Methylphenidate and its ethanol transesterification metabolite ethylphenidate: brain disposition, monoamine transporters and motor activity
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Ethylphenidate is formed by metabolic transesterification of methylphenidate and ethanol. Study objectives were to (a) establish that ethylphenidate is formed in C57BL/6 (B6) mice; (b) compare the stimulatory effects of ethylphenidate and methylphenidate enantiomers; (c) determine methylphenidate and ethylphenidate plasma and brain distribution and (d) establish in-vitro effects of methylphenidate and ethylphenidate on monoamine transporter systems. Experimental results were that: (a) coadministration of ethanol with the separate methylphenidate isomers enantioselectively produced l-ethylphenidate; (b) d and dl-forms of methylphenidate and ethylphenidate produced dose–responsive increases in motor activity with stimulation being less for ethylphenidate; (c) plasma and whole-brain concentrations were greater for ethylphenidate than methylphenidate and (d) d and DL-methylphenidate and ethylphenidate exhibited comparably potent low inhibition of the dopamine transporter, whereas ethylphenidate was a less potent norepinephrine transporter inhibitor. These experiments establish the feasibility of the B6 mouse model for examining the interactive effects of ethanol and methylphenidate. As reported for humans, concurrent exposure of B6 mice to methylphenidate and ethanol more readily formed l-ethylphenidate than d-ethylphenidate, and the l-isomers of both methylphenidate and ethylphenidate were biologically inactive. The observed reduced stimulatory effect of d-ethylphenidate relative to d-methylphenidate appears not to be the result of brain dispositional factors, but rather may be related to its reduced inhibition of the norepinephrine transporter, perhaps altering the interaction of dopaminergic and noradrenergic neural systems. Behavioural Pharmacology 18:39–51 © 2007 Lippincott Williams & Wilkins.

Introduction
The recognition given to adult attention-deficit hyperactivity/disorder (ADHD) has increased in recent years (Kessler \textit{et al.}, 2005; Resnick, 2005). For the past 50 years, dl-threo-methylphenidate (MPH) has been the most commonly prescribed medication for the treatment of ADHD (Dodson, 2005; Spencer \textit{et al.}, 2005). As a result of lifestyle differences between adults and children, the suitability of using psychostimulants to treat adult ADHD patients warrants special consideration. For example, in contrast to children, adults diagnosed with ADHD have an increased likelihood of comorbid substance abuse disorder (Barrett and Pihl, 2002; Secnik \textit{et al.}, 2005). In addition, ethanol (EtOH) commonly consumed by adults is now recognized to interact with MPH (Markowitz \textit{et al.}, 1999, 2000; Patrick \textit{et al.}, 2005a, 2006). Owing to such differences between child and adult populations, treatment strategies over the life cycle are under investigation.

MPH is metabolized primarily to the inactive de-esterification product ritalinic acid. A smaller portion of the drug is converted to lactam metabolites, as well as to the active compound para-hydroxy-MPH. Recent findings indicate that coadministration of EtOH and MPH also yields the transesterification product ethylphenidate (EPH). EPH was first detected as a metabolite in two cases of fatal drug overdose, in which unknown amounts of MPH and EtOH were coingested (Markowitz \textit{et al.}, 1999). In a subsequent controlled study (Patrick \textit{et al.}, 2005a, 2006) using adult volunteers, a clinically relevant dose of dl-MPH (0.3 mg/kg) given with EtOH (0.6 g/kg) produced primarily l-EPH. The $C_{\text{max}}$ values for l-EPH reached 1.01 and 0.55 ng/ml for men and women, respectively, in comparison with corresponding values of 0.07 and 0.05 ng/ml for d-EPH. In addition, EtOH elevated blood concentrations of d-MPH (i.e. $C_{\text{max}}$ and area under curve values) compared with values obtained from MPH alone. The formation of EPH...
following MPH–EtOH coadministration appears to be analogous to the formation of cocaethylene following concomitant exposure to cocaine and EtOH. As noted above for MPH, the cocaine–EtOH combination also elevates cocaine levels in plasma (Perez-Reyes and Jeffcoat, 1992). In laboratory animals, the stimulatory effects of cocaethylene administration alone (Jatlow et al., 1991; Katz et al., 1992; Sable et al., 2004), or cocaine administered with EtOH (Masur et al., 1989), differs from cocaine injections alone. As the structure of the pharmacophore of cocaine and MPH are similar (Froimowitz et al., 1995), and the two drugs have similar inhibitory action on the dopamine transporter (DAT) (Ding et al., 1994; Gatley et al., 1995, 1999; Volkow et al., 1998, 1999; Patrick et al., 2005a), it was postulated that the stimulatory effects of the EPH transesterification product from MPH and EtOH may likewise differ from its parent compound MPH.

Studies on rats (Patrick et al., 1987; Gerasimov et al., 2000) and mice (Gatley et al., 1999) consistently indicate that d and dl-MPH produce dose-related increases in motor activity, whereas the l-form has little or no stimulatory effect (Ding et al., 2004). It is noted though, that interstudy variables such as animal strain (Yang et al., 2003; Amini et al., 2004), route of drug administration (Gerasimov et al., 2000), dose range as well as the use of the racemic form versus separate enantiomers of MPH, can profoundly impact the response to MPH and complicate comparisons of results from different laboratories (Risch et al., 1980; Tirelli and Witkin, 1995; Yuan et al., 1997). The use of racemic versus d-MPH might account for some of the observed variability across studies at similar doses, as the racemic form contains only 50% of the active d-form. A recent report from our laboratory (Patrick et al., 2005a), however, indicated that the stimulatory effects of dl-MPH injected into B6 mice was 25% rather than 50% less than that produced by d-MPH.

