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The psychostimulant *d-threo*-(*R,R*)-methylphenidate binds as an agonist to the $5HT_{1A}$ receptor

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The present study was undertaken to determine whether *d-threo*-(*R,R*)-methylphenidate (MPH) was exerting binding activity as an agonist or antagonist of 5-HT_{1A} and 5-HT_{2B} receptors. [^{35}S]guanosine5'[gamma-thio]triphosphate ([^{35}S]GTPγS) binding assay and field-stimulated Guinea pig ileum assay were used to determine 5-HT_{1A} receptor agonism and antagonism activity of *d-threo*-(*R,R*)-MPH. The results suggested *d-threo*-(*R,R*)-MPH induced 5-HT_{1A} receptor agonist activity at 100 μM. The Guinea pig ileum functional assay showed that *d-threo*-(*R,R*)-MPH produced agonist-like reduction of neurogenic twitch with an EC_{50} 5.65 $\pm$ 0.36 μM. At 30 μM concentrations, *d-threo*-(*R,R*)-MPH produced 171 $\pm$ 4.24% of the relaxation relative to that caused by 0.12 μM 8-OH-DPAT. However, *d-threo*-(*R,R*)-MPH exhibited no significant pharmacological activity in rat stomach fundus 5-HT_{2B} receptor functional assay. Thus, *d-threo*-(*R,R*)-MPH appears to act as a selective 5-HT_{1A} receptor agonist *in vitro*. It is speculated that the activation of 5-HT_{1A} receptor might play a partial role in *d-threo*-(*R,R*)-MPH mediated dopamine (DA) release in the brain.

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a complex neurobehavioral disorder characterized by varying degrees of inattention, hyperactivity, and impulsivity (Biederman 2005). It is reportedly the single most common chronic health problem afflicting school-age children with an estimated worldwide prevalence of 8–12% (Faraone et al. 2003). The most widely used pharmacological treatment of ADHD is the psychostimulant methylphenidate (MPH) consisting of the racemic (50 : 50) mixture of *d-threo*-(*R,R*)-MPH and *l-threo*-(*S,S*)-MPH isomers. Numerous lines of evidence indicate the *d-threo*-(*R,R*)-MPH, available as an enantiopure dosage formulation, is responsible for the therapeutic benefits of the drug (Patrick et al. 2005; Markowitz and Patrick 2008).

Recently, we reported the results of an exhaustive *in vitro* screening of both *d-threo*-(*R,R*)-MPH and *l-threo*-(*S,S*)-MPH isomers in an array of approximately 100 receptor binding, ion channels, membrane transporters, and functional cellular assays pertinent to the CNS pharmacology of MPH (Markowitz et al. 2006). One of the novel findings from this investigation was stereoselective binding of *d-threo*-(*R,R*)-MPH to two serotonin (5-hydroxytryptamine; 5-HT) receptor subtypes, 5-HT_{1A} and 5-HT_{2B} (Table). Although the degree of potency was relatively low

Table: *In vitro* receptor binding of *d*-MPH and *l*-MPH

Receptor	Ligand	Reference compound	Methylphenidate compound (% inhibition of control specific binding)			
			d-threo-(*R,R*)-MPH		*l-threo*-(*S,S*)-MPH	
5-HT_{1A} (*human*)	[^{3}H]8-OH-DPAT	8-OH-DPAT	64		7	
5-HT_{2B} (*human*)	[^{125}I](±)DOI	(±)DOI	60		8	
IC_{50} and K_i determinations (μM)			IC_{50}	K_i	IC_{50}	K_i
5-HT_{1A} (*human*)			6.8	3.4	>100	>100
5-HT_{2B} (*human*)			4.9	4.7	>100	>100

[^{125}I](±)DOI = 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; [^{3}H]8-OH-DPAT = (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene; IC_{50} = one-half maximal drug concentration; K_i = dissociation constant
* Adapted from: Markowitz et al. (2006)

(Ki values 3.4, 4.7 μM respectively, vs. >100 μM for *l-threo-(S,S)*-MPH at both receptors), stereoselectivity of the interaction, the involvement of serotonergic neurotransmission in numerous psychiatric disorders, and the fact that MPH is known to interact with the DA and NET, but not the 5-HT transporter (Markowitz et al. 2006) led us to further investigate the nature of MPH binding to 5-HT receptors. Accordingly, a series of complementary cellular and tissue response assays were carried out to assess 5-HT binding characteristics (agonist vs. antagonist) with particular interest in the much better characterized 5-HT_{1A} receptor subtype.

