The Role of the Polymorphic Efflux Transporter P-glycoprotein on the Brain Accumulation of $d$-Methylphenidate and $d$-Amphetamine

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List of non-standard abbreviations:

AMP: amphetamine; d-AMP: dextroamphetamine; l-AMP: levoamphetamine; MPH: methylphenidate; d-MPH: dexmethylphenidate; l-MPH: levomethylphenidate
ABSTRACT

The psychostimulant medications methylphenidate (MPH) and amphetamine (AMP), available in various ratios or enantiopure formulations of their respective active dextrorotary isomers, constitute the majority of agents employed in the treatment of attention-deficit/hyperactivity disorder (ADHD). Substantial inter-individual variability occurs in their pharmacokinetics and tolerability. Little is known regarding the potential role of drug transporters such as P-glycoprotein (P-gp) in psychostimulant PK and response. Accordingly, experiments were carried out in P-gp knockout (KO) mice versus wild-type (WT) mice following intraperitoneal dosing (2.5 mg/kg) of \(d\)-MPH or (3.0 mg/kg) of \(d\)-AMP. Following the administration of each psychostimulant, locomotor activity was assessed at 30-min intervals for two hours. Total brain-to-plasma drug concentration ratios were determined at 10, 30, 80 min post-dosing time points. The results showed no statistically supported genotypic difference in \(d\)-AMP-induced locomotor activity stimulation or in brain-to-plasma ratio of \(d\)-AMP. As for \(d\)-MPH, the P-gp KO mice had 33% higher brain concentrations (\(p<0.05\)) and 67.5% higher brain-to-plasma ratios (\(p<0.01\)) than WT controls at the 10 min post-dosing time point. However, in spite of elevated brain concentrations, \(d\)-MPH-induced locomotor activity increase was attenuated for P-gp compared to that for WT mice. These data indicate that P-gp has no apparent effect on the pharmacokinetics and pharmacodynamics of \(d\)-AMP. Additionally, \(d\)-MPH is a relatively weak P-gp substrate, and its entry into the brain may be limited by P-gp. Furthermore, the mechanism by which \(d\)-MPH-induced locomotor activity was attenuated in P-gp KO mice remains to be elucidated.
Attention-deficit/hyperactivity disorder (ADHD) is the most common neurobehavioral disorder affecting school-aged children. ADHD is generally characterized by varying degrees of inattention, hyperactivity, and impulsivity (Biederman and Faraone, 2005). Although recently established practice guidelines, and algorithms have not identified a specific medication of first choice (Greenhill et al., 2002; Pliszka et al. 2000a; Am Acad Pediatrics, 2001), essentially all of these published guidelines identify one of the primary psychostimulants, methylphenidate (MPH) or amphetamine (AMP) as initial pharmacotherapy. Of these two compounds, various formulations exist in differing biopharmaceutical delivery systems and isomeric content (Greenhill et al., 1999; Pliszka et al., 2000b; Faraone et al., 2002; Patrick et al., 2005) and aggregately constitute approximately 80% of all medications presently employed in the treatment of ADHD. Large interindividual differences in drug response and dose have been noted (Wilens and Biederman 1992; Greenhill et al., 2002) and predicting a therapeutic response in individual patients remains problematic with most research finding no neurological, psychological or physical characteristics as reliable predictors of response (Greenhill et al., 2002).