It is well documented that MPH inhibits the uptake of dopamine (DA) and norepinephrine (NE) by its action on their respective transporters, that is, DAT and NET (Patrick et al., 1987, 2005a; Kuczenski and Segal, 1997, 2001, 2002), thus elevating extracellular transmitter levels and receptor binding. The role of DA systems in the motor stimulatory effects of MPH is also well established (Ross, 1979; Patrick et al., 1987, 2005a; Aoyama et al., 1997; Gatley et al., 1999; Kuczenski and Segal, 2001; Ding et al., 2004), and some reports suggest that NE systems might also contribute to the drug's stimulatory action (Tyler and Tessel, 1980; Kuczenski and Segal, 2001; Bymaster et al., 2002; Oades et al., 2005; Patrick et al., 2005a), perhaps via interaction of NE and DA systems (Xu et al., 2000; Kuczenski and Segal, 2001, 2002; Bymaster et al., 2002; Moran-Gates et al., 2005; Patrick et al., 2005a). Furthermore, the extent to which the differential effects of MPH and EPH on motor activity might be accounted for by different effects on monoaminergic neurotransmitter systems has not been well established.

The focus of this study was to examine the utility of the B6 mouse model for investigating the interactive effects of MPH and EtOH. The experimental objectives were (a) to determine whether EPH is formed in the B6 mouse model as noted for humans; (b) to further characterize EPH as an active metabolite of MPH by comparing the stimulatory effect of the enantiomers and racemic forms of the two compounds; (c) to compare brain accumulation of the two compounds to determine whether whole-brain concentrations might contribute to the reduced stimulatory effect of EPH compared with MPH and (d) to characterize the in-vitro effects of MPH enantiomers on monoamine transporters for comparison with the previously established activities of the EPH enantiomers (Patrick et al., 2005a). The overall objective was to determine a potential mechanism for the differences in the stimulatory effects of MPH relative to EPH which may be associated with differences in whole-brain concentrations or in binding properties to transporter proteins.

Methods

Subjects

Male B6 mice were obtained from the Jackson Laboratories (Bar Harbor, Maine, USA) at 49 days of age. They were individually housed in a colony room on a 12-h light/dark cycle (light: 07.00–19.00 h) with free access to food and water for at least 4 days before the start of the behavioral tests. All experiments were approved by and conducted within the guidelines of the Institutional Animal Care and Use Committee at the Medical University of South Carolina and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80–23, revised 1996).

Ethylphenidate formation

Murine formation of EPH was assessed by injecting animals separately with 5.0 mg/kg d-MPH or l-MPH and a 2.5 g/kg dose of EtOH. Animals were immediately placed in metabolism chambers to collect urine. Urine was collected for 6–8 h and stored at −70°C until analysis.

Apparatus

Motor activity was assessed with a Digiscan Animal Activity Monitor system, model RXYZCM(8) TAO with a two-animal option (Omnitech Electronics, Columbus, Ohio, USA). Each activity chamber contained a total of 16 photo beams spaced 5 cm apart, with eight beams located on the x-axis and eight on the y-axis. Photocells were activated when the photo beams on the wall directly
opposite to the cells were interrupted. The Digiscan analyzer recorded the interruption of each beam and provided the total distance (in centimeters) each animal traveled during testing. Each activity chamber was partitioned into 20 × 20 cm quadrants with acrylic dividers to allow simultaneous testing of two mice. Three activity chambers allowed testing of six mice per session. Each of the activity chambers were enclosed in 90 × 54 × 35 cm sound-attenuated boxes. The Digiscan analyzer was interfaced with an IBM XT computer using ILAM software (Coulbourn Instruments, Lehigh Valley, Pennsylvania, USA).

Procedures for motor activity assessment

Activity was assessed for a 3-h period 4 days/week between 12:00 and 16:00 h over a 10-week period. One animal from each of the 18 treatment groups was tested in each week of the experiment, with the exception of the saline group in which two animals were tested each week. The order of treatment groups within each week and the particular test chamber used to test the different groups was counterbalanced across the entire experiment to eliminate any contribution of possible differences in activity monitors or days of testing to observed effects on motor activity. Each animal was subjected to only one treatment and 1 day of testing to minimize confounds that may result from multiple exposures to MPH or EPH, or from differences in the extent of habituation to the activity chambers. Mice received an intraperitoneal (i.p.) injection of the assigned treatment and were placed in the open-field activity chamber. Horizontal movements (measured in centimeters traveled) were recorded in 5-min bins for the entire 180 min session. Following the conclusion of the activity session, animals were returned to their home cages and returned to the colony room. Animals were allowed at least 1 week between injections for motor activity testing and injections for plasma and brain sample collection.

Plasma and brain sample collection

On the basis of differences in motor activity for the different treatment groups, two time points (20 and 60 min) were selected to collect plasma and brain samples following injections of MPH or EPH. The 20-min time point was selected on the basis of potential group differences in motor activity, and the 60 min collection was selected to assess potential differences in the rates of elimination of MPH and EPH. The 5.0 mg/kg dose was chosen because motor activity was not altered by the 2.5 mg/kg dose and the 10.0 mg/kg dose produced near maximum stimulatory effect. Animals previously injected with 2.5 mg/kg dose of each compound were used. At 15 or 55 min after MPH or EPH injections, mice were injected i.p. with pentobarbital 100 mg/kg at a volume of 1.5 × 0.1 ml/g body weight. After induction of anesthesia, a vertical midline incision was made along the abdomen and the thoracic cavity was opened to expose to the heart. The right atrium was cut and 0.3 ml of blood was drawn via 22-gauge needle. Blood samples from three animals were pooled into 4 ml vacutainers containing sodium-fluoride to prevent clotting of blood and hydrolysis of MPH and EPH. Samples were then centrifuged at 3500 r.p.m. for 10 min, followed by aspiration of the plasma and storage at −70°C until analysis. Immediately following collection of blood samples, the brains from each animal were removed, pooled and stored at −70°C until analysis.

