6 animals in each group and expressed as percent of control. Different from control, ** $p < 0.01$ and * $p < 0.05$ (Student's t test). MPH, methylphenidate

to respond to stressing agents and play a role in the protection of neurons. Sadasivan et al. (2012) showed that MPH administration sensitizes dopaminergic neurons to the MPTP (parkinsonian agent).

IF cytoskeletal proteins are known to respond to a cell disturbance through alterations in its phosphorylation system (hyper and hypophosphorylation), observed in animal models of neurometabolic diseases (de Almeida et al. 2003; de Mattos-Dutra et al. 1997; Funchal et al. 2005; Loureiro et al. 2010; Pierozan et al. 2012, 2014). They are also ascribed to be directly or indirectly involved with human disease (Omary et al. 2006). Changes in protein phosphorylation lead to improper brain cytoskeletal regulation and neural cell death, which reinforces the criticality of the cytoskeleton in neurodegeneration (Lee et al. 2011). Based on these findings, we suggest that MPH may cause neural dysfunction associated with cytoskeletal disruption in the hippocampus of juvenile rats.

Whereas that MPH was administered in a period characterized by rapid development of CNS, intense cellular proliferation and growth (Rice and Barone Jr 2000), and a progressive and physiological accumulation of lipids (Adibhatla and Hatcher 2007; Pfrieger 2003). And also taking into account that membrane/lipid rafts and the cytoskeleton interact dynamically and regulate many facets of eukaryotic cell function and their adaptation to changing environments (Head et al. 2014), we investigated the effect of chronic MPH administration on the lipid content in hippocampus of juvenile rats. Results showed that MPH treatment reduces cholesterol content, total ganglioside content and the major brain gangliosides GM1, GD1a and GD1b. In addition, the total phospholipid content and the major brain phospholipids sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol + phosphatidylserine are reduced in hippocampus of juvenile rats. In line with this, we have recently showed that MPH treatment reduced

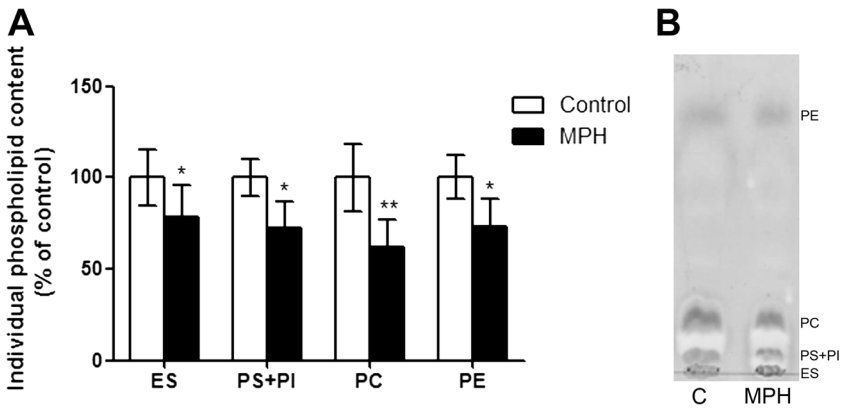

Fig. 5 The effects of chronic methylphenidate treatment on sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol + phosphatidylserine contents in the hippocampus of juvenile rats are displayed (**a**). Representative bands of the phospholipids studied are shown (**b**). Results are expressed with the mean ± standard deviation for 5–6 animals in each group and expressed as percent of control. Different from control, ** $p < 0.01$ and * $p < 0.05$ (Student's t test). ES, sphingomyelin; PC, phosphatidylcholine; PE phosphatidylethanolamine; PS + PI, phosphatidylserine + phosphatidylinositol; MPH, methylphenidate

Na^+,K^+-ATPase activity in hippocampus of juvenile rats (Schmitz et al. 2016b) and the activity of this enzyme is sensitive to membrane lipids composition changes (Cornelius et al. 2015). Corroborating our data, Phan et al. (2015), showed that methylphenidate dramatically affected both the distribution and abundance of lipids and their derivatives, particularly fatty acids, diacylglycerides, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in the fly brains.

Nervous tissue is capable of synthesizing cholesterol in a developmentally regulated manner. Cholesterol is vital to normal brain functions such as signaling, synaptic plasticity, learning and memory (Pfrieger 2003). Moreover, cholesterol is an essential component of cellular membranes and is required for viability and cellular proliferation. One of its important functions is the modulation of physicochemical properties of cellular membranes (Ohvo-Rekilä et al. 2002). Disturbances in cholesterol synthesis or metabolism have significant consequences on brain functions (Pfrieger 2003; Ohvo-Rekilä et al. 2002). Corroborating our study, Kabara (1975) showed that MPH caused a decrease in brain cholesterol concentration. Synapses are particularly sensitive to cholesterol level and a reduction of its content by MPH treatment, may be associated, at least in part, with synapse loss, and ultimately neurodegeneration. In addition, cholesterol is an important component of myelin (Pfrieger 2003). This suggests that chronic MPH treatment could compromise axonal myelination. We suggest that the decreased cholesterol level could be related to a lack of available energy, as there is evidence that MPH increases energy demand (Fagundes et al. 2007; Fagundes et al. 2010a, b; Réus et al. 2013, 2015).

Phospholipids constitute the backbone of neural membranes and provide the membrane with a suitable environment, fluidity, ion permeability, and are required for structural functions (phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine). In addition, they take part in cellular signaling (phosphatidylinositol and sphingomyelin) (Ohvo-Rekilä et al. 2002). The decreased phospholipid content observed might be a consequence of the phospholipase cascade activation and an insufficient energy supply to oligodendrocytes leading to impairments in fatty acid synthesis and myelin sheath formation (Ferriero 2001). Corroborating our finding, a significant reduction in the resonance of phosphatidylcholine metabolites in the anterior cingulum following chronic MPH in adult ADHD has been observed and a decrease in choline-containing compounds may indicate membrane rupture and is associated with decreased synaptic plasticity (Kronemberg et al. 2008). Besides, Quansah et al. (2017b) showed that creatine (metabolite associated with energy), myo-inositol (marker of membrane turnover) and phosphocoline (precursor of various membrane phospholipids) were increased in cerebral extracts of adolescent male rats treated with MPH (5.0 mg/kg). Taking all this into account, we can suggest that altered lipid turnover rate might be involved in the action of the MPH, affecting the suitable environment for the membrane, fluidity, ion permeability, as well as structural functions and cellular signaling. In addition, it is important not to neglect that alterations in the brain phospholipid composition have also been shown in models of brain injury, such as hypoxia/ischemia (Ramirez et al. 2003), schizophrenia (du Bois et al. 2005) and Alzheimer's disease (Farooqui et al. 2004).

Gangliosides are present in high concentrations in neuronal membranes acting on proliferation, neuronal differentiation, myelination, and synaptic transmission (Mocchetti 2005). They also play a significant role in learning/memory mechanisms (She et al. 2005). Gangliosides in the hippocampus are closely associated with synaptogenesis and myelinogenesis, and they participate in many neuronal functions (Mocchetti 2005). Thus, a decrease in ganglioside levels suggests that chronic MPH treatment could impair the plethora of functions gangliosides perform. Decreased gangliosides have also been reported in other models of brain injury like undernutrition (Trindade et al. 1992), organic acidaemia (Trindade et al. 2002) and neurodegeneration (Schneider et al. 1998). The mechanisms involved in the decrease in ganglioside levels need further investigation; however, the high energy demand required for its synthesis could be an important factor involved.

A better understanding of the neurobiology associated with chronic MPH exposure on early stages of brain development is critical, as this psychostimulant is widely misused by children and adolescents who do not meet full diagnostic criteria for ADHD (Akay et al. 2006; Dafny and Yang 2006; Gonçalves et al. 2014; Loureiro-Vieira et al. 2017). Although other brain regions, such as prefrontal cortex and striatum are involved in MPH mechanisms, the hippocampus may be the region most affected by long-term MPH treatment (Motaghinejad et al. 2016; Lagace et al. 2006). This may be because the treatment with this psychostimulant in adolescent rodents increases norepinephrils in the hippocampus in a dose-dependency pattern (Kuczenski and Segal 2002).

Summing up, in this study we showed that cytoskeletal and lipid homeostasis are affected by chronic MPH treatment in rat immature brain. These results contribute to the understanding of the effects and consequences associated with chronic use of this psychostimulant during the development of the CNS and emphasize the need for proper use of this psychostimulant in youth.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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3.5 Capítulo III

Methylphenidate causes behavioral impairments and neuron and astrocyte loss in the hippocampus of juvenile rats

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Methylphenidate Causes Behavioral Impairments and Neuron and Astrocyte Loss in the Hippocampus of Juvenile Rats

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Abstract Although the use, and misuse, of methylphenidate is increasing in childhood and adolescence, there is little information about the consequences of this psychostimulant chronic use on brain and behavior during development. The aim of the present study was to investigate hippocampus biochemical, histochemical, and behavioral effects of chronic methylphenidate treatment to juvenile rats. Wistar rats received intraperitoneal injections of methylphenidate (2.0 mg/kg) or an equivalent volume of 0.9 % saline solution (controls), once a day, from the 15th to the 45th day of age. Results showed that chronic methylphenidate administration caused loss of astrocytes and neurons in the hippocampus of juvenile rats. BDNF and pTrkB immunocontents and NGF levels were decreased, while TNF-α and IL-6 levels, Iba-1 and caspase 3 cleaved immunocontents (microglia marker and active apoptosis marker, respectively) were increased. ERK and PKCaMII signaling pathways, but not Akt and GSK-3β, were decreased. SNAP-25 was decreased after methylphenidate treatment, while GAP-43 and synaptophysin were not altered. Both exploratory activity and object recognition memory were impaired by methylphenidate. These findings provide additional evidence that early-life exposure to methylphenidate can have complex effects, as well as provide new basis for understanding of the biochemical and behavioral consequences associated with chronic use of methylphenidate during central nervous system development.

Keywords Methylphenidate · Neurotrophins · Cytokines · Cell survival pathways · Synaptic proteins · Memory of object recognition

Abbreviations

ADHD	Attention-deficit/hyperactivity disorder
Akt	Protein kinase B
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
ERK	Extracellular signal-regulated kinase ½
GAP-43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
GSK-3β	Glycogen synthase kinase 3 beta
Iba-1	Ionized calcium-binding adapter molecule 1
IL-6	Interleukin 6
MAP	Mitogen-activated protein
MPH	Methylphenidate
NGF	Nerve growth factor
PI3K	Phosphatidylinositol-3 kinase
PKCaMII	Calcium/calmodulin-dependent protein kinase II
pTrkB	Tyrosine kinase B receptor phosphorylated

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SNAP-25 Synaptosomal-associated protein 25
TNF-α Tumor necrosis factor alpha

Introduction

Methylphenidate (MPH), an amphetamine-like psychostimulant, is widely used for the treatment of attention-deficit/hyperactivity disorder (ADHD) in adolescent and adults [1]. ADHD is a complex neuropsychiatric disease characterized mainly by high levels of inattention, hyperactivity, and impulsivity [1–3]. However, recent studies have reported a large increase in the incidence of MPH misuse among young adults and students who do not meet the criteria for ADHD, in search of cognitive enhancement [4, 5], in preschool children with 2–4 years of age [6, 7]. Treatment with MPH is usually well tolerated, and its efficacy in reducing symptoms of ADHD is associated with an increase of dopamine and norepinephrine levels in synapses due to blockage of their carriers [8, 9]. However, the increasing misuse of MPH creates the need to further investigate the long-term consequences of its use on brain development and behavior [10, 11].

Young rats treated with MPH may have notable changes on behavioral, neurophysiological, and biochemical parameters in adulthood. Juvenile rats exposed to MPH show decreased responses to rewarding stimuli, as well as increased depressive- and anxiety-like behaviors [12–17]. In this context, Chase and colleagues [18] showed that MPH administration alters c-fos expression in the brain of juvenile rats. Decreased activity of dopaminergic neurons [19, 20], increase in the levels of cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein and Homer 1A transcript [13, 14], as well as increase in corticosterone levels following restraint stress [15] have also been reported after MPH treatment. Recently, it has been shown that chronic treatment with MPH at early age causes excitotoxicity associated to inhibition of glutamate uptake and disturbances in the Na^+,K^+-ATPase in the prefrontal cortex [21]. Although the mechanisms through which MPH affects the developing brain are still poorly understood, it is reasonable to hypothesize the involvement of hippocampus development, since this psychostimulant dose-dependently increases norepinephrine levels in the hippocampus of adolescent rodents [22].

MPH also increases dopamine levels in various brain regions, including the hippocampus; this monoamine is reported to play an important modulatory effect on neurogenesis during brain development [23, 24]. In this period, the establishment of appropriate synaptic connections is essential for the survival of neural cells that are dependent on trophic factors such as brain-derived neurotrophic factor (BDNF) [25] and nerve growth factor (NGF) [26]. At the molecular level, the neurotrophins promote neuronal survival by interaction with the tyrosine kinases (Trk) receptor and activation of mitogen-activated protein/extracellular signal-regulated kinase ½ (MAP/ERK) and/or phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) pathways [27]. In addition to that, synaptic proteins such as synaptosomal-associated protein 25 (SNAP-25), growth associated protein 43 (GAP-43), and synaptophysin are also very important for the process of synaptogenesis and maturation of functional synaptic connectivity during brain development [28, 29]. Interestingly, mice carrying a deletion of SNAP-25 gene have been used as ADHD animal model [30].

Free dopamine triggers an inflammatory response in the brain characterized by the increase of cytokines and chemokines [31] leading to microgliosis. Inflammatory mediators can exert both beneficial and deleterious effects in the central nervous system (CNS); immune protection and aid in removal of dead neurons are the beneficial ones. Conversely, when present in large quantities in neurogenic niches, they can impair hippocampal-dependent forms of synaptic plasticity leading to cognitive dysfunction in neuropsychiatric and neurodegenerative disorders such as depression, Alzheimer's disease, and Parkinson's disease [32]. Supporting that, Sadasivan and colleagues [33] showed that pre-treated rats with MPH have higher neuronal loss in striatum when subjected to MPTP (a drug used to induce experimental Parkinson's disease) than control animals, probably due to the inflammatory response. In addition, studies show that long-term use of methamphetamine, a psychostimulant that also increases the levels of dopamine in the synaptic cleft, causes behavioral changes and neurodegeneration [34] and increases the risk of developing Parkinson's disease [35].

Considering the need to understand the consequences of the chronic use of MPH during CNS development, the present study investigates biochemical and histochemical parameters, such as neuron and astrocyte loss, the levels of neurotrophins (NGF and BDNF), pro-BDNF and pTrkB (precursor and activated receptor of BDNF, respectively), synaptic proteins (SNAP-25, GAP-43, and synaptophysin), inflammatory mediators [TNF-α and IL-6 levels; and Iba-1 (a microglia marker)], caspase 3 cleaved immunocontent (an active apoptosis marker), and signaling pathways in the hippocampus of juvenile rats treated chronically with MPH. Functional behavioral effects of MPH treatment were assessed in the elevated plus maze, in the open field test and in the object recognition memory task.

Experimental Procedures

Animals and Reagents

Rats were obtained from the Central Animal House of the Department of Biochemistry of the University Federal of Rio Grande do Sul, Porto Alegre, Brazil. Litters were culled

to eight pups on postnatal day (PD) 3 (day of parturition = PD 0) and were kept with the dam until weaning on PD 21. After weaning, the rats were re-housed in boxes containing up to four male rats. Animals were maintained on a 12–12 light–dark cycle at a constant temperature of 22 ± 1 °C, with free access to water and commercial protein chow. Sample sizes were determined as 7 and 14 animals per group for biochemical/histochemical and behavioral testing, based on standard deviation values of previous studies, with power estimation of 0.80 and alpha = 0.05. A total of 95 male Wistar rats were used. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Ethics Statement

All animal experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (No. 80–23, revised 1996) and a research protocol approved by the University's Ethics Committee (No. 24812). All efforts were made to minimize the number of animals used and their suffering.

Chronic Early Treatment with Methylphenidate

Beginning on PD 15, rats were weighted and injected intraperitoneally with 0.9 % saline solution (control group) or 2.0 mg/kg of methylphenidate (MPH group), once a day, for 30 consecutive days. MPH was dissolved in 0.9 % saline solution and injected at a volume of 1 ml/100 g of body weight. Control group received equivalent volume of saline solution [36, 37]. This dose and route of administration were selected because they mimic the therapeutic doses in terms of magnitude of neurochemical and behavioral effects [38]. MPH administrations started at 15 days of life because this period is characterized by intense synaptogenesis, myelination, and gliogenesis, comparable to early childhood in humans [29]. Treatment lasted for 30 days because it has been shown that this period mimics chronic use in humans [39]. Twenty-four hours after the last administration of MPH (PD 45), the rats were decapitated (for biochemical studies) or perfused (for histochemical studies) or subjected to the behavioral assessment (Fig. 1).

Biochemical and Histochemical Assessment

Flow Cytometry Analysis

Fourteen rats were used for this assay (seven per group). Hippocampus was dissociated with PBS/Collagenase/DNase, washed once with PBS then suspended in PBS/collagenase containing 10 μg/ml propidium iodide (PI). Cells were incubated at room temperature in the dark for 30 min, washed with PBS and centrifuged at 3000 rpm for 5 min at 4 °C to remove the free PI. The integrity of plasma membrane was assessed by determining the ability of cells to exclude PI. Afterwards, the cell was permeabilized with 0.2 % PBS Triton X-100 in for 10 min at room temperature and blocked for 15 min with BSA 5 %. After blocking, cells were incubated for 2 h in blocking solution containing the monoclonal antibodies anti-NeuN diluted 1:100 or anti-GFAP diluted 1:100. Cells were then washed twice with PBS and incubated for 1 h in blocking solution containing fluorescein isothiocyanate (FITC)-anti-rabbit IgG diluted or Alexa 488-anti-mouse IgG diluted 1:200. The levels of PI incorporation and numbers of positive NeuN and GFAP cells were determined by flow cytometry [40]. FITC or Alexa Fluor 488 and PI dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples with the secondary antibody) were included for setting up the machine voltages. Controls stained with a single dye (Alexa fluor 488 or FITC and PI) were used to set compensation. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: green (FL-1; 530 nm/30) and red (FL-3; 670 nm long pass). Fluorescence emissions were collected using logarithmic amplification. In brief, data from 10,000 events (intact cells) were acquired and the mean relative fluorescence intensity was determined after exclusion of debris events from the data set. All flow cytometric acquisitions and analyses were performed using Flow Jo software 7.6.3 (Treestar, Ashland, OR). Flow cytometry data were analyzed and plotted by density as a dot plot which shows the relative FL1 fluorescence on the *x*-axis and the relative FL3 fluorescence on the *y*-axis. The quadrants to determinate the negative and positive area were placed on unstained samples. The number of cells in each quadrant was computed and the proportion of cells stained with PI, GFAP, and NeuN were expressed as percentage of PI uptake.

Immunofluorescence and FJC Staining

Fourteen rats were used for this assay (seven per group). After MPH treatment, each rat was overdosed with sodium thiopental and perfused intracardially with 0.9 % saline followed by 4 % paraformaldehyde. The brain was removed and postfixed overnight in 4 % paraformaldehyde at 4 °C. The tissue was then rinsed in 0.1 M phosphate buffer and transferred to the 15 and 30 % sucrose solution in NaCl/Pi at 4 °C. After the brain had sunk (2–3 days), it was frozen by immersion in isopentane cooled with CO_2 and stored in a freezer (−80 °C) for later analyses. Serial coronal sections (30 μm) of the hippocampus were obtained with a cryostat at −20 °C (Leica, St. Louis, MO, USA). A set of eight sections, taken from the same region in all groups throughout the rostrocaudal axis of the dorsal hippocampus, was used for histochemical analysis. The sections were incubated, according Pierozan and colleagues [41], with polyclonal rabbit with GFAP Ig (clone 6 F2) or mouse NeuN Ig (clone A60) for 48 h, and diluted 1:3000 and 1:1000,

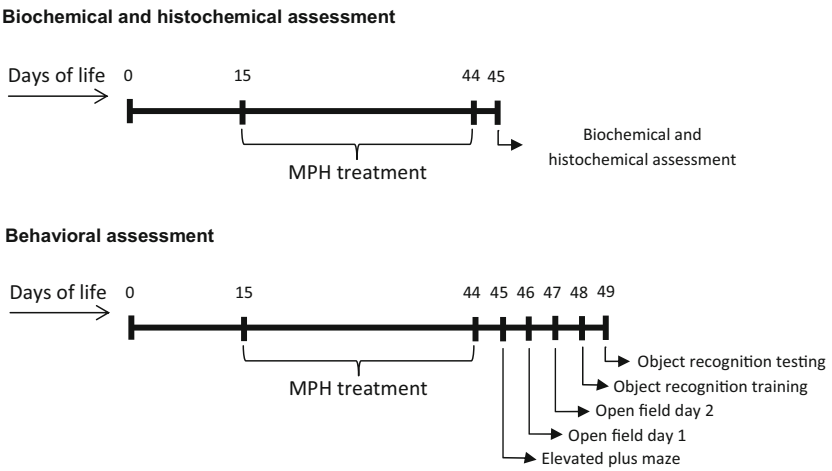


Fig. 1 Timeline of experimental procedures. *MPH* methylphenidate

respectively, in NaCl/Pi (0.3 % Triton X-100) and BSA 2 %. Negative controls were carried out with omission of the primary antibodies. After being washed repeatedly in NaCl/Pi, tissue sections were incubated with rabbit Ig or mouse Ig Cy3 (F(ab')2 fragment), both diluted 1:500 in NaCl/Pi, 0.3 % Triton X-100 and 2 % BSA for 1 h at room temperature. The sections were then extensively washed in NaCl/Pi and transfered to gelatinized slides. To visualize degenerative neurons, the slides were air-dried and subjected to FJC staining, with a method adapted from Ehara and Ueda [42]. Briefly, slides were rinsed for 5 min in distilled water and then incubated in 0.06 % potassium permanganate solution for 10 min. Following a 2-min water rinse, slides were incubated for 10 min in the FJC staining solution with 0.001 % DAPI. The slides were washed, dried, coverslipped in acidic mount media (DPX), and examined under an epifluorescence microscope. The images were obtained with an Olympus IX-81 confocal FV-1000 microscope and analyzed with OLYMPUS FLUOVIEW software (Shinjuku, Tokyo, Japan).

