Acta Neuropsychiatrica 2013

DOI: 10.1111/acn.12015

ACTA NEUROPSYCHIATRICA

Short Communication

Methylphenidate treatment affects mitogen-activated protein kinase activation in the striatum of young rats

Comim CM, Pereira JG, Ribeiro SJ, Quevedo J, Boeck CR. Methylphenidate treatment affects mitogen-activated protein kinase activation in the striatum of young rats.

Objective: Methylphenidate (MPD) is a drug prescribed for the treatment of attention deficit/hyperactivity disorder and its therapeutic effect is attributed to the inhibition of dopamine.
Methods: Young male Wistar rats were administered MPD (1, 2, 5, or 10 mg/kg) once a day or an intraperitoneal injection of saline for 28 days (chronic treatment) or for 1 day (acute treatment). Two hours after the last administration the animals were decapitated and their striatum was dissected.
Results: In this work, we show that continued treatment with MPD is capable of modifying the levels of phosphorylation of proteins JNK1/2 (c-Jun amino-terminal kinases 1 and 2) and ERK1/2 (extracellular signal-regulated kinases 1 and 2). Whereas the level of phosphorylation of protein ERK increased significantly, that of proteins JNK1/2 diminished.
Conclusion: The alteration in the level of activation of mitogen-activated protein kinases can be a molecular mechanism through which MPD exerts its therapeutic effect.

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Keywords: ERK, JNK, MAPK, methylphenidate, striatum

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Accepted for publication July 11, 2012

First published online 7 March, 2013

Significant outcomes

- Methylphenidate (MPD) increases the level of phosphorylation of protein extracellular signal-regulated kinase 1 (ERK1) at a dose of >1 mg/mg.
- MPD decreases the level of phosphorylation of c-Jun amino-terminal kinase 1 (JNK1) at a dose of >2 mg/kg.
- MPD decreases the level of phosphorylation of JNK2 at 2 mg/kg.

Limitation

- This study could have used other brain structures like the hippocampus and cortex, which are also involved in attention deficit/hyperactivity (ADHD).

Introduction

ADHD disorder is the most commonly diagnosed neuropsychiatric disorder in childhood, characterised by excessive levels of inattentiveness, impulsivity, and hyperactivity (1). Most ADHD patients benefit from treatment with MPD, irrespective of the aetiology of the disorder, which effectively reduces symptoms in up to 70% of paediatric patients (2). However, recognition that ADHD may persist into adulthood has led to an increased use of MPD in adult patients; extended use of MPD can have chronic effects in the organism (3).

Studies on humans suggest that blockade of the dopamine transporter (DAT) produces the therapeutic effect of MPD by increasing the concentration of dopamine in the central nervous system (CNS) (4). However, it is not yet clear whether the effects on the CNS are due to prolonged use of MPD.

In this context, the CNS is well known for its involvement in the pathway of mitogen-activated protein kinases (MAPKs) (5). The three main MAPKs characterised in the brain of mammals are the protein kinase regulated by ERK1/2, proteins JNK1/2, and the protein p38. Activation of these proteins regulates several cellular events ranging from proliferation and cellular differentiation to apoptosis (6). In addition, dopamine also positively regulates ERK by acting through multiple signalling mechanisms that involve D1 and/or D2-class dopamine receptors. Although ERK has been shown to participate in acute responses to cocaine, it appears to be mostly involved in the development of long-term changes in gene expression, synaptic plasticity, and locomotor responses following repeated exposure to this drug (7).

Because critical actions of psychostimulants appear to involve the dopaminergic inputs into the striatum and the nucleus accumbens, these structures should also be investigated. To understand the brain alterations caused by long-term exposure to MPD, the aim of this work was to investigate the influence of the medicament MPD on the expression and phosphorylation of MAPKs in the striatum of young rats subjected to continued treatment and to try establish the molecular basis of its mechanism of action and, consequently, possible processes in the physiopathology and neurochemistry responsible for the manifestation of ADHD.

Materials and methods

Animals

Young [postnatal day (PD) 25; 75–85 g] male Wistar rats obtained from our breeding colony were housed five to a cage with food and water available *ad libitum* and maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). All experimental procedures were in accordance with the recommendations of the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) for animal care.

