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Interactive Effects of Methylphenidate and Alcohol on Discrimination, Conditioned Place Preference and Motor Coordination in C57BL/6J mice

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Abstract

Introduction—Prior research indicates methylphenidate (MPH) and alcohol (ethanol, EtOH) interact to significantly affect responses humans and mice. The present studies tested the hypothesis that MPH and EtOH interact to potentiate ethanol-related behaviors in mice.

Methods—We used several behavioral tasks including: drug discrimination in MPH-trained and EtOH-trained mice, conditioned place preference (CPP), rota-rod and the parallel rod apparatus. We also used gas chromatographic methods to measure brain tissue levels of EtOH and the *d* and *l* isomers of MPH and the metabolite, ethylphenidate (EPH).

Results—In discrimination, EtOH (1g/kg) produced a significant leftward shift in the MPH generalization curve (1-2mg/kg) for MPH-trained mice, but no effects of MPH (0.625-1.25mg/kg) on EtOH discrimination in EtOH-trained mice (0-2.5g/kg) were observed. In CPP, the MPH (1.25mg/kg) and EtOH (1.75g/kg) combination significantly increased time on the drug paired side compared to vehicle (30.7%), but this was similar to MPH (28.8%) and EtOH (33.6%). Footslip errors measured in a parallel rod apparatus indicated that the drug combination was very ataxic, with footslips increasing 29.5% compared to EtOH. Finally, brain EtOH concentrations were not altered by 1.75g/kg EtOH combined with 1.25mg/kg MPH. However, EtOH significantly increased *d*-MPH and *l*-EPH without changing *l*-MPH brain concentrations.

Conclusions—The enhanced behavioral effects when EtOH is combined with MPH are likely due to the selective increase in brain *d*-MPH concentrations. These studies are consistent with observations in humans of increased interoceptive awareness of the drug combination and provide new clinical perspectives regarding enhanced ataxic effects of this drug combination.

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Conflict of Interest:

The authors have no conflicts of interest to disclose

Keywords

psychostimulant; methylphenidate; ethanol; interoceptive; reinforcement; ataxia; mouse; drug-drug interaction

Introduction

Alcohol drinking commonly occurs with the use of other drugs and evidence indicates that psychostimulants such as methylphenidate (MPH) are extensively used/misused with alcohol (ethanol, EtOH) (Barrett *et al.* 2006; Martin 2008; McCabe *et al.* 2006). In fact, MPH has been reported to be the most common prescription stimulant used for nonmedical purposes in a national survey (Kroutil *et al.* 2006). The combination of MPH and EtOH has been reported in the context of abuse or misuse (Barrett *et al.* 2002; Darredeau *et al.* 2007; Markowitz *et al.* 1999) and epidemiology studies routinely report co-abuse of stimulants and EtOH in adolescents and young adults (Godfrey 2009; Hasin *et al.* 2007; Johnston *et al.* 2008; Mallonee *et al.* 2005; McCabe *et al.* 2004; Teter *et al.* 2006). MPH abuse appears to be in part driven by the belief that Attention-Deficit/Hyperactivity Disorder (ADHD) stimulant medications permit the consumption of more EtOH, thus facilitating late-night partying [see (Godfrey 2009)]. Additionally, the combination of MPH and EtOH can be an important consideration in patients with ADHD since the use of MPH in patients of legal drinking age has substantially risen as a consequence of the increasing recognition that ADHD commonly persists into adulthood (Biederman *et al.* 2005; Biederman *et al.* 2002). It is noteworthy that approximately one third of prescribed ADHD medications are to individuals at least 20 years of age, with the most common prescriptions being the racemic form of MPH (i.e., the 50:50 *dl*-isomeric mixture)(Okie 2006). Evidence has emerged that the co-ingestion of EtOH and MPH is associated with both pharmacokinetic and pharmacodynamic drug-drug interactions in humans. The interaction of MPH and EtOH involves unique stereochemical considerations. In a normal volunteer study of men and women, it was established that concentrations of the pharmacologically active *d*-isomer of MPH were significantly elevated when combined with EtOH. Further, the mean plasma *l*-MPH concentrations were elevated 3-fold when dosing with a typical therapeutic dose of *dl*-MPH and an EtOH drink (0.6 g/Kg) (Patrick *et al.* 2007). Additionally, the combination of MPH and EtOH leads to the production of the EtOH transesterification metabolite, ethylphenidate (EPH), in humans (Markowitz *et al.* 2000; Patrick *et al.* 2007; Zhu *et al.* 2011a), enantioselectively forming the pharmacologically inactive *l*-isomer of EPH (Patrick *et al.* 2007; Patrick *et al.* 2005).