Nerve Growth Factor Measurement

Fourteen rats were used for this assay (seven per group). The NGF levels in the hippocampus were determined using a sandwich-ELISA assay with monoclonal antibody specific to NGF (CYT304—Milliopore, USA and Canada). Briefly, hippocampus was homogenized in phosphate buffer solution with a protease inhibitor cocktail. Microtiter plates were coated for 24 h with the samples diluted 1:2 in sample diluent and the standard curve ranged from 7.8 to 500 pg/ml of NGF. The plates were then washed four times with sample diluent and a monoclonal anti-NGF (diluted 1:1000 in sample diluent) was then added to each well and incubated for 3 h at room temperature. After washing, a peroxidase-conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After the addition of the streptavidin-enzyme, substrate, and stop solution, the amount of NGF was determined in 450 nm.

Western Blot Analyses

Hippocampi were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4 % SDS. For electrophoresis analysis, samples were dissolved in 25 % (*v*/*v*) of a solution containing 40 % glycerol, 5 % mercaptoethanol, and 50 mM Tris–HCl, pH 6.8. Equal protein concentrations were loaded onto 10 % polyacrylamide gels and analyzed by SDS-PAGE. The gels were transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20 % methanol, and 0.25 % SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5 % bovine serum albumin, fraction V). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05 % Tween-20 (T-TBS). After blocking with Tris-buffered saline 0.1 % Tween-20 (TBS-T) containing 3 % bovine serum albumin for 1 h, the membranes were incubated for 24 h at 4 °C with the primary antibodies listed in Table 1. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:10000. The blot was washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). Membranes were re-probed for anti-β-actin immunoreactivity. The membranes were developed in a photodocumenter, and band intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the web site (http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-actin as a loading control to assess protein levels. The data used in statistical analysis were obtained from the ratio of the protein studied and β-

Table 1 Primary antibodies used for Western blot analyses

Primary antibody	Dilution	Purchased from
Mouse anti-GFAP	1:2000	Sigma
Mouse anti-pro-BDNF	1:1000	Sigma
Mouse anti-BDNF	1:1000	Sigma
Rabbit anti-pTrkB	1:2000	Abcam
Rabbit anti-SNAP-25	1:2000	Sigma
Mouse anti-GAP-43	1:2000	Abcam
Rabbit anti-synaptophysin	1:2000	Sigma
Rabbit anti-Iba-1	1:2000	Sigma
Rabbit anti-ERK	1:1000	Cell signaling
Rabbit anti-pERK	1:1000	Cell signaling
Mouse anti-Akt	1:1000	Cell signaling
Mouse anti-pAkt	1:1000	Cell signaling
Rabbit anti-PKCaMII	1:1000	Cell signaling
Rabbit anti-GSK-3β	1:1000	Cell signaling
Rabbit anti-pGSK-3β	1:1000	Cell signaling
Rabbit anti-caspase 3 cleaved	1:1000	Sigma

Abbreviations: *GFAP* glial fibrillary acidic protein, *BDNF* brain-derived neurotrophic factor, *pTrkB* tyrosine kinase B receptor, *SNAP-25* synaptosomal-associated protein 25, *GAP-43* growth associated protein 43, *Iba-1* ionized calcium-binding adapter molecule 1, *ERK* extracellular signal-regulated kinase ½, *pERK* extracellular signal-regulated kinase ½ phosphorylated, *Akt* protein kinase B, *pAkt* protein kinase B phosphorylated, *PKCaMII* calcium/calmodulin-dependent protein kinase II, *GSK-3β* glycogen synthase kinase 3 beta, *pGSK-3β* glycogen synthase kinase 3 beta phosphorylated

actin density unit lines. For the determination of Western blot analyses, 14 rats were necessary (seven per group).

Cytokines (TNF-α and IL-6) Assay

Hippocampi were homogenized in 1:5 (*w*/*v*) saline solution (0.9 % NaCl). The homogenized was centrifuged at 800 × *g* for 10 min at 4 °C and the supernatant was used in the assays. For determination of cytokines, 14 rats were necessary (seven per group).

TNF-α and IL-6 levels in the hippocampus were quantified by rat high-sensivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Invitrogen®). The ELISA was performed following the manufacture's instruction, and the concentrations of cytokine were measured by optical densitometry at 450 nm in SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Protein Determination

The protein concentration was determined by the method of Lowry and colleagues [43] using serum bovine albumin as the standard.

Behavioral Assessment

Twenty-four hours after the last injection, 25 rats (14 MPH-treated and 11 control) were subjected to the following behavioral tests: elevated plus maze, open field, and object recognition.

Elevated plus maze. The plus maze apparatus is a pharmacologically validated anxiety measure in rodents [44] and consisted of a black painted Plexiglas, with two open arms 65 cm long and 14 cm wide and two closed arms enclosed by 45 cm walls, with an open roof arranged in such a way that the two arms of each type were opposite each other. Each mouse was placed in the central square facing an open arm. The total number of entries into the four arms (when all four paws had entered the arm), the number of entries and the time spent into the open arms were recorded for 5 min session. In accordance with Benetti et al. [45], the larger the number of entries into the open arm and the longer the time spent there, less anxious is the animal.

Habituation and locomotion. The motor activity, exploratory, and the habituation of rats were evaluated in the open field test. The open field was made of wood covered with impermeable wood, had a black floor measuring 600 cm^2, and was surrounded by 60-cm-high walls. In the habituation task, the rats were allowed to explore the open field for 10 min on two consecutive days. The distance traveled was registered on the first day as an index of general activity [46]. The rats were individually placed in the center of the open field, and behavioral parameters were recorded and subsequently elaborated with an automated activity-monitoring system (Any-maze; Stoelting, Wood Dale, IL, USA).

Object recognition task. The object recognition task was conducted in the open field, as previously described by Ennaceur and Delacour [47], and adapted by Pamplona and colleagues [48]. It consisted of three distinct phases: habituation, sample, and discrimination. In the habituation phase, the rats were allowed to explore the open field for 10 min on two consecutive days. In the sample phase, two identical objects (A1 and A2; cubes) were placed in opposite corners of the open field, 20 cm distant from the walls and ~60 cm apart from each other, and the rats were allowed to explore them for 10 min. After the end of the sample phase, the rats were removed from the open field and kept in the home cage. After a delay period of 24 h, in the discrimination phase, an identical copy of the familiar object (A1) and a novel object (B) were placed in the locations previously occupied by A1 and A2, and the rats were allowed to explore the objects for 10 min. All of the objects were constructed with plastic LEGO blocks. The locations of the objects were counterbalanced in each

session. The time spent by the rats in exploring each object was monitored with a video system placed in an adjacent room. Exploration of an object was defined as directing the nose to the object at a distance of ≤2 cm and/or touching it with the nose. Analyses were performed on the following measures: the total time spent exploring the two objects in the sample phase (A1 + A2) and the discrimination index, which is defined by the difference in exploration time between the novel and the familiar objects, divided by the total time spent exploring these two objects in the discrimination phase [(B − A1)/(B + A1)].

Statistical Analysis

Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer. Student's *t* test was used to evaluate all different parameters after checking data normal distribution with Shapiro–Wilk test. Results are expressed as means ± standard deviation or standard error mean, and differences were considered statistically significant when $p < 0.05$. In all cases, litter effects were controlled by assigning not more than two subjects from a litter to a particular group.

Results

Neuron and Astrocyte Loss Caused by Chronic MPH Treatment

Flow cytometry analysis with anti-NeuN and anti-GFAP antibodies to identify and quantify neurons and astrocytes, respectively, was run to investigate the effects of chronic treatment with MPH in the hippocampus of juvenile rats (Fig. 2). MPH caused a significant decrease in neurons ($p < 0.01$) and astrocytes ($p < 0.01$) of treated rats as compared to the control group (Fig. 2a, b). We also tested anti-NeuN and anti-GFAP antibodies co-stained with PI for assessing cell viability. Results showed that PI incorporation into GFAP and NeuN positive cells was not altered in response to MPH ($p > 0.05$), (Fig. 2c, d). PI positive cells were also not altered ($p > 0.05$) (Fig. 2e).

To further assess neuronal and astrocyte loss induced by chronic MPH treatment to rats, hippocampus sections were stained with anti-GFAP or anti-NeuN, as well as for nuclear staining with 40,6-diamidino-2-phenylindole (DAPI) and Fluoro-Jade C (marker of degenerating neurons). The absence of Fluoro-Jade C-labeled cells (data not shown) and a decrease in NeuN and GFAP immunostaining in the hippocampus of juvenile rats chronically treated with MPH (Fig. 3a) were observed. Corroborating with the data flow cytometry and immunofluorescence, a reduction in GFAP immunocontent ($p < 0.05$) when compared to the control group (Fig. 3b) was also observed. These results strongly suggest that chronic treatment with MPH promotes the loss of neurons and astrocytes in the hippocampus by some mechanism that does not involve necrosis, since no changes were observed when the cells were co-stained with PI.

Neurotrophins and Cytokines Are Affected by Chronic MPH Treatment

As depicted in Fig. 4, chronic treatment with MPH decreased BDNF immunocontent ($p < 0.05$) and pTrkB immunocontent (its activated receptor) ($p < 0.05$) but did not change the immunocontent of pro-BDNF (its precursor) ($p > 0.05$). The levels of NGF, another important neurotrophin, were also decreased after chronic MPH treatment.

The effects of chronic MPH treatment on synaptogenesis and maturation of functional synaptic connectivity during brain development were also evaluated by investigation of immunocontent of SNAP-25, GAP-43, and synaptophysin. Figure 5 shows that MPH decreased SNAP-25 ($p < 0.05$) but did not alter GAP-43 and synaptophysin ($p > 0.05$) immunocontent in hippocampus of juvenile rats.

Since the inflammatory response can affect cell survival, we also tested the effects of chronic treatment with MPH on some inflammatory parameters in the hippocampus of juvenile rats. Figure 6 shows that MPH treatment promoted an increase in TNF-α ($p < 0.05$) and IL-6 ($p < 0.001$) levels, as well as increased the Iba-1 immunocontent ($p < 0.05$) in hippocampus of MPH-treated rats.

ERK and PKCaMII, But Not PI3K/Akt Signaling Pathways, Are Involved in the Effects of MPH Treatment

In an attempt to determine signaling mechanisms involved in the neuronal and astrocyte loss promoted by chronic MPH treatment, we initially evaluated the involvement of ERK and PKCaMII in the actions of this psychostimulant in hippocampus of juvenile rats chronically treated. Results showed that the ERK signaling is inhibited in hippocampus, as suggested by decreased in pERK immunocontent (active form) ($p < 0.05$). Total ERK immunocontent was not changed by MPH treatment ($p > 0.05$) (Fig. 7a). In addition, as depicted in Fig. 7b, MPH promoted a decreased of PKCaMII immunocontent ($p < 0.05$) in hippocampus of juvenile rats chronically treated with MPH.

To examine whether the chronic treatment with MPH affect the PI3K/Akt signaling pathway, we assessed the Akt and pAkt immunocontents (total and active form, respectively). MPH did not alter the immunocontent of these proteins in hippocampus of juvenile rats ($p > 0.05$) (Fig. 7d). It also evaluated the glycogen synthase kinase 3 beta (GSK-3-β) activity, since it is described as a kinase that can be modulated by Trk

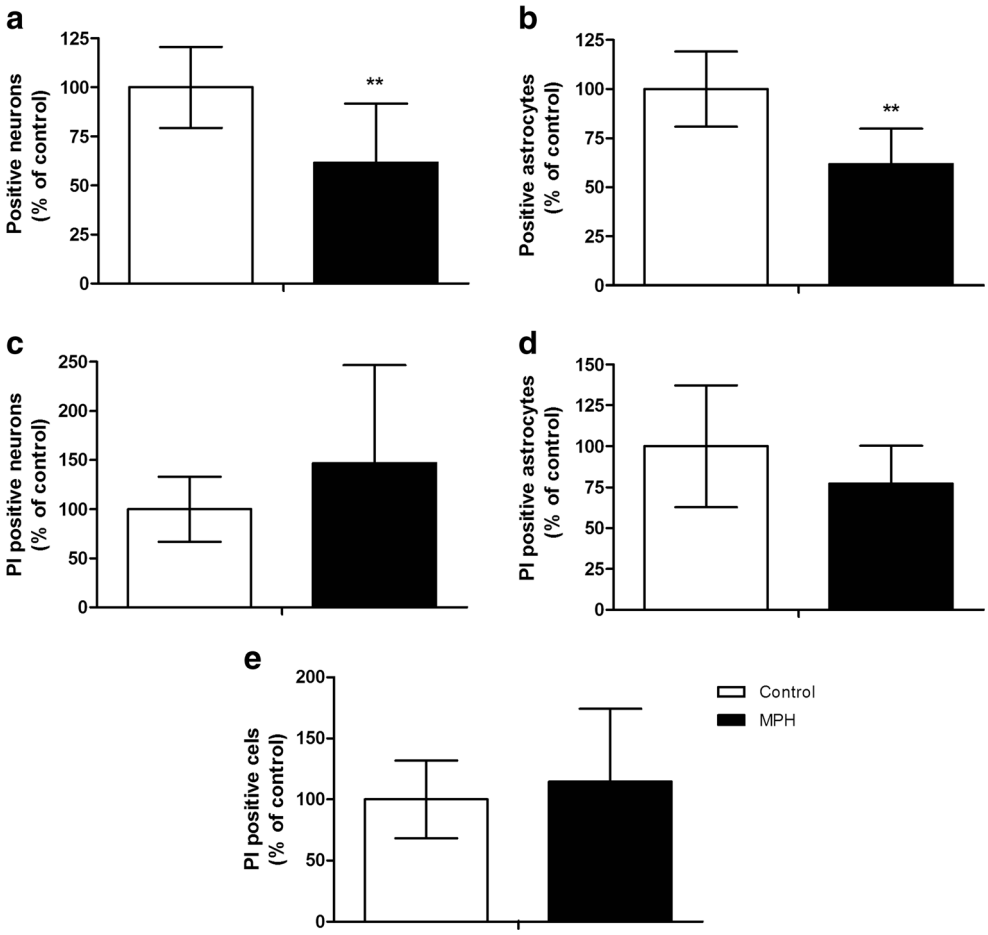


Fig. 2 Effect of chronic treatment with methylphenidate on NeuN positive cells (**a**), GFAP positive cells (**b**), propidium iodide plus NeuN positive cells (**c**), propidium iodide plus GFAP positive cells (**d**), and on the total propidium iodide positive cells (**e**) was measured by flow cytometry analysis on hippocampus of juvenile rats. Data are reported as mean ± standard deviation for 6–7 animals in each group and expressed as percent of control. Different from control, $^{**}p < 0.01$ (Student's *t* test). *PI* propidium iodide, *MPH* methylphenidate

receptor and it is involved in many cellular functions, contributing to the regulations of apoptosis, cell cycle, cell polarity and migration, gene expression, and other functions [49]. However, we observed that total GSK-3β (active form) and pGSK-3β (inactive form) (Ser9) were not altered in the hippocampus of MPH-treated rats ($p > 0.05$), suggesting that this kinase is not directly implicated in the effects of this psychostimulant (Fig. 7e). Caspase 3 cleaved was also evaluated; chronic MPH treatment promoted an increased in the immunocontent of this protein in hippocampus of juvenile rats when compared to the control group ($p < 0.01$), suggesting apoptotic cell death (Fig. 7c). Altogether, these results suggest that chronic treatment with MPH causes a decrease in cell survival and increased cell death by apoptosis in the hippocampus of juvenile rats.

Exploratory Activity and Object Recognition Memory Are Impaired by MPH Treatment

Behavioral evaluation was carried out 24 h after the last injection of MPH and saline, and the tests were performed on the 5 following days. The behavioral performance of the animals was evaluated on the following tasks: elevated plus maze, open field, and object recognition memory.

The results for anxiety-like behavior assessed in the elevated plus maze are summarized in Table 2. We observed that rats chronically treated with MPH showed a decrease in the total number entries ($p < 0.05$), in the entries in open arms ($p < 0.01$) and in the % of the time spent in open arms ($p < 0.01$) when compared to the control group.

Motor and exploratory activities and the habituation of rats were evaluated in the open field test (open field day 1 and open field day 2). As shown in Table 2, in open field day 1, rats chronically treated with MPH revealed a decrease in distance traveled ($p < 0.05$), average speed ($p < 0.05$), distance traveled on the periphery ($p < 0.01$), average speed on the periphery ($p < 0.05$), and on time spent at the periphery ($p < 0.05$) when compared to the control group. Although there is a trend of crossing number in treated rats, the difference did not reach statistical significance ($p = 0.075$). No differences were seen in open field day 2 ($p > 0.05$) (Table 2).

To further demonstrate the exploratory behavior, the distance traveled in 30 s intervals for 10 min in the first and 2nd day (Fig. 8) were monitored. In the open field day 1, MPH-treated animals showed lower locomotor activity in almost all times, with a significant difference in the ranges of 60–90, 120–150, and 240–270 s ($p < 0.05$) (Fig. 8a). In the

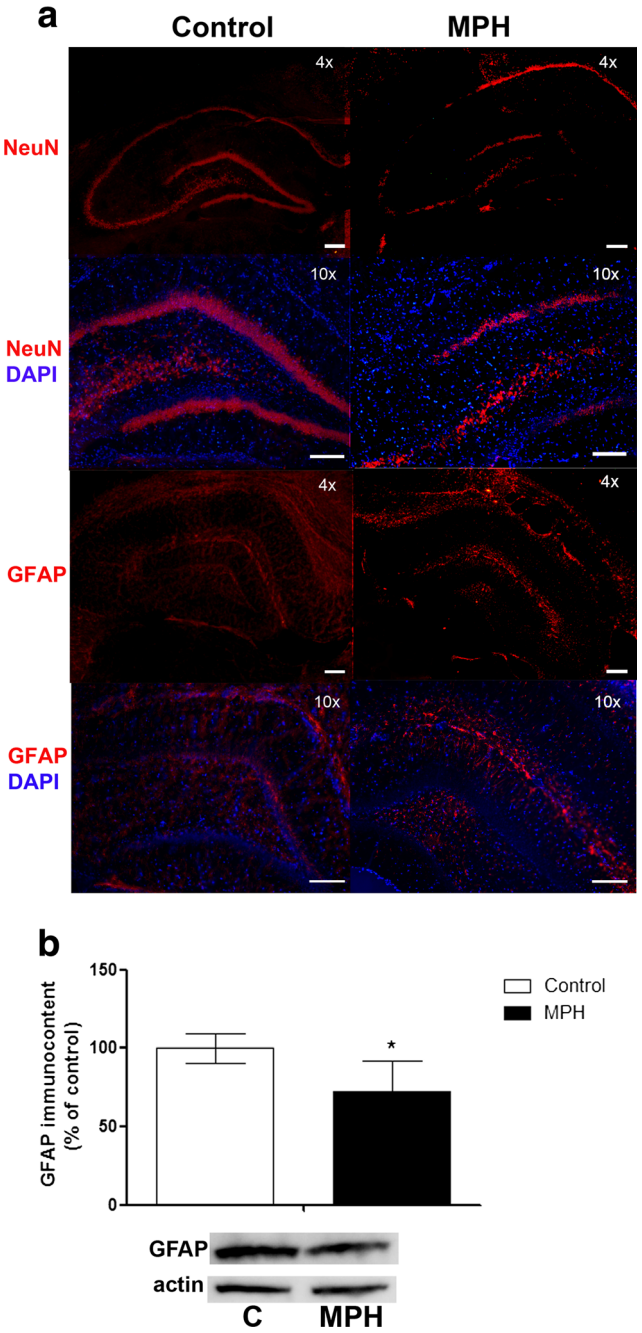


Fig. 3 Effect of chronic treatment with methylphenidate on NeuN and GFAP immunohistochemistry (**a**) and GFAP immunocontent (**b**) on hippocampus of juvenile rats. Representative images of six rats. Bar scale 4 × = 2 mm and 10 × = 200 μm. Results of GFAP immunocontent are expressed as mean ± standard deviation for 6 animals in each group and expressed as percent of control. Different from control, $*p < 0.05$ (Student's *t* test). *GFAP* glial fibrillary acidic protein, *MPH* methylphenidate

open field day 2, MPH-treated rats did not show any difference when compared to the control group (Fig. 8b). The difference observed in the open field 1 suggests that MPH treatment reduces the curiosity to explore novel environments.