2. Investigations and results

The results of [^{35}S]GTPγS binding assay suggested *d-threo-(R,R)*-MPH induced 5-HT_{1A} agonist activity per predefined study criteria, but only at the highest concentration tested (i.e. 100 μM). With regard to functional tissue assay assessing 5-HT_{1A} based on electric field stimulated Guinea pig ileum, various concentrations of *d-threo-(R,R)*-MPH were evaluated for their ability to induce agonist-like reduction in neurogenic twitch of ileum. The results were compared with the effect of 0.12 μM of the established 5-HT_{1A} agonist, and positive control, 8-OH-DPAT, by which the reduction effect was defined as 100%. Experimental results indicated that *d-threo-(R,R)*-MPH produced a concentration dependent, agonist-like reduction of neurogenic twitch with an EC_{50} of 5.65 ± 0.36 μM (Fig. 1). At 30 μM concentrations, *d-threo-(R,R)*-MPH produced 171 ± 4.24% of the relaxation relative to that caused by 0.12 μM 8-OH-DPAT alone. In contrast, with regard to the described 5-HT_{2B} tissue response assay, *d*-MPH exhibited no significant pharmacological activity in either the presence or absence of 0.1 μM 5-HT_{2B} receptor agonist, α-methyl serotonin.

3. Discussion

Using heterologously expressed 5-HT_{1A} and 5-HT_{2B} receptors and radioligand binding assays, we recently provided evidence for a stereoselective binding of *d-threo-(R,R)*-MPH (relative to *l-threo-S,S*-MPH) to both 5-HT_{1A} and 5-HT_{2B} receptors (Markowitz et al. 2006; Markowitz and Patrick 2008). We sought to advance these novel binding data by investigating whether *d-threo-(R,R)*-MPH would exhibit agonist or antagonist effects on 5-HT_{1A} and 5-HT_{2B} receptors utilizing heterozygous cell model as well as native biological assays. The functional assay indicated that *d-threo-(R,R)*-MPH acts as a 5-HT_{1A} receptor agonist with the EC_{50} value of 5.65 ± 0.36 μM determined by a tissue response assay. No agonist/antagonist activity of *d-threo-(R,R)*-MPH on 5-HT_{2B} receptor was observed.

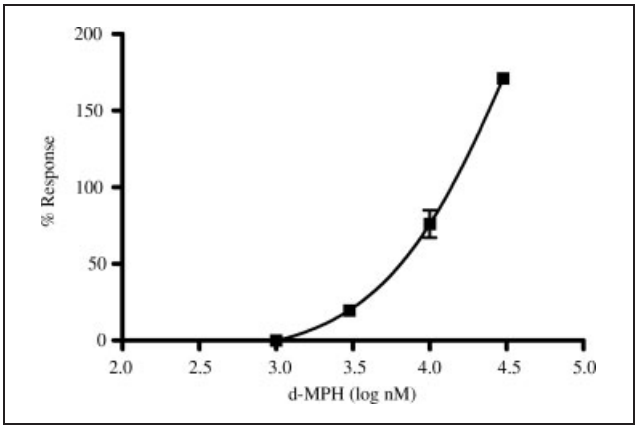

Fig.: *d-threo-(R,R)*-MPH induced reduction of isometric tension caused by electric field stimulation. The ileum segments obtained from Guinea pigs were subjected to electric field stimulation (60% of maximum voltage, 0.05 Hz, 0.3 msec) to produce isometric tension. *d-threo-(R,R)*-MPH was added at indicated concentrations and induced reduction of tension was recorded. Reduction in contractions by 0.12 μM 8-OH-DPAT was defined as 100%. Data are expressed as mean ± SD from two independent experiments.

Although MPH has been widely used for the treatment of ADHD for five decades, the pharmacologic mechanism of MPH is not completely understood. It is generally believed that MPH exerts its therapeutic effects by increasing extracellular DA concentrations in the brain via blockade of the DA transporter as well as the NE transporter (NET) (Solanto 1998; Dougherty et al. 1999). The response to MPH therapy is directly related to the enhancement of extracellular DA release in the brain. The DA transporter is known to be the major factor regulating DA release and re-uptake. However, there is growing evidence that, in addition to the DA transporter, the 5-HT_{1A} receptor also plays a crucial role in regulating DA release. For instance, Sakaue and colleagues demonstrated that the selective 5-HT_{1A} receptor agonist, MKC-242, significantly increased extracellular DA levels in the prefrontal cortex of rat, and pretreatment with a selective 5-HT_{1A} receptor antagonist, WAY100635, inhibited MKC-242 induced DA release (Sakaue et al. 2000). Furthermore, another selective 5-HT_{1A} receptor agonist, *R*(+)-8-OH-DPAT, was demonstrated to enhance DA levels in the prefrontal cortex via activation of the 5-HT_{1A} receptor (Rollema et al. 1997, 2000; Assie et al., 2005). It is well known that the prefrontal cortex is critical for the regulation of behavior, attention, and is the region where MPH exerts at least a portion of its therapeutic effect on ADHD by regulating DA levels (Arnsten 2006). Thus, besides the direct blockage of DA and NE transporters, the activation of the 5-HT_{1A} receptor could play a role in the regulation DA levels by MPH. Additionally, the 5-HT_{1A} receptor has increasingly become a potential target of therapeutic agents due to accumulating evidence of its role in learning and memory (King et al. 2008).