Experimental design

Young rats ($n = 10$) were administered MPD [1, 2, 5, or 10 mg/kg; Ritalin® (Novartis; East Hanover, NJ, USA) – racemic dextroand levo-MPD mixture dissolved in saline solution] once a day or an intraperitoneal injection of saline for 28 days (chronic treatment) or for 1 day (acute treatment). The acute treatment was at PD25 and the chronic treatment between PD25 and PD53 (8–12). Two hours after the last administration of MPD or saline, the animals were killed and their striatum was dissected and rapidly solubilised with SDS-stopping solution (4% SDS, 2 mM EDTA, 8% β-mercaptoethanol, and 50 mM Tris, pH 6.8). Aliquots of proteins were quantified and extracts were stored at −80°C until the preparation of samples for immunoblot analysis.

Immunoblot analysis

Equal amounts of protein (50 μg) were separated for SDS–PAGE using a 12% polyacrylamide gel, followed by their transfer to nitrocellulose membranes (Hybond ECL, GE Healthcare, Piscataway, NJ, USA). Protein loading and efficiency of blot transfer were monitored by staining with Ponceau S (0.5% ponceau/1% acetic acid; Sigma Chemical Co., St Louis, MO, USA). The membranes were blocked for 45 min with TBS-T (tris-buffered saline and Tween 20 0.1%, pH 7.4) plus 5% non-fat milk. Membrane blots were incubated with polyclonal anti-phospho-p44/42 MAPK or anti-phospho SAPK/JNK (both 1 : 1000 – Cell Signaling Technology, MA, USA) diluted in TBS-T and stored overnight at 4°C. After washing, the membranes were incubated for 1 h with goat anti-rabbit IgG (1 : 2000 – Cell Signaling Technology, MA, USA) conjugated with horseradish peroxidase. Immunocomplexes were visualised using the enhancing chemiluminescence detection system (LumiGLO™ – Cell Signaling Technology) as described by the manufacturer and visualised on ImageQuant. Densitometric analysis was performed using Scion Image Software, version Beta 4.0.2 (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Results were analysed by STATISTICA, version 7.0 (StatSoft Inc., Tulsa, OK, USA). All data are presented as a percentage of control ± SEM (standard error of the mean), and each value reflects the mean of 4–5 animals per group for immunoblot analysis. In all cases, statistical analyses were performed using two-way analysis of variance (ANOVA) with chronic and acute treatments as the main factors, followed by the Duncan test for immunoblot analysis. The level of significant difference was accepted at $p < 0.05$.

Results

Figure 1 shows that chronic treatment with MPD is capable of modifying the levels of phosphorylation of proteins ERK1/2 (a) and JNK1/2 (b). Our results

https://doi.org/10.1111/acn.12015 Published online by Cambridge University Press

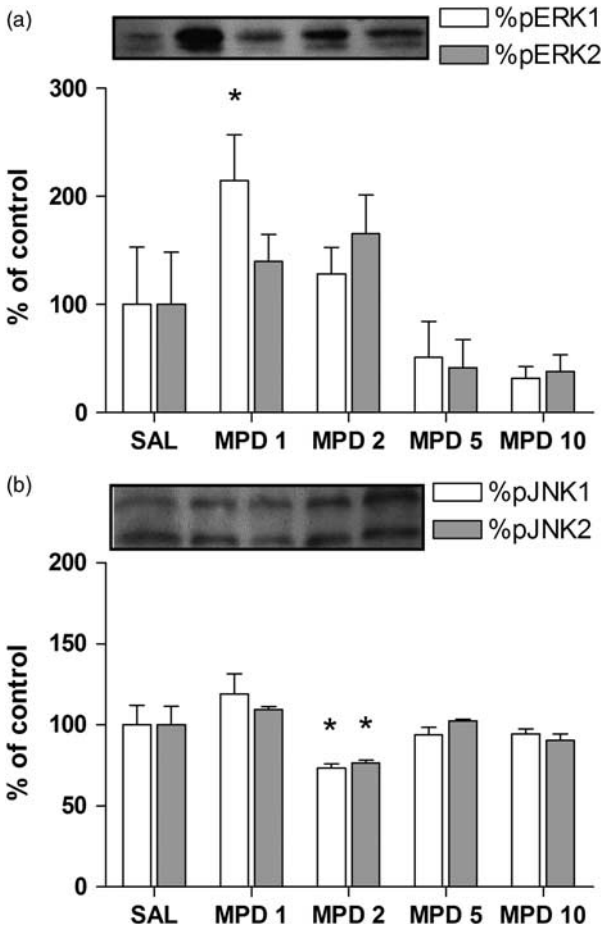

Fig. 1. Phosphorylation of proteins JNK1/2 and ERK1/2 in the striatum as a result of chronic treatment with MPD (1, 2, 5, or 10 mg/kg) or saline. Immunodetections were determined as described in the 'Materials and methods' section. (a) A summary of optical density data expressed as a percentage of control of pERK1/2 contents ± SEM. (b) A summary of optical density data expressed as a percentage of control of pJNK1/2 contents ± SEM. *$p < 0.05$ versus the control group; two-way ANOVA and *post hoc* Duncan test. ERK1/2, extracellular signal-regulated kinases 1 and 2; JNK1/2, c-Jun amino-terminal kinases 1 and 2; MPD, methylphenidate; SAL, saline.