Finally, we evaluated the performance of animals chronically treated with MPH on the long-term object recognition memory (Fig. 9). Results show that MPH-treated rats showed smaller exploration times of objects both in the training ($p < 0.05$) and the test days ($p < 0.01$) (Fig. 9a). Control animals were able to distinguish the new object from the previous one ($p < 0.001$), whereas rats chronically treated with MPH were not able to do so ($p > 0.05$) (Fig. 9c), expressing a lower object recognition index ($p < 0.05$) when compared to the control group (Fig. 9d). As expected, Fig. 9b shows there were no differences in the exploration of objects on the training day ($p > 0.05$).

Discussion

The present study investigated the effects of chronic MPH treatment on the number of neurons and astrocytes in the hippocampus of juvenile rats. Results of flow cytometry, immunohistochemistry, and immunocontent analysis showed a reduction in neuron and astrocyte numbers in the hippocampus, suggesting that chronic administration of MPH affects the cell survival.

Generation and differentiation of neurons and glial cells are dependent upon cell-cell interactions mediated by a wide variety of growth factors and cytokines. In an attempt to investigate the mechanisms through MPH treatment decreases the number of astrocytes and neurons, we started testing whether MPH affects pivotal neurotrophins, such as BDNF and NGF. Results demonstrated that chronic MPH treatment decrease BDNF and pTrkB immunocontent, as well as NGF levels in hippocampus of juvenile rats. Neurotrophins, such as NGF and BDNF, activate Trk receptor tyrosine kinases through receptor dimerization, followed by autophosphorylation and resultant intracellular signaling. pTrkB is coexpressed with astroglia, neurons and neural precursors, suggesting a role for TrkB in newborn hippocampal cells. In addition, it is well known that BDNF signaling through its receptor TrkB can influence the morphology and synaptic connectivity of developing neurons [50, 51]. In this context, rats submitted to maternal separation, an animal model of vulnerability to drug dependence, anxiety, stress-induced illness, and depression, showed that BDNF levels were decreased in amygdala and nucleus accumbens, and NGF levels were decreased in the hippocampus, amygdala, and nucleus accumbens [52].

In order to evaluate the effects of MPH on neurotrophins and pTrkB in the hippocampus of juvenile rats, we investigated some signaling pathways that are regulated by these neurotrophins and are involved in cell death and survival mechanisms. We found that Akt and GSK-3β pathways were not altered, whereas pERK and PKCaMII were reduced after chronic MPH treatment in rat hippocampus. Agreeing with our findings, Lagace et al. [53] demonstrated that juvenile rats

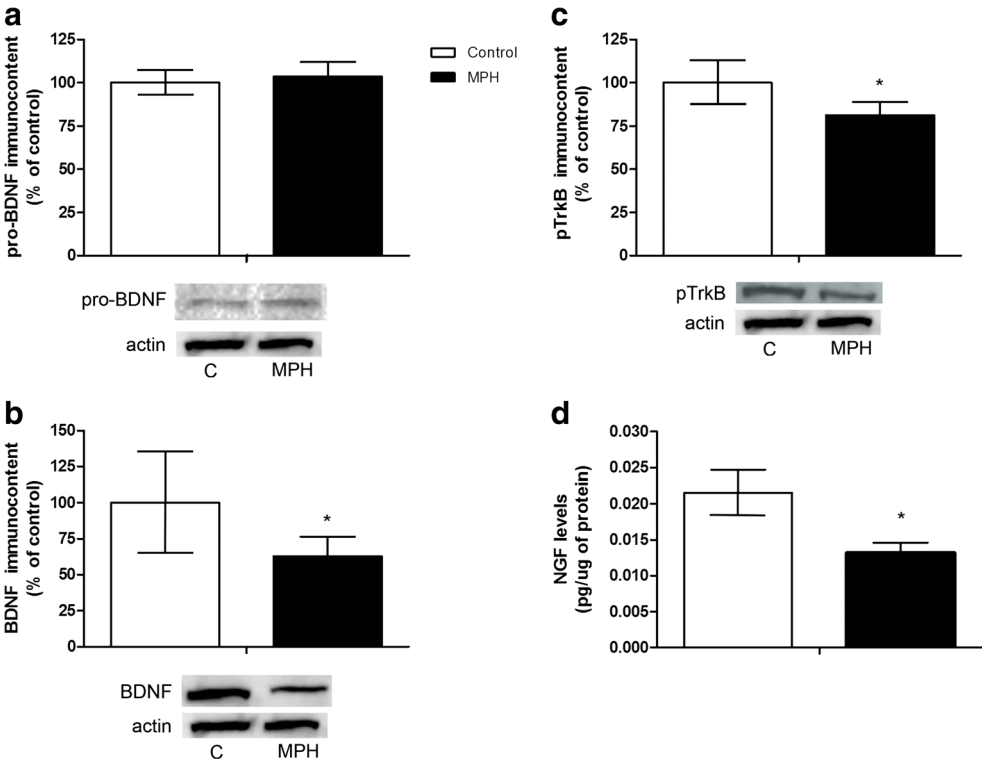


Fig. 4 Effect of chronic treatment with methylphenidate on immunocontent of the pro-BDNF (**a**), BDNF (**b**), pTrkB (**c**), and NGF levels (**d**) on hippocampus of juvenile rats. All *lanes* received equivalent amounts (30 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 6–7 animals in each group and expressed as percent of control. Different from control, $*p < 0.05$ (Student's *t* test). *BDNF* brain-derived neurotrophic factor, *TrkB* tyrosine kinase B receptor, *MPH* methylphenidate, *C* control, *NGF* nerve factor growth

treated with MPH (2 mg/kg twice a day for 15 days) showed a decrease in survival of new cells of the hippocampus, without altering cell proliferation. In addition, Schaeffer et al. [54] reported that chronic treatment with MPH (5 mg/kg for 30 days) did not alter the number of proliferating cells. In contrast, it recently reported an increase in cell proliferation and differentiation of neuroblasts after MPH administration (10 mg/kg once daily for 28 days) [55]. Based on this, our results suggest that the decrease in neural cells observed in hippocampus of juvenile rats could be explained, at least in part, by a decrease in BDNF-pTrkB immunocontent and NGF levels and this could underlie ERK and PKCaMII survival pathways inhibition. SNAP-25 is a pivotal component of the complex trans-SNARE, which is involved in the fusion of synaptic vesicles in the presynaptic neuron and is related to synaptogenesis and synaptic maturation of functional connectivity during brain development [28, 56]. In this study, we demonstrated that chronic MPH treatment reduces SNAP-25 immunocontent in hippocampus of juvenile rats. Dysfunction of SNAP-25 is linked to various human mental disorders, such as ADHD, schizophrenia, and early-onset bipolar disorder [57–59]. Based on this, our result could compromise cell events such as exocytosis, neurite outgrowth, neuronal development, as well as hormone and neurotransmitter release. All these events could be related to detriment of synaptogenesis and maturation of functional synaptic connectivity in the hippocampus of juvenile rats treated with MPH. In agreement with our result, Cheng et al. [60] showed that SNAP-25 has important role on behavioral, electrophysiological, and biochemical changes promoted by MPH. However, we cannot rule out the hypothesis that the reduction of SNAP-25 immunocontent in the hippocampus of juvenile rats exposed to chronic treatment with MPH may be a way that the body found to protect themselves from the toxic effects of increased release of neurotransmitters (glutamate, dopamine, noradrenaline, and others) in the synaptic cleft and/or a consequence of the reduction of neurons.

Neuroinflammation has been associated with increased expression of cytokines in the neurogenic niches, which directly impairs hippocampal-dependent forms of synaptic plasticity, potentially leading to cognitive impairment [32]. In the present study, we demonstrated that chronic MPH treatment increased the TNF-α and IL-6 levels, as well as caspase 3 cleaved and Iba-1 immunocontent in hippocampus of juvenile rats subject to chronic MPH treatment. It has been shown that TNF-α contributes to inhibition of synaptic plasticity and memory consolidation, as reported in patients with major disease disorder or in experimental models of depression, Parkinson's disease, and Alzheimer's disease, which are conditions characterized by progressive neurodegeneration as well as by an abnormal immune response due to hyper stimulation of microglia to produce inflammatory cytokines. Moreover, TNF-α reduced neurogenesis in adult rat hippocampus and promoted apoptosis in adult rats. Elevated levels of IL-6 reduce proliferation, gliogenesis, and neurogenesis and increase apoptosis in rat hippocampus by reducing soluble factors, such as the protein sonic hedgehog, which is known to promote neuronal differentiation [31]. In agreement with our

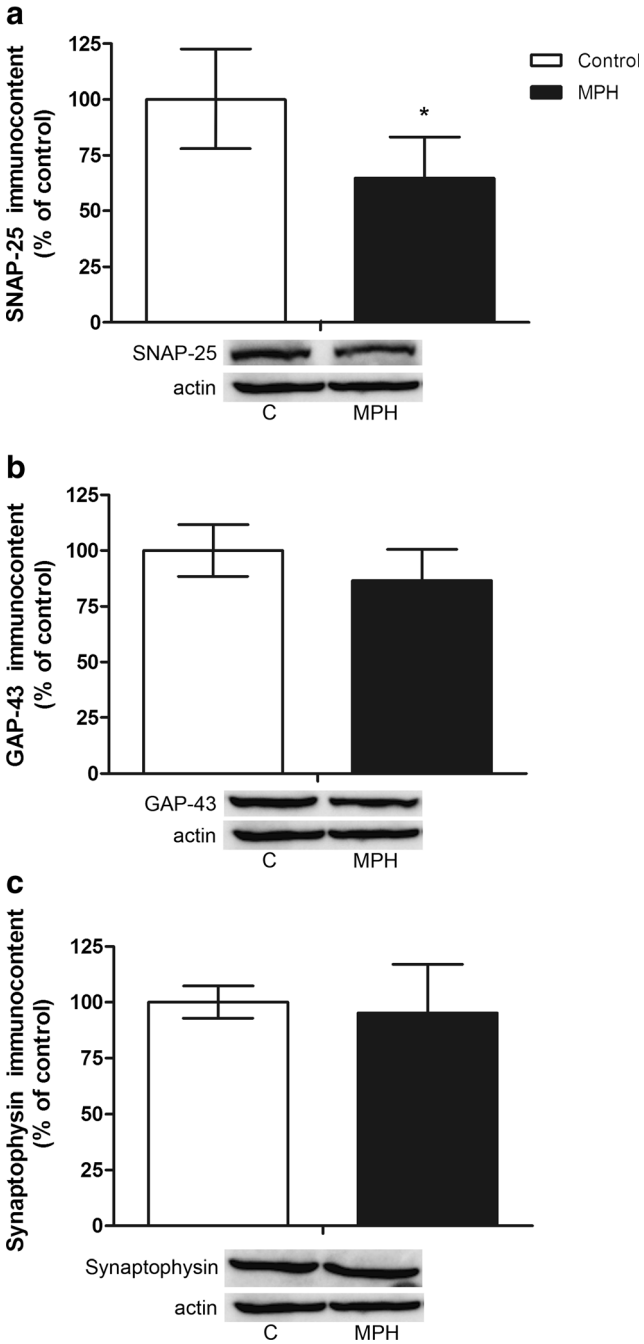


Fig. 5 Effect of chronic treatment with methylphenidate on immunocontent of the SNAP-25 (**a**), GAP-43 (**b**), and synaptophysin (**c**), on hippocampus of juvenile rats. All *lanes* received equivalent amounts (30 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 6–7 animals in each group and expressed as percent of control. Different from control, $*p < 0.05$ (Student's *t* test). *MPH* methylphenidate, *C* control, *SNAP-25* synaptosomal-associated protein 25, *GAP-43* growth associated protein 43

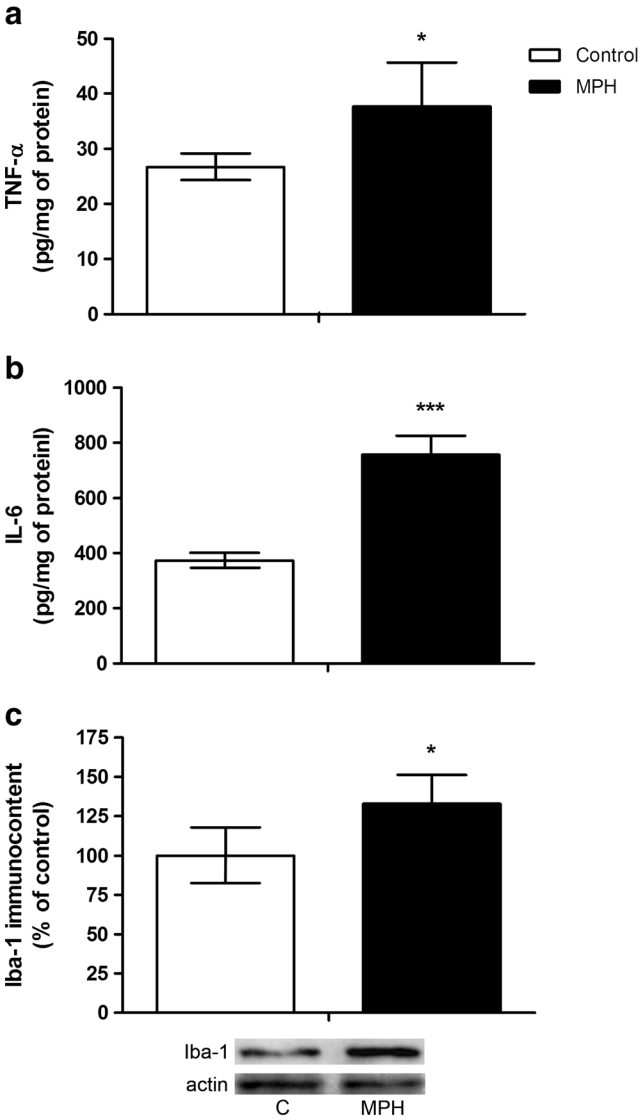


Fig. 6 Effect of chronic treatment with methylphenidate on TNF-α (**a**) and IL-6 (**b**) levels as well as on Iba-1 immunocontent (**c**) in the hippocampus of juvenile rats. Results are expressed as mean ± standard deviation for 5–6 animals in each group. Different from control, $*p < 0.05$ and $***p < 0.001$ (Student's *t* test). *C* control, *MPH* methylphenidate, *TNF-α* tumor necrosis factor alpha, *IL-6* interleukin-6, *Iba-1* ionized calcium-binding adaptor molecule 1

study, it has been shown that MPH induces microglial activation and increases of mRNA levels of IL-6 and TNF-α in the striatum of rats after treatment with MPH [33]. In addition, Bahcelioglu et al. [61] showed that MPH (5–20 mg/kg, 5 days a week for 3 months) induces astrogliosis, increasing the thickness of the basal membrane and increases endothelial vesicles in cells, suggesting that the MPH causes changes in the function of the blood–brain barrier. In this context, it is suggested that the increase in inflammatory markers and caspase 3 cleaved observed in the hippocampus of juvenile rats treated with MPH appear to be associated with neuronal and astrocyte loss.

In order of identify whether the immunohistochemical and biochemical alterations in hippocampus caused by chronic

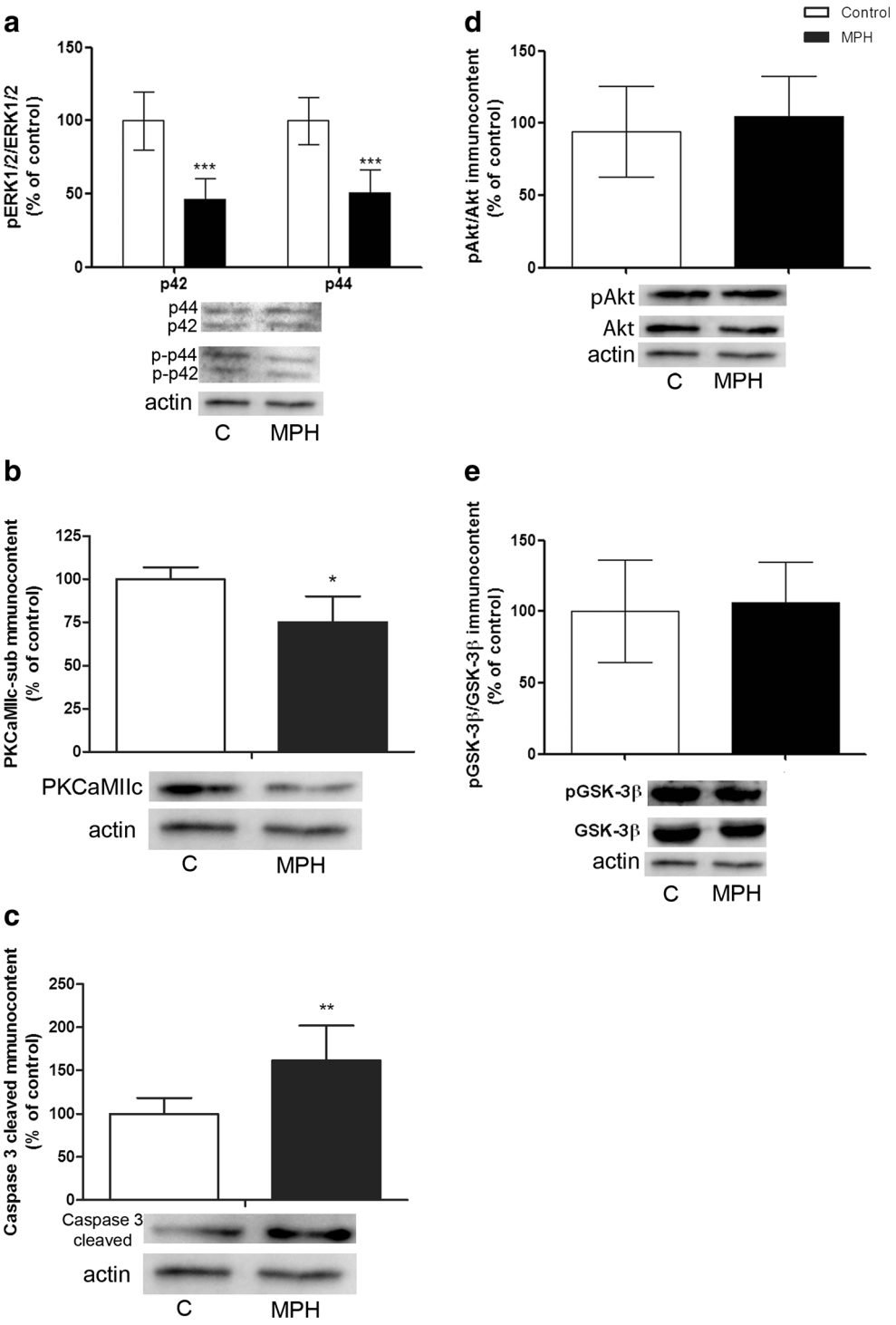


Fig. 7 Effect of chronic treatment with methylphenidate on immunocontent of the total ERK and pERK (**a**), PKCaMII (**b**), caspase 3 cleaved (**c**), total Akt and pAkt (**d**), and total GSK-3β and pGSK-3β (**e**), on hippocampus of juvenile rats. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 5–6 animals in each group and expressed as percent of control. Different from control, $*p < 0.05$ and $**p < 0.01$ (Student's *t* test). *MPH* methylphenidate, *C* control, *ERK* extracellular signal-regulated kinase ½, *pERK* extracellular signal-regulated kinase ½ phosphorylated, *Akt* protein kinase B, *pAkt* protein kinase B phosphorylated, *PKCaMII* calcium/calmodulin-dependent protein kinase II, *GSK-3β* glycogen synthase kinase 3 beta, *pGSK-3β* glycogen synthase kinase 3 beta phosphorylated

MPH administration in juvenile rats observed in this study coincides with impaired in hippocampal-dependent behavioral tasks, we initially assessed the performance of MPH-treated rats on elevated plus maze and open field. The findings showed that juvenile rats treated with MPH explored less the open arms in the elevated plus maze. The results obtained in the open field task reinforced the slightest interest in exploring the new environment. Moreover, we observed impairment of long-term memory in the object recognition task in MPH-treated rat. There are conflicting results regarding the anxiety-like behavioral and exploratory activity caused by MPH treatment. Britton et al. [62, 63] showed that MPH triggered anxiety-like behavior and enhanced the exploratory activity of the animals. Another study showed that MPH administered during development up to 35 days old caused a decrease in the anxiety-like behavior when rats were tested at

Table 2 Effect of chronic treatment with methylphenidate on the performance on elevated plus maze, open field day 1 and open field day 2

	Control	MPH
Elevated plus maze		
Total number entries	9.81 ± 2.86	7.35 ± 2.56*
Entries in open arms	3.09 ± 0.94	1.92 ± 0.99**
% of the time spent in open arms	4.94 ± 1.51	3.08 ± 1.60**
Open field day 1		
Distance traveled (m)	37.7 ± 5.6	31.6 ± 5.7*
Average speed (m/min)	3.7 ± 0.5	3.1 ± 0.5*
Crossings number	480.8 ± 69.1	422 ± 88.3
Maximum speed (m/min)	28.5 ± 15.6	20.9 ± 2.73
Distance traveled on the periphery (m)	32.8 ± 4.8	26.8 ± 5.2**
Average speed on the periphery (m/min)	3.53 ± 0.5	2.93 ± 0.5*
Mobile time on the periphery (min)	8.5 ± 0.4	7.9 ± 0.7*
Immobile time on the periphery (min)	0.8 ± 0.3	1.2 ± 0.8
Open field day 2		
Distance traveled (m)	28.6 ± 7.3	26.5 ± 9.9
Average speed (m/min)	2.8 ± 0.7	2.6 ± 1.0
Crossings number	366.2 ± 98.4	367.9 ± 130.4
Maximum speed (m/min)	21.9 ± 4.3	23.4 ± 9.7
Distance traveled on the periphery (m)	24.6 ± 5.3	22.4 ± 8.1
Average speed on the periphery (m/min)	2.6 ± 0.6	2.4 ± 0.9
Mobile time on the periphery (min)	7.3 ± 0.8	6.7 ± 1.9
Immobile time on the periphery (min)	2.2 ± 1.0	2.7 ± 2.1

Results are expressed as means ± standard deviation for 11–14 animals in each group. Different from control, *$p < 0.05$ and **$p < 0.01$ (Student's *t* test)

MPH methylphenidate

adulthood [64]. McFadyen-Leussis et al. [65] showed that prenatal exposure to MPH increased exploratory activity but also evoked anxiety-related behavior with no benefits on learning and memory tasks in mice. We have demonstrated previously that rats presented lower performance in the Water maze test after they had received MPH (2 mg/kg) from the 15th to the 45th day of age [66]. On the other hand, acute treatment in adult mice of 5 mg/kg of MPH improved, while the dose 50 mg/kg worsened object recognition memory and aversive memory [67]. Both dosing regimens did not change locomotor activity of animals and increased the number of entries and the time spent in open arms [68]. Our results suggest that rats chronically treated with MPH have less interest in exploring new environments and objects and thus the problems observed in memory could be explained, at least in part by acquisition deficits. In addition, we cannot rule out the possibility that MPH has affected motivation, attention, and sensorimotor function since the total time exploration was affected by MPH. In agreement with our study, Bolaños et al. [15] showed that adult rats exposed to repeated doses of MPH during their juvenile were less responsive with respect to motor activation exhibited by animals when first exposed to a novel environment. However, behavioral findings of the administration of MPH are clearly distinct and dependent on the schedule of administration and age of the animals. It is important to emphasize that our study aimed to investigate the effects of the MPH administered during development on behavior of these animals.

