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The impact of methylphenidate and its enantiomers on dopamine synthesis and metabolism in vitro

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ABSTRACT

Methylphenidate (MPH), a psychostimulant, is an effective first-line treatment for the symptoms associated with Attention-Deficit/Hyperactivity Disorder (ADHD). Although most MPH formulations are composed of the racemic 1:1 mixture of the two enantiomers (D- and L-*threo*), converging lines of evidence indicate that D-*threo* MPH seems to be superior to the L-isomer. We aimed to investigate whether MPH racemic mixture or pure enantiomers influence the enzyme activity of tyrosine hydroxylase (TH), monoamine oxidase B (MAO-B), catechol-*O*-methyltransferase (COMT), and aldehyde dehydrogenase (ALDH) in vitro in homogenates of rat PC12 cells incubated with racemic, D- and L-*threo* MPH (1 nM up to 100 μM), or a vehicle for control. We could observe dose dependent enhancement of TH activity with D-*threo* MPH, probably due to its higher affinity to the enzyme, which we could confirm for D-*threo* versus L-*threo* MPH via docking and molecular dynamic simulations analysis. MAO-B enzyme activity was found to be enhanced when incubated with both D- and L-isomers but not with the racemic mixture. This conflicting result was hypothesized to be due to possible aggregation of the two enantiomers or other molecular conformations. Such a possible interaction was observed indirectly, when TH was incubated with constant D-*threo* MPH while increasing L-isomer (increasing total MPH concentrations). Hence, TH activity was slightly decreased with increased L-isomer. In conclusion, the current in vitro investigation points to the stereoselectivity of the investigated enzymes and pharmacological effects of MPH enantiomers.

1. Introduction

Psychostimulant drugs, such as methylphenidate (MPH) and amphetamines (AMP), are the first line treatment for Attention-Deficit/Hyperactivity Disorder (ADHD) in Europe (MPH) and in the US and Canada (AMP). Both drugs show comparatively high effect sizes in the treatment of psychiatric disorders and are by far the most widely studied class of drugs for ADHD. MPH, with a history of use spanning approximately 60 years, is the standard of care treatment for ADHD and reduces all of the three disorder's core symptoms, attention deficit, hyperactivity and impulsivity (Gunther et al., 2010; Wilens, 2008). The chemical structure of MPH possesses two chiral centers, which give rise to four possible isomers. The earliest MPH formulation contained all four isomers: D-(*R*,*S*)-erthyro-, L-(*S*,*R*)-erythro, D-(*R*,*R*)-*threo*-, and L-(*S*,*S*)-*threo*-MPH (Patrick et al., 1981; Patrick et al., 2009). As the D/L-*erythro* MPH components exacerbated cardiovascular toxicity without contributing to the desired central nervous system stimulant effects, they were eliminated from the formulation to improve the margin of safety for MPH products (Patrick et al., 1981). Available data suggest that many of

Abbreviations: ADHD, attention-deficit hyperactivity disorder; ALDH, aldehyde dehydrogenases; AMP, amphetamine; ANOVA, analysis of variance; COMT, catechol-*O*-methyltransferase; DA, dopamine; DAT, dopamine transporter; DHAP, 3,4-dihydroxyacetophenone; DHBA, 3,4-dihydroxybenzylamin; DOPA, 3,4-dihydroxy-L-phenylalanine; ELISA, enzyme-linked immunosorbent assay; HBI, 7,8-dihydrobiopterin; MAO-B, monoamine oxidase B; MD, molecular dynamics; MPH, methylphenidate; NET, norepinephrine transporter; PC12, rat pheochromocytoma cells; PDB, protein data bank; RMSD, Root Mean Square Distance; SAMe, *s*-adenosyl-L-methionin; TH, tyrosine hydroxylase

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the behavioral effects of MPH are conferred by D-*threo* MPH (Davids et al., 2002; Heal and Pierce, 2006), although most MPH formulations are composed of a racemic mixture of D-*threo* MPH and L-*threo* MPH (Markowitz et al., 2003; Quinn et al., 2004). The contribution of the different MPH-isomers to the overall pharmacological profile of the racemic mixture remains unclear; owing to the suggestion that L-*threo* MPH may not be merely inert isomeric ballast. Although the chemical structure of MPH and its function as a stimulant has been known since 1954 (Meier et al., 1954), the mechanisms of action underlying the clinical effects of MPH (racemic mixture and isomers) used in the therapy of ADHD are only partially known. MPH has a neuropharmacological profile with some similarities to AMP, which is well known as an indirect dopamine (DA) agonist causing a striatal DA overflow, probably via DA transporter (DAT)- and partially norepinephrine transporter (NET)-inhibition (Challman and Lipsky, 2000; Easton et al., 2007). However, there are also some known differences between MPH and AMP; MPH for instance exhibits a higher affinity to DAT than AMP, while the latter is a substrate stimulating DA efflux (Walitza et al., 2014).

Due to the fact that MPH is acting in the catecholamine system, especially in the DA system, the present approach used isolated enzymes predominantly involved in DA synthesis and degradation to mimic the possible influence of MPH in the synthesis/degradation pathway of DA. We intended to monitor the acute effect of MPH on DA turnover and DA synthesis capacity, hypothesized to be through inhibition of metabolizing enzymes activities, monoamine oxidase (MAO)-B, catechol-*O*-methyltransferase (COMT), and aldehyde dehydrogenases (ALDH), and/or as a result of activating the rate limiting synthesis enzyme tyrosine hydroxylase (TH) (Fig. 1).

