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Research article

Chronic methylphenidate regulates genes and proteins mediating neuroplasticity in the juvenile rat brain

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HIGHLIGHTS

- Chronic methylphenidate upregulated Arc, IRSp53, Cdc42 and Arp2 expression in cerebral areas associated with reward, cognition and addiction.
- Arc and IRSp53 were down-regulated in the cerebellum following chronic methylphenidate administration.
- The IRSp53 pathway should to be studied further to establish its role in methylphenidate induced neuronal plasticity and long term behavioural effects.

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ABSTRACT

Methylphenidate (MPH) is the front-line psychostimulant medication prescribed for alleviating the symptoms associated with attention deficit hyperactivity disorder (ADHD) in children. Here, we investigated the effects of chronic MPH (2.0 mg/kg, twice daily for 15 days) exposure to young rats (20–25 days old at start of treatment) on the expression of genes and proteins associated with neuroplasticity, such as activity regulated cytoskeleton-associated protein (Arc), insulin receptor substrate protein 53 (IRSp53), cell division control protein 42 (Cdc42), and actin-related protein 2 (Arp2). Chronic MPH increased Arc expression in areas of the cerebrum including, the striatum, nucleus accumbens and hippocampus. In addition, chronic MPH also increased the expression of IRSp53 in the striatum, while Cdc42 and Arp2 were specifically increased in the nucleus accumbens. Conversely, chronic MPH decreased Arc and IRSp53 protein expression in the cerebellum, indicating differential effects of the drug in cerebral areas relative to the cerebellum. Overall, our results indicate that chronic MPH treatment increases expression of genes and proteins associated with dendritic spine formation and neuronal plasticity in target areas of the cerebrum while it decreases the expression in the cerebellum.

1. Introduction

Methylphenidate (MPH) is the main drug treatment for attention deficit hyperactivity disorder (ADHD) [1] and the limited information available regarding the chronic effects of MPH on the maturing brain, has fuelled growing concerns over possible long-term effects of the drug [2]. Dysfunctional dopamine and noradrenaline systems are often associated with the disorder and MPH works by restoring extracellular dopamine and noradrenaline levels via dopamine and noradrenaline transporter blockade [1,3].

Existing evidence suggest that modifications of dopaminergic function in some brain areas such as the frontal cortex and hippocampus are important in mediating memory and learning, while corresponding changes in the nucleus accumbens may underlie reward and addictive behaviour [4,5]. Given the known interaction between dopamine and glutamate in the nucleus accumbens [6], glutamate also contributes to psychostimulant-induced behaviours such as drug-seeking, behavioural sensitisation and relapse [7–9]. In this context, the dendritic spines of the GABAergic medium spiny neurons in the nucleus accumbens and striatum receive dopaminergic inputs from the midbrain, as well as glutamatergic projections from the frontal cortex and the thalamus [10]. Interestingly, chronic exposure to psychostimulants including MPH, amphetamine and cocaine have been shown to increase dendritic branching and dendritic spine density in the nucleus accumbens [5,11,12]. Although MPH has been shown to cause such morphological changes of dendritic spines and thereby induce behavioural changes, the effect of chronic MPH on genes and proteins driving these morphological changes remain largely unknown.

Abbreviations: Arc, activity regulated cytoskeleton associated protein; Arp2, actin related protein 2; Cdc42, cell division control protein 42; IRSp53/58, insulin receptor substrate protein 53/58; NMDA, *N*-methyl-D-aspartate; RT-PCR, real time – polymerase chain reaction.

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Alterations of dendritic spine density are related to the expression patterns of actin-associated genes [13] and actin filaments form dendritic spines from spine precursors (i.e. filopodia). Moreover, the spines covering the surface of the dendrites for the GABAergic medium spiny neurons represent the major contact sites for dopamine and glutamate and hence of importance for the mechanism of action by MPH [14]. Genes and proteins controlling the formation of dendritic spines include the Arc protein, as well as the filament nucleating Arp2/3 complex and its Rho family of GTPase regulators, including Cdc42 that promotes the formation of the spine precursor, filopodia [15]. Another protein that has attracted attention recently is the insulin receptor tyrosine kinase substrate protein 53 (IRSp53). IRSp53 is a multi-domain adaptor protein that interacts with Cdc42 and other effector molecules to regulate membrane and actin dynamics at actin-rich subcellular structures such as filopodia [16–18]. IRSp53 has also been implicated in several brain disorders including ADHD [19], autism spectrum disorders [20], and schizophrenia [21]. Notably, studies on the behavioural phenotypes of mice lacking IRSp53 suggest that such mice exhibit social and cognitive deficits, as well as hyperactivity, which are among the core symptoms of ADHD [5,22].

In this study, the effect of chronic MPH exposure on the mRNA and protein levels of dendritic spine- and plasticity-associated markers such as Arc, IRSp53, Cdc42 and Arp2 were investigated in young rats. The expression of these markers were evaluated in brain regions implicated in the symptoms of ADHD and the mechanism of action of MPH. The study provides novel insights regarding the effect of prolonged MPH exposure on these synaptic plasticity-associated markers in the maturing brain.