The understanding of the consequences of chronic treatment with MPH on early stages of brain development is very important since this psychostimulant has been used extensively in preschool age children and young adults who do not meet full diagnostic criteria for ADHD [4–7]. Experimental studies have shown changes on the dopaminergic system, redox status, mitochondrial function, glutamatergic/GABAergic systems, neuroinflammation, blood–brain barrier, and neurogenesis after MPH treatment [69], but still, it is unknown how early-life exposure to MPH affects adult hippocampal function [10]. The hippocampus plays a prominent role in affective behaviors, drug taking, and relapse [70–72] and MPH actions in the hippocampus may be central to MPH long-term effects since the treatment with this psychostimulant in adolescent rodents increases

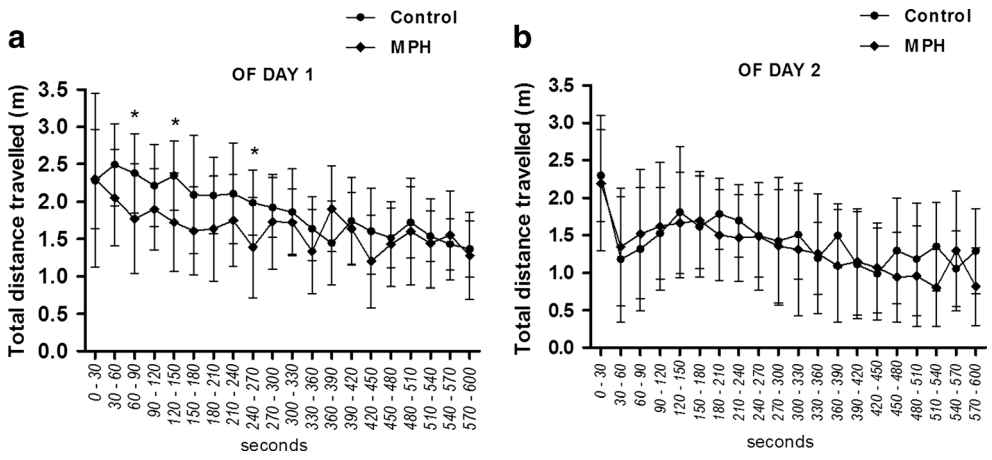
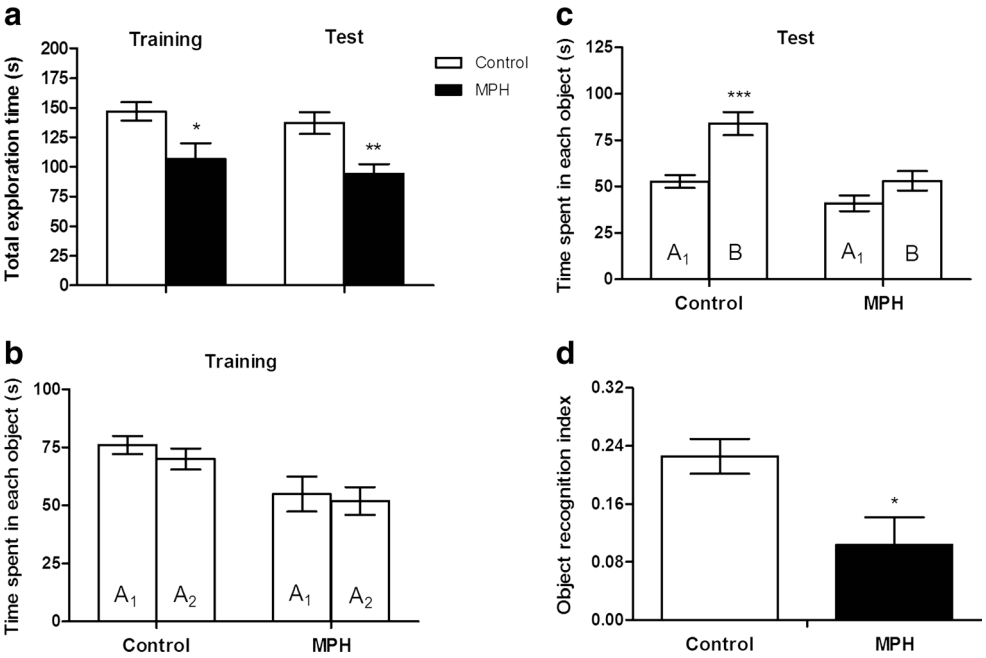


Fig. 8 Effect of chronic treatment with methylphenidate on the distance traveled in the open field task. The rats were exposed for 10 min to the open field on day 1 (**a**), and 24 h later, they were exposed again to the open field on day 2 (**b**). Results are expressed as mean ± standard deviation for 11–14 animals in each group. Different from control, *$p < 0.05$ (Student's *t* test). *OF* open field, *MPH* methylphenidate



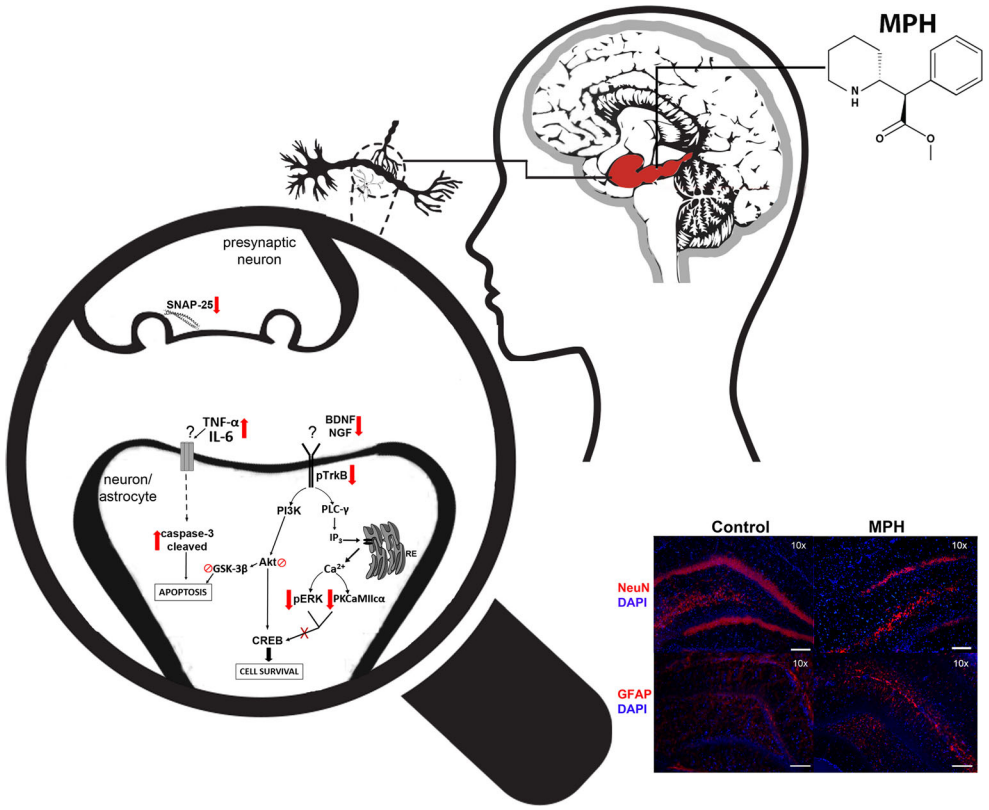


Fig. 9 Effects of chronic treatment with methylphenidate in the performance of juvenile rats on the object recognition task. Time total exploration (**a**), time spent in each object during the training (**b**), time spent in each object during the test (**c**), and object recognition index (**d**) are represented. Results are expressed as mean ± standard error mean for 11–14 animals in each group. Different from control, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ (Student's *t* test). *MPH* methylphenidate

Fig. 10 Graphical summary of the processes that were quantified throughout the investigation, highlighting the main effects of chronic early treatment with methylphenidate on crucial parameters for the proper functioning of central nervous system in hippocampus. In summary, we demonstrated that methylphenidate reduces the number of neurons and astrocytes as well as inhibit cell survival and active cell death pathways regulated by cytokines and neurotrophins in hippocampus of juvenile rats. *MPH* methylphenidate, *BDNF* brain-derived neurotrophic factor, *NGF* nerve growth factor, *pTrkB* tyrosine kinase B receptor phosphorylated, *SNAP-25* synaptosomal-associated protein 25, *TNF-α* tumor necrosis factor alpha, *IL-6* interleukin 6, *Akt* protein kinase B, *ERK* extracellular signal-regulated kinase ½, *PKCaMII* calcium/calmodulin-dependent protein kinase II, *CREB* cAMP response element-binding protein, *GSK-3β* glycogen synthase kinase 3 beta, *PI3K* phosphatidylinositol-3 kinase

norepinephrine levels in the hippocampus in a dose-dependency pattern [22]. Thus, identify MPH-induced changes in hippocampal physiology or function may be critical in interpreting of mechanisms that contribute to the persistent in behavior and in neurochemical changes associated to MPH exposure.

Summarizing, present study suggests that chronic MPH treatment caused a decrease in new environment and object exploration and thus the memory impairment observed could be explained, at least in part by acquisition deficits. These behavioral effects are associated to astrocyte and neuron loss and inhibition of cell survival and activation of cell death pathways regulated by cytokines and neurotrophins, as shown in Fig. 10. These findings provide additional evidence that early-life exposure to MPH can have complex effects, as well as offer new basis for the understanding of biochemical and behavioral consequences of chronic use of MPH during CNS development.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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3.6 Capítulo IV

Methylphenidate alters Akt-mTOR signaling in rat pheochromocytoma cells

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Methylphenidate alters Akt-mTOR signaling in rat pheochromocytoma cells

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ABSTRACT

The exponential increase in methylphenidate (MPH) prescriptions in recent years has worried researchers about its misuse among individuals who do not meet the full diagnostic criteria for attention-deficit/hyperactivity disorder (ADHD) such as young children and students in search of cognitive improvement or for recreational reasons. The action of MPH is based mainly on inhibition of dopamine transporter, but the complete cellular effects are still unknown. Based upon prior studies, we attempted to determine whether the treatment with MPH (1μM) influences protein kinase B-mammalian target of rapamycin complex 1 signaling pathways (Akt-mTOR), including translation repressor protein (4E-BP1) and mitogen activated protein kinase (S6K), in rat pheochromocytoma cells (PC12), a well characterized cellular model, in a long or short term. MPH effects on the Akt substrates [cAMP response element-binding protein (CREB), forkhead box protein O1 (FoxO1), and glycogen synthase kinase 3 beta (GSK-3β)] were also evaluated. Whereas short term MPH treatment decreased the pAkt/Akt, pmTOR/mTOR and pS6K/S6K ratios, as well as pFoxO1 immunocontent in PC12 cells, long term treatment increased pAkt/Akt, pmTOR/mTOR and pGSK-3β/GSK-3β ratio. Phosphorylation levels of 4E-BP1 were decreased at 15 and 30 min and increased at 1 and 6 h by MPH. pCREB/CREB ratio was decreased. This study shows that the Akt-mTOR pathway, as well as other important Akt substrates which have been described as important regulators of protein synthesis, as well as being implicated in cellular survival, synaptic plasticity and memory consolidation, was affected by MPH in PC12 cells, representing an important step in exploring the MPH effects.

1. Introduction

Methylphenidate (MPH), a central nervous system (CNS) stimulant in use for approximately 70 years, is the most frequently prescribed drug for the symptomatic treatment of attention-deficit/hyperactivity disorder (ADHD) (Johnston et al., 2011a,b). Most recently, researchers have been quite concerned about the increased incidence of its misuse among individuals not meeting the criteria for ADHD, as a "cognitive enhancer" and as an alternative to other psychostimulants for recreational use (Johnston et al., 2011a,b).

The MPH neuropharmacological profile is similar to amphetamine and cocaine (Challman and Lipsky, 2000), but the complete cellular effects of MPH are still unknown (Grünblatt et al., 2013; Gumustas et al., 2017). Functionally, MPH has been proposed as a high-affinity inhibitor of dopamine (DA) and norepinephrine transporters, blocking the inward transport of DA and norepinephrine (Kuczenski and Segal, 2001; Volkow et al., 2001). DA transporter (DAT) plays an essential role in terminating DA signaling, since DA levels are primarily regulated by removing extracellular DA and recycling it back to the neuron (Amara and Kuhar, 1993).

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; β-Arr2, β-arrestin 2; CNS, central nervous system; DA, dopamine; DAT, dopamine transporter; D2R, dopamine D2 receptor; MPH, methylphenidate; Akt, protein kinase B; pAkt, protein kinase B phosphorylated; mTOR1, mammalian target of rapamycin complex 1; pmTOR1, mammalian target of rapamycin complex 1 phosphorylated; 4E-BP1, translation repressor protein; p70, S6K mitogen activated protein kinase; pp70, S6K mitogen activated protein kinase phosphorylated; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; pCREB, cAMP response element-binding protein phosphorylated; GSK-3β, glycogen synthase kinase 3 beta; pGSK-3β, glycogen synthase kinase 3 beta phosphorylated; pFoxO1, forkhead box protein O1 phosphorylated; PC12, pheochromocytoma cells; PP2A, phosphatase-2A

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DA is a major modulatory neurotransmitter that controls a number of important physiological activities and behavioral states of the mammalian brain, including motor control, motivation, and mood (Gingrich and Caron, 1993). Dysregulation of dopaminergic neurons has been implicated in the pathogenesis of Parkinson's disease, schizophrenia, and drug addiction (Sotnikova et al., 2006). Approximately 80% of the DA found in the CNS is localized in the striatum where it binds to DA receptors on medium sized spiny projection neurons (Carlsson, 1959), which specifically express the DA receptors, D1R and D2R (Gingrich and Caron, 1993). In this sense, Sadasivan et al. (2012) showed that rats pre-treated with MPH have a higher neuronal loss in striatum when subjected to MPTP (a drug used to induce experimental Parkinson disease).

There are several studies suggesting a specific role of D2R in regulating DAT function (Meiergerd et al., 1993; Batchelor and Schenk, 1998; Dickinson et al., 1999), as well as a physical interaction between them (Bolan et al., 2007). DAT blocking by MPH is widely established (Challman and Lipsky, 2000), however the MPH effects through D2R have not been explored so far. In this context, recent *in vivo* studies revealed that striatal D2-class receptors exert their action in a cAMP-independent manner by promoting the formation of a signaling complex composed of protein kinase B (Akt), protein phosphatase-2 A (PP2 A), and β-arrestin 2 (β-Arr2) (Beaulieu et al., 2004, 2005). Formation of this complex leads to the inactivation of Akt after the dephosphorylation of its regulatory threonine 308 (Thr-308) residue by PP2 A (Beaulieu et al., 2005). Inactivation of Akt in response to DA results in the activation of glycogen synthase kinase 3β (GSK-3β), which in turn contributes to the expression of DA-associated behaviors (Beaulieu et al., 2004). In this context, decreased responses to rewarding stimuli, as well as increased depressive- and anxiety-like behaviors were displayed by juvenile rats exposed to MPH (Achat-Mendes et al., 2003; Adriani et al., 2006; Andersen et al., 2002; Bolanos et al., 2003; Carlezon et al., 2003; Mague et al., 2005).

The mTORC1 (mammalian target of rapamycin complex 1) pathway is a well-described regulator of protein synthesis downstream of Akt action. It is of particular interest, since synaptic proteins involved in synaptic signaling and mTORC1-dependent translation which has been implicated in synaptic plasticity, memory consolidation (Antion et al., 2008; Hoeffer et al., 2011; Bhattacharya et al., 2012; Santini and Klann, 2011). Akt activation relieves inhibition of mTORC1 activity, which, in turn, promotes cap-dependent translation by phosphorylating and inhibiting 4E-BP (4E-binding protein). p70 S6 kinase (S6K), another downstream effector of mTORC1, phosphorylates ribosomal protein S6, which is also associated with increased translation (Meyuhas, 2008; Ruvinsky and Meyuhas, 2006). In this sense, there is evidence that dopaminergic neurotransmission is influenced by signaling pathways involving S6 (Welsh et al., 1998; Oak et al., 2001).

Other important factors that can regulate protein synthesis, including synaptic proteins, have been also involved with psychostimulant treatment (Bartl et al., 2010; Zhenga et al., 2013). In this context, Bartl et al. (2010) showed that the levels of cAMP (cyclic adenosine monophosphate) response element binding (CREB) have been increased after MPH treatment. On the other hand, the levels of pFoxO1 in the striatum, transcription factor forkhead box (class O1), were decreased after d-amphetamine administration to Sprague-Dawley rats (Zhenga et al., 2013).

The use and misuse of MPH is increasing in children and adolescents. However, there is little information about the effects of this psychostimulant on cellular signaling proteins. Here, we investigated the effects of MPH on the Akt-mTOR signaling and on the mTORC1 substrates, 4E-BP1 and S6K, in PC12 cells. We have chosen PC12 cells, which is an important cellular model to study signal transduction. MPH effects on CREB, pFoxO1, and GSK-3β activities were also investigated. We hypothesized that changes in signaling proteins may be involved in the cellular MPH effects since their activities are regulated by D2R, which is widely expressed in brain, as well as is tightly regulated by DAT, MPH's main target.

Table 1
Primary antibodies used for western blot analyzes.

Primary antibody	Dilution	Purchased from
rabbit anti-pAkt (Thr308)	1:1000	Cell Signaling (9275 L)
rabbit anti-Akt	1:1000	Cell Signaling
rabbit anti-pmTOR (Ser2448)	1:1000	Cell Signaling (2971)
rabbit anti-mTOR	1:1000	Abcam (ab2732)
rabbit anti-4E-BP1	1:1000	Cell Signaling (9452)
rabbit anti-pp70 S6 K (Thr389)	1:1000	Cell Signaling (9205)
rabbit anti-p70 S6 K	1:1000	Millipore (03-490)
rabbit anti-pCREB (Ser133)	1:1000	Cell Signaling (9198S)
mouse anti-CREB	1:1000	Abcam (ab32515)
rabbit anti-pGSK-3β (Ser9)	1:1000	Cell Signaling (9336)
rabbit anti-GSK-3β	1:1000	Transduction (G22320)
rabbit anti-pFoxO1 (Ser256)	1:1000	ThermoFisher (17907)
mouse anti-β-actin	1:3000	Sigma

pAkt, protein kinase B phosphorylated; Akt, protein kinase B; pmTOR; mammalian target of rapamycin phosphorylated; mTOR; mammalian target of rapamycin; 4E-BP1; repressor of mRNA translation; pp70 S6 K; mitogen activated protein kinase phosphorylated; p70 S6 K; mitogen activated protein kinase; pCREB; cAMP response element-binding protein phosphorylated; CREB; cAMP response element-binding protein; pGSK-3β, glycogen synthase kinase 3 beta phosphorylated; GSK-3β, glycogen synthase kinase 3 beta; pFoxO1; forkhead box protein O1 phosphorylated.