Taken together, the present data for the first time demonstrate that *d-threo-(R,R)*-MPH produces selective agonist-like activity at the 5-HT_{1A} receptor. However, the concentrations required to exert these effects within the *in vitro* systems studied were high relative to other known 5-HT_{1A} agonist compounds. The potential for the induction of meaningful *in vivo* activity, especially the regulation of DA levels in the brain, mediated via 5-HT_{1A} receptor activation following dosing with *d-threo-(R,R)*-MPH, has not been investigated and conclusions of any potential clinical implications of these findings are premature. Further *in vivo* investigations employing appropriate 5-HT_{1A} receptor agonists/antagonists are warranted to elucidate the pharmacologic consequences of *d-threo-(R,R)*-MPH mediated 5-HT_{1A} receptor activation in influencing the balance of DA levels in the brain and therefore altering other physiological functions.

4. Experimental

4.1. Chemical compounds

The highest analytical grade of *d-threo-(R,R)*-MPH that was commercially available was utilized in the studies. *d-threo-(R,R)*-MPH was purchased from Sigma-Aldrich (St. Louis, MO). The purity of the compound as assessed by HPLC was 98% and the specific optical rotation of the isomer was +91.4 degrees (conditions = 1% in methanol at 20 °C).

4.2. Functional cellular and tissue response assays

All cellular and tissue response assays were performed in duplicate by CEREP (Celle l'Evescault, France). Further detail of all assays performed may be accessed at the CEREP web site (www.cerep.com).

4.3. 5-HT$_{1A}$ Cellular assay

An established cellular assay specific to 5-HT$_{1A}$ receptors using heterozygous cells models, Chinese Hamster Ovary cells that stably express human 5-HT$_{1A}$ receptor by measuring ([^{35}S]GTPγS) binding of five different *d-threo*-(*R,R*)-MPH concentrations (1, 10, 30, 50 and 100 μM) was performed in duplicate. *Agonists* were defined *a priori* as agents producing a ≥50% increase in bound [^{35}S]GTPγS relative to 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT, positive control) response. Any compound producing ≥50% inhibition of serotonin-induced [^{35}S]GTPγS binding is considered an *antagonist*.

4.4. 5-HT$_{1A}$ Tissue response assay

Agonist and antagonist effects of *d-threo*-(*R,R*)-MPH on functional 5-HT$_{1A}$ receptors were investigated using ileum from Dunkin Hartley derived male or female Guinea pigs. Changes in isometric tension were recorded in response to test compounds to evaluate the functional effect of 5-HT$_{1A}$ receptors as described by Connor et al. (1989). The ileum segments obtained from CO_2-anesthetized Dunkin Hartley derived male or female Guinea pigs (325–350 g) were placed under 0.5 g tension in a 10 mL bath containing Krebs solution pH 7.4 at 32 °C. The ileum was subjected to electric field stimulation (60% of maximum voltage, 0.05 Hz, 0.3 ms) and the change in isometric tension was recorded. Test compounds, at indicated concentrations, were added and induced reduction of isometrically recorded tension. Reduction in contractions by 50 percent or more (≥50%) within 5 min, relative to the control response from 0.12 μM 8-OH-DPAT, indicates possible 5-HT$_{1A}$ receptor agonist activity. At a test substance concentration where no significant relaxation activity is observed, the ability to inhibit 0.12 μM 8-OH-DPAT-induced relaxant response by ≥50% indicated 5-HT$_{1A}$ receptor antagonist activity according to the experimental protocol.

4.5. Serotonin 5-HT$_{2B}$ tissue response assay

Agonist and/or antagonist effect of *d-threo*-(*R,R*)-MPH on 5-HT$_{2B}$ receptor was evaluated using rat stomach fundus isometric contractions as described by Cohen and Fludzinski (1987). Briefly, Wistar rats (275–300 g) were anaesthetized by CO_2 exposure. Longitudinal sections of the stomach fundus were prepared and placed in organ baths containing 10 ml of modified Krebs buffer (118.2 mM NaCl, 4.6 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM dextrose, 24.8 mM $NaHCO_3$, pH 7.4), saturated with 95% O_2/5% CO_2 at 37 °C. The stomach fundus strips were placed under 4 g resting tension and allowed 1 h to equilibrate prior to drug exposure. Isometric contractions were recorded as changes in grams of force. Contractile dose-responsive curves for agonists were obtained using various concentrations (30 μM to 1 μM). EC_{50} values were defined as the concentration that produced 50% of its maximal contraction. Test substances (30 μM)-induced isometrically recorded contraction by ≥50% within 5 min, relative to the positive control, 0.1 μM α-methyl-serotonin, indicated possible serotonin 5-HT$_{2B}$ receptor agonist activity. At a test substance concentration where no significant agonist activity is elicited, the ability to reduce 0.1 μM α-methyl-serotonin-induced contractile responses by ≥50% indicates 5-HT$_{2B}$ receptor antagonist activity.

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