2. Methodology

2.1. Animal treatment

Young (80–100 g; 25 days postnatal i.e. P25) male Sprague-Dawley rats were purchased from Charles River (UK). All animals were housed (n = 6 per cage) under controlled conditions of light (12/12 h light-dark cycle) and temperature (22–25 °C, 45% humidity), and allowed free access to food and water. Experiments in this study were performed in strict accordance with the UK Home Office guidelines and the Animal Scientific Procedures Act (1986). Rats were injected intraperitoneally (i.p.) twice daily at 9 am and 5 pm with 2.0 mg/kg MPH (treatment group) or 1.0 ml/kg saline (control group) for 15 days (1.4% of a 3 year predicted life span) starting from P25 and ending at P40 (n = 6 rats/group). At the end of the treatment (~P40) body weights (180–200 g) of rats treated chronically with MPH were not different from their corresponding controls. The 2.0 mg/kg MPH dose administered to the experimental animals in this study has previously been shown to reach peak plasma levels within the therapeutic range in humans [23]. Rats were sacrificed 24 h after the last injection, brain regions corresponding to the nucleus accumbens, frontal cortex, striatum, hippocampus and cerebellum were dissected, snap frozen and stored at −80 °C.

2.2. Quantitative RT-PCR analysis

Total RNA was isolated from the brain samples using RNeasy® Mini kit (Qiagen). TURBO DNA-free™ DNase treatment kit (Ambion Inc, USA) was used to remove genomic DNA contamination. The concentration and quality of the RNA samples were then verified by spectrometry using A260/280 ratios, with a Nanodrop Lite Spectrophotometer (Thermo Scientific, UK). cDNA was synthesised from the total RNA sample using iScript™ cDNA synthesis kit (Bio-Rad, UK). To verify the integrity of the synthesised cDNA and to test the primer conditions prior to quantitative RT-PCR, standard PCR amplifications were performed and the products analysed via gel electrophoresis (2% agarose gel; ThermoFisher Scientific, UK) and visualised on a Gel Doc™ EZ Imager (Bio-Rad, UK). Quantitative RT-PCR analysis was carried out using the StepOnePlus™ Real-Time PCR system (AB Applied Biosystems, UK). Each reaction contained 5 μl SensiFAST™ SYBR Hi-ROX Mix (Bioline, UK), 0.5 μl cDNA, 0.5 μM each of the forward and reverse primer pair, in a total volume of 10 μl made up with sterile distilled water. Three technical replicates were used for each sample. The amplification conditions were as follows: 95 °C for 2 min followed by 40 cycles of 5 s at 95 °C and 30 s at the primer's specific annealing temperature (see Table 1). To obtain the melting curve, the amplified template was melted from 65 °C to 95 °C increasing the temperature by 0.5 °C per cycle. The amplification efficiencies for all pairs of primers were evaluated via tenfold serial dilutions of pooled cDNA. A standard curve for each of the target genes was generated to confirm that the quantitative RT-PCR reactions were run in a linear range. The Ct for each target gene was normalised to the Ct for both *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenase*) and *β-actin*. To control for differences arising from running samples in different 96-well plates, an inter-run calibration was performed using the internal standard samples that were included in all plates. Analyses were conducted according to MIQE guidelines [24]. Normalised gene expression levels were determined by the geometric mean of the relative quantities for all reference targets using target and run specific amplification with qBase Plus software version 2.5 (http://www.biogazelle.com/qbaseplus).

2.3. Western blot analysis

The brain tissues were homogenised in RIPA lysis buffer (Sigma Aldrich, UK) containing protease inhibitor at 4 °C. After centrifu-

Table 1
Target primers and their annealing temperatures.

Gene (GenBank accession number)	Primer Sequence	Ta	Product size
Arc (XM_008765590.1)	F: GGGAGTTCAAACAGGGCTCGGT R: TCCTCCTCCTCAGCGTCCACAT	65 65	199
IRSp53 (AY037934)	F: GACCAAGATGCGGGGCTGGT R: AGTCAGGAGGAGGGAGGGCC	61 63	152
Cdc42 (XM_008764287.1)	F: GCAGGGCAAGAGGATTATGACAG R: TCAATTTGGGTCCCGACAAGCAA	65 63	179
Arp2 (FQ219838.1)	F: GTGGCGCAGTCCTAGCAGACAT R: TTAGAGGCGTGATGGGGACAAGCC	65 71	147
β-actin (NM_031144.3)	F: TCCGTAAAGACCTCTATGCC R: GGACTCATCGTACTCCTGCTT	55 59	230
GAPDH (NG_028301.2)	F: AAACCCATCACCATCTTCCA R: GGCGGAGATGATGACCCTTT	53 57	156

F, Forward primer; R, Reverse primer; Ta, Annealing temperature.