This study was conducted with different doses of racemic mixture of MPH (1:1 mix of D- and L-*threo* MPH) or of the enantiomers D-*threo* and L-*threo* MPH treatment to obtain possible differential regulatory effects of MPH and specifically its enantiomers on DA metabolism in vitro.

2. Material and methods

2.1. Cell culture

The rat pheochromocytoma cells (PC12) used in this study originated from the laboratory of Dr. Silvia Mandel, Technion Faculty of Medicine, Haifa, Israel (old generation originating from ATCC). Cells were grown in buffered 4.5 mg/ml glucose Dulbecco's modified Eagle medium (Life Technologies, Switzerland) supplemented with 10% fetal bovine serum (Life Technologies, Switzerland), 5% horse serum (Life Technologies, Switzerland), and 0.3% gentamycin (50 mg/ml; Life Technologies, Switzerland) in a humidified incubator (5% CO_2) at 37 °C. To eliminate any external monoamines (e.g. serotonin in serum) in the later prepared cell homogenates for the activity assays, cells were sequentially adapted to neurobasal medium (Life Technologies, Switzerland) supplemented with N2 (Life Technologies, Switzerland) and 0.03% gentamycin to reach nearly serum free conditions of 0.25% serum.

2.2. Protein extractions from PC12 cells

Pellets of cultivated PC12 were dissolved in Complete-Lysis-M buffer (Roche, Grenzach, Germany) and homogenized via tissue lyser (Qiagen, Germany) with a frequency of 1/20 per second and for 25 s. After measuring the protein concentration with Bradford reagent (Bradford, 1976) (Sigma Aldrich, Switzerland), the solution was diluted to a finale concentration of 1 mg protein/ml and used for TH, MAO-B, and ALDH enzyme activity assays.

2.3. TH activity assay

The method was modified according to Nagatsu et al. (1979) and composed of the following ingredients (total volume 500 µl): 25 µl

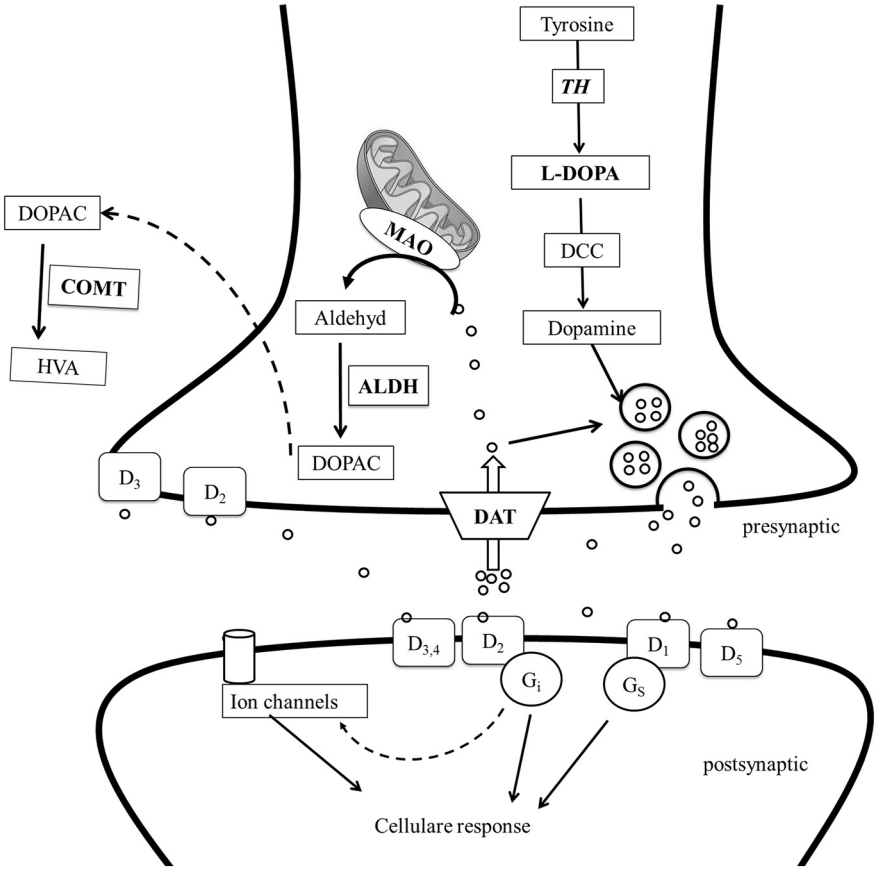

Fig. 1. A representative dopaminergic synapse, including the pre- and post-synaptic terminals. Dopamine is synthesized out of tyrosine via the rate limiting enzyme tyrosine hydroxylase. Once dopamine is released into the synapse, this neurotransmitter can bind to the postsynaptic dopamine receptor families, including the D1, D2, and D3 dopamine receptors. Dopamine D2 receptors are also localized at the presynaptic terminal, acting as a feedback mechanism to regulate dopamine release. The dopamine transporter is located perisynaptically and functions to terminate the actions of dopamine via a transport mechanism. Dopamine is metabolized into DOPAC and HVA via MAO, ALDH. and COMT. Abbreviation: ALDH, aldehyde dehydrogenase; COMT, catechol-*O*-methyltransferase; DDC, DOPA decarboxylase; DOPAC, dihydroxyphenylacetic acid; DAT, Dopamine transporter; HVA, homovanillic acid; MAO, monoamine oxidase; TH, tyrosine hydroxylase.