The pharmacologically active *d*-isomer of EPH is only detected in the picogram/ml range in humans after a therapeutic MPH dose and standard EtOH drink (Patrick *et al.* 2007), but could possibly be more important in overdose situations when higher levels of both drugs are present (Markowitz *et al.* 1999). Behaviorally, it was also established that the MPH and EtOH combination interacted to increase self-reports of positive subjective effects, e.g., feel “good”, “like”, “stimulated” and “high” in normal subject volunteers (Patrick *et al.* 2007). The increased subjective pleasure of the drug combination is consistent with other studies using survey methodology (Barrett *et al.* 2002), and since both MPH and EtOH have abuse potential in their own right, the increased self-reported pleasure with the drug combination carries a heightened risk of abuse liability. The increased self-reports of pleasurable feelings obviously point to the possibility of enhanced reinforcing effects of the drug combination but could also simply indicate increased interoceptive awareness of the discriminative stimulus effects of the drugs consistent with “feel any drug effect.” Our data indicate that either interpretation could be explained by increases in the circulating concentrations of the active *d*-isomer of MPH (Patrick *et al.* 2007).

In several previous studies, we have used the inbred strain of C57BL/6J (B6) mice to investigate the pharmacological and pharmacokinetic interactions of the MPH and EtOH combination. We found that concomitant MPH and EtOH significantly stimulates locomotor activity more than administration of either drug alone (Bell *et al.* 2011a; Bell *et al.* 2011b; Griffin *et al.* 2010). Further, like in humans, we have also shown that B6 mice biotransform MPH and EtOH to EPH following either intraperitoneal (ip) (Williard *et al.* 2007), oral or transdermal administration (Bell *et al.* 2011a; Bell *et al.* 2011b). Accordingly, this particular mouse strain exhibits certain pharmacological effects which appropriately model humans in investigating MPH-EtOH interactions *in vivo*. In the present series of studies, we examined the effects of the drug combination in several behavioral tests and also examined the enantioselective aspects associated with this drug interaction as possible pertinent to the mechanisms underlying influences of EtOH on MPH behavioral effects. As with the human studies, we hypothesized that the combination of MPH and EtOH potentiates behavioral responses which occur as EtOH increases the central nervous system availability of the *d*-isomer of MPH.

Materials and Methods

Subjects

Adult male C57BL/6J mice were used in these experiments (Jackson Laboratories, Bar Harbor, ME). Mice arrived at 8-9 weeks of age and acclimated at least 1 week before beginning studies. All mice were singly housed under standard conditions (12h light cycle) in a AAALAC accredited facility with free access to food and water, except that some mice were food restricted as noted below. The use of specific groups of mice is detailed in Table 1. All procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee.

Drugs

Drugs were given by the intraperitoneal (ip) route using 0.9% saline as the vehicle at a volume of 0.02ml/g of body weight in all experiments. *d,l*-MPH was used as the HCl salt obtained from Sigma-Aldrich Inc (Saint Louis, MO) and 95% ethanol was obtained from AAPER (Shelbyville, KY). Before use, the ethanol was diluted with 0.9% saline [12.2% (v/v) for a 1.75 g/kg dose, and 6.6% (v/v) for a 1 g/kg dose]. When combined, MPH and EtOH were co-administered in the same ip injection.