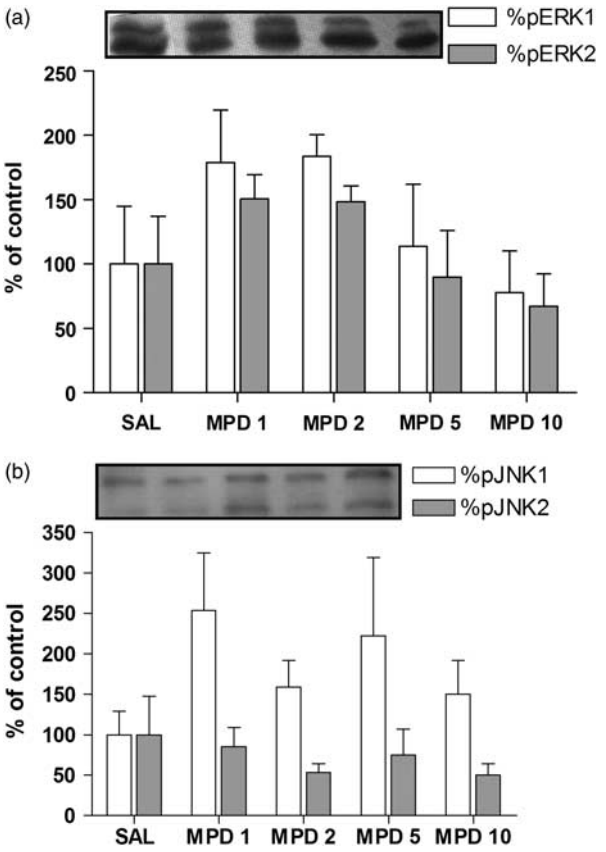

Fig. 2. Phosphorylation of proteins JNK1/2 and ERK1/2 in the striatum as a result of acute treatment with MPD (1, 2, 5, or 10 mg/kg) or saline. Immunodetections were determined as described in the 'Materials and methods' section. (a) A summary of optical density data expressed as a percentage of control of pERK1/2 contents ± SEM. (b) A summary of optical density data expressed as a percentage of control of pJNK1/2 contents ± SEM; two-way ANOVA and *post hoc* Duncan test. ERK1/2, extracellular signal-regulated kinases 1 and 2; JNK1/2, c-Jun amino-terminal kinases 1 and 2; MPD, methylphenidate; SAL, saline.

showed that MPD increased phosphorylation of the protein ERK1 at a dose of 1 mg/mg ($p < 0.05$) (Fig. 1a) and decreased phosphorylation of JNK1/2 at 2 mg/kg ($p < 0.05$), when compared as a percentage of control (Fig. 1b). However, Fig. 2 demonstrates that acute treatment with MPD did not alter the phosphorylation of proteins ERK1/2 (Fig. 2a) and JNK1/2 (Fig. 2b; $p > 0.05$).

Discussion

The prolonged time course of the initiation and maintenance of psychostimulant dependence has raised the question of whether long-term drug-induced alterations in gene expression play a critical role. In this study, we observed that chronic treatment with MPD is capable of modifying the levels of phosphorylation of proteins ERK1 (at a dose of 1 mg/kg) and JNK1/2 (at a dose of 2 mg/kg). The alteration in the index of activation of MAPKs can be a molecular mechanism through which MPD exerts its therapeutic effect. Previous studies demonstrate that amphetamine increases the phosphorylation of cAMP-responsive element binding protein (CREB) in the striatum (13). A recent study shows that both cocaine and methamphetamine upregulate pERK1/2 in the striatum (14). These findings suggest that both dopaminergic and glutamatergic inputs are capable of regulating the ERK1/2 signalling pathways leading to the phosphorylation of CREB and Elk-1 and thus to alterations in gene expression in striatal neurons.