Gas chromatography–mass spectrometry analysis of samples

Samples were analyzed for d-MPH, l-MPH, d-EPH and l-EPH as pentafluoropropionyl (PFP) derivatatives using a novel GC–MS-negative ion chemical ionization method. d 3-d-MPH (methyl labeled, Sigma Chem; St. Louis, Minnesota, USA) was incorporated as the internal standard. An Agilent Model 6890 GC-5973 MS was fitted with a 5% phenylmethylpolysiloxane column (HP-5MS, J&W Scientific, 30 m × 0.25 mm, 0.25 μm/l film) and 2 μl injections were made by the pulsed-splitless mode. The helium-carrier gas linear velocity was 50 cm/s. 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)], m/z 342 for the internal standard [M-(2HF)] and m/z 353 for EPH–PFP [M-(2HF)]. These eluted at 8.97, 8.96 and 9.18 min, respectively.

Plasma

Blank plasma samples were used as calibrators and run in parallel with the unknown plasma samples. Plasma calibrators were made by transferring 100 μl of plasma to silanized 4-ml vials and fortified with MPH-HCl (Cerilliant Science, 5 ng/ml in methanol as free base-0, 2, 10, 20 μl respectively) to give 0, 100, 500, 1000 ng/ml MPH HCl concentrations. The internal standard, d 3-MPH (12.5 μl at 100 ng/ml in CH3CN) was added to 20 ml of distilled water and 400 μl of this solution was added to the each plasma sample. 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. Pentafluoropropionic anhydride (Aldrich, Milwaukuee, Wisconsin, USA 75 μl) 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 transferred to spring-loaded microvial inserts contained in autosampler vials.

Brain

Calibrators used 1/2 untreated mouse brain ( ~0.5 g, cut down midline) placed in centrifuge tubes, and then
fortified to contain 0.5, 1 and 4 μg/gm of either dl-MPH or dl-EPH. Calibrators were run in parallel with the 1/2 brain unknowns. Distilled water (1 ml) containing 1 μg d3-MPH was added to each sample, followed by the addition of ammonium hydroxide (28%, 100 μl), then homogenization (Polytron) and centrifugation (2000g). The supernatants were transferred to silanized 4 ml vials, 0.5 g of NaCl was added to prevent emulsion. The samples were then extracted and derivatized in the manner used for plasma (above).

Detection of metabolically formed l-ethylphenidate in mouse urine after l-methylphenidate and ethanol

Mouse urine (0.5 ml) analysis used a modification of the above method for plasma with the following methodological changes. During urine sample preparation, the internal standard (d3-MPH) was omitted. During GC–MS analysis of samples, the oven 90°C hold-time was increased from 1.5 to 4 min, which increased the retention times for MPH and EPH to 9.15 and 9.34 min, respectively. As a result of these changes, the ions monitored for EPH and the qualifier ion were m/z 393 (M) and 373 (M-HF). These l-EPH metabolite ions were detected at the same relative abundance as those of an authentic reference standard (Patrick et al., 2005a). The m/z 379 (M) ion was monitored for MPH.

Methylphenidate and ethylphenidate effects on monoamine transporter binding and uptake inhibition

Mean transporter binding (Ki) and transmitter uptake inhibition (IC50) values (mean ± SEM) for each compound tested on dopaminergic, noradrenergic and serotonergic systems in transfected human embryonic kidney cells. For detailed methods, please see (Patrick et al., 2005a). Each compound was tested in three separate experiments with duplicate (binding assays) or triplicate (uptake inhibition) determinations. When the Ki or IC50 was > 10 μmol/l, only two experiments were conducted and uptake inhibition experiments were not performed. Cocaine was used as a reference standard for all studies. MPH and EPH experiments were conducted at separate times, so cocaine data from each of these sets of experiments were averaged and presented for reference.

Drugs

The d, l, and dl-EPH used in this experiment were synthesized according to the methods described in an earlier report (Patrick et al., 2005a). The d and l-MPH were provided by Dr Steve Teo and Ms Elizabeth Pensabene of the Celgene Corporation. The dl-MPH was provided by National Institute on Drug Abuse NIDA. Animals were assigned to one of 18 treatment groups (n = 10 per group). Treatment groups consisted of low (2.5), medium (5.0) and high (10.0 mg/kg) doses of each of the six compounds (d, l, dl-MPH and d, l, dl-EPH). All compounds were dissolved in 0.9% saline. An additional control group (n = 20) received injections of saline and provided basal levels of motor activity during motor activity tests. All compounds were administered at a volume of 0.01 ml/g body weight i.p. immediately before motor activity testing.

Data analysis

Motor activity was collected in 5-min bins throughout the study. As motor activity had reached constant low levels for all compounds and doses by 120 min after injection, only activity before this time point was subject to analysis. Data were collapsed into 10-min bins to facilitate presentation and statistical analysis. Statistical analysis was conducted using SPSS (SPSS I.; Chicago, Illinois, USA) 12.0. To emphasize comparison of MPH and EPH with respect to vehicle controls, data from the separate enantiomers and racemic forms were analyzed with 2 (drug) × 4 (dose) × 12 (time, 10-min period) mixed factorial analyses for d, l and dl-forms. Significant interactions of the primary factors were resolved with subsequent univariate analysis of variances (ANOVAS) and post-hoc tests. All tests were conducted at a P < 0.05 level of significance.

Results

Ethylphenidate formation

EPH was detected in mouse urine after coadministration of EtOH with l-MPH, but not with d-MPH. The identification of l-EPH was determined by comparing the molecular ion and qualifier ion relative abundances with those of an l-EPH reference standard (Patrick et al., 2005a, b). For emphatic comparison of MPH and EPH with respect to vehicle controls, data from the l-isomer are shown in Fig. 1. Peaks for l-MPH (upper chromatogram) and l-EPH (lower chromatogram) were detected at 9.15 and 9.34 min, respectively. The chromatographic peak area of metabolite l-EPH was 9% that of l-MPH.