MPH Discrimination apparatus and procedures

The apparatus and procedures used to train and test mice on MPH discrimination have been recently described (McGovern *et al.* 2011). Briefly, behaviorally naive mice (n=16) were food-restricted to 85% of their free-feeding body weight and given their daily food ration 2-3 hr after completing behavioral procedures. Injections of 5mg/kg MPH or vehicle were given 15 min before entry to the chamber. Reinforcement was 0.1 ml sucrose (5% w/v in tap water). The mice were trained using the same schedule previously described (McGovern *et al.* 2011) but modified so mice experienced as many as 3 training sessions per day as described by (Schechter 1988). Based on the schedule, mice could have three sessions in the same day if the training order for the next three sessions was Vehicle/Vehicle/MPH. Alternatively, if the first daily session was scheduled to be MPH, the mice would have only one session that day. Thus, a vehicle training session never followed an MPH session. Training continued until mice demonstrated >80% overall responding on the drug-paired lever and >80% responding when completing the first fixed ratio (FFR) for 3 consecutive sessions. Once acquisition criteria were met, mice were tested in a single 2 min session under extinction conditions (McGovern *et al.* 2011) and the following day, returned to

training until at least 3 consecutive sessions of criterion performance were reached prior to another test.

EtOH discrimination apparatus and procedures

The apparatus and procedures used to train and test mice on the ability to distinguish EtOH (1.5 g/kg) from vehicle has been described (Becker *et al.* 2006; Crissman *et al.* 2004). Briefly, behaviorally naïve mice (n=24) were food restricted to 85% of their free-feeding body weight and given food rations several hr after completing daily sessions. Reinforcement was a 20 mg food pellet (BioServ, #F0163). In this study, mice were trained only one session per day. Training continued in this manner until mice demonstrated >80% overall responding on the drug-paired lever and >80% when completing the first fixed ratio (FFR) for 8 out of 10 sessions. Once acquisition criteria were met, mice were tested with and without MPH using a cumulative EtOH dosing procedure. Briefly, mice were injected with vehicle and 5 min later placed in the operant chamber for a 2 min test with no reinforcement for responses. The first test was followed rapidly by 5 additional 2 min tests each occurring 5 min after an EtOH injection of 0.5 g/kg, producing a total cumulative dose of 2.5 g/kg. Thus, including vehicle injection, each mouse had a series of 6 tests within 45 min in the context of increasing blood EtOH concentrations (Becker *et al.* 2006; Crissman *et al.* 2004). When MPH was included with the EtOH injection, the amount included produced a total cumulative dose of either 0.625 or 1.25 mg/kg. The blood levels of MPH for this procedure were not empirically determined, but the half-life of MPH (3mg/kg orally) in B6C3F1 mice has been reported to be 1.1 hr (Manjanatha *et al.* 2008). Therefore, over the 45 min testing procedure, it was assumed that the cumulative dosing procedure produced comparable systemic concentrations as a single bolus dose of MPH.

Locomotor activity apparatus and procedures

The apparatus for measuring locomotor activity was a Digiscan Animal Activity Monitor (Accuscan Instruments, Columbus, OH) (Griffin *et al.* 2010). Briefly, behaviorally naïve mice (n=44) were habituated to the procedure by injecting them with vehicle for 2 consecutive days and then returned to their home cage for a latency period of 5 min. After 5 min in their home cage, the mice were placed into the activity chamber for 15 min. On the third consecutive day, the same procedure was repeated but mice were divided into 4 groups and injected as follows: VEH, MPH 1.25 mg/kg, EtOH 1 g/kg and the combination of MPH 1.25 mg/kg + EtOH 1 g/kg. Horizontal and vertical beam breaks were the primary dependent variables.

Conditioned place preference (CPP) apparatus and procedures

The Med Associates (St. Albans, VT, USA) apparatus consisted of 6 activity chambers (ENV-510) equipped with two compartment place preference inserts (ENV-512). The walls of the inserts are made of plastic and the floor on one side of stainless steel wire mesh (6x6mm) and the other side, parallel rods (3mm radius, 8mm center to center spacing). Each side of the insert was 13cm wide × 24cm long × 15cm deep. Each CPP chamber was contained within a sound attenuating cabinet (ENV-022MD-027) equipped with fans and lights turned on every session. Data were collected by computer using 2 sets of photobeams (ENV-256T), one set positioned 1 cm above the floor to capture horizontal activity and another set positioned 5 cm above the floor to capture vertical activity (rearing behavior). For these studies, the dependent variable used was time spent on the drug paired side.