2. Experimental Procedures

2.1. Cell line and reagents

The cell culture model chosen for this investigation was the rat PC12 cells derived from rat pheochromocytoma, a tumor arising from the chromaffin cells of the adrenal medulla and a very important and well known model for studying neurobiochemical and neurobiological events (Jaeger, 1985). These cells have been widely used as an *in vitro* experimental model to study the effects of various neuroactive compounds and contain many membrane-bound and cytosolic neuron-associated macromolecules (Shafer and Atchison, 1991). Previously it was reported that differentiated PC12 cells highly express D2R (Zhu et al., 1997) and DAT (Kadota et al., 1996), which makes this cell line an appropriated model to study the effects of MPH.

Antibodies were obtained according to Table 1. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Cell culture and treatment

The PC12 cell culture was performed as previously described (Rodriguez-Blanco et al., 2008). Briefly, PC12 cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, 100 U/mL benzyl penicillin, and 100 mg/l streptomycin (Gibco, Grand Island, NY, USA). For all experiments, the cells were seeded on 6-well plates at a density of 1.0×10^5 cells/mL. Cells were treated with DMEM or MPH (1μM) for a long (15 min, 30 min, 1 h, 6 h and/or 24 h) or short (5 min, 10 min, 15 min, 20 min, 25 min and 30 min) term treatment with six wells in each group. All of the experiments were repeated three times in different batches of cells.

This dose (1μM) was selected because has been showed that micromolar doses of MPH produces an extracellular DA level higher than the intracellular level, which is in accordance with previous reports showing that MPH increases the extracellular level of DA (Kuczenski and Segal, 1997; Easton et al., 2007; Volz et al., 2008).

MPH was protected from light for all treatments, and the dilutions were always freshly prepared. The cells were incubated with MPH under dark conditions in order to reduce MPH degradation.

2.3. Western blot analysis

After MPH treatment, the cells were harvested using cell scrapers and washed in ice-cold PBS; following which, they were lysed with two different ice-cold lysis buffers (Rodriguez-Blanco et al., 2008). The concentration of total protein isolated was measured using the Bradford assay (Bio-Rad). 20–30 µg of the protein was separated by 4–12% Bis-Tris precast gels (Invitrogen). 17.5% Tris-Tricine precast gels (Bio-Rad) were used to separate 4E-BP1 different phosphorylation levels. The proteins were transferred to a 0.2-µm nitrocellulose membrane (GE Healthcare). The membrane was blocked by 3% BSA in TBST for 1 h at room temperature (22 °C) and then incubated overnight with primary antibodies (Table 1) diluted in 3% BSA in TBST (Sigma-Aldrich). The blot was then washed with TBST followed by incubation with HRP-conjugated secondary antibody in 3% BSA in TBST for 1 h at room temperature diluted 1:10,000. The blot was washed twice for 5 min with TBST and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). Membranes were re-probed for anti-β-actin immunoreactivity. The membranes were developed in a photodocumenter, and band intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the web site (http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-actin as a loading control to assess protein levels. The data used in the statistical analysis were obtained from the ratio of the protein studied and β-actin density unit lines.

2.4. Protein determination

The protein content of samples was determined using bovine serum albumin as standard, according to Bradford (Bradford, 1976).

2.5. Statistical analysis

Statistical analyses of the Western blot data were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer. Statistical tests included one-way ANOVA followed by Tukey's post hoc analysis. Shapiro-Wilk test was used to evaluate whether data were presenting a normal distribution. P-values of < 0.05 were regarded as statistically significant. For a better comparison of the data, we normalized the results and converted the data into percentages. The control group was set as 100% and the results are expressed as means ± standard deviation.

3. Results

3.1. Effect of MPH on Akt

Initially, we evaluated the effects of long term MPH treatment on the pAkt(Thr308)/Akt ratio in PC12 cells. Fig. 1A shows that MPH significantly decreased the pAkt(Thr308)/Akt ratio in 15 min ($p < 0.001$), increased in 1 h ($p < 0.05$) but did not alter in 30 min, 6 h and 24 h ($p > 0.05$) when compared to controls.

To closely observe the effect of MPH treatment, we also evaluated the effects of short term MPH treatment on the pAkt(Thr308)/Akt ratio. As we can see in Fig. 1B, MPH decreased the pAkt(Thr308)/Akt ratio in PC12 cells after 5 min ($p < 0.05$), 10 min ($p < 0.05$), 15 min ($p < 0.001$), 20 min ($p < 0.01$), and 25 min ($p < 0.05$), but did not alter in 30 min ($p > 0.05$).

3.2. Effect of MPH on mTOR

The next step was to evaluate the effects of long and short term MPH treatment on the pmTOR/mTOR ratio in PC12 cells since the Akt-mTOR pathway is the one well-described cell signaling pathway (Antion et al., 2008; Hoeffer et al., 2011; Bhattacharya et al., 2012; Santini and Klann, 2011). As could be expected, Fig. 2A shows that MPH treatment reduced pmTOR/mTOR ratio at 15 min ($p < 0.05$) and increased at 1 h ($p < 0.05$), without altering at 30 min and 6 h ($p > 0.05$). Closely, as we can see in Fig. 2B, MPH decreased the pmTOR/mTOR ratio at 5, 10 and 15 min ($p < 0.05$) in PC12 cells.

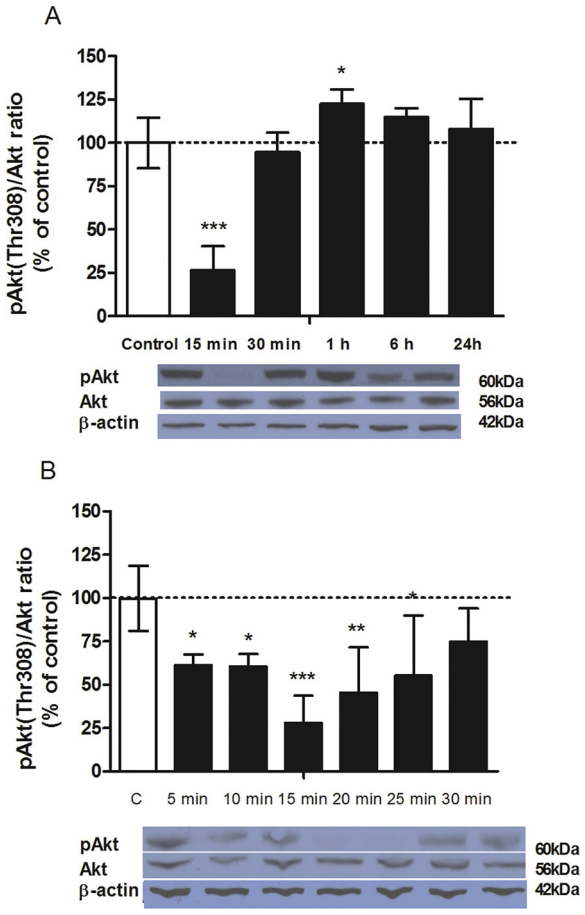

Fig. 1. Effect of MPH on pAkt(Thr308)/Akt ratio after a long (A) and a short (B) term treatment in PC12 cells. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 5–6 treatments in each group and expressed as percent of control. Different from control, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (One-way ANOVA). MPH, methylphenidate; Akt, protein kinase B; pAkt, protein kinase B phosphorylated.

3.3. Effect of MPH on the 4E-BP1 phosphorylation levels

Since dephosphorylation/phosphorylation balance of the 4E-BP1 may be affected by Akt-mTOR pathway, 4E-BP1 phosphorylation levels were investigated in PC12 cells after a long and short term MPH treatment. First, we separated the different 4E-BP1 phosphorylation levels in 17.5% Tris-Tricine precast gels (Bio-Rad). After, we quantified the top and bottom bands, which correspond to the fraction more and less phosphorylated, respectively.

Fig. 3A shows that long term MPH treatment decreased the top band immunocontent (more phosphorylated one) in 15 min ($p < 0.001$) and increased at 1 and 6 h ($p < 0.001$). MPH did not affect this parameter at 30 min ($p > 0.05$) when compared to PC12 cells not treated with MPH. As supposedly expected, MPH treatment increased the bottom band immunocontent (less phosphorylated one) at 15 and 30 min ($p < 0.001$) and decreased at 1 h ($p < 0.01$). No significant effects were observed after 6 h of MPH treatment ($p > 0.05$) (Fig. 3B).

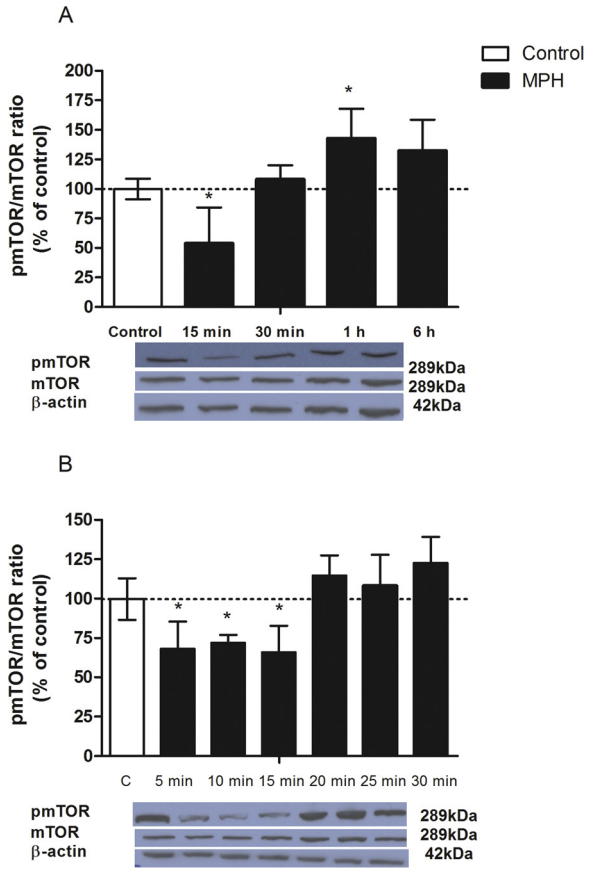

Fig. 2. Effect of MPH on pmTOR/mTOR ratio after a long (A) and a short (B) term treatment in PC12 cells. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 4–6 treatments in each group and expressed as percent of control. Different from control, *$p < 0.05$ (One-way ANOVA). MPH, methylphenidate; mTOR, mammalian target of rapamycin; pmTOR, mammalian target of rapamycin phosphorylated.

Fig. 3C shows that short term MPH treatment decreased the top band immunocontent at 5, 10, 15, 20 and 25 min ($p < 0.001$), when compared to PC12 cells not MPH treated. No significant effect was observed in 30 min after MPH treatment ($p > 0.05$). On the other hand, Fig. 3D shows that MPH treatment increased the bottom band immunocontent at 5, 10, 15, 20, 25 ($p < 0.001$) and 30 min ($p < 0.01$). These results suggest that MPH promotes a 4E-BP1 hypophosphorylation after a short term treatment and a 4E-BP1 hyperphosphorylation after a long term treatment, which are in agreement with the Akt-mTOR results showed above.

3.4. Effect of MPH on the p70 S6K

In order to investigate the MPH effect on another downstream effector of mTORC1, phospho p70 S6K/p70 S6K ratio was evaluated in PC12 cells. Fig. 4 shows that MPH significantly decreased the phospho p70 S6K/p70 S6K ratio in PC12 cells after 15 min ($p < 0.05$), but did not change in any of the other evaluated times ($p > 0.05$).

3.5. Effect of MPH on CREB, GSK3β and FoxO1

Finally, we evaluated whether CREB, GSK-3β, and FOXO1, which are also Akt substrates, respond to MPH treatment in PC12 cells. As we can see in Fig. 5A, MPH significantly decreased pCREB/CREB ratio at 30 min and 1 h ($p < 0.01$), but did not alter at 15 min and 6 h ($p > 0.05$). pGSK-3β/GSK-3β ratio was increased by MPH in PC12 cells at 1 h ($p < 0.05$), but not altered at 15 min, 30 min and 6 h ($p > 0.05$) (Fig. 5B). Fig. 5C shows that MPH decreased pFoxO1 immunocontent at 15 min ($p < 0.05$), but did not alter at 30 min, 1 h and 6 h ($p > 0.05$), when compared to PC12 cells not MPH treated.

4. Discussion

PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla, has been widely used as an *in vitro* experimental model to study Parkinson's disease (Pan et al., 2012; Xu et al., 2013), and ADHD (Grünblatt et al., 2013). PC12 cells are also used to study the effects of various neuroactive compounds such as cocaine (Shafer and Atchison, 1991; Imam et al., 2005), bromocriptine and quinpirole (dopamine D2 agonists) (Chiasson et al., 2006), himantane (antiparkinsonian drug) (Abaimov et al., 2008), 6-hydroxydopamine (Xu et al., 2013), and MPH (Grünblatt et al., 2013; Bartl et al., 2010), since it is a dopaminergic neuronal cell and can synthesize, store, secrete, and take up DA (Pan et al., 2012).

Based on that, we investigated the PC12 cells response to MPH treatment, analyzing Akt-mTOR pathway, as well as the mTOR substrates, 4E-BP1 and S6K kinase. CREB, GSK-3β, and FoxO1 response to MPH treatment were also investigated. Results show that short term MPH treatment decreased pAkt(Thr308)/Akt, pmTOR/mTOR, and pS6K/S6K ratio, as well as pFoxO1 immunocontent in PC12 cells; on the other hand, long term treatment increased pAkt(Thr308)/Akt, pmTOR/mTOR and pGSK-3β/GSK-3β ratio. Phosphorylation levels of 4E-BP1 were decreased at 15 and 30 min and increased at 1 and 6 h after MPH treatment. pCREB/CREB ratio was decreased by MPH. These findings suggest that MPH alters cell signaling in PC12 cells and its responses differ according to the time of exposure to this psychostimulant.

It is known that MPH increases DA levels in various brain regions through DAT blocking and this monoamine is an important modulatory effect on neurogenesis (Reinoso et al., 1996; Todd, 1992). An important physical interaction between DAT and D2R has been demonstrated to regulate DAT cell surface expression (Bolan et al., 2007). Activation of D2R is widely associated with inhibition of Akt by dephosphorylation of its regulatory threonine 308 residue after the formation of a signaling complex composed of Akt, PP2 A, and β-Arr2 (Beaulieu et al., 2004, 2005). Here, we found that MPH decreased the pAkt(Thr308)/Akt ratio in PC12 cells probably by D2R/PP2 A activation promoted by increased DA levels triggered by MPH treatment in a short term treatment. However, pAkt(Thr308)/Akt ratio was increased after a long term MPH treatment (1 h) in PC12 cells. These results are intriguing. Bartl et al. (2010) showed that extracellular DA metabolites DOPAC and HVA levels are higher and correlated with a bigger breakdown of DA after MPH treatment. They have also suggested that the degradation of DA seems to be enhanced after MPH treatment through the activation of monoamine oxidase-B or catechol-O-methyltransferase activities. Based on this, we suggest that the decrease in pAkt(Thr308)/Akt ratio in the first minutes, followed by its increase in PC12 cells after MPH treatment, may be associated with increased DA levels and D2R/PP2 A activation, followed by its increased degradation and, consequently, a decrease in their levels and D2R/PP2 A inactivation. According to these findings, it was showed that D2R can regulate Akt dephosphorylation at relatively high drug doses (e.g., 3 mg/kg amphetamine or 6 mg/kg apomorphine) (Beaulieu et al., 2007a, 2007b). Still in this sense, we have shown that MPH increased PP2 A immunocontent in rat hippocampus after a chronic MPH exposure (Schmitz et al., 2018).

The cellular mTORC1 kinase is a key target of the D2R/Akt pathway that plays important roles in neuronal functions, including control of synaptic plasticity, long term potentiation, axonal growth, and regeneration (Willis and Twiss, 2006; Lin and Holt, 2008; Richter and Klann, 2009). These processes require protein synthesis, which can be potentially regulated by the mTORC1 substrates S6K and eIF4E binding proteins (4E-BPs) (Ma and Blenis, 2009). Whereas S6K is activated upon

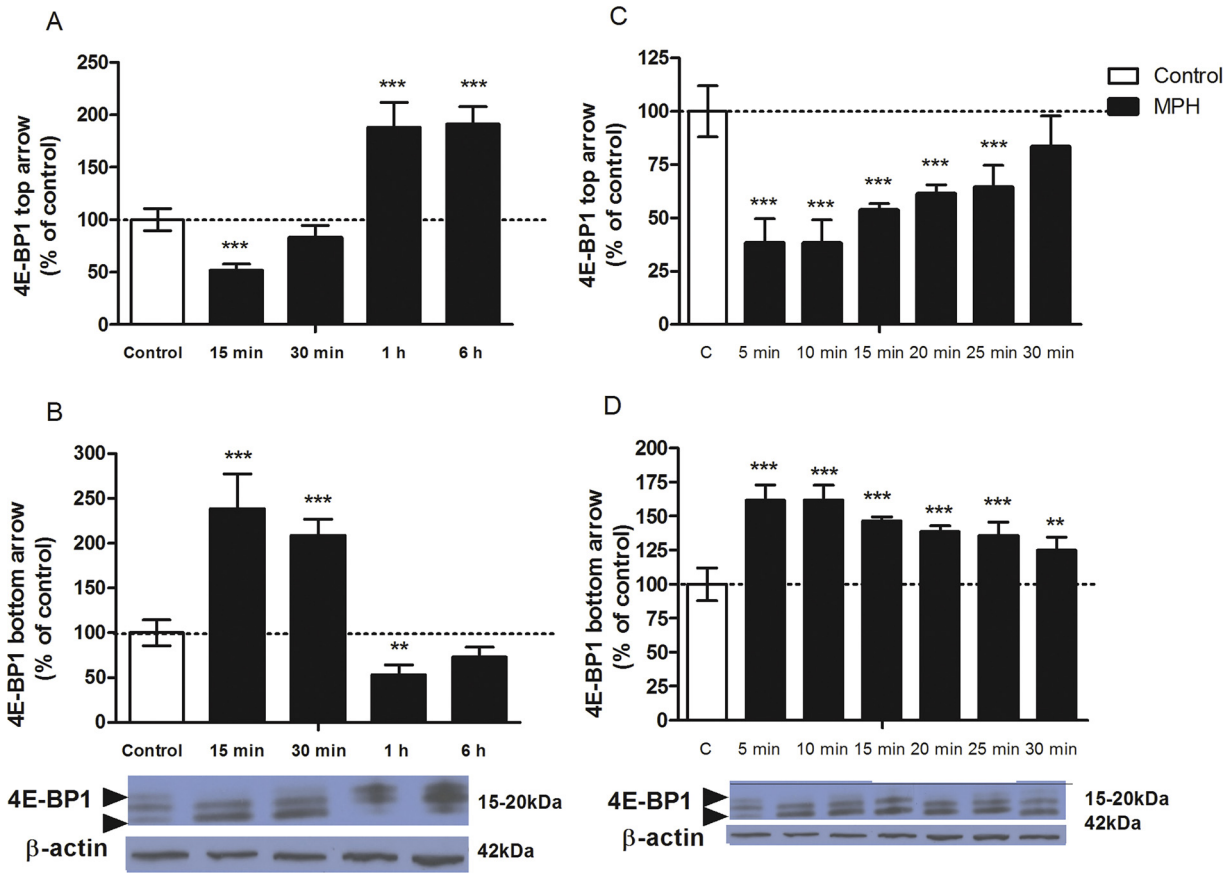

Fig. 3. Effect of MPH on 4E-BP1 phosphorylation levels after a long (A,B) and a short (C,D) term treatment in PC12 cells. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Slow migrating hyperphosphorylated (top arrow) and faster-migrating hypophosphorylated (bottom arrow) 4E-BP1 forms are indicated at the left. Results are expressed as mean ± standard deviation for 4–6 treatments in each group and expressed as percent of control. Different from control, $^{**}p < 0.01$ and $^{***}p < 0.001$ (One-way ANOVA). MPH, methyphenidate; 4E-BP1, repressor of mRNA translation.

phosphorylation by mTORC1, 4E-BP1 is inhibited. S6K stimulates translation elongation mainly by phosphorylating ribosomal protein S6. Alternatively, 4E-BP1 selectively represses cap-dependent translation by associating with the cap-binding initiation factor eIF4E and inhibiting 40S ribosome recruitment to mRNA (Ma and Blenis, 2009). 4E-BP1 is inactivated by mTORC1 phosphorylation on T37/46 by mTORC1 (Gingras et al., 1999). Our results show that MPH decreases pmTORC1/mTORC1, as well as phospho p70 S6K/p70 S6K ratio in PC12 cells after a short term treatment. We also found that 4E-BP1 hyperphosphorylated levels were decreased, whereas 4E-BP1 hypophosphorylated levels were increased in PC12 cells after a short term treatment. These results corroborate the decreased in pAkt(thr308)/Akt ratio observed, as well as suggest a mTORC1-dependent translation impairment induced by MPH treatment, affecting processes which require protein synthesis as synaptic plasticity, axonal growth, and regeneration. On the other hand, after a long term treatment, corroborating the results observed for Akt, the pmTORC1/mTORC1 ratio was increased by MPH. We also observed that the 4E-BP1 hyperphosphorylated levels were increased after a long term MPH treatment, 1 h and 6 h, with no alterations in phospho p70 S6K/p70 S6K ratio. Consistently, we observed that the response to MPH treatment differs according to the time of treatment probably due to changes in DA levels caused by this psychostimulant. Also, we cannot rule out the hypothesis that these effects re-establish the basal protein synthesis regulated by Akt-mTORC1, as well as S6K and 4E-BP. The observed results in a short term treatment suggest that processes which require protein synthesis as synaptic plasticity, axonal growth, and regeneration are impaired by MPH. On the other hand, after a long term treatment, we observed the opposite effects. In this context, antipsychotics treatment like haloperidol, which are DA receptor antagonists (mainly D2R), have been associated with Akt phosphorylation and increased mTORC1-dependent translation, as well as with an increase in branching, spine number (Bowling et al., 2014), and morphological complexity (Navari and Dazzan, 2009; Benes et al., 1983; Meredith et al., 2000).