Motor activity data

The effects of various doses of d, dl and l-MPH and EPH on motor activity are summarized in Figs 2–5 and in Table 1. As motor activity had reached constant low levels for all compounds and doses by 120 min after injection, only activity before this time point was subject to analysis. Data were collapsed into 10-min bins to facilitate presentation and statistical analysis.

Motor activity of saline versus l-isomer-injected mice

To provide a balanced design for statistical analysis, the inactive l-isomer of MPH and EPH for each dose was used as a control. The absence of l-MPH and l-EPH effects on motor activity relative to saline at any of the three doses tested is illustrated in Fig. 2 and is supported
Effects of d and dl-methylphenidate versus d and dl-ethylphenidate on motor activity

To facilitate the comparison of EPH with its parent drug MPH, results are organized to compare the d (Fig. 3a–c) and dl (Fig. 4a–c) forms. Data for each form (d, dl) were analyzed with 2 (drug) × 4 (dose) × 12 (time, 10-min period) ANOVAs. Significant interactions of the primary factors were resolved with subsequent one-way ANOVAs and post-hoc tests.

### d-methylphenidate versus d-ethylphenidate

Motor activity data from the 2-h test period generated by mice injected with various doses of the d-isomers of MPH and EPH are summarized in Fig. 3a–c. Figure 3a illustrates the different dose–responsive increases in motor activity produced by the d-forms of MPH [dose: $F(3,111) = 63.56, P < 0.001$] and that activity was lower for d-EPH than for d-MPH-injected mice [drug: $F(1,111) = 4.74, P < 0.05$]. The apparent difference in the dose–response function for the two compounds approached significance [drug × dose: $F(3,111) = 2.37, P = 0.074$]. As the dose–response curves for MPH and EPH were somewhat different, activity...
across dose was analyzed with univariate ANOVA and compared with its vehicle (Dunnett’s post-hoc test) for both MPH and EPH. These tests indicated that in comparison with their vehicle controls, motor activity was greater for mice injected with the two higher doses of MPH and EPH (Dunnett’s, $P < 0.05$). Additional comparison of MPH and EPH within each dose group indicated that activity was greater for MPH than EPH at the 5.0 mg/kg dose [$F(1,18) = 4.604$, $P < 0.05$].

The effects of the different doses of MPH and EPH on motor activity across time are summarized in Fig. 3b for d-MPH and Fig. 3c for d-EPH, and indicate that motor activity depended on a complex drug/dose/time interaction [$F(33,309) = 2.03$, $P < 0.001$]. The most apparent difference between the two compounds was a different pattern of stimulation at the 10 mg/kg dose. At this dose, motor activity decreased more rapidly during the early stages of testing for d-MPH (Fig. 3a) than for d-EPH (Fig. 3b). It is possible that the rapid decrease in motor activity for d-MPH at this high dose was due to the induction of stereotypic behavior, locomotor activity and stereotypic behavior were analyzed across the first 40 min of testing. During this time period, motor activity for mice injected with the high dose of MPH declined (3847–2380–2235–2354 cm) at 10-min intervals. The measure of stereotypic behavior also declined across this time period (1150–854–813–722 cm), hence cannot account for the more rapid decline in motor activity. A caveat to this interpretation is that the activity monitoring system in our experiments measured only gross motoric activity. It is possible that assessing finer movement, such as gnawing, would detect stereotypy, as noted in a study that assessed MPH-induced gnawing behavior in B6 mice (Tirelli and Witkin, 1995).
dl-methylphenidate (MPH) and dl-ethylphenidate (EPH) effects on motor activity over a 2-h test period following injections of 2.5, 5.0 and 10.0 mg/kg doses. Compared with the inactive l-form, dl-MPH and dl-EPH produced dose associated increases in motor activity (a). dl-MPH produced a slightly greater stimulatory effect than dl-EPH at the 5.0 mg/kg dose (P = 0.111). The time course of stimulation at 10-min intervals is summarized in (b) for dl-MPH and (c) for dl-EPH. dl-MPH and dl-EPH differed in the stimulation produced over time as indicated by a three-way interaction (drug × dose × time interaction [F(33,312) = 1.507, P < 0.05]).

**Fig. 4**

**Fig. 5**

Direct comparison of 5.0 mg/kg dose of d-methylphenidate (MPH) and ethylphenidate (EPH) (a) and dl-MPH and EPH (b) effects on motor activity over 2-h test period. The time course of drug effects on motor activity depended on both drug and dose, as indicated by a significant drug × dose × time interaction for both d (P < 0.001) and dl (P < 0.05) MPH and EPH (see Figs 3 and 4). d-MPH and d-EPH produced different durations of stimulation as indicated by a significant drug × time interactions (d-form: drug × time, P < 0.05). Subsequent univariate analysis of variance at 20 and 60 min after injection revealed that the stimulatory effects of d-MPH and d-EPH differ at 20 but not 60 min.

**Methylphenidate and ethylphenidate in mice**

Williard et al. 1994

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**dl-methylphenidate versus dl-ethylphenidate**

Motor activity data from mice injected with the racemic forms of MPH and EPH are summarized in Fig. 4a–c. As noted for the d-isomers, the racemic forms of MPH and EPH produced dose-dependent increases in motor activity. The dose–responsive stimulation is presented in Fig. 4a [Dose: F(3,112) = 20.78, P < 0.001] with
stabilization being less for dl-EPH than for dl-MPH [drug: \( F(1,112) = 3.94, P = 0.05 \)]. Owing to a reduced stimulatory effect of the racemic form of the compounds, the drug × dose interaction only approached significance [drug × dose: \( F(3,112) = 2.05, P = 0.11 \)]. As a result of the near significant interaction, univariate ANOVAs and Dunnett’s post-hoc tests were used to determine how the doses of MPH and EPH may have differed. Analyses indicated that the two higher doses produced a significantly greater stimulatory effect for both MPH and EPH (Dunnett’s, \( P < 0.05 \)) compared with their vehicle controls. Further analysis indicated that at the 5.0 mg/kg dose, the difference between dl-MPH and dl-EPH approached significance [\( F(1,119) = 2.80, P = 0.11 \)].