There were two CPP experiments in which behaviorally naïve mice were similarly handled and assigned to one of four groups: VEH, MPH 1.25 mg/kg, EtOH 1.75 g/kg, and the combination of MPH 1.25 mg/kg + EtOH 1.75 g/kg. This dose of EtOH readily produces place preference after 16 sessions (Nocjar *et al.* 1999) and also increases locomotor activity

in B6 mice (Middaugh 1992) which can be potentiated by the 1.25 mg/kg dose of MPH (Griffin *et al.* 2010). Briefly, mice were given a Pre-Test and two Post-tests, the first post-test occurred after 8 conditioning sessions (Test 1) and the second occurred after 16 conditioning sessions (Test 2). Mice were initially placed in the apparatus for the Pre-Test and data from this session (time on either side) were used to balance the groups for the remainder of the study. Drug/Vehicle conditioning sessions commenced 24hrs later with 8 conditioning sessions prior to Test 1 and 8 additional sessions prior to Test 2 for a total of 16 sessions (8 vehicle, 8 drug-paired sessions). Conditioning sessions were 10 min and the Pre-Test, Test 1 and Test 2 were 15 min in duration. Drug- and Vehicle-Injected subjects were counter-balanced across the contextual cues on either side of the CPP insert, with injections given 5 min prior to chamber placement. Further, vehicle injections were given 5 min before the Pre-Test, Test 1 and Test 2 so that t Testing for each Pre-Test was conducted under drug-free conditions with all mice receiving vehicle injections 5-min prior to the test. Two tests were used in these experiments because of the possibility that the addition of MPH to EtOH reinforcement could reduce the number of sessions from 16 that was previously reported (Nocjar *et al.* 1999). The experiments were conducted Monday through Friday with no events on Saturday or Sunday.

The two CPP experiments differed according to the characteristics of the CPP insert providing contextual cues for conditioning and the side-bias exhibited by the mice. For the first CPP experiment shown in Figure 3A, the place preference inserts provided by the manufacturer (rod vs wire-mesh floors, see above). When introduced to these inserts, mice showed a clear preference for the mesh side over the parallel rods. Therefore, all mice were conditioned to the parallel bar side, consistent with our previous reports using a different apparatus that promoted a side bias by the mice (Nocjar *et al.* 1999; Szumlinski *et al.* 2002). As described more fully in the Results section, significant ataxia was observed in the EtOH and EtOH+MPH treated mice while exposed to the parallel rods in the apparatus. Thus, for the second CPP experiment (Figure 3B) the inserts were modified so that the floor was the same on each side and the walls and floor were white on one side and black on the other, creating distinct visual contexts (Cunningham *et al.* 2007; Cunningham *et al.* 2006). This done by cutting heavy paper stock (B221, Smooth Black Berkshire Matboard, Crescent, Inc) to fit exactly to the exposed areas on the exterior walls and floors of the activity chambers and was visible through the clear plastic walls of the insert. The floor covers were the same material but covered with clear plastic sheet protectors (Chrystar, No. 41102, Samsill, Fort Worth, TX) secured on the underside with tape. These modifications reduced side bias by the mice (see results), although there was a slight tendency for mice to prefer the black side. Because of the relatively unbiased preference, half the mice in each group were conditioned to the black side and the other half of the mice were conditioned to the white side.

Footslip apparatus and procedures

For these studies, we used the CPP insert (ENV-512) described above and confined the mice to the parallel bar side. Mice were placed into the inserts, 5 min after the appropriate drug injection according to group assignment and footslips recorded for 2 min. Footslips were defined as any hindfoot or forefoot slip between the parallel bars of the floor. In the first footslip experiment, the data were collected by simple observation in real time while the CPP insert remained inside the apparatus with the doors to the sound attenuating chamber open for observation. The mice used in this experiment had been used in the CPP experiment 1 and were administered drug according their group assignment in that experiment. For second footslip experiment (Figure 4) the place preference insert was set up on a laboratory bench under ambient lighting conditions in a quiet room. A video camera was positioned next to the insert to record each session from the side and footslips were counted later by a blinded observer. The mice used in this study (Figure 4) were from CPP

experiment 2 and the rota-rod test (see below) and were administered drug according to their original assignment.