The ability of drug transporters to significantly influence drug absorption and disposition, and potential role in explaining interindividual differences in therapeutic response or toxicity is increasingly recognized (Ho and Kim 2005). The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of efflux transport proteins contains the subfamily B of which the most thoroughly studied is P-glycoprotein (P-gp).
The multidrug resistance (MDR) gene ABCB1 encodes for P-gp, which is present in numerous normal tissues including the apical membrane of the gastrointestinal tract, the biliary canalicular membranes of hepatocytes, and luminal membranes of endothelial cells in cerebral capillaries forming the blood-brain barrier (BBB) (Lin and Yamazaki, 2003). The biological function of P-gp is thought to be serving a protective role for major organs by limiting cellular uptake of xenobiotics by extruding these compounds into bile, urine, and intestinal lumen, thus limiting brain accumulation (Löscher and Potschka, 2005). P-gp has broad substrate specificity and is able to actively transport a wide variety of structurally unrelated compounds out of cells. P-gp is also highly polymorphic (Schwab et al., 2003) and its expression in the BBB limits substrate access to the central nervous system (CNS) thereby influencing activity of some psychoactive drugs (Lee and Bendayan, 2004; Löscher and Potschka, 2005). Therefore individual differences in P-gp expression could have therapeutic implications for patients receiving pharmacotherapy for ADHD. Further, since a variety of medications and other substances may inhibit or induce the expression of P-gp, substrates of this transporter may become subject to potential drug interactions that may compromise outcomes (Lin 2003; Fricker and Miller 2004; Balayssac et al., 2005)

Although drug transporters can have profound affects on the disposition of, and response to various CNS therapeutic agents (Wang et al., 2004a; 2004b; Löscher and Potschka, 2005), to date, there are little data available with regard to psychostimulants as substrates for P-gp or other drug transporters. In the mouse, P-gp is encoded by both
mdr1a (localized in brain capillaries) and mdr1b (localized in brain parenchyma), which have 90% sequence homology to each other and 80% to human MDRI (Ambudkar et al., 1999; Löscher and Potschka, 2005). Thus, double-knockout mice (i.e. lacking both mdr1a, and mdr1b) are completely devoid of P-gp and allow for the assessment of the influence of P-gp upon candidate substrates (Schinkel et al., 1999).

Accordingly, the present BBB transport studies utilized Abcb1ab(-/-, -/-) double knock-out (KO) mice lacking P-gp, and genetically matched wild-type (WT) Abcb1ab(+/+, +/+) FVB mice as an in vivo model for studying the role of P-gp in brain penetration or accumulation of the major active isomers of MPH and AMP, d-MPH and d-AMP, respectively (Fig. 1). Additionally, these pharmacokinetic studies were complemented by standard pharmacodynamic measures that assessed differences in animal response to psychostimulant dosing potentially mediated by P-gp status.
MATERIALS AND METHODS

Compounds and Reagents

(+)-amphetamine or d-AMP and (-)-amphetamine or l-AMP, as their respective sulfate salts, were gifts from the National Institute on Drug Abuse (NIDA) Research Resource Drug Supply System, (+)-methylphenidate (d-MPH) and (-)-methylphenidate (l-MPH), d3-dl-MPH (methyl labeled), 1-Methyl-3-phenylpropylamine (MPPA), and pentafluoropropionic anhydride were obtained from Sigma-Aldrich (St. Louis, MO). 4-(4,5-diphenyl-1H-imidazol-2-yl) benzoyl chloride (DIB-Cl) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), pentobarbital (Nembutal®) was from Abbott Laboratories (Abbott Park, Ill). All other agents were of high analytical grade and obtained through Fisher Scientific Co. (Fairlawn, NJ).

Research Animals

Eight to nine week-old male Abcb1ab(-/-, -/-) KO mice functionally devoid of P-gp, and genetically matched wild-type (WT) Abcb1ab(+/-, +/-) FVB mice aged 8-10 weeks weighing 20-30 g were obtained from Taconic (Germanton, NY). Animals were housed in groups in translucent polypropylene cages with wood chip bedding. A separate rodent colony room was maintained at 70-74°F on a 12-hour light cycle (lights on 0700 hr; lights off 1900 hr) and all animals had ad libitum access to tap water and a standardized rodent chow (Harlan Teklad Rodent Diets, Indianapolis, IN). Animals were allowed to acclimate
to their cages and colony room for a minimum of 7 days following their arrival prior to any study procedure. All animal studies and procedures were performed in accordance with the US Public Health Service Policy for the Care and Use of Laboratory Animals. Additionally, the Medical University of South Carolina Animal Care Committee approved all experimental studies.