Figure 4b and c, respectively, summarize the effects of the different doses of racemic MPH and EPH on motor activity across time. A significant drug × dose × time [\( F(33,312) = 10507, P < 0.05 \)] interaction indicated the effects of the various doses of dl-MPH and dl-EPH on motor activity differed across time; however, the difference was not as remarkable as observed for the d-isomers.

**Methylphenidate versus ethylphenidate: 5.0 mg/kg**

As the 5.0 mg/kg dose most clearly distinguished the reduced stimulatory effect of d and dl-EPH relative to MPH, in a 2 (drug) × 2 (dose) × 12 (time) ANOVA, MPH and EPH at this dose were compared with their inactive l-isomer controls. These data are presented in Fig. 5a (d-form) and b (dl-form). For the d-isomers (Fig. 5a) MPH was more stimulatory than EPH [drug × dose: \( F(1,75) = 7.55, P < 0.01 \)]. As the stimulatory effects of MPH and EPH depended upon time [drug × dose × time: \( F(1,75) = 2.03, P < 0.025 \)], two time points (20 and 60 min) were selected to assess differences in the stimulation at this dose. At 20 min, the greater stimulation produced by d-MPH versus dl-EPH approached significance [\( F(3,75) = 13.26, P < 0.001 \)] (Tukey’s honest significant difference \( P = 0.067 \)), whereas at 60 min, there was no significant difference. As noted for the d-form of MPH and EPH, the relative effects of the racemic forms at this dose (Fig. 5b) was also greater for dl-MPH relative to dl-EPH [drug × dose: \( F(1,76) = 4.48, P < 0.05 \)]. In contrast to the d-isomers, the drug × dose ×

**Table 2** Effects of MPH and EPH enantiomers on monoamine transporter binding and uptake inhibition

<table>
<thead>
<tr>
<th>IC50/Ki (nmol/l)</th>
<th>d-MPH</th>
<th>d-EPH</th>
<th>l-MPH</th>
<th>l-EPH</th>
<th>dl-MPH</th>
<th>dl-EPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT binding</td>
<td>161 ± 22</td>
<td>370 ± 580</td>
<td>2380 ± 830</td>
<td>&gt; 10 000</td>
<td>7540 ± 400</td>
<td>392 ± 22</td>
</tr>
<tr>
<td>NET binding</td>
<td>206 ± 32</td>
<td>2380 ± 830</td>
<td>&gt; 10 000</td>
<td>&gt; 10 000</td>
<td>7540 ± 400</td>
<td>392 ± 22</td>
</tr>
<tr>
<td>SERT binding</td>
<td>&gt; 10 μmol/l</td>
<td>&gt; 10 000</td>
<td>&gt; 10 000</td>
<td>&gt; 10 000</td>
<td>7540 ± 400</td>
<td>392 ± 22</td>
</tr>
<tr>
<td>DA uptake inhibition</td>
<td>23.3 ± 8.7</td>
<td>1600 ± 740</td>
<td>1730 ± 180</td>
<td>19.9 ± 7.7</td>
<td>95 ± 18</td>
<td>250 ± 29</td>
</tr>
<tr>
<td>NE uptake inhibition</td>
<td>38.7 ± 6.7</td>
<td>980 ± 300</td>
<td>&gt; 10 μmol/l</td>
<td>&gt; 10 000</td>
<td>480 ± 200</td>
<td>392 ± 58</td>
</tr>
<tr>
<td>SHT uptake inhibition</td>
<td>&gt; 10 μmol/l</td>
<td>ND</td>
<td>&gt; 10 μmol/l</td>
<td>&gt; 10 000</td>
<td>ND</td>
<td>253 ± 22</td>
</tr>
</tbody>
</table>

ND, indicates uptake experiment was not conducted when IC50 > 10 μmol/l.

**Table 3** Normalized Kt and IC50 values for MPH and EPH

<table>
<thead>
<tr>
<th>IC50/Ki (nmol/l)</th>
<th>d-MPH</th>
<th>d-EPH</th>
<th>l-MPH</th>
<th>l-EPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT binding</td>
<td>139</td>
<td>276</td>
<td>105</td>
<td>382</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>0.86</td>
<td>1.2</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>NET binding</td>
<td>408</td>
<td>2479</td>
<td>1560</td>
<td>4824</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>1.98</td>
<td>0.67</td>
<td>1.98</td>
<td>0.6</td>
</tr>
<tr>
<td>DA uptake inhibition</td>
<td>28</td>
<td>24</td>
<td>24</td>
<td>82</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>1.2</td>
<td>0.87</td>
<td>1.2</td>
<td>0.87</td>
</tr>
<tr>
<td>NE uptake inhibition</td>
<td>46</td>
<td>247</td>
<td>31</td>
<td>408</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>1.2</td>
<td>0.85</td>
<td>1.2</td>
<td>0.85</td>
</tr>
</tbody>
</table>

ND, indicates uptake experiment was not conducted when IC50 > 10 μmol/l.