Rota-Rod performance

A rota-rod from UgoBasile (Model 47600) was used. Mice were acclimated to the device one time and tested 24 hours later. On acclimation day, mice were administered vehicle, rested in the home cage for 5 min and then placed on the rota-rod for a 5 min session with the rotation speed at 4 RPM. Testing used the same procedure except mice were administered drug solutions according to previous group assignment. Additionally, during testing the rotation speed advanced from 4 to 40 RPM over the 5 min test. The test ended when the mouse fell and stopped the timer. The time on the rod and the final rotation speed were recorded.

Tissue collection for measurement of brain EtOH, MPH and EPH concentrations

Immediately following the 2 min footslip observations shown in Figure 5, mice were rapidly and deeply anesthetized with isoflurane (>15%) in a bell jar. After checking reflexes, the brain was extracted and cut into left and right hemispheres, including the cerebellum and brainstem. A small piece of cortex was removed from the left hemisphere (14-16mg), placed in a tared vial and 50 volumes ice-cold deionized water was added according to weight. Lastly, both hemispheres were placed separately into tared, glass scintillation vials, the weights recorded and then frozen immediately in dry ice. The entire tissue extraction procedure was completed within 3 min for each mouse. The small tissue sample for EtOH determination was stored at -20°C and the hemispheres were stored at -80°C until analysis.

Brain EtOH concentrations

Brain samples were thawed and homogenized by brief sonication and 10 µl was placed into a 6 ml headspace vial. The concentrations of EtOH in brain tissue were determined using headspace gas chromatography (GC) with flame-ionization detection (FID) and extrapolation from standards prepared using untreated brain tissue spiked with known amounts of EtOH. The instrument was a Shimadzu Model GC2010 Gas Chromatograph equipped with a Model AOC 5000 headspace autosampler. The carrier gas was helium (215.7 ml/min; total pressure 84.6) combined with a mixture of air (400 ml/min) and hydrogen (40ml/min) for sample combustion. The vial containing the sample was heated to 60°C for 5 min and then 2 ml of headspace withdrawn and injected into the instrument. EtOH was separated from the sample using a 30 meter × 0.53 µmHP-FFAP column and eluted in 1 min. The limit of detection is 0.032 mM EtOH.

Enantiospecific brain MPH and EPH concentrations

We have developed methods to enantiospecifically quantify concentrations of the *d*- and *l*-isomers of MPH and the *d*- and *l*-isomers of its transesterification metabolite, EPH, from a variety of biological matrices (LeVasseur *et al.* 2008; Patrick *et al.* 2007; Zhu *et al.* 2011a). The brain tissue extraction procedures for MPH and EPH have been described recently (Bell *et al.* 2011a; Bell *et al.* 2011b) as well as the gas chromatography-mass spectrometric analyses.

Data Analysis

Comparison of group means was by Student's T-test or ANOVA as appropriate. For data sets with repeated measures (*e.g.* CPP experiments), we used a mixed model analysis with Group as the between subjects factor and Time as a repeated measure. The mixed model analysis used a heterogeneous first-order autoregressive covariance structure. To analyze the effects of EtOH or MPH on generalization curves for the training drug in drug

discrimination experiments, we compared generalization curves using a residual f-test in a constrained non-linear regression. Maximum responses were constrained to be less than or equal to 100 and minimum response set to a constant of 0. A fit in which all parameters (logistic slope, maximum response, and ED₅₀) were shared was compared with a fit in which only the logistic slope was shared. All analyses were conducted in IBM®SPSS® Statistics Version 19 (IBM Corp, Armonk, NY) with alpha set to 0.05.

Results

Methylphenidate (MPH) Discrimination

As in an earlier report (McGovern *et al.* 2011), most mice (11 of 16) acquired the MPH discrimination task and accurately discriminated the training dose of MPH (5 mg/kg) from vehicle. The 4 mice that never met acquisition criterion were excluded from further analysis. Interestingly, an additional 4 of the remaining 11 mice, required that the training dose to be lowered (2.5 mg/kg) in order to meet MPH discrimination criteria and were also excluded from the final analysis. The remaining 7 mice were used to examine the interactive effects of MPH and EtOH and required 20 ± 2.59 sessions to meet criteria for discrimination, Although the number of sessions to meet discrimination criteria is consistent with our previous report (McGovern *et al.* 2011), the duration of the experiment was shorter because multiple training sessions/day.