**Study Design and Drug Dosing**

All behavioral studies utilized a DigiScan® Animal Activity Monitor system (model RXYZCM(8) TAO; Omnitech Electronics, Columbus, OH) which employs computer-interfaced photocell monitoring. These chambers can differentiate three distinct types of rodent behaviors; 1) locomotion (recorded as distance traveled (cm) during a given test period), 2) stereotypic movements, and 3) vertical movements (rearing responses, which provide an index of a coordinated complex motor response). The latter two behaviors were recorded as frequencies.

**Dose Finding Studies**

Prior to performing the primary pharmacokinetic and pharmacodynamic experiments, preliminary dose-finding studies were conducted to determine doses of the respective psychostimulants which would best capture any pharmacodynamic consequences of P-gp deficiency and might allow correlation of behavioral effects with pharmacokinetic measurements in blood and brain. The goal of these preliminary dosing experiments was to identify a dose of each compound that would allow detection of either enhancement or attenuation of drug-induced locomotor stimulation for P-gp deficient, relative to control mice. Since dose-response studies indicate that higher doses of each drug produce
stereotypic behavior that results in a reduction of locomotor activity, stereotypy was assessed to insure that reductions in locomotor activity reflected attenuated rather than enhanced pharmacodynamics. Thus, in separate dose finding experiments, following a 30 min habituation period, three groups of WT control mice (n=6 each) were dosed i.p. with saline vehicle (0.01 ml/g bodyweight), 2.5 mg/kg, or 5.0 mg/kg doses of d-MPH (Exp-1) and d-AMP (Exp-2). Doses ultimately selected were 2.5 mg/kg for d-MPH and 3.0 mg/kg for d-AMP.

Locomotor Activity Determinations

Following the determination of optimal doses of each psychostimulant (2.5 mg/kg for d-MPH and 3.0 mg/kg d-AMP), naïve WT control mice [Abcb1ab(+/+ , +/+)] (n=18) and P-gp KO mice [Abcb1ab(-/- , -/-)] (n=18) were used to assess the influence of P-gp reduction on the stimulatory effects of d-MPH and d-AMP. The mice in each experiment were naïve to experimental manipulations. The experimental design was a 2(WT vs KO) x 2(Psychostimulant vs Vehicle) factorial. Mice were habituated to the activity chambers for 30 min, removed and injected with the respective psychostimulant, and placed back into the activity chamber for an additional 120 min. Activity data was collected in 5 min increments. Data were analyzed with an analysis of variance (ANOVA) appropriate for the 2 x 2 factorial design.

Dosing and Blood and Brain Sample Collection

Pharmacokinetic studies to compare blood and brain tissue drug concentrations in Abcb1ab(-/- , -/-) mice versus the control animals (n= 6 mice per time point/per group) were completed on mice used for the motor activity experiments at 10, 30, or 80 min post i.p. dosing with 2.5 mg/kg for d-MPH and 3.0 mg/kg for d-AMP. At the selected time
points following psychostimulant dosing, mice were injected i.p. with pentobarbital 100mg/kg to produce deep anesthesia prior to exposure of the heart. The right atrium was then punctured and approximately 0.3 ml of blood was withdrawn via syringe equipped with a 22-gauge needle (Becton Dickinson, Rutherford, NJ). Direct heart blood was obtained in order to avoid possible contamination with gastric contents associated with trunk blood sampling that could influence drug concentration measurements. Collected whole blood samples were immediately transferred to light gray-stoppered glass collection tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ) stored on ice that contained sodium oxalate to prevent clotting and sodium fluoride to prevent hydrolysis of MPH. Blood samples were then centrifuged at 3000g x 20 min to separate plasma from other blood components. The plasma portion was then removed and stored at -70º C until analysis. Whole brains were likewise immediately collected by dissection, weighed, and placed in plastic conical tubes on ice, and subsequently transferred to a -70º C freezer prior until analysis. Stored whole brains were later thawed and homogenized in a five-fold volume of an HBSS buffer containing 0.02M HEPES, pH 7.2 with a Polytron PT 1200 handheld homogenizer (Brinkmann Instruments, Westbury NY) prior to extraction procedures.