**Table 3 Nonlinear Least-Squares Analysis of the Relationship Between Plasma/Whole-Brain Concentrations and Motor Activity**

**Table 4** Nonlinear Least-Squares Analysis of the Relationship Between Plasma/Whole-Brain Concentrations and Motor Activity

<table>
<thead>
<tr>
<th>IC50/Ki (nmol/l)</th>
<th>d-MPH</th>
<th>d-EPH</th>
<th>l-MPH</th>
<th>l-EPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT binding</td>
<td>139</td>
<td>276</td>
<td>105</td>
<td>382</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>0.86</td>
<td>1.2</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>NET binding</td>
<td>408</td>
<td>2479</td>
<td>1560</td>
<td>4824</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>1.98</td>
<td>0.67</td>
<td>1.98</td>
<td>0.6</td>
</tr>
<tr>
<td>DA uptake inhibition</td>
<td>28</td>
<td>24</td>
<td>24</td>
<td>82</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>1.2</td>
<td>0.87</td>
<td>1.2</td>
<td>0.87</td>
</tr>
<tr>
<td>NE uptake inhibition</td>
<td>46</td>
<td>247</td>
<td>31</td>
<td>408</td>
</tr>
<tr>
<td>Ratio (MPH/EPH)</td>
<td>1.2</td>
<td>0.85</td>
<td>1.2</td>
<td>0.85</td>
</tr>
</tbody>
</table>

**Plasma/whole-brain concentrations for d and l-methylphenidate and ethylphenidate, and effect on motor activity**

Plasma and brain concentrations of d and l-MPH and d and l-EPH at 20 and 60 min after injection are summarized in Table 1. Mean motor activity at these time points is included for comparison. Concentrations in blood and whole-brain were higher for EPH than MPH at both the time points for both isomers. In contrast, motor activity of mice injected with EPH was less than that of mice injected with MPH at each time point indicating that the reduced stimulatory effect of EPH was not because of lower whole-brain concentrations of EPH compared with MPH.

**Methylphenidate and ethylphenidate effects on monoamine transporters: in-vitro experiments on binding and uptake**

Binding of the d and dl-forms of MPH and EPH to the monoamine transporter proteins and their effects on uptake of their respective monoamine transmitters are summarized in Tables 2 and 3. The EPH data included in Table 2 were previously reported (Patrick et al., 2005a); however, the MPH enantiomeric data are unpublished. Taken together, these data are of special value in gauging the effects of the racemic forms of the compounds, the reduced stimulatory effect of d and dl-EPH relative to MPH, and the relative effects of the racemic forms at this dose (Fig. 5b) was also greater for dl-MPH relative to dl-EPH [drug × dose: \( F(1,76) = 4.48, P < 0.05 \)]. In contrast to the d-isomers, the drug × dose ×
relative effects of EPH and MPH on binding to the transporters and inhibition of transmitter uptake. Regardless of form, d or dl, EPH and MPH had similar binding affinity for and similar inhibitory effects on the DAT and the serotonin transporter (SERT). Both MPH and the EPH metabolite had high affinity for the DAT and inhibited DA uptake, whereas exhibiting low affinity for the SERT and little effect on serotonin uptake. Although MPH and EPH had similar potency on measures of DAT function, they differed in their binding affinity for the NET and inhibition of NE uptake. Whereas d and dl-MPH had substantial affinity for the NET and strong inhibition of NE uptake, d and dl-EPH did not. In addition, noted in the table, the l-forms of the drug and metabolite had little activity on the transporters, which is consistent with fundamental MPH structure–activity relationships (Patrick et al., 1987, 2005a).

The isomers of MPH and EPH were submitted to NIDA’s Cocaine Treatment Discovery Program for standardized testing at separate times. Consequently, the cocaine data presented in Table 2, which are the only data collected in both experiments, represent an average of the values obtained in the separate MPH and EPH experiments. To further confirm the observed differential effects of EPH and MPH on monoamine transporters, noted in Table 2, binding and uptake data for DAT and NET were normalized with a ratio of cocaine data obtained from both experiments and are summarized in Table 3. Specifically, we calculated a ratio for cocaine binding to DAT and NET during the MPH and EPH experiments, on the basis of the mean determined from the two. The normalized values for d-MPH, d-EPH, dl-MPH and dl-EPH binding and uptake inhibition for DAT and NET presented in the table were normalized by multiplying the values in Table 2 by the cocaine ratios calculated from the two experiments. Examination of the normalized values in Table 3 confirms the interpretation of Table 2 data indicating that the d and dl-forms of MPH and EPH exhibit similar binding affinity and uptake inhibition for DAT; however, in comparison to its EPH metabolite, MPH had more potent activity at the NET.

**Discussion**

This study contributes to an understanding of MPH–EtOH interaction, which can help identify the possible risks involved with the use of MPH for treating adults diagnosed with ADHD and to the validation of a B6 mouse model for investigating this drug interaction. As noted for humans (Markowitz et al., 1999, 2000; Patrick et al., 2006), the coadministration of MPH and EtOH in B6 mice forms EPH and this metabolic pathway exhibits a stereoselectivity-favoring formation of the l-isomer. Furthermore, as noted for humans, the l-isomers of both MPH and EPH were biologically inactive; and racemic MPH and EPH were less stimulatory than the d-isomer alone. This study also indicates that the d and dl-EPH metabolic compounds were less stimulatory than their parent MPH compounds. In spite of the attenuated stimulatory effect of EPH, its concentrations in blood and whole-brain were greater than those of MPH, indicating that lower concentrations of EPH could not account for its reduced stimulatory effect. In-vitro experiments indicated that a primary difference between d and dl-EPH and their parent MPH isomeric forms was a substantially reduced binding to NET and inhibition of NE uptake. MPH and its EPH metabolite had similar effects on DA and serotonin systems. Thus, the lower potency of EPH on NE neural pathways, perhaps by influencing DA tone (Kuczenski and Segal, 2001), may account for the reduced stimulatory effect of d and dl-EPH relative to d and dl-MPH.