The discrimination tests were restricted to one on a given day. Interestingly, the drug combination significantly reduced response rates, particularly when combining 1 g/kg EtOH with MPH at doses of 3.5 mg/kg and greater. In fact, some mice made fewer than the 15 lever responses required for reinforcement on the FR15 during training. Because the discrimination training sessions required 15 responses on the correct lever to earn food-pellet reinforcement, mice that did not meet this learning criteria during the test were eliminated from the study. This resulted in the following group sizes for EtOH + MPH test sessions: 0mg/kg (6), 1 mg/kg (7), 2mg/kg (4), 3.5 mg/kg (3), 5 mg/kg (3). Three additional mice were excluded for low response rates with the MPH alone conditions at the highest dose (7.5 mg/kg).

As shown in Figure 1A, EtOH administration with increasing doses of MPH produced a clear leftward shift in the generalization curve compared to administration of MPH alone. Data for the two MPH generalization curves were compared with constrained non-linear curve fitting Which indicated a strong overall difference between the curves [residual F-test, $F(2,54)=11.39$, $p<.001$]. Since slopes and maxima were the same in the two conditions, the data were modeled again with the curve in the presence of EtOH as a parallel shifted version of the MPH only curve. EtOH produced a significant 1.73 ± 0.06 -fold shift in the curve. These analyses confirm that EtOH produced a significant leftward shift in the MPH generalization curve in Figure 1A.

Response rates for the mice included in the above analysis for Figure 1A are summarized in Figure 1B and differed according to whether EtOH was included with MPH as indicated by significant factor interaction [$F(4, 51) = 3.224$, $p = 0.02$]. Post-hoc analysis indicated that response rates for the three lowest doses of MPH (0, 1 and 2 mg/kg) were significantly lower when combined with EtOH (1 g/kg) than when given alone ($*p<0.05$). Collectively, these results indicate that EtOH interacts with MPH to significantly increase MPH discrimination at doses lower than the training dose, but this comes at the expense of reduced response rates.

EtOH Discrimination

All 24 mice in the experiment met criterion-level discrimination responding on the EtOH-paired lever within 30 sessions. When given alone in a cumulative dose of 2.5 g/kg, EtOH produced a dose-dependent increase in responses on the EtOH-paired lever, indicating the mice clearly discriminated EtOH from vehicle. The inclusion of MPH with EtOH produced no effect on the EtOH-generalization curve. Further, when given only MPH the mice responded almost exclusively on the vehicle-paired lever. The absence of MPH effect on EtOH discrimination was confirmed by comparing the EtOH generalization curves generated in the presence and absence of MPH with the constrained non-linear curve-fitting as described above. The overall logistic slope was 2.38 ± 0.604 in the unconstrained model versus 2.25 ± 0.59 in the constrained fit. The natural log ED_{50} 's for (the three conditions), respectively, were -0.170 ± 0.160 , -0.117 ± 0.255 , and -0.119 ± 0.217 corresponding to ED_{50} 's of 0.85, 0.89, and 0.89. Maximum responses in the three conditions were 100 ± 10.9 , 82.84 ± 13.0 , and 99.6 ± 13.85 . The residuals of the constrained and unconstrained fits did not differ [$F(4,233)=0.720$, $p=0.58$] indicating no reliable differences in the dose-response curve across conditions. Finally, as shown in and, MPH did not alter response rates of EtOH discrimination trained mice when given in combination with EtOH or alone (both $F < 0.7$ for the factor interactions).

Locomotor activity

The finding in the MPH discrimination study that MPH (1-2 mg/kg) could interact significantly with a low dose of EtOH (1 g/kg) to affect behavior prompted a second study to evaluate the effects of 1.25 mg/kg MPH and 1 g/kg EtOH on locomotor activity, another test commonly used to evaluate the effects of stimulants and EtOH on behavior of mice. These data are summarized in Figure 2. Five mice were removed from the final analysis due to procedural errors during testing. Consistent with previous reports from our laboratory, neither the 1.25 mg/kg MPH dose (Griffin *et al.* 2010), nor the 1 g/kg EtOH dose (Middaugh *et al.* 1992) increased locomotor activity above that after vehicle injections. Importantly, however, when given in combination, the 1.25 mg/kg MPH and 1 g/kg EtOH increased horizontal activity (photobeam breaks) compared to that of mice given either drug alone, or vehicle. These observations were supported by a significant one way ANOVA [$F(3,27) = 5.388$, $p = 0.005$]. Post-hoc multiple comparisons tests with Bonferroni's correction indicated that the drug combination group had greater horizontal activity than vehicle and MPH treatments ($*p < 0.05$), while the difference with the EtOH group approached significance ($p = 0.053$).