**Gas Chromatographic-Mass Spectrometry Analysis (GC-MS)**

MPH determinations were made for both *d-* and *l-*MPH as pentafluoropropionyl (PFP) derivatives using a GC-MS-negative ion chemical ionization (NICI) method. *d*-dl-MPH was incorporated as the internal standard. Instrumentation was an Agilent Model 6890
DMD 9605

GC-5973 MS fitted with a 5% phenylmethylpolysiloxane column (HP-5MS, J & W Scientific, 30 M x 0.25 mm, 0.25 µM film). In brief, 100 µl of plasma per sample time point and 100 µl of brain sample homogenate were analyzed. $d_3$-MPH was added as the internal standard at 0.25 µg per plasma sample and 1 µg per brain sample. Sodium chloride (0.5 gm) was added to brain samples to prevent emulsion. Ammonium hydroxide (28%, 100 µl) was added, followed by extraction with butyl chloride: acetonitrile (4:1, 2 ml). The organic phase was transferred to silanized 4 ml vials and evaporated to dryness under nitrogen. Pentfluoropropionic anhydride (50 uL) was added and upon sealing with Teflon-lined caps, the vials were heated at 50°C for 25 min, cooled to 25°C, and then evaporated to dryness under nitrogen. The residue was reconstituted with heptane (50 µl) and 2 µl injections were made by the pulsed-splitless mode. The helium carrier gas linear velocity was 50 cm/sec. The oven was held at 90°C for 1.5 min, then ramped to 280°C at 20°C/min and held for 5 min. Methane was used as the ionization buffer. Detection was by selected ion monitoring of m/z 339 for MPH-PFP [M-(2HF)] and m/z 342 for the internal standard [M-(2HF)]. These compounds eluted at 8.97 and 8.96 min, respectively. The lower limit of quantitation for $d$-MPH in plasma and brain was 10ng/ml and 40 ng/g, respectively.

High Performance Liquid Chromatography (HPLC) Analysis

Both $d$- and $l$-AMP determinations were made using an HPLC method based on that of Al-Dirbashi and associates (al-Dirbashi et al., 1998). In brief, 100 µl of the sample, 50 µl of internal standard MPPA (2 µM) were added and mixed followed by the addition of
500 µl of 0.05 M borate buffer (pH 10.0) and 1.5 ml of ethyl acetate. Samples were mixed for 5 min and then centrifuged for 10 min at 2000g. The organic layer (1 ml) was transferred to fresh tubes and evaporated to dryness under nitrogen. The sample residues were derivatized by adding 10 µl of carbonate buffer (10 mM, pH 9.0) and 180 µl of 0.1 mM DIB-Cl. The mixture was vortexed and left at room temperature for 10 min. The reaction was terminated by the addition of 10 µl of 28% ammonia solution and 20 µl of resultant mixture were subjected to HPLC analysis. The HPLC system consisted of a Waters 2690 Separations module (Waters, Milford, MA), a C18 reversed-phase column (250×4.6mm, 5 µm, Phenomenex, Torrance, CA) and a Waters 474 scanning fluorescence detector (Waters, Milford, MA). The mobile phase was a mixture of 10mM of citrate buffer (pH 4.0): acetonitrile (34:66, v:v) with the flow rate set at 1.0 ml/min. The detection wavelengths were set at 330 nm (λex), 440 (λem). The lower limit of d-AMP detection in plasma and brain was 0.9 ng/ml and 7.4 ng/g, respectively.