CREB, a transcription factor that activates target genes through cAMP response elements, is activated by phosphorylation by various signaling pathways including Akt (Du and Montminy, 1998). CREB is able to mediate signals from numerous physiological stimuli, resulting in regulation of a broad array of cellular responses, such as playing a key role in promoting neuronal survival, precursor proliferation, neurite outgrowth and neuronal differentiation in certain neuronal populations (Redmond et al., 2002), as well as is involved in learning and memory in several organisms (Guzowski and McGaugh, 1997). Here, we showed that MPH decreased pCREB/CREB ratio in PC12 cells. This result suggests that MPH treatment triggers a loss in the key functions of CREB in PC12 cells. In this context, Chase et al., (2003) showed that MPH administration alters c-fos expression in the brain of juvenile rats. Decrease in gene expression level of synaptotagmin 1 and 4, syntaxin 1a, and synaptic vesicle glycoprotein 2C in PC12 cells were also associated with MPH treatment (Bartl et al., 2010).

Considering that GSK-3β is a critical downstream element of the Akt cell survival pathway and brain Akt-GSK-3β signaling has been shown to be responsive to DA (Beaulieu et al., 2004, 2005, 2007a,b), typical/atypical antipsychotics (Beaulieu et al., 2007a, 2007b; Emamian et al., 2004; Cyr et al., 2003), antidepressants (Cyr et al., 2003), and mood stabilizers (Beaulieu et al., 2004; Gould et al., 2004). In this study, we

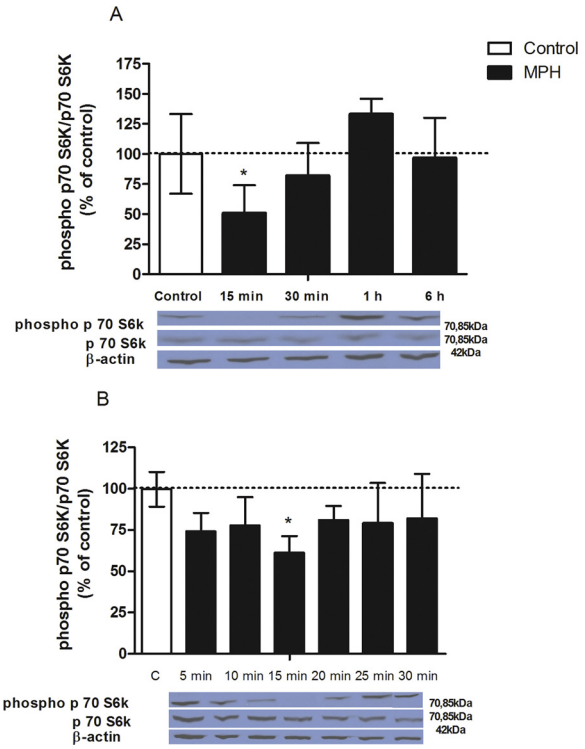

Fig. 4. Effect of MPH pS6K/S6K ratio after a long (A) and a short (B) term treatment in PC12 cells. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 4–6 treatments in each group and expressed as percent of control. Different from control, *p < 0.05 (One-way ANOVA). MPH, methylphenidate; p70 S6K, mitogen activated protein kinase; phospho p70 S6K, mitogen activated protein kinase phosphorylated.

investigated whether MPH treatment affects pGSK-3β/GSK-3β ratio in PC12 cells. Contrary to expected, since pAkt(Thr308)/Akt ratio was decreased by MPH in a short term treament, pGSK-3β/GSK-3β was not altered in 15 and 30 min. However, we found that pGSK-3β/GSK-3β ratio was increased at 1 h, corroborating the Akt response at this time, as well as suggest a possible involvement of the Akt–GSK-3β pathway in the effects of MPH. In this context, genetic inactivation of Akt1 or GSK-3β, administration of GSK-3 inhibitors, or uncoupling of Akt from dopamine receptors in β-Arr2 knock-out mice have been shown to affect DA-related changes in locomotor activity or sensory motor gating (Beaulieu et al., 2004, 2005; Emamian et al., 2004).

FoxO1, a downstream target of Akt, promotes apoptosis and cell-cycle inhibition (van der Horst and Burgering, 2007; Wen et al., 2012). Here, we showed that pFoxO1 immunocontent (inactive form) was decreased at 15 min after MPH treatment in PC12 cells. This result corroborate the decreased in pAkt(thr308)/Akt ratio observed, as well as suggest a possible involvement of the Akt–FoxO1 pathway in the effects of MPH. Corroborating our finding, Zheng et al. (2013) suggested that the Akt–FoxO1 pathway may be a common target for the action of dopaminergic drugs, and its modulation may be relevant to the treatment of neuropsychiatric disorders. These authors showed that the administration of d-amphetamine to Sprague–Dawley rats resulted in a concomitant decrease in levels of pAkt and pFoxO1 in the striatum, whereas lithium chloride (a drug used primarily in the treatment of bipolar disorder, which is reported to interact with the DA system and antagonize some DA-related behaviors) exerted the opposite effect. They also showed that pretreatment of animals with lithium attenuated amphetamine-induced locomotor activity and decreased prepulse inhibition (Zheng et al., 2013).

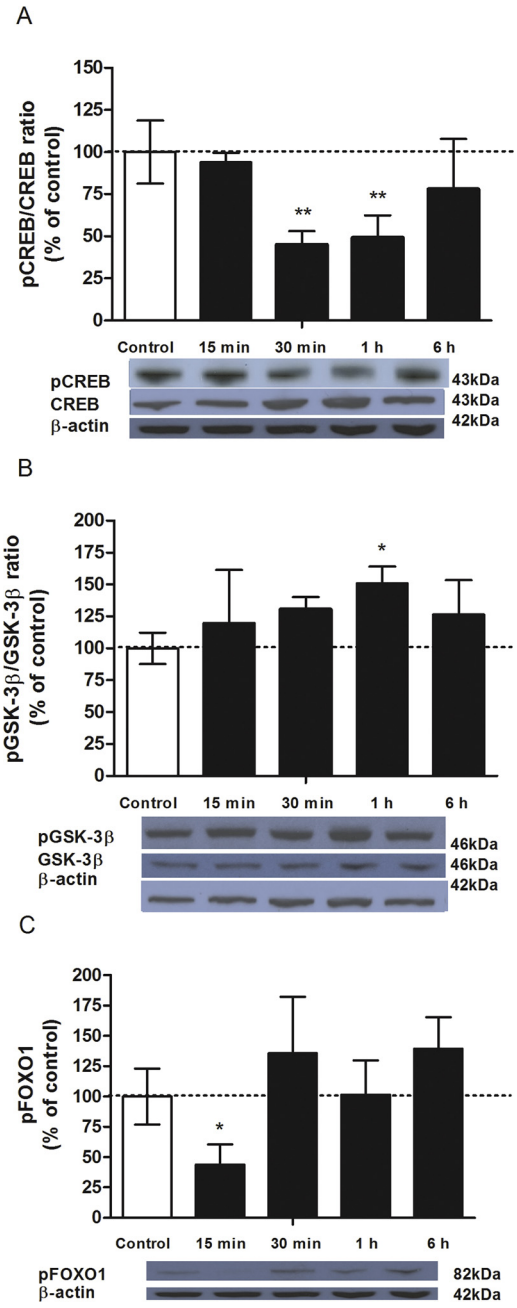

Fig. 5. Effect of MPH on pCREB/CREB (A) and pGSK-3β/GSK-3β (B) ratio and pFoxO1 (C) immunocontent in PC12 cells. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± standard deviation for 4–6 treatments in each group and expressed as percent of control. Different from control, *p < 0.05 and **p < 0.01 (One-way ANOVA). MPH, methylphenidate; CREB, cAMP response element-binding protein; pCREB, cAMP response element-binding protein phosphorylated; GSK-3β, glycogen synthase kinase 3 beta; pGSK-3β, glycogen synthase kinase 3 beta phosphorylated; pFoxO1, forkhead box protein O1 phosphorylated.

Although MPH is used successfully in reducing symptoms of ADHD, several studies have shown that young rats treated with MPH may have notable changes on behavioral, neurophysiological, and biochemical parameters in adulthood (Schmitz et al., 2015, 2016a,b, 2018; Brandon et al., 2003; Federici et al., 2005). Lagace et al. (2006) demonstrated that juvenile rats treated with MPH (2 mg/kg twice a day for 15 days) showed a decrease in survival of new cells of the hippocampus, without

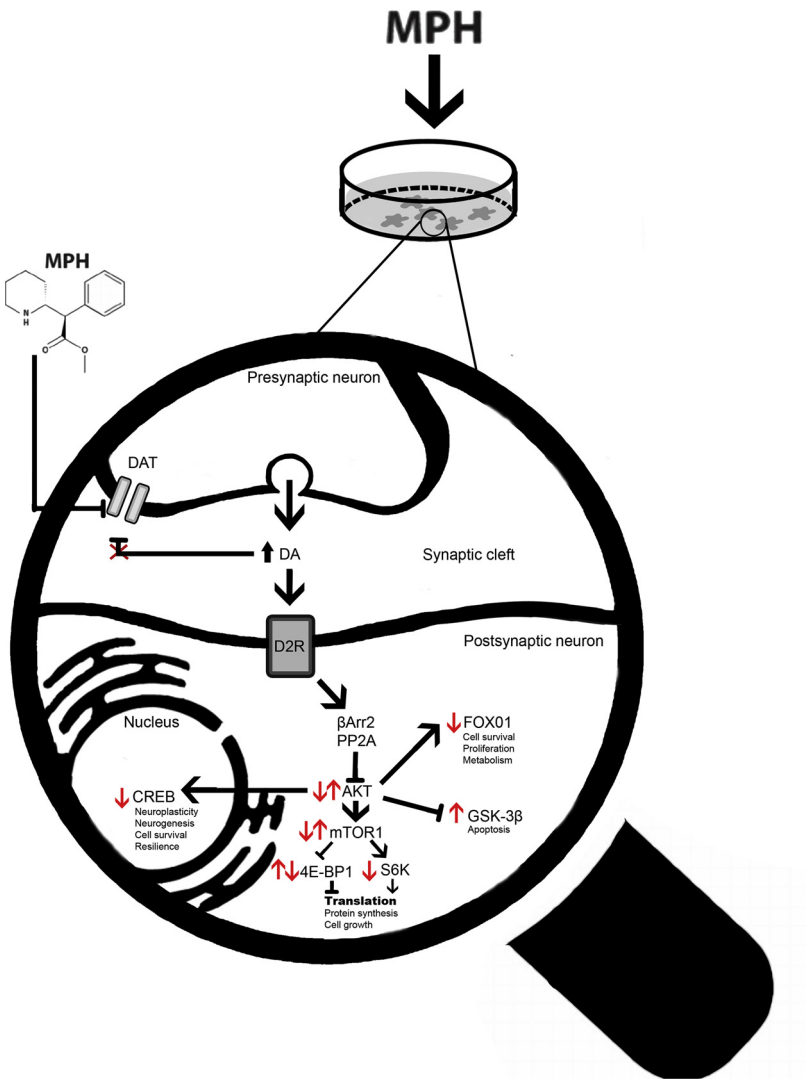

Fig. 6. Graphical summary of the processes that were quantified throughout the investigation, highlighting the main effects of long and/or short term MPH treatment on crucial parameters in PC12 cells. Summing up, we demonstrated that Akt-mTOR pathway, as well as other important signaling proteins like CREB, FoxO1 and GSK-3β are involved in the response to MPH in PC12 cells. Akt, protein kinase B; βArr2, β-arrestin 2; CNS, central nervous system; CREB, cAMP response element-binding protein; DA, dopamine; DAT, dopamine transporter; D2R, dopamine D2 receptor; GSK-3β, glycogen synthase kinase 3 beta; MPH, methylphenidate; mTOR, mammalian target of rapamycin; 4E-BP1, repressor of mRNA translation; S6K, mitogen activated protein kinase; FoxO1, forkhead box protein O1; PP2 A, phosphatase-2 A.

altering cell proliferation. Schaeffers et al. (2009), on the other hand, reported that chronic treatment with MPH (5 mg/kg for 30 days) did not alter the number of proliferating cells. In contrast, it was recently reported an increase in cell proliferation and differentiation of neuroblasts after MPH administration (10 mg/kg once daily for 28 days) (Lee et al., 2012). Furthermore, studies show that long-term use of amphetamine, a psychostimulant that also increases the levels of DA in the synaptic cleft, causes behavioral changes and neurodegeneration (Fukami et al., 2004) and increases the risk of developing Parkinson's disease (Callaghan et al., 2012). For all these, the study of the cellular mechanisms of MPH in order to elucidate the effects of drug therapy is fundamental, since many of their mechanism of action and their adverse effects are not yet fully understood (Challman and Lipsky, 2000; Grünblatt et al., 2013). In addition, the understanding of the cell mechanisms of treatment with MPH on cell signaling is very important since this psychostimulant is widely misused by children and adolescents who do not meet full diagnostic criteria for ADHD (Akay et al., 2006; Dafny and Yang, 2006; Gonçalves et al., 2014; Loureiro-Vieira et al., 2017).

Summing up, we showed that Akt-mTOR pathway in PC12 cells, as well as other important proteins involved in translation, protein synthesis, cell growth, survival, proliferation, neurogenesis, and neuroplasticity respond to MPH according to the time of exposure (Fig. 6). This study represents an important step in exploring the effects of MPH, since it provides additional evidence that exposure to MPH can have complex effects. In addition, it gives new basis for understanding the biochemical effects associated with MPH treatment which may conduct futures studies.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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4. DISCUSSÃO

A tentativa de compreender as consequências do tratamento crônico com MFD é muito importante desde que esse psicoestimulante tem sido amplamente utilizado por crianças de idade pré-escolar, adolescentes e jovens adultos. Além disso, pouco se sabe sobre os mecanismos envolvidos nas alterações neuroquímicas e comportamentais associadas à sua utilização em longo prazo, bem como suas consequências na fase adulta quando utilizado durante a fase de desenvolvimento do SNC.

Nesse contexto, recentemente foi mostrado que o MFD alterou as concentrações de aminoácidos no líquido cefalorraquidiano, aumentando os níveis de glutamato, provavelmente pela inibição da captação de glutamato no córtex pré-frontal (Schmitz et al., 2015). O glutamato é um neurotransmissor envolvido em diversas funções fisiológicas importantes, tais como aprendizado, memória, desenvolvimento e envelhecimento (Reiner e Levitz, 2018).

No presente estudo, nós inicialmente investigamos os efeitos da exposição crônica de MFD sobre o perfil de aminoácidos e a captação de glutamato no hipocampo de ratos jovens. Os resultados mostraram que a exposição crônica ao MFD diminuiu os níveis de glutamina e diminuiu a captação de glutamato no hipocampo de ratos jovens, sugerindo que o MFD provoca um desequilíbrio no sistema glutamatérgico que pode levar à excitotoxicidade.

O sistema dopaminérgico e glutamatérgico estão intrinsecamente interligados, por meio de vias de sinalização intracelulares e interações anatômicas (Cepeda, 2006). No entanto, ainda é desconhecido como a

exposição crônica ao MFD durante o desenvolvimento do SNC pode comprometer a homeostase glutamatérgica no hipocampo. A fim de investigar o prejuízo na captação de glutamato provocada pelo tratamento crônico com MFD, nós avaliamos o imunoconteúdo dos transportadores de glutamato, GLAST e GLT-1, responsáveis por retirar a maioria do glutamato extracelular (Berridge et al., 2006). Os resultados sugerem que a diminuição da captação de glutamato induzida por MFD não envolve uma mudança quantitativa nos transportadores de glutamato, mas uma possível alteração na sua função, uma vez que não se observou qualquer alteração significativa no imunoconteúdo dessas proteínas.

O adequado funcionamento da captação de glutamato depende do gradiente de Na^+ gerado pela Na^+,K^+-ATPase (Rose et al., 2009). A atividade dessa enzima foi avaliada e encontra-se diminuída em hipocampo de ratos jovens tratados cronicamente com MFD. A fim de investigar os mecanismos envolvidos na diminuição da Na^+,K^+-ATPase, nós estendemos nosso estudo e avaliamos se o tratamento crônico com MFD poderia alterar o imunoconteúdo das subunidades catalíticas dessa enzima. Os resultados mostraram que o imunoconteúdo das subunidades catalíticas, α_1, α_2 e α_3, não foram alterados significativamente pelo MFD no hipocampo desses animais. A diminuição na atividade da Na^+,K^+-ATPase pode estar relacionada com a diminuição da captação de glutamato e essas alterações podem contribuir, pelo menos em parte, com o aumento dos níveis de glutamato no líquido cefalorraquidiano observado (Schmitz et al., 2015). No entanto, são necessários mais estudos para investigar os mecanismos envolvidos nessas alterações desde que o

imunoconteúdo dos transportadores de glutamato e o imunoconteúdo das subunidades catalíticas da Na^+,K^+-ATPase parecem não estar envolvidos.

A Na^+,K^+-ATPase é uma proteína transmembrana altamente sensível ao ataque de radicais livres (Zakharaova et al., 2007) e o transporte do glutamato também pode ser afetado pelo estresse oxidativo (Pedersen et al., 1999; Volterra et al., 1994). A fim de estudar mais profundamente as alterações observadas na atividade da Na^+,K^+-ATPase e na captação de glutamato causada pela exposição crônica ao MFD, nós investigamos os efeitos desse psicoestimulante sobre alguns parâmetros de equilíbrio redox. Os resultados mostraram que o MFD não alterou nenhum dos parâmetros avaliados no hipocampo de ratos jovens cronicamente expostos, a saber: produção de espécies reativas, dano à lipídeos e proteínas, bem como as atividades das enzimas antioxidantes (SOD, CAT e GPx). Uma diminuição na formação de espécies reativas e de peroxidação de lipídeos no hipocampo de ratos jovens tratados cronicamente com MFD 2 h após a última injeção foi mostrada (Schmitz et al., 2012). Martins e colaboradores (2006) mostraram que a administração aguda de MFD (2 mg/kg) a ratos jovens de 25 dias de idade e a administração crônica de MFD em ratos adultos (do 60º ao 88º dia de idade) diminuiu a peroxidação lipídica; enquanto que a administração crônica de MFD em ratos jovens (do 25º ao 53º dia da idade) aumentou esse parâmetro no hipocampo 2 horas após a última injeção. Os níveis de carbonilas (um indicativo de dano à proteína) (Martins et al., 2006), a atividade da SOD e da CAT (Gomes et al., 2008) e a produção de ânion superóxido (Gomes et al., 2009) não foram alterados pelo MFD em hipocampo. Além disso, mostrou-se que o estriado foi mais suscetível a danos ao DNA do que o hipocampo

(Andreazza et al., 2007). Nesse contexto, pode-se sugerir que o hipocampo de ratos jovens tratados cronicamente com MFD parece ser uma estrutura pouco suscetível a alterações na homeostase redox.

A Na^+,K^+-ATPase consome cerca de 40-50% do ATP produzido no cérebro para bombear Na^+ e K^+ através da membrana celular (Kaplan, 2002; Mobasheri et al., 2000). Além disso, a ativação dos receptores de glutamato aumenta o consumo de energia e os níveis neuronais de ATP são rapidamente diminuídos (Greenwood e Connolly, 2007; Greenwood et al., 2007). Com base nessas considerações e a fim de compreender melhor as alterações observadas na captação de glutamato e na atividade da Na^+,K^+-ATPase causadas pelo MFD, nós investigamos o efeito da exposição crônica ao MFD sobre os níveis de ATP no hipocampo de ratos jovens. Os resultados mostraram uma diminuição significativa nos níveis de ATP. Esse resultado pode explicar o prejuízo na captação de glutamato, já que isso depende do gradiente de Na^+ gerado pela Na^+,K^+-ATPase que, por sua vez, é dependente de ATP.