Vertical activity for mice in the different treatment groups (Mean $\pm$ S.E.M.) were: vehicle 122 ± 9 ; MPH 129 ± 23 ; EtOH 68 ± 9 ; drug combination 127 ± 26 . Vertical activity data were analyzed as described for horizontal activity. Although the group means suggest that EtOH reduced vertical activity and the addition of MPH to EtOH prevented the reduction, the one way ANOVA did not reach statistical significance [$F(3,27) = 2.509$, $p = 0.083$]. Thus, MPH or EtOH given at doses which neither alone impacts locomotor activity, when combined interact to significantly increase locomotor activity.

Place preference

The data from the two place preference experiments are summarized in Figure 3. Both data sets were analyzed using the mixed model analysis of variance procedures described in the methods. In the first experiment (Figure 3A), the Pre-Test established that mice spent only 300 sec of the 900 sec test session on side of the chamber with the parallel rods floor, hence this side was paired with drug administration during the biased conditioning procedure design. At the conclusion of the experiment, we found that only the MPH group showed increased time on the drug paired side after 8 conditioning sessions. These observations

were supported by a significant interaction of the Group and Time factors [$F(6, 47.1) = 3.39, p = 0.007$]. Post-hoc comparisons using Bonferroni's corrected multiple T-test showed that only the MPH group had increased time on the drug paired side compared with the vehicle group (Test 2, $p=0.012$). Interestingly, significant conditioning for the MPH group was evident after only 4 conditioning sessions compared to vehicle (Test 1, $p=0.002$). The p values approached significance for the EtOH group at Test 1 and 2 were $p=0.054$ and $p=0.077$, respectively, compared to the vehicle group. Finally, the p values for the remaining comparisons all exceeded values of 0.08. Thus, only the MPH group demonstrated significant place preference and there was no evidence that the drug combination increased reinforcing value of the drug-paired compartments.

During the first CPP experiment, we observed that the mice in the two groups administered EtOH exhibited significant ataxia while ambulating on the parallel rods. This was further investigated by measuring footslips, which established significant ataxia in EtOH treated mice and even greater ataxic effects in mice administered EtOH and MPH (see Results below). Following this observation, conducted a second CPP experiment on a different group of mice using a modified the conditioning apparatus with floors that did not present an ambulation challenge. The results of this experiment are summarized in Figure 3B. The data analysis indicated a significant interaction of the Group and Time factors [$F(6,66.6) = 2.29, p = 0.045$]. Post-hoc analyses indicated a significant place preference during Test 2 for all three active drug groups relative to the vehicle group (all $p's < 0.05$), an effect not found during Test 1. Therefore, significant place preference for the MPH, EtOH and drug combination groups required eight conditioning sessions, and none of the active drug groups demonstrated significantly different place-preference relative to each other.

Footslips (ataxia)

As noted in the methods section, the first quantification of footslips was conducted within the CPP apparatus in real time using mice that had completed the first CPP experiment. In this first set of observations, we found that mean footslips varied as a function of drug treatment as follows (footslips $\pm$ S.E.M.): 1.3 ± 0.3 VEH; 2.9 ± 0.6 MPH; 18.1 ± 2.4 EtOH; and 32 ± 3.3 for the drug combination. These data were analyzed using a one way ANOVA which found a significant effect of group [$F(3,36) = 52, p < 0.0001$]. Post-hoc analyses using Bonferroni's corrected multiple T-test found that the drug combination increased footslips to a greater extent than either drug alone (all $p < 0.05$) and vehicle. Thus, the initial observation indicated that the combination of MPH and EtOH increased footslips more than either EtOH or MPH alone.

Figure 