Data Analysis

Quantitation of d-MPH and d-AMP was compared by comparing peak area ratios of each psychostimulant to their respective internal standards with ratios derived from calibration curves of standards containing known amounts of drug extracted from spiked mouse plasma and brain as described above. Three time points were examined following dosing of each drug expressed as ng/ml for blood concentrations and ng/g for whole brain. An unpaired t-test was used to compare the differences between concentrations of the respective psychostimulants in the Abcb1ab(-/-, -/-) mice versus the control animals (i.e.
Abcb1ab[+/+, +/-]) and their brain-to-plasma ratios. A two-tailed t test was used with a level of statistical significance was set at $P<0.05$. With regard to behavioral activity measures of Abcb1ab(-/-, -/-) mice versus control animals, motor activity was expressed as total distance traveled (cm) during a 30-min habituation prior to drug or vehicle injections to determine potential genotypic differences in basal motor activity; and then at 30-min intervals for two hours following injections of drug or vehicle. Basal motor activity was analyzed with a one-way ANOVA for the four groups: wild-type controls injected with vehicle (WTV) or with drug (WTD) and P-gp KO mice injected with vehicle (P-gp KOV) or drug (P-gp KOD). For the first experiment, the drug was d-MPH, for the second, d-AMP. Data following drug or vehicle injections were analyzed with 2(Genotype) x 2(Drug) x 4(30-Min Time Interval) ANOVAs for each experiment. Significant interaction terms were resolved with additional one-way ANOVAs and Tukey tests of differences between means. Sample size for the different groups in the d-MPH experiment were: 8 for WTV, 7 for P-gp KOV, 6 for WTD, and 6 for P-gp KOD. For the d-AMP experiment, sample sizes were: 8 for WTV, 8 for P-gp KOV, 8 for WTD, and 8 for P-gp KOD.
RESULTS

Locomotor Activity

Locomotor activity during the 30 min habituation period and the two-hour test period following psychostimulant injections are summarized in Fig 2 for d-MPH and Fig 3 for d-AMP. To determine if P-gp deletion influenced basal motor activity levels, data generated during the 30-min habituation period prior to injection of psychostimulant were analyzed with One-way ANOVAs for each experiment. To assess the impact of P-gp deletion on the stimulatory effect of d-MPH and d-AMP, data generated after drug injection were analyzed with 2(Genotype) × 2(Drug) × 4(30-Min Time Interval) ANOVAs for each experiment.

Basal motor activity during the 30-min habituation period did not differ for Abcb1ab(−/−, −/−) P-gp KO mice and Abcb1ab(+/+, +/+ WT controls. The ANOVA on the habituation data indicated no significant difference among the four groups for either the d-MPH [F(3,23)= 1.203, p= 0.331] (Fig. 2) or the d-AMP [F(3,30)= 2.020, p= 0.135] (Fig. 3) experiments.

Mice injected with d-MPH had elevated, motor activity relative to that of vehicle-injected mice regardless of genotype; however the degree of stimulation was somewhat attenuated for the P-gp KO mice during the earlier testing times (Fig. 2). This different pattern of activity across time for the four different groups following the d-MPH or vehicle injections was supported by a significant Genotype × Drug × Time Interval interaction [F(3,69)= 4.906, p < 0.01]. Additional analysis to resolve this interactive
effect of the three factors on motor activity indicated differences in activity among the four groups at the 30-min \( [F(3,23)= 18.510, p< 0.001] \), the 60 min \( [F(3,23)= 10.448, p< 0.001] \) and the 90 min \( [F(3,23)= 5.134, p<0.007] \) intervals following injections. The groups did not differ during the final 30-min interval of assessment \( [F3,23)= 1.055, p = 0.387] \). Tukey HSD tests were used to statistically compare individual groups at each of the 30-min intervals. These tests indicated that activity of vehicle-injected WT and P-gp mice did not differ at any of the time points. \textit{d}-MPH dosed WT mice were more active than their vehicle controls during the first two 30-min intervals \([p< 0.01]\) whereas \textit{d}-MPH-injected P-gp KO mice were more active than their vehicle controls during the first three time periods. Importantly, and contrary to our hypothesis, \textit{d}-MPH elevated motor activity of P-gp KO mice to a lesser, rather than greater degree than observed for WT control mice. This genotypic difference was statistically supported for the first 30-min interval.