In this context, ERK1 and ERK2 can also be activated by a variety of extracellular signalling

https://doi.org/10.1111/acn.12015 Published online by Cambridge University Press

molecules, including growth factors, hormones, and neurotransmitters. Calcium influx in particular has gained importance because of an increase in ERK activity as a result of *N*-methyl-D-aspartate (NMDA) glutamate receptor-stimulated calcium influx. Thus, it is attractive to speculate that NMDA receptor activation may be the initial trigger leading to activation of the ERK pathway; calcium influx may also lead to activation of the JNK pathway through other signalling mechanisms (15). It is known that pERK is increased by high levels of reactive oxygen species. In this study, only 1 mg/kg increased the phosphorylation of ERK1 and 2 mg/kg decreased the phosphorylation of JNK1/2. Previous findings showed that MPD doses of <5 mg/kg administered intraperitoneally better reflect those used clinically in patients (16) and previous studies showed that a dose of 2 mg/kg is utilised in studies on animals to investigate alterations in the CNS (8–12).

In contrast to ERK and Akt, JNK is more commonly considered a pro-death signalling molecule. In addition to its well-established role in the execution of apoptosis, the JNK pathway is likely to be involved in the neurodegenerative cascade and has been implicated in the loss of dopamine neurons (17). Our study shows that chronic MPD treatment with 2 mg/kg decreases phosphorylation of JNK1/2 in the striatum. Inflammatory signals, changes in levels of reactive oxygen species, ultraviolet radiation, protein synthesis inhibitors, and a variety of stress stimuli can activate JNK. One way this activation may occur is through disruption of the conformation of sensitive protein phosphatase enzymes; specific phosphatases normally inhibit the activity of JNK itself and the activity of proteins linked to JNK activation. Downstream molecules that are activated by JNK include c-Jun, ATF2, ELK1, SMAD4, p53, and HSF1. The downstream molecules that are inhibited by JNK activation include NFAT4, NFATC1, and STAT3. By activating and inhibiting other small molecules in this manner, JNK activity regulates several important cellular functions including cell growth, differentiation, survival, and apoptosis [for a review, see Vlahopoulos and Zoumpourlis (18)]. Studies have shown that MPD is capable of modifying the expression of immediate genes c-fos and zif 268 (19), whose expression and activation are regulated by MAPKs (20).

Although MPD has been used extensively, there is limited knowledge on its potential for producing molecular changes in the brain, especially in intracellular signalling events triggered by receptor activation, and their implications in functional effects. In other studies conducted by our group using MPD, chronic treatment led to the following changes in the striatum: (1) increase in TBARS content and protein carbonyl formation (1, 2, and 10 mg/kg) (21); (2) increase in mitochondrial respiratory chain enzyme activities (2 and 10 mg/kg) (22); (3) increase in DNA damage (10 mg/kg) (8); (4) increase in creatine kinase activity (2 and 10 mg/kg) (23); and (5) cognitive impairment (2 mg/kg) (10–12). Recently, another research group observed that a low dose of MPD enhanced the learning and memory capacity of rats, rather than deteriorating it, supporting the use of MPD as a drug to treat attention deficit hyperactive disorder (24). In addition, an overdose of MPD was seen to upregulate adenosine A(1) receptors in the frontal cortex, but this receptor was not involved in anxiolytic effects (25). A reduction in the immunocontent of brain-derived neurotrophic factor and increased acetylcholinesterase activity in the prefrontal cortex but not in the hippocampus of rats were also observed after treatment with MPD (26). As the phosphorylation of ERK1 leads to proliferation and differentiation and the phosphorylation of JNK1/2 leads to inflammation and apoptosis, we believe that chronic treatment with MPD at low doses is beneficial to the central nervous system during the course of development.

Acknowledgements

This research was supported by grants from CNPq (C.R.B. and J.Q.) and UNESC (C.R.B. and J.Q.), both from Brazil. C.R.B. and J.Q. are CNPq Research Fellows. C.M.C. is the holder of a CNPq Studentship.

References

1. Peterson K, McDonagh MS, Fu R. Comparative benefits and harms of competing medications for adults with attention-deficit hyperactivity disorder: a systematic review and indirect comparison meta-analysis. Psychopharmacology 2008;**197**:1–11.
2. Wilens TE. Pharmacotherapy of ADHD in adults. CNS Spectr 2008;**13**:11–13.
3. Quinn D. Does chirality matter? Pharmacodynamics of enantiomers of methylphenidate in patients with attention-deficit/hyperactivity disorder. J Clin Psychopharmacol 2008;**28**:S62–S66.
4. Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S. The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. J Neurosci 2000;**20**:4563–4572.
5. Sweatt JD. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J Neurochem 2001;**76**:1–10.
6. Wang YZ, Bonner JC. Mechanism of extracellular signal-regulated kinase (ERK)-1 and ERK-2 activation by vanadium pentoxide in rat pulmonary myofibroblasts. Am J Respir Cell Mol Biol 2000;**22**:590–596.
7. Beaulieu JM, Sotnikova TD, Gainetdinov RR, Caron MG. Paradoxical striatal cellular signaling responses to