A fim de determinar a causa da diminuição nos níveis de ATP no hipocampo de ratos jovens tratados com MFD, nós investigamos a atividade da citrato sintase (enzima que catalisa a condensação de acetil-CoA com oxaloacetato para formar citrato e controla o primeiro passo do ciclo de Krebs) e a atividade da SDH, bem como a atividade dos complexos da cadeia respiratória mitocondrial (II, II-III e IV). Os resultados mostraram que a exposição crônica ao MFD não alterou nenhum desses parâmetros no hipocampo de ratos jovens. A massa e o potencial de membrana mitocondrial também não foram alterados. Tem sido mostrado que o tratamento crônico com

MFD em ratos jovens e adultos (do 25º ao 53º e do 60º ao 88º dia de idade, respectivamente; 2mg/kg) diminuiu a atividade da citrato sintase e da isocitrato desidrogenase, mas não alterou a atividade da malato desidrogenase em hipocampo (Réus et al., 2013). A atividade do complexo IV encontra-se aumentada no hipocampo de ratos jovens (Fagundes et al., 2007) e diminuída em ratos adultos cronicamente expostos ao MFD (Fagundes et al., 2010a). A captação de glicose e a liberação de lactato não foram alteradas pelo MFD em hipocampo (Réus et al., 2015). Por outro lado, Fagundes e colaboradores (2010b) mostraram uma inibição da cadeia respiratória mitocondrial (complexo I, II, III e IV) no hipocampo de ratos adultos (60 dias de idade) submetidos a uma única injeção de MFD (2 mg/kg). Tomados em conjunto, nossos resultados sugerem uma falha na produção de energia no hipocampo de ratos jovens tratados com MFD desde que não foram observadas alterações nos parâmetros avaliados no sentido de reestabelecer o baixo nível de ATP. Corroborando nossos achados, Scaini e colaboradores (2008) mostraram que o tratamento crônico com MFD (2 mg/kg, do 25º ao 53º dia de idade) promoveu um aumento na atividade da creatina cinase no hipocampo. Essa enzima é uma fonte secundária de ATP e é ativada quando o consumo de energia é grande e a produção de ATP a partir da fosforilação oxidativa está prejudicada ou não é suficiente (Scaini et al., 2008).

Embora a glicose seja a principal fonte de energia para o encéfalo, a oxidação dos aminoácidos também pode servir como fonte de energia. Nesse contexto, nós mostramos uma redução nas concentrações de leucina e isoleucina no hipocampo de ratos jovens tratados cronicamente com MFD. Corroborando esse resultado, nós mostramos que a exposição crônica ao MFD

diminuiu os níveis de leucina e serina no córtex pré-frontal e aumentou os níveis de ornitina no líquido cefalorraquidiano (Schmitz et al., 2015). Esses resultados sugerem uma maior degradação de aminoácidos no hipocampo de ratos submetidos ao tratamento com MFD a fim de suprir a demanda de energia, mas sem sucesso desde que os níveis de ATP permaneceram diminuídos.

A histamina é um autacóide que no SNC atua como neurotransmissor controlando uma ampla variedade de funções neurobiológicas e comportamentais e é sintetizada em neurônios histaminérgicos a partir do aminoácido essencial histidina (Huang et al., 2018; Provensi et al., 2018). A histamina facilita diretamente a ativação do receptor NMDA contendo a subunidade NR2B por meio da sua ligação ao sítio modulatório de poliaminas presentes nesse receptor (Williams, 1994). Além disso, a maior liberação de histamina está associada com a melhora da cognição em modelos experimentais (Leurs et al., 2005; Provensi et al., 2018). Nesse estudo, verificou-se um aumento na concentração de histidina no hipocampo de ratos jovens submetidos à exposição crônica com MFD. Nós também demonstramos um aumento dos níveis desse aminoácido no córtex pré-frontal e no líquido cefalorraquidiano de ratos jovens (Schmitz et al., 2015). Esses resultados sugerem que a exposição crônica com MFD inibiu a conversão de histidina a histamina, a fim de evitar uma hiperestimulação do sistema glutamatérgico. Por outro lado, o papel benéfico da histamina sobre a cognição, atenção e alerta, estaria comprometido.

A morte neuronal e perda celular são as características chaves de neurodegeneração em todas as doenças neurodegenerativas, sendo a

apoptose e necrose os processos centrais envolvidos (Pekny e Nilsson, 2005). A neurodegeneração tem sido relacionada a perturbações da homeostase do citoesqueleto das células neurais (Perrot e Eyer, 2009). Os NFs são os componentes mais abundantes do citoesqueleto de grandes axônios mielinizados do SNC e periférico (Perrot e Eyer, 2009). Na sequência da sua síntese e montagem no corpo da célula, os NFs são transportados ao longo do axônio. Esse processo é finamente regulado por meio de fosforilação da extremidade carboxila terminal das duas subunidades do NF de alto peso molecular. A formação correta da rede do NF é crucial para o estabelecimento e manutenção do calibre axonal e consequentemente, para a otimização da velocidade de condução. Por conseguinte, a frequente desorganização das redes dos NFs é observada em diversas doenças do SNC (Perrot et al., 2008). No presente estudo, nós mostramos que o MFD desencadeia a hipofosforilação do NF-M e NF-H no hipocampo de ratos jovens sem alterar o imunoconteúdo dessas proteínas. Nossos dados também sugerem que PP1 e PP2A participam desses efeitos, já que seus imunoconteúdos foram aumentados pelo MFD. A menor fosforilação de GFAP foi acompanhada de uma redução no seu imunoconteúdo.

O equilíbrio entre fosforilação e defosforilação desempenha um papel importante na regulação da organização estrutural e função dos FIs (Brownlees et al., 2000; Pierozan e Pessoa-Pureur, 2018; Sihag et al., 2007) e NFs hipofosforilados são mais suscetíveis à degradação proteolítica (Goldstein et al., 1987; Pant 1988; Pierozan e Pessoa-Pureur, 2018). A hipofosforilação das repetições de Lys-Ser-Pro (KSP) de NF-M e NF-H foi observada no presente trabalho. Esse resultado está de acordo com o aumento do imunoconteúdo de

PP1 e PP2A. Considerando que esses sítios de fosforilação estão implicados na regulação do calibre axonal e transporte (Pierozan e Pessoa-Pureur, 2018), é possível sugerir que o MFD afete o citoesqueleto, prejudicando o transporte axonal e, consequentemente, interferindo em processos altamente regulados durante o desenvolvimento do SNC.

As Ser-Thr fosfatases mais frequentemente envolvidas na modulação dos níveis de fosforilação dos FIs são a PP1, PP2A e PP2B (Pierozan e Pessoa-Purer, 2018). A PP1, neste caso, poderia ser regulada pelo DARPP-32, um importante regulador endógeno da atividade da PP1, cujos efeitos bioquímicos dependem do seu nível de fosforilação em locais específicos (Heimfarth et al., 2012; Hakansson et al., 2004). Estudos mostraram que a cascata DARPP-32/PP1 é um alvo importante para fármacos psicoestimulantes, e a DA pode alterar a fosforilação de DARPP-32 (Klein et al., 2018). Corroborando nossos achados, Souza e colaboradores (2009) mostraram que ratos jovens submetidos ao tratamento crônico com MFD (2 mg/kg, do 25º ao 58º dia de idade) apresentam uma diminuição no imunoconteúdo de DARP-32 no hipocampo. No entanto, mais investigações são necessárias para elucidar os mecanismos envolvidos.

No que diz respeito à regulação negativa do imunoconteúdo da GFAP. Não podemos descartar o fato de que a hipofosforilação de GFAP possa ser uma consequência do seu menor imunoconteúdo e não simplesmente o resultado de um sistema de fosforilação desregulado associado ao citoesqueleto de astrócitos. A GFAP é necessária para a manutenção da forma celular, motilidade/migração, proliferação, homeostase do glutamato e proteção do SNC (Middeldorp e Hol, 2011). Foi descrito que astrócitos de camundongos

knockout para GFAP$^{-/-}$ são menos eficientes em lidar com lesões agudas no SNC (Pekny e Pekna, 2004). Os astrócitos são particularmente adaptados para responder a agentes estressantes e desempenham um papel na proteção dos neurônios. Sadasivan e colaboradores (2012) mostraram que a administração de MFD aumentou a sensibilidade dos neurônios dopaminérgicos ao MPTP (um agente parkinsoniano).

Sabe-se que os FIs do citoesqueleto respondem as necessidades celulares por meio de alterações no seu sistema de fosforilação (hiper e hipofosforilação), observados em modelos animais de doenças neurometabólicas (de Almeida et al., 2003; de Mattos-Dutra et al., 1997; Funchal et al., 2005, Loureiro et al., 2010; Pierozan et al., 2012; Pierozan et al., 2014; Pierozan e Pessoa-Pureur, 2018). A perda do equilíbrio entre fosforilação e defosforilação está associada com um prejuízo nas funções do citoesqueleto e à morte da célula neural, o que reforça o papel do citoesqueleto na neurodegeneração (Lee et al., 2011). Além disso, alterações na fosforilação dos FIs têm sido correlacionadas direta ou indiretamente com transtornos clínicos (Omary et al., 2006). Com base nesses achados, sugerimos que o MFD pode causar disfunção neural associada à ruptura da homeostase do citoesqueleto no hipocampo de ratos jovens.

O MFD foi administrado em um período caracterizado por rápido desenvolvimento do SNC, intensa proliferação e crescimento celular (Rice e Barone, 2000), caracterizados por um acúmulo progressivo e fisiológico de lipídeos (Adibhatla e Hatcher, 2007; Pfrieger, 2003). E também deve-se levar em conta que as *rafts* de membranas/lipídeos e o citoesqueleto interagem dinamicamente e regulam muitas facetas da função das células eucarióticas,

bem como medeiam sua adaptação a mudanças ambientais (Head et al., 2014). Desse modo, nós investigamos o efeito da administração crônica de MFD sobre o conteúdo lipídico de hipocampo de ratos jovens. Os resultados mostraram que o tratamento com MFD reduziu o conteúdo total de colesterol, bem como o conteúdo total de gangliosídeos e o conteúdo dos principais gangliosídeos encefálicos (GM1, GD1a e GD1b). Além disso, o conteúdo total de fosfolipídeos e o conteúdo dos principais fosfolipídeos encefálicos (esfingomielina, fosfatidilcolina, fosfatidiletanolamina e fosfatidilinositol + fosfatidilserina) foram reduzidos no hipocampo desses animais. Corroborando nossos dados, Phan e colaboradores (2015) mostraram que o MFD afetou dramaticamente a distribuição e abundância de lipídeos e seus derivados, particularmente ácidos graxos, diacilglicerídeos, fosfatidilcolina, fosfatidiletanolamina e fosfatidilinositol nos encéfalos de moscas. Além disso, esses achados corroboram a redução da Na^+,K^+-ATPase no hipocampo de ratos jovens, uma vez que a atividade dessa enzima é sensível a alterações na composição dos lipídeos de membrana (Cornelius et al., 2015).

O tecido nervoso é capaz de sintetizar o colesterol de maneira regulada de acordo com o seu desenvolvimento. O colesterol é vital para as funções normais do encéfalo, como sinalização, plasticidade sináptica, aprendizagem e memória (Pfrieger et al., 2003). Além disso, o colesterol é um componente essencial das membranas celulares e é necessário para a viabilidade e proliferação celular. Uma de suas funções importantes é a modulação das propriedades físico-químicas das membranas celulares (Ohvo-Rekilä et al., 2002). Alterações na síntese ou metabolismo do colesterol têm consequências significativas nas funções cerebrais (Pfrieger et al., 2003; Ohvo-Rekilä et al.,

2002). Corroborando nosso estudo, Kabara (1975) mostrou que o MFD causou uma diminuição na concentração de colesterol no encéfalo. As sinapses são particularmente sensíveis ao nível de colesterol e a redução de seu conteúdo pelo tratamento com MFD pode estar associada, pelo menos em parte, à perda da plasticidade sináptica e, em última instância, à neurodegeneração. Além disso, o colesterol é um componente importante da mielina (Pfrieger et al. 2003), o que suge que o tratamento crônico com MFD poderia comprometer a mielinização axonal. Além disso, nós sugerimos que a diminuição do nível de colesterol pode estar relacionada aos baixos níveis de ATP, desde que há evidências de que o MFD aumenta a demanda energética (Fagundes et al., 2007; Fagundes et al., 2010a, b; Réus et al., 2013; Réus et al., 2015).

Os fosfolipídeos constituem a “espinha dorsal” das membranas neurais proporcionando a essa um ambiente adequado, fluidez e permeabilidade a íons. Enquanto fosfatidilcolina, fosfatidilserina e fosfatidiletanolamina desempenham funções estruturais, fosfatidilinositol e esfingomielina participam da sinalização celular (Ohvo-Rekilä et al., 2002). A redução no conteúdo de fosfolipídeos observado no presente estudo pode ser uma consequência da ativação da cascata de fosfolipases, bem como um fornecimento insuficiente de energia aos oligodendrócitos, levando a prejuízos na síntese de ácidos graxos e na formação da bainha de mielina (Ferriero, 2001). Corroborando nossos achados, foi observada uma redução significativa na ressonância dos metabólitos de fosfatidilcolina no cíngulo anterior de pacientes adultos com TDAH após tratamento crônico com MFD (Kronemberg et al., 2008). Uma diminuição nos compostos contendo colina pode indicar ruptura da membrana, bem como uma diminuição da plasticidade sináptica (Kronemberg et al., 2008).

Além disso, Quansah e colegas (2017b) mostraram que a creatina (metabólito associado à energia), o mioinositol (marcador de *turnover* da membrana) e a fosfocolina (precursor de vários fosfolipídeos da membrana) estavam aumentados em encéfalo de ratos machos tratados com MFD (5,0 mg/kg). Levando tudo isso em consideração, pode-se sugerir que alterações no *turnover* lipídico podem estar envolvidas nos efeitos do MFD, comprometendo o adequado ambiente para a membrana, a fluidez, a permeabilidade iônica, bem como as funções estruturais e a sinalização celular. Além disso, alterações na composição dos fosfolipídeos encefálicos também foram descritas em modelos de lesão cerebral, tais como hipóxia/isquemia (Ramirez et al., 2003), esquizofrenia (du Bois et al., 2005) e doença de Alzheimer (Farooqui et al., 2004).

Os gangliosídeos estão presentes em altas concentrações nas membranas neuronais e atuam na proliferação, diferenciação neuronal, mielinização e transmissão sináptica (Mocchetti, 2005). Eles também desempenham um papel significativo nos mecanismos de aprendizagem/memória (She et al., 2005). No hipocampo, os gangliosídeos participam de muitas funções neuronais e estão intimamente associados com sinaptogênese e mielinogênese (Mocchetti, 2005). Assim, uma diminuição nos níveis de gangliosídeos sugere que o tratamento crônico com MFD poderia estar comprometendo as múltiplas funções que os gangliosídeos estão envolvidos. Uma diminuição no conteúdo dos gangliosídeos também tem sido observada em outros modelos de lesão cerebral, como desnutrição (Trindade et al., 1992), acidemia orgânica (Trindade et al., 2002) e neurodegeneração (Schneider et al., 1998). Os mecanismos envolvidos na diminuição dos níveis

de gangliosídeos em hipocampo de ratos tratados cronicamente com MFD ainda precisam ser estudados; no entanto, a alta demanda de energia necessária para sua síntese pode ter sido um fator importante.

Nós também investigamos o efeito do tratamento crônico com MFD sobre o número de neurônios e astrócitos no hipocampo de ratos jovens. Os resultados mostram uma redução nos neurônios e astrócitos em hipocampo desses animais, sugerindo que a exposição crônica a esse fármaco afeta a sobrevivência celular.

A geração e diferenciação de neurônios e células gliais são dependentes de interações célula-célula e essas interações são mediadas por uma ampla variedade de fatores de crescimento e citocinas. A fim de investigar os mecanismos pelos quais o tratamento com MFD diminuiu o número de neurônios e astrócitos, avaliamos primeiramente o efeito do tratamento sobre duas importantes neurotrofinas, NGF e BDNF, bem como o precursor e o receptor ativo do BDNF, pro-BDNF e pTrkB, respectivamente. Os resultados demonstraram que o MFD diminuiu o imunoconteúdo de BDNF e pTrkB, bem como os níveis de NGF em hipocampo de ratos jovens. Essas neurotrofinas ativam Trk, promovendo autofosforilação e a ativação e/ou inibição de vias de sinalização intracelular. pTrkB é co-expresso com glia, neurônios e precursores neuronais, sugerindo que o TrkB possui importante papel na diferenciação das células hipocampais. Além disso, sabe-se que a sinalização do BDNF por meio do seu receptor pode influenciar a morfologia e a conectividade sináptica dos neurônios em desenvolvimento (Huang e Reichardt, 2001; Poo, 2001). Neste contexto, ratos submetidos à separação materna, um modelo animal que induz a uma maior predisposição à dependência química, ansiedade, stress e

depressão, apresenta níveis de BDNF diminuídos em amígdala e no núcleo accumbens e níveis de NGF diminuídos em hipocampo, amígdala e núcleo accumbens (Della et al., 2013).

A fim de avaliar as consequências dos efeitos do MFD sobre as neurotrofinas e sobre o pTrkB no hipocampo de ratos jovens, algumas vias de sinalização que são reguladas por essas neurotrofinas e estão envolvidas nos mecanismos de morte e sobrevivência celular foram investigadas. O tratamento com MFD não alterou a razão pAkt/Akt e pGSK-3β/GSK-3β, mas diminuiu a razão pERK/ERK e o imunoconteúdo de PKCaMII em hipocampo de ratos jovens. Corroborando com esses achados, Lagace e colaboradores (2006), demonstraram que ratos jovens tratados com MFD (2 mg/kg, duas vezes por dia, durante 15 dias) apresentaram uma menor sobrevivência de novas células do hipocampo, mas sem alterar a proliferação celular. Schaeffers e colaboradores (2009) também relataram que o tratamento crônico com MFD (5 mg/kg durante 30 dias) não alterou o número de células em proliferação. Por outro lado, foi recentemente relatado um aumento na proliferação celular e diferenciação de neuroblastos após a administração de MFD (10 mg/kg, uma vez por dia, durante 28 dias) (Lee et al., 2012). Nesse contexto, os nossos resultados sugerem que a diminuição das células neurais observadas em hipocampo de ratos jovens pode ser explicada, pelo menos em parte, pela diminuição do imunoconteúdo de BDNF-pTrkB e do nível de NGF, bem como pela inibição das vias de sobrevivência, ERK e PKCaMII.

SNAP-25 é uma importante proteína envolvida na fusão das vesículas sinápticas no neurônio pré-sináptico e está relacionada com a sinaptogênese e a maturação da conectividade sináptica durante o desenvolvimento do SNC

(Davletov et al., 2007; Hussain et al., 2018; Verderio et al., 1999). Nessa tese mostramos que o tratamento crônico com MFD reduziu o imunoconteúdo de SNAP-25 no hipocampo de ratos jovens. Alterações funcionais de SNAP-25 têm sido associadas a uma série de desordens mentais, tais como TDAH, esquizofrenia e transtorno bipolar precoce (Noor e Zahid, 2017). Desse modo, níveis diminuídos de SNAP-25 podem comprometer eventos celulares, tais como exocitose, crescimento de neuritos, desenvolvimento neuronal, bem como liberação de hormônios e neurotransmissores. Todos esses eventos podem estar relacionados ao detrimento da sinaptogênese e da maturação da conectividade sináptica no hipocampo de ratos jovens tratados com MFD. Corroborando nosso estudo, Cheng e colaboradores (2014) mostraram que a SNAP-25 tem papel importante nas mudanças comportamentais, eletrofisiológicas e bioquímicas promovidas pelo MFD. No entanto, não é possível descartar a hipótese de que a redução do imunoconteúdo de SNAP-25 seja um mecanismo adaptativo do organismo com o objetivo de se proteger dos efeitos tóxicos por meio da contenção do aumento da liberação de neurotransmissores excitatórios (glutamato, DA, noradrenalina e outros) na fenda sináptica e/ou uma consequência da redução dos neurônios.

A neuroinflamação tem sido associada com o aumento de citocinas em nichos neurogênicos e isso pode prejudicar diretamente a plasticidade sináptica em hipocampo, induzindo disfunção cognitiva (Borsini et al., 2015). Na presente tese, foi demonstrado que o tratamento com MFD aumentou os níveis de TNF-α e IL-6, bem como o imunoconteúdo de caspase 3 clivada e Iba-1 no hipocampo de ratos jovens. O TNF-α contribui para a inibição da plasticidade sináptica e da consolidação da memória, como visto em pacientes e em

modelos experimentais de depressão, doenças de Parkinson e Alzheimer. Essas doenças são caracterizadas por neurodegeneração progressiva, bem como por uma resposta imune anormal devido à hiperestimulação microglial e, consequentemente, maior produção de citocinas inflamatórias. Além disso, o TNF-α reduziu a neurogênese e promoveu apoptose em hipocampo de ratos adultos. Níveis elevados de IL-6 reduzem a proliferação, a gliogênese, a neurogênese e aumentam a apoptose em hipocampo de ratos por meio da redução de fatores solúveis, tais como a proteína sonic hedgehog, que é conhecida por promover a diferenciação neuronal (Jang et al., 2012). Corroborando dados do estudo, Sadasivan e colaboradores (2012) observaram uma ativação microglial e aumento dos níveis de mRNA de IL-6 e TNF-α no estriado de ratos após tratamento com MFD. Além disso, Bahcelioglu e colaboradores (2009), demonstraram que o tratamento com MFD (5-20 mg/kg, 5 dias por semana, durante 3 meses) induz astrogliose, aumento da espessura da membrana basal e aumento de vesículas endoteliais em células, sugerindo que o MFD provoca alterações na função da barreira sangue-cérebro. Nesse contexto, nós sugerimos que o aumento dos marcadores inflamatórios e da caspase 3 clivada no hipocampo de ratos jovens tratados com MFD parecem estar associados, pelo menos em parte, com a perda de neurônios e astrócitos observada.