As noted for \textit{d}-MPH, \textit{d}-AMP significantly elevated locomotor activity in comparison to vehicle-injected mice for both genotypes, and the stimulation was for a longer time period than that produced by \textit{d}-MPH (Fig. 3). In contrast to the \textit{d}-MPH effect, genotype did not influence the \textit{d}-AMP effect on motor activity \([\text{Genotype } \times \text{ Drug } \times \text{ Time Interval}: F(3,81)= 0.395]\). The ANOVA, however, did indicate a significant Drug effect \([F(1,27)= 53.122, p< 0.001]\) which depended upon time interval \([F(3,81)= 8.347, p< 0.001]\). Follow-up analyses indicated significantly elevated activity for drug injected mice relative to vehicle controls at all four time intervals \([\text{T-30: } F(3,27)= 8.720, p< 0.001; \text{T-60: } F(3,27)= 13.267, p< 0.001; \text{T-90: } F(3,27)= 23.60, p<0.001; \text{T-120: } F(3,27)= 23.9960, p<0.001]\) intervals following injections. Comparison of individual groups at
each time interval indicated no genotypic difference between the two vehicles or between the two $d$-AMP injected groups at any time point.

**Blood and Brain Drug Measurements and Ratios**

As expected, in the case of the P-gp KO mice vs control animals, plasma concentrations were not significantly different at any time point following i.p. injection of $d$-MPH and $d$-AMP since this dosing route does not expose the molecules to absorption processes also mediated by P-gp which could affect systemic concentrations ($\text{Fig. 4a, 5a}$). However, with regard to brain concentrations, the P-gp KO mice had 33% higher whole brain $d$-MPH concentrations ($p<0.05$) than controls at the 10 min post-dosing time point ($\text{Fig. 4b}$), and numerically higher values at 30 and 80 min time points but did not reach statistical significance. Additionally, the brain to plasma ratios of $d$-MPH were 67.5% higher in the P-gp KO mice vs control animals ($p<0.01$) while the 30 and 80 min time points again yielded numerically higher values but failed to reach statistical significance ($\text{Fig. 4c}$). For $d$-AMP, no differences in either brain concentration or brain to plasma ratios were noted at any post-dosing time point for P-gp KO vs WT mice ($\text{Fig. 5b, 5c}$).
DISCUSSION

For any therapeutic agent intended to act as a psychotherapeutic agent, adequate drug concentrations must be attained in the brain to achieve the desired response. P-gp expressed in BBB provides a protective physiological barrier capable of limiting a variety of CNS drugs from entering brain and prevents or minimizes their pharmacological effects. The mdr1a/b knockout mouse model has been widely used in investigating the function of P-gp in drug disposition. Comparison of the brain-to-plasma concentration ratio in P-gp KO animals versus WT animals has become a standard experimental approach to determine whether the tested drugs are P-gp substrates and limited from entering into brain. At least one previous study assessed the AMP derivative methamphetamine, an infrequently prescribed FDA-approved agent for treating ADHD in the US (Desoxyn®) in a mouse P-gp KO model (i.e. single knockout, mdr1a -/-) relative to control animals (Mann et al., 1997). In this investigation, differential effects on the dopamine transporter were observed in the P-gp deficient mice relative to control animals suggesting AMPs and structurally related compounds could serve as P-gp substrates. However, unlike the present study, no drug concentrations were measured. Doran and associates (2005) recently reported the results of a comprehensive screening of over 30 CNS active agents in the Abcb1ab(-/-, -/-) mouse model including racemic MPH (i.e. dl-MPH) at subcutaneous doses of 3 mg/kg which effectively delivers only 1.5 mg/kg of the active d-isomer per dose. These investigators collected blood (plasma), brain, and cerebral spinal fluid (CSF) samples at 4 time points post dosing, 0.5, 1.0, 2.5, and 5 hrs.
and calculated the area under the time versus concentration curves (AUCs) for \( Abcb1ab(-/-, -/-) \) mice versus control animals. No statistically significant difference in drug concentration in any sampled tissues were reported when comparing the two groups. However, significant differences were found when they compared the ratio of brain-to-plasma and CSF-to-plasma concentrations of \( dl \)-MPH.