**Ethylphenidate formation in B6 mice**

EPH was formed enantiopreferences in B6 mice with only L-EPH detected in significant concentrations. As L-EPH was the predominant isomer formed from dl-MPH in a human study (Patrick et al., 2006), this result supports the validity of the B6 mouse model for exploring the MPH–EtOH interaction. Although, the specific enzyme(s) mediating metabolic transmethylation from l-MPH to L-EPH are not established in mice, studies on hepatocytes from rats indicate that the carboxylesterase-1 isoform mediates the transmethylation of MPH–EPH in the presence of EtOH (Bourland et al., 1997). Furthermore, studies using hepatocytes from humans (Redinbo et al., 2003) and rats (Bourland et al., 1997, 1998) indicate that carboxylesterase-1 mediates EtOH-induced transmethylation of cocaine to cocaethylene. As mice express the carboxylesterase-1 (Boyer and Petersen, 1992) and the same enzyme mediates the transmethylation of MPH to EPH in rat hepatocytes (Bourland et al., 1997, 1998), it is reasonable to postulate a similar mechanism for the EPH formation in the B6 mice of this experiment.

**Methylphenidate versus ethylphenidate: effects on motor activity and plasma and whole-brain concentrations**

The current report provides a detailed comparison of the time course of motor activity changes produced by various doses of racemic and enantiomeric MPH and EPH, thus extending our earlier report on the cumulative stimulatory effects of MPH versus EPH (Patrick et al., 2005a). The comparison of the stimulatory effect of the various isomers and doses of MPH and EPH in a single experiment eliminates the possible confounding effects of differences in species and strain, doses, activity measure methods, etc. that can occur when experiments are conducted in different laboratories. Results from the present comparison of EPH and MPH indicated that (a) the l-forms of both MPH and EPH had no effect on motor activity, (b) d and dl-MPH and EPH dose-
dependently elevated motor activity, (c) MPH and EPH did not differ significantly in their time course of action and (d) EPH produced less of a stimulatory effect than MPH. The greatest difference in the stimulatory effects of MPH and EPH was at the 5 mg/kg dose (Fig 5a and b). At this dose, motor activity over the entire 2-h test period was approximately 25% less for EPH than MPH for both the d and dl-forms. In addition, during the time of peak activity (i.e. the first 10-min interval) EPH produced a smaller increase relative to their controls than noted for MPH for both the d-form and the racemic at the 5.0 and 10.0 mg/kg doses (5.0 mg/kg: d-MPH = 162% versus d-EPH = 119%; dl-MPH = 134% versus dl-EPH = 120%; 10 mg/kg: d-MPH = 173% versus d-EPH = 161%, dl-MPH = 142% versus dl-EPH = 127%). Furthermore, the effect of MPH and EPH on motor activity did not differ at either the 2.5 mg/kg or the 10 mg/kg dose of either the d or dl forms. The absence of a difference between the drugs at the 10.0 mg/kg dose is possibly owing to a “ceiling effect” since the activity at the 5 and 10 mg/kg doses did not differ for either the d or dl forms of either drug.

Whole-brain concentrations of d and l-EPH were greater than those for the parent MPH compounds; thus, whole-brain concentrations of EPH cannot account for its attenuated motor stimulation compared with MPH. The reason(s) for the higher concentrations of EPH compared with MPH are speculative. One possibility is that the larger aliphatic feature of an ethyl-ester compared with a methyl-ester might render EPH more lipophilic, thereby enhancing its transport across the blood–brain barrier. Alternatively, the additional methyl group on EPH could decrease its rate of hydrolysis compared with MPH (Portoghese and Malspeis, 1961), an interpretation consistent with the longer half-life of cocaethylene (cocaine ethyl ester) compared with cocaine (Lau, 1992). We originally considered the possibility that MPH might be removed from the brain more rapidly by serving as a substrate for the membrane efflux transporter protein, p-glycoprotein; however, a recent study indicates a low substrate affinity of p-glycoprotein for MPH (Zhu et al., 2006). Although this experiment cannot account for the higher whole-brain concentration of EPH compared with MPH, it does clearly establish that EPH enters the brain in amounts greater than the parent compound. Although reduced transport of EPH from the periphery into brain cannot account for its attenuated stimulatory effect, the distribution of EPH and MPH across different brain regions or tissues remains a possible explanation and will need to be determined in future studies.

MPH and cocaine have similarities in their pharmacophore, mechanism of action and interactions with EtOH. Although the metabolites cocaethylene and EPH are formed by similar transesterification processes, comparison of these transesterification products with their parent compounds suggests substantial differences in pharmacokinetic parameters. For example, in comparisons of brain concentrations of cocaine and cocaethylene, some studies indicate higher concentrations of cocaine (Dean et al., 1992; Bradberry et al., 1993; Pan and Hedaya, 1999), whereas others indicate higher concentrations of the transesterification product, cocaethylene (Fowler et al., 1992; Bailey, 1996), as was observed for EPH in our experiment. Furthermore, although cocaine and cocaethylene produced comparable stimulatory effects in rats (Jatlow et al., 1991; Katz et al., 1992), cocaethylene was found to be less stimulatory than cocaine in Swiss Webster mice (Katz et al., 1992) which parallels the reduced stimulatory effect of EPH and MPH for B6 mice in our study.

**Methylphenidate versus ethylphenidate: in-vitro monoamine transporter effects**

Comparison of the current results of MPH effects on monoamine transporters with the previously reported effects of EPH (Patrick et al., 2005a) reveals that both MPH and EPH have high affinity for the DAT and block DA uptake; whereas neither had appreciable activity at the SERT. Compared with MPH, EPH had low binding affinity and uptake inhibition at the NET, providing evidence of significant differential selectivity of ester homologs MPH and EPH for these transporter proteins. These comparative effects of MPH and EPH are consistent with a report that cocaine and its transesterification metabolite, cocaethylene, had similar effects on the DAT, but that cocaethylene had reduced effect on the NET compared with cocaine (Hearn et al., 1991). The present results also demonstrated the anticipated enantioselective influence on the monoamine transporters, that is high activity for the d-isomers and low activity for the l-isomers. MPH activity on monoamine systems in this experiment is consistent with previous reports using the racemic form of the drug, which indicate appreciable activity at the DAT and NET, but not the SERT (Han and Gu, 2006).