A fim de identificar se as alterações neuroquímicas promovidas pelo tratamento crônico com MFD em hipocampo de ratos podem comprometer tarefas comportamentais dependentes do hipocampo, avaliamos o desempenho dos animais submetidos ao tratamento crônico com MFD no labirinto em cruz elevado, campo aberto e na tarefa de memória de

reconhecimento de objetos. Os resultados obtidos no labirinto em cruz elevado mostraram que ratos jovens tratados com MFD exploram menos os braços abertos que os controles demonstrando um comportamento do tipo ansioso. Na tarefa de campo aberto, observou-se que os animais tratados com MFD apresentaram um menor interesse em explorar o ambiente. Além disso, observou-se um prejuízo da memória de longo prazo na tarefa de reconhecimento de objetos. Ainda não há um consenso acerca do comportamento do tipo ansioso na atividade exploratória após o tratamento com MFD. Nesse contexto, Britton e colaboradores (2007, 2009) mostraram que o MFD desencadeou comportamento do tipo ansioso e aumentou a atividade exploratória dos animais. Outro estudo mostrou que quando o MFD foi administrado durante o desenvolvimento até o 35º dia de idade, os animais apresentaram menos comportamentos do tipo ansioso na idade adulta (Gray et al., 2007). McFadyen-Leussis e colaboradores (2004) mostraram que a exposição pré-natal ao MFD aumentou a atividade exploratória e o comportamento do tipo ansioso, sem benefícios em tarefas de aprendizagem e memória em camundongos. Nosso grupo mostrou que ratos tratados com MFD apresentaram desempenho inferior aos animais controles no teste do labirinto aquático de Morris depois de terem recebido MFD (2 mg/kg) do 15º ao 45º dia de idade (Scherer et al., 2010). O tratamento agudo com MFD na dose de 5 mg/kg melhorou a memória de reconhecimento e a memória aversiva em camundongos adultos, enquanto que o tratamento agudo com MFD na dose de 50 mg/kg prejudicou esses parâmetros (Mioranzza et al., 2010). Ambas as doses não alteraram a atividade locomotora dos animais e aumentaram o número de entradas e o tempo de permanência nos braços abertos (Mioranzza

et al., 2011). Nossos resultados sugerem que os ratos tratados cronicamente com MFD apresentam menor comportamento exploratório e prejuízo na memória de reconhecimento de objetos o que poderia ser explicado, pelo menos em parte, por um prejuízo na etapa de aquisição. Além disso, não podemos descartar a possibilidade de que o MFD afetou a motivação, atenção e a função sensório-motora dos animais desde que o tempo total de exploração foi diminuído pelo tratamento. Corroborando nosso estudo, Bolaños e colaboradores (2003) demonstraram que ratos adultos expostos a repetidas doses de MFD durante a juventude apresentaram menor atividade motora quando expostos pela primeira vez a um novo ambiente. Contudo, as alterações comportamentais encontradas na literatura após a administração de MFD são algumas vezes distintas e isso pode ser resultante de diferentes protocolos de administração, dose e idade dos animais. É importante ressaltar que nosso estudo teve como objetivo investigar os efeitos comportamentais da administração do MFD durante o desenvolvimento do SNC.

Apesar do grande aumento no uso de MFD nas últimas décadas, seu mecanismo de ação e efeitos sobre a sinalização celular ainda não estão totalmente esclarecidos. Nesse contexto, os modelos celulares tem se mostrado importantes ferramentas não só na compreensão da fisiopatologia de doenças, mas também na investigação dos mecanismos de ação de substâncias e seus efeitos celulares.

As células PC12, por exemplo, uma linhagem celular derivada de feocromocitoma da medula adrenal de rato, tem sido amplamente utilizada como modelo experimental *in vitro* para o estudo de doenças como a doença de Parkinson (Pan et al., 2012; Xu et al., 2013) e o TDAH (Grünblatt et al.,

2013). Essas células também tem sido utilizadas para estudar os efeitos de vários compostos neuroativos como a cocaína (Shafer et al., 1991; Imam et al., 2005), a bromocriptina e o quinpirole (agonistas dopaminérgicos D2) (Chiasson et al., 2006), o himantano (fármaco antiparkinsoniano) (Abaimov et al., 2008), 6-hidroxidopamina (Xu et al., 2013) e o MFD (Grünblatt et al., 2013; Bartl et al., 2010), uma vez que são células neuronais dopaminérgicas capazes de sintetizar, armazenar, secretar e captar DA (Pan et al., 2012).

Com base nisso, o presente estudo investigou a resposta das células PC12 ao tratamento com MFD, analisando a via Akt-mTOR, bem como os substratos: 4E-BP1 e S6K. Os efeitos do tratamento sobre CREB, GSK-3β e FoxO1 foram também investigados. Os resultados mostram que o tratamento com MFD em curto prazo diminuiu a razão pAkt(Thr308)/Akt, pmTOR/mTOR e pS6K/S6K, bem como o imunoconteúdo de pFoxO1 em células PC12. Por outro lado, o tratamento em longo prazo aumentou a razão pAkt(Thr308)/Akt, pmTOR/mTOR e pGSK-3β/GSK-3β. Os níveis de fosforilação de 4E-BP1 foram diminuídos aos 15 e 30 minutos e aumentados em 1 e 6 h após o tratamento com MFD. A razão pCREB/CREB foi diminuída. Esses achados sugerem que o MFD altera a sinalização celular nas células PC12 e suas respostas são diferentes de acordo com o tempo de exposição a esse psicoestimulante.

O aumento dos níveis de DA em várias regiões do encéfalo por meio do bloqueio do DAT pelo MFD está amplamente descrito. Esse neurotransmissor tem um importante efeito modulador na neurogênese (Klein et al., 2018; Reinoso et al., 1996; Tood, 1992). Uma interação física entre o DAT e o D2R demonstrou ser importante na regulação da expressão de DAT na superfície celular (Bolan et al., 2007). A ativação de D2R está amplamente associada à

inibição da Akt pela defosforilação de seu resíduo regulatório de treonina após a formação de um complexo de sinalização composto por Akt, PP2A e β-Arr2 (Beaulieu et al., 2004, 2005). No estudo observou que o MFD diminuiu a razão pAkt(Thr308)/Akt nas células PC12 provavelmente pela ativação de D2R/PP2A promovida pelo rápido aumento dos níveis de DA ocasionado pelo tratamento com MFD. No entanto, após 1h, a razão pAkt(Thr308)/Akt aumentou em células PC12. Esses resultados são intrigantes. Bartl e colaboradores (2010) mostraram que os níveis extracelulares dos metabólitos da DA (DOPAC e HVA) são mais altos e estão correlacionados com uma maior degradação de DA após o tratamento com MFD. Eles também sugeriram que a degradação de DA parece estar aumentada após o tratamento com MFD pelo aumentado na atividade da monoamina oxidase-B e/ou catecol-O-metiltransferase. Com base nisso, pode-se sugerir que a diminuição da razão pAkt(Thr308)/Akt nos primeiros minutos e o aumento após 1h, pode ser explicado pelos aumentados níveis de DA e ativação de D2R/PP2A, seguido do incremento da sua degradação e, consequentemente, uma diminuição nos seus níveis e inativação de D2R/PP2A. Corroborando com nossos achados, foi demonstrado que o D2R pode regular a defosforilação da Akt após tratamento com anfetamina (3 mg/kg) e apomorfina (6 mg/kg) (Beaulieu et al., 2007a).

A mTORC1 é um alvo importante da via D2R/Akt que pode desempenhar importantes papéis nas funções neuronais, incluindo controle da plasticidade sináptica, potenciação de longa duração, crescimento e regeneração axonal (Willis e Twiss, 2006; Lin e Holt, 2008; Richter e Klann, 2009). Esses processos requerem síntese proteica, que pode ser potencialmente regulada pelos substratos: S6K e 4E-BP1 (Ma e Blenis, 2009).

Enquanto a S6K é ativada após a fosforilação pela mTORC1, a 4E-BP1 é inibida. O S6K estimula o alongamento da tradução principalmente pela fosforilação da proteína ribossômica S6. Alternativamente, 4E-BP1 reprime seletivamente a tradução associando-se ao fator de iniciação de ligação, eIF4E (Ma e Blenis, 2009). Nossos resultados mostram que o MFD diminuiu a razão de pmTORC1/mTORC1, bem como a razão de pp70 S6K/p70 S6K em células PC12 após um tratamento de curta duração. Nós também mostramos que os níveis de 4E-BP1 hiperfosforilados estavam diminuídos, enquanto os níveis de 4E-BP1 hipofosforilados estavam aumentados nas células PC12 após um curto período de tratamento. Esses resultados corroboram a diminuição da razão pAkt(thr308)/Akt, bem como sugerem um comprometimento da tradução dependente de mTORC1 induzido pelo tratamento com MFD, podendo comprometer processos que requerem síntese proteica como plasticidade sináptica, crescimento axonal e regeneração. Por outro lado, após longa duração, assim como para pAkt(thr308)/Akt, a razão pmTORC1/mTORC1 foi aumentada pelo MFD. Observamos também que os níveis de 4E-BP1 hiperfosforilados estavam aumentados após um tratamento de longa duração com MFD (1 e 6 h), sem alterações na razão pp70 S6K/p70 S6K. Os resultados observados em um tratamento de curto prazo sugerem que os processos que requerem síntese proteica como plasticidade sináptica, crescimento e regeneração axonal são prejudicados pelo MFD. Por outro lado, o efeito oposto foi observado após um longo período de tratamento. As diferentes respostas ao MFD de acordo com o tempo de tratamento podem ser explicadas, pelo menos em parte, pelas alterações promovidas por esse psicoestimulante sobre os níveis de DA. Nesse contexto, o tratamento com antipsicóticos, como o

haloperidol, que são antagonistas do receptor de DA (principalmente D2R), tem sido associado à fosforilação da Akt e aumento da tradução dependente da mTORC1, bem como aumento da ramificação, número de espinhos dendríticos (Bowling et al., 2014) e complexidade morfológica (Navari e Dazzan, 2009; Benes et al., 1983; Meredith et al., 2000).

O CREB é ativado por fosforilação através de várias vias de sinalização, incluindo Akt (Wang et al., 2017). Esse fator de transcrição é capaz de mediar sinais de inúmeros estímulos fisiológicos, resultando na regulação de uma ampla gama de respostas celulares, desempenhando um papel-chave na promoção da sobrevivência neuronal, crescimento de neuritos e diferenciação neuronal em certas populações, bem como na aprendizagem e memória em vários organismos (Wang et al., 2017). Nesse estudo nós mostramos que o MFD diminuiu a razão de pCREB/CREB nas células PC12. Esse resultado sugere que as funções de CREB podem estar comprometidas em células PC12 após o tratamento com MFD. Nesse contexto, Chase e colegas (Chase et al., 2003) mostraram que a administração de MFD altera a expressão de c-fos no encéfalo de ratos jovens. A diminuição do nível de expressão gênica de sinaptotagmina 1 e 4, sintaxina 1a e glicoproteína 2C de vesículas sinápticas também foram associadas ao tratamento com MFD em células PC12 (Bartl et al., 2010).

A GSK-3β exerce um importante papel na sobrevivência celular e a sinalização da via Akt-GSK-3β no encéfalo pode ser regulada por DA (Beaulieu et al., 2004, 2005, 2007b; Klein et al., 2018), antipsicóticos típicos/atípicos (Beaulieu et al., 2007b; Emamian et al., 2004; Cyr et al., 2003), antidepressivos (Cyr et al., 2003) e estabilizadores de humor (Beaulieu et al., 2004; Gould et al.,

2004). Nesse contexto, foi mostrado que a inativação genética de Akt1 ou GSK-3β, bem como a administração de inibidores de GSK-3 ou o desacoplamento de Akt dos receptores de DA em camundongos *knockout* para β-Arr2 afetam comportamentos relacionadas à DA (Beaulieu et al., 2004, 2005; Emamian et al., 2004). Nesse estudo, nós investigamos se o tratamento com MFD afeta a razão entre pGSK-3β/GSK-3β em células PC12. Ao contrário do esperado, uma vez que a razão entre pAkt(Thr308)/Akt foi diminuída por MFD em um tratamento de curto prazo, pGSK-3β/GSK-3β não foi alterado em 15 e 30 min. No entanto, encontramos que a razão entre pGSK-3β/GSK-3β foi aumentada em 1 h, corroborando o resultado observado para a Akt.

O FoxO1 é um substrato da Akt e pode promover apoptose e inibição do ciclo celular (van der Horst e Burgering, 2007; Wen et al., 2012). Nesse estudo o imunoconteúdo de pFoxO1 (forma inativa) foi diminuído em 15 min após o tratamento com MFD em células PC12. Esse resultado corrobora a diminuição observada na razão pAkt(thr308)/Akt, bem como sugere um possível envolvimento da via Akt-FoxO1 nos efeitos do MFD. Nossos dados corroboram dados de Zheng e colaboradores (2013) que sugeriram que a via Akt-FoxO1 pode ser um alvo comum para a ação de fármacos dopaminérgicos e sua modulação pode ser relevante para o tratamento de transtornos neuropsiquiátricos. Eles também mostraram que a administração de d-anfetamina resultou em uma diminuição concomitante nos níveis de pAkt e pFoxO1 no estriado, enquanto o cloreto de lítio, um fármaco usado principalmente no tratamento do transtorno bipolar e conhecido por interagir com o sistema dopaminérgico e inibir alguns comportamentos relacionados a DA, exerceu o efeito oposto. Também foi demonstrado que o pré-tratamento de

animais com lítio atenuou as alterações comportamentais induzidas pela anfetamina (Zheng et al., 2013).

Embora o MFD seja usado com sucesso na redução dos sintomas do TDAH, a compreensão das consequências do tratamento crônico com MFD em estágios iniciais do desenvolvimento do encéfalo é muito importante desde que esse psicoestimulante tem sido amplamente utilizado em crianças de idade pré-escolar e jovens adultos que não possuem todos os critérios para serem diagnosticados com TDAH (Johnston et al., 2011a,b; Zito et al., 2010). Nesse contexto, estudos experimentais têm mostrado alterações no sistema dopaminérgico, estado redox, função mitocondrial, sistemas glutamatérgico/gabaérgico, neuroinflamação, barreira sangue-cérebro e neurogênese após o tratamento com MFD (Gonçalves et al., 2014; Quansah et al., 2017a,b; Loureiro-Vieira et al., 2017); porém, pouco se sabe sobre como a exposição precoce ao MFD pode afetar o funcionamento do hipocampo na vida adulta (Andersen, 2005; Carias et al., 2018; Oakes et al., 2018). O hipocampo desempenha um papel importante em comportamentos afetivos, dependência química e recaída (Carlezon et al., 2005; Duman et al., 1997; Nestler et al., 2002). Além disso, as ações do MFD sobre o hipocampo podem ser centrais nos efeitos em longo prazo uma vez que o tratamento com esse psicoestimulante em jovens roedores aumenta os níveis de noradrenalina no hipocampo em um padrão dose-dependente (Kuczenski e Segal, 2002). Portanto, identificar as alterações induzidas pelo MFD no hipocampo pode ser crucial para a interpretação dos mecanismos que contribuem para as alterações comportamentais e neuroquímicas associadas ao tratamento com MFD. Além disso, a elucidação dos efeitos celulares do MFD é fundamental,

uma vez que seu mecanismo de ação e efeitos adversos ainda não estão completamente elucidados (Grünblatt et al., 2000; Gusmutas et al., 2017).

Em resumo, o presente estudo sugere que o tratamento crônico com MFD prejudicou a captação de glutamato e a atividade da Na^+,K^+-ATPase no hipocampo de ratos jovens e esees resultados, podem estar associados, pelo menos em parte, com a diminuição nos níveis de ATP. Além disso, mostramos que a homeostase do citoesqueleto e dos lipídeos foi afetada pelo tratamento crônico com MFD durante o desenvolvimento cerebral de ratos. O MFD também promoveu uma perda de astrócitos e neurônios, provavelmente pela inibição de vias de sobrevivência e ativação de vias de morte celular reguladas por citocinas e neurotrofinas. Além disso, ratos tratados com MFD apresentaram menor interesse em explorar novos ambientes e objetos, afetando a memória de reconhecimento de objetos, provavelmente, por um déficit na fase de aquisição. Também mostramos que a via Akt-mTOR nas células PC12, bem como outras proteínas importantes envolvidas na tradução, síntese proteica, crescimento celular, sobrevivência, proliferação, neurogênese e neuroplasticidade, respondem ao MFD de acordo com o tempo de exposição.

Acreditamos que os achados dessa tese representam um importante passo na exploração dos efeitos do MFD, desde que fornece evidências adicionais de que a exposição precoce ao MFD pode ter efeitos complexos durante o desenvolvimento do SNC, alterando a neuroquímica e o desempenho em tarefas comportamentais em ratos. Além disso, os resultados fornecem uma nova base para a compreensão dos mecanismos celulares associados ao tratamento com esse psicoestimulante.

5. CONCLUSÕES

A partir do desenvolvimento dessa tese, observamos que a administração crônica de MFD em ratos jovens promoveu em hipocampo:

1. Aumento da concentração de histidina e diminuição de glutamina, leucina e isoleucina;
2. Diminuição da captação de glutamato;
3. Diminuição da atividade da Na^+,K^+-ATPase;
4. Diminuição do nível de ATP;
5. Hipofosforilação e diminuição do imunoconteúdo de GFAP;
6. Hipofosforilação de NF-H e NF-M;
7. Aumento do imunoconteudo de PP1 e PP2A;
8. Diminuição do conteúdo total de fosfolipídeos, bem como o conteúdo individual de esfingomielina, fosfatidilcolina, fosfatidiletanolamina, fosfatidilinositol e fosfatidilserina;
9. Diminuição do conteúdo total de gangliosídeos, bem como o conteúdo individual de GM1, GD1a e GD1b;
10. Diminuição do conteúdo total de colesterol;
11. Diminuição no número de neurônios e astrócitos em hipocampo;
12. Diminuição do imunonconteúdo de BDNF-pTrkB, bem como os níveis de NGF;
13. Diminuição do imunoconteúdo de SNAP-25;
14. Aumento nos níveis de TNF-α e IL-6, bem como do imunoconteúdo de Iba-1 e caspase 3 clivada;

15. Diminuição da razão pERK/ERK e o imunonconteúdo de PKCaMII;
16. Uma menor exploração de novos ambientes e objetos;
17. Prejuízo na tarefa de memória de reconhecimeto de objetos.

Além disso, verificamos que o tratamento de células PC12 com MFD promoveu:

1. Diminuição da razão pAkt/Akt, pmTOR/mTOR e pS6K/S6K em curto prazo;
2. Aumento da razão pAkt/Akt, pmTOR/mTOR e pGSK-3β/GSK-3β em longo prazo;
3. Diminuição dos níveis de fosforilação de 4E-BP1 em 15 e 30 minutos e aumento em 1 e 6 h;
4. Diminuição do imunoconteúdo de pFoxO1;
5. Diminuição da razão pCREB/CREB.

Concluindo, os resultados da presente tese sugerem que o tratamento crônico com MFD diminui a captação de glutamato e a atividade da Na^+,K^+-ATPase no hipocampo de ratos jovens, bem como os níveis de ATP. Além disso, a homeostase do citoesqueleto e dos lipídeos foi afetada pelo MFD. O MFD também promoveu uma perda de astrócitos e neurônios, provavelmente pela inibição de vias de sobrevivência e ativação de vias de morte celular reguladas por citocinas e neurotrofinas. Além disso, ratos tratados com MFD apresentaram menor interesse em explorar novos ambientes e objetos, bem como um prejuízo na memória de reconhecimento de objetos, provavelmente,

por um déficit na fase de aquisição. Por fim, mostramos que a via Akt-mTOR nas células PC12, bem como outras proteínas importantes, respondem ao MFD de acordo com o tempo de exposição.

Esses resultados fornecem evidências adicionais de que a exposição crônica precoce ao MFD pode ter efeitos complexos, bem como fornece novas bases para a compreensão das consequências neuroquímicas e comportamentais associadas ao tratamento com esse psicoestimulante. Além disso, fornece uma nova base para a compreensão dos mecanismos associados ao tratamento com esse psicoestimulante, que pode conduzir estudos futuros.

6. PERSPECTIVAS

1. Avaliar o comportamento social e afetivo de animais submetidos ao tratamento crônico com MFD;
2. Investigar se as alterações apresentadas nessa tese persistem até a idade adulta de ratos (~80 dias);
3. Explorar o papel dos receptores de DA, especialmente o D2R, nos efeitos do MFD em células PC12 e cultura primária de neurônios através do uso de antagonistas; bem como o papel de Akt, GSK-3β, CREB e mTOR com o uso de inibidores;
4. Fazer *screnning* sobre a síntese proteica.

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