In the present study, the concentrations of \( d \)-MPH and \( d \)-AMP in brain and plasma were measured at 10, 30 and 80 min post-dosing time point in P-gp KO and WT mice. The results showed that, only at 10 min post-dosing time point, P-gp KO mice had significant higher values of the \( d \)-MPH brain concentrations and brain-to-plasma ratios than WT control. However, these increments in P-gp KO mice were minor relative to other CNS drugs known to be P-gp substrates. With regard to \( d \)-AMP, no significant differences in the drug concentrations of brain and plasma and the ratios of brain to plasma were observed at any time points between P-gp KO mice and WT mice. These data indicate that \( d \)-AMP is not likely to be a P-gp substrate, while \( d \)-MPH may be a relatively weak P-gp substrate. The present data are consistent with, and support, previous findings with racemic MPH.

Locomotor activity assessment is a well-known and widely accepted research paradigm to evaluate behavioral effects of stimulants in rodents. Coupled measures of motor activity with brain and blood concentrations of the respective psychotimulants were investigated in order to document the potential behavioral consequences of any P-gp mediated differences in the brain penetration of the studied compounds in \( Abcb1ab(-/-, -/-) \).
mice versus matched control animals. As expected, in a comparison of the increase of locomotor activity stimulated by d-AMP in a 120 min period, there was no statistical difference between P-gp KO and WT mice. However, in spite of elevated d-MPH concentrations in the brain of P-gp KO mice compared to WT mice, drug-induced stimulation was attenuated rather than enhanced as was hypothesized. Possible reasons for this unanticipated result are speculative but include the possibility that regioselective differences in d-MPH concentrations could have differed among the genotypes that would have gone undetected in our whole brain analyses. An alteration in the volume of distribution and/or residence time could also potentially contribute to the increase of the brain:plasma ratio of d-MPH in KO animals versus WT animals. Additionally, as has been recently reported, the possibility of compensatory increases in genetic expression and synthesis of other transporters in P-gp deficient mice cannot be excluded (see Cisternino et al., 2004). Other possible explanations include the down-regulation of other systems affecting d-MPH disposition and pharmacodynamic response, or attenuated activity in Abcb1a/b(-/-) mice could be a physiological role for P-gp in removing neuroactive metabolites.

Although l-isomers of MPH and AMP are viewed as being pharmacologically inactive to mildly active relative to their respective d-isomer counterparts (Patrick and Markowitz, 1997), the P-gp substrate properties of l-MPH and l-AMP, as well as d-isomers of MPH and AMP, were initially studied in vitro utilizing the human MDR1 cDNA-transfected cell line LLC-PK1/MDR1 and its parental cell line LLC-PK1 (the experimental details and results for these and other ADHD therapeutic agents are being
presented in full elsewhere). No differences in the accumulation of tested compounds were observed between LLC-PK1/MDR1 and LLC-PK1 cells except that a 97% increase of the accumulation of \( d \)-MPH in LLC-PK1 cells occurred compared to that in LLC-PK1/MDR1 cells. These results corroborate our \textit{in vivo} findings and suggest that neither \( d \)- nor \( l \)-AMP nor \( l \)-MPH are P-gp substrates, and that \( d \)-MPH may be at best, a relatively weak P-gp substrate (unpublished observation). Considering the minor differences between \( d \)- and \( l \)-isomers of MPH and AMP with regard to being P-gp substrates coupled with the weak pharmacological activity of the respective \( l \)-isomers, no further \textit{in vivo} experiments of \( l \)-MPH and \( l \)-AMP in P-gp KO mice were carried out. Both MPH and AMP are known to be biotransformed into CNS-active compounds. However, the amounts of these metabolites produced relative to circulating parent drugs in humans is so small as to be generally viewed as insignificant (see Patrick and Markowitz, 1997). Thus, further evaluation of their respective metabolites was not deemed worthwhile at present and not undertaken. In conclusion, these data indicate that P-gp has no effect on the pharmacokinetics and pharmacodynamics of \( d \)-AMP. Additionally, \( d \)-MPH appears to be a relatively weak P-gp substrate, and its entry into the brain may be limited to a minor extent by P-gp. Finally, the mechanism by which \( d \)-MPH-induced locomotor activity was attenuated in P-gp KO mice remain unknown. It appears that if active transport of \( d \)-AMP and \( d \)-MPH does occur within the BBB, one or more drug transporters other than P-gp governs brain penetration.
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LEGENDS FOR FIGURES