The values obtained for DA and NE transporter-binding and transmitter-uptake inhibition in both the EPH experiment (Patrick et al., 2005a) and the MPH experiment were evaluated in reference to the prototypic monoamine transporter blocker, cocaine. Comparison of previous reports on cocaine binding to monoamine transporters indicate that binding and uptake inhibition values can differ by several orders of magnitude (Han and Gu, 2006), likely because of methodological differences. Therefore, the data were normalized to confirm that MPH and EPH bind with high affinity to the DAT, but not the SERT, and that EPH has low affinity for the NET. The normalized data confirmed the raw data and the results of each are consistent with previous findings (Eshleman et al., 1999; Han and Gu, 2006). Additionally, binding and uptake inhibition values fall within the range...
that is considered to be acceptable for these types of experiments.

Integration of behavior, whole-brain concentration and monoamine transporter experiments

In a previous report it was noted that in comparison to MPH, EPH had approximately 20% less ‘centrally stimulating activities’ (Portoghese and Malspeis, 1961). This study substantiates the reduced central nervous system activity of the EPH metabolite and extends previous work by providing information about the stimulatory characteristics, whole-brain concentrations and monoamine transporter activity of various forms of MPH and EPH. The current results indicate that the attenuated stimulatory effect of EPH compared with MPH is not because of limited availability of EPH in the central nervous system or reduced activity at the DAT. Several lines of experimental evidence support the hypothesis that compounds which directly or indirectly increase activity of dopaminergic systems produce an increase in motor activity (Aoyama et al., 1997; Duvauchelle et al., 2000; Gerasimov et al., 2000; Kuczenski and Segal, 2001, 2002; Uhl et al., 2002). Results from studies on the role of noradrenergic systems in stimulating motor activity are conflicting (Tyler and Tessel, 1980; Xu et al., 2000; Kuczenski and Segal, 2001; Berridge and Waterhouse, 2003; Moran-Gates et al., 2005). Kuczenski reported that a low dose of MPH (2.5 mg/kg), which increase NE release in the hippocampus, but not DA release in the nucleus accumbens, stimulates motor activity (Kuczenski and Segal, 2001), suggesting a noradrenergic component to MPH stimulation. In contrast, Tyler and Tessel (1980) reported that systemic administration of NET inhibitors attenuated motor activity increases induced by psychostimulants such as cocaine and MPH suggesting that higher levels of NE reduce motor activity. Furthermore, the selective NET uptake-inhibitor, atomoxetine, had no stimulatory effect on motor activity, but produced a slight decrease in motor activity of rats during the first 30 min of a 90-min test (Moran-Gates et al., 2005). Despite these divergent findings, collective interpretation of these results suggests that NE plays a modulatory role in motor stimulation. Therefore, the reduced stimulatory effect of EPH relative to MPH in our study could be related to its reduced effect on noradrenergic systems.

Although the focus of this study was a systematic comparison of EPH with its parent compound, consideration of d versus dl-MPH comparison warrants consideration. The effects of the MPH isomers on motor activity of B6 mice in this study are consistent with most reports of MPH stimulation in rats (Patrick et al., 1987; Gatley et al., 1995; Aoyama et al., 1996, 1997; Kuczenski and Segal, 1997, 2001; Gerasimov et al., 2000; Teo et al., 2003; Amini et al., 2004; Ding et al., 2004). Such studies indicate that although the l-form of MPH has no effect on locomotion, the d and dl-forms of the drug systematically increase motor activity, with dl-MPH being less stimulatory than the d-form at equivalent doses, perhaps due to half the amount of the active d-isomer. Consistent with the earlier reports, racemic MPH was less stimulatory than the d-isomer. Stimulation produced by the racemic mix in our experiment was, however, 60–75% that of the d-isomer rather than the 50% predicted on the basis of a completely inactive l-isomer suggesting the possibility of an isomeric interaction. The reason(s) for the smaller than expected reduction in stimulation by dl-MPH in comparison to d-MPH are not clear, but may be related to the particular doses and the particular characteristics of the activity measurement used in the study. Although the highest dose of the d-isomer was approaching maximum stimulation under the conditions of the experiment, this ceiling effect cannot account for the less than expected difference between d-MPH and dl-MPH because the highest dose of racemic MPH was not more stimulatory than the middle dose. Current literature suggests possible enantiomeric interaction between the d and l-forms, although whether it enhances or attenuates effects of the d-isomer and other details remain to be elaborated (see Patrick et al., 2005b). For example, the l-form of MPH reduced cocaine and apomorphine induced stimulation in rats (Davids et al., 2002), but enhanced cocaine stimulation in mice (Ding et al., 2004).

Summary

The objectives of this study were to (a) determine whether B6 mice form EPH following concomitant injections of MPH and EtOH, (b) systematically compare the separate enantiomers of MPH with its transesterification metabolite, EPH, (c) evaluate potential differences in blood and whole-brain concentrations of MPH and EPH and (d) compare monoamine transporter binding and uptake inhibition of the separate enantiomers of MPH and EPH. The present findings augment previously reported EPH effects on motor activity (Patrick et al., 2005a). We have established that concomitant administration of MPH and EtOH results in the enantioselective production of the transes- terification product l-EPH in B6 mice. Compared with the parent drug MPH, EPH produced less motor stimulation in the B6 mouse. EPH i.p. administered attained higher whole-brain concentrations than i.p. MPH at the 5 mg/kg dose. In-vitro studies demonstrated that EPH and MPH had similar action on the DAT, but that EPH was considerably less active than MPH at the NET. These results further establish that the attenuated stimulation of EPH is not due to reduced whole-brain concentrations or its lack of activity on dopaminergic systems, but may be due to reduced activity on noradrenergic systems.

The consequences of EtOH consumption on the therapeutic effects or abuse liability of MPH remains
an area of active clinical investigation (Patrick, 2005; Patrick et al., 2006). The results of this study indicate that the B6 mouse models may be useful for assessing some aspects of the MPH–EtOH interaction in humans.

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