psychostimulants in hyperactive mice. J Biol Chem 2006; **281**:32072–32080.
8. Andreazza AC, Frey BN, Valvassori SS, et al. DNA damage in rats after treatment with methylphenidate. Prog Neuropsychopharmacol Biol Psychiatry 2007;**31**: 1282–1288.
9. Scherer EB, Matté C, Ferreira AG, et al. Methylphenidate treatment increases Na(+), K(+)-ATPase activity in the cerebrum of young and adult rats. J Neural Transm 2009; **116**:1681–1687.
10. Gomes KM, Souza RP, Valvassori SS, et al. Chronic methylphenidate-effects over circadian cycle of young and adult rats submitted to open-field and object recognition tests. Curr Neurovasc Res 2009;**6**:259–266.
11. Gomes KM, Comim CM, Valvassori SS, et al. Diurnal differences in memory and learning in young and adult rats treated with methylphenidate. J Neural Transm 2010;**117**: 457–462.
12. Gomes KM, Souza RP, Inácio CG, et al. Evaluation of light/dark cycle in anxiety- and depressive like behaviors after regular treatment with methylphenidate hydrochloride in rats of different ages. Rev Bras Psiquiatr 2011;**33**:55–58.
13. Konradi C, Cole RL, Heckers S, Hyman SE. Amphetamine regulates gene expression in rat striatum via transcription factor CREB. J Neurosci 1994;**14**:5623–5634.
14. Adams DH, Hanson GR, Keefe KA. Psychostimulants activate p42/44 MAPK in dorsal and ventral striatum. Soc Neurosci Abstr 2001;**27**:445–452.
15. Shen C, Tsimberg Y, Salvadore C, Meller E. Activation of Erk and JNK MAPK pathways by acute swim stress in rat brain regions. BMC Neurosci 2004;**5**:1–13.
16. Bolaños CA, Barrot M, Berton O, Wallace-Black D, Nestler EJ. Methylphenidate treatment during pre- and periadolescence alters behavioural responses to emotional stimuli at adulthood. Biol Psychiatry 2003;**54**:1317–1329.
17. Saporito MS, Hudkins RL, Maroney AC. Discovery of CEP-1347/KT-7515, an inhibitor of the JNK/SAPK pathway for the treatment of neurodegenerative diseases. Prog Med Chem 2002;**40**:23–62.
18. Vlahopoulos S, Zoumpourlis VC. JNK: a key modulator of intracellular signaling. Biochemistry (Moscow) 2004;**69**: 844–854.
19. Brandon CL, Steiner H. Repeated methylphenidate treatment in adolescent rats alters gene regulation in the striatum. Eur J Neurosci 2003;**18**:1584–1592.
20. Eriksson M, Taskinen M, Leppä S. Mitogen activated protein kinase dependent activation of c-Jun and c-Fos is required for neuronal differentiation but not for growth and stress response in PC12 cells. J Cell Physiol 2007;**210**: 538–548.
21. Martins MR, Reinke A, Petronilho FC, Gomes KM, Dal-Pizzol F, Quevedo J. Methylphenidate treatment induces oxidative stress in young rat brain. Brain Res 2006; **1078**:189–197.
22. Fagundes AO, Rezin GT, Zanette F, et al. Chronic administration of methylphenidate activates mitochondrial respiratory chain in brain of young rats. Int J Dev Neurosci 2007;**25**:47–51.
23. Scaini G, Fagundes AO, Rezin GT, et al. Methylphenidate increases creatine kinase activity in the brain of young and adult rats. Life Sci 2008;**5**:795–800.
24. Veetil PK, Mukkadan JK. Effect of methylphenidate on enhancement of spatial learning by novel alternated dual task. Indian J Physiol Pharmacol 2011;**55**:176–182.
25. Mioranzza S, Costa MS, Botton PH, et al. Blockade of adenosine A(1) receptors prevents methylphenidate-induced impairment of object recognition task in adult mice. Prog Neuropsychopharmacol Biol Psychiatry 2011; **15**:169–176.
26. Scherer EB, da Cunha MJ, Matté C, Schmitz F, Netto CA, Wyse AT. Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in the rat. Neurobiol Learn Mem 2010;**94**:247–253.

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