**Figure 1.** Chemical structures of *d*-MPH and *d*-AMP.

**Figure 2.** Locomotor activity of P-gp KO and WT mice at 30 min intervals prior to and following i.p. injections of *d*-MPH (2.5 mg/kg) or saline. Locomotor activity of the four groups did not differ prior to *d*-MPH injection. *d*-MPH elevated locomotor activity to a greater extent in the WT relative to P-gp KO mice.

**Figure 3.** Locomotor activity of P-gp KO and WT mice at 30 min intervals prior to and following i.p. injections of *d*-AMP (3.0 mg/kg) or saline. Locomotor activity of the four groups did not differ prior to *d*-AMP injection. *d*-AMP elevated locomotor activity to the same degree for P-gp KO mice and control animals.

**Figure 4.** Blood (a), brain (b) concentrations and brain-to-plasma ratios (c) of *d*-MPH in P-gp KO mice (solid bars) and WT mice (open bars) at 10, 30, 80 min after i.p. injection of 2.5 mg/kg of *d*-MPH. The concentrations of *d*-MPH were determined using GC-MS analysis. Values are shown as mean ± SD (n=6). *p*<0.05, **p*<0.01 compared with WT mice.

**Figure 5.** Blood (a), brain (b) concentrations and brain-to-plasma ratios (c) of *d*-AMP in P-gp KO mice (solid bars) and WT mice (open bars) at 10, 30, 80 min after i.p. injection of 3.0 mg/kg of *d*-AMP. The concentrations of *d*-AMP were determined using HPLC analysis. Values are shown as mean ± SD (n=6).
Figure 1

*d-threo*-methylphenidate

*d*-amphetamine
Figure 2

This figure illustrates the total distance (cm) as a function of post-injection time (min) for different groups: WT-Vehicle (N=8), KO-Vehicle (N=7), WT-MPHD (N=6), and KO-MPHD (N=6). The graph shows a decrease in total distance over time for all groups, with the KO-Vehicle group having the least distance and the WT-Vehicle group having the most distance at baseline.
Figure 3
Figure 4a

The figure shows the d-MPH plasma concentration (µg/ml) over time (min) for two genotypes: Abcb1ab -/-,-/- and Abcb1ab +/+,+/+. The concentration is measured at 10, 30, and 80 minutes, with error bars indicating variability.
Figure 4b

![Graph showing d-MPH brain concentration (µg/g) over time (min) for Abcb1ab -/-,-/- and Abcb1ab +/-,+/+. The graph includes error bars indicating variability.](image)
Figure 4c

![Bar graph showing the ratio of d-MPH Brain to Plasma concentration over time (10, 30, and 80 minutes) for Abcb1ab -/-,-/- and Abcb1ab +/+,+/

**Statistical significance:**

- **Abcb1ab -/-,-/-**
  - 10 minutes: 4.5 ± 0.5
  - 30 minutes: 6.0 ± 0.8
  - 80 minutes: 30.0 ± 2.0

- **Abcb1ab +/+,+/**
  - 10 minutes: 3.0 ± 0.3
  - 30 minutes: 5.5 ± 0.7
  - 80 minutes: 25.0 ± 2.5

**Note:** The graph indicates a significant difference between the two groups at 80 minutes (***).
Figure 5a
Figure 5b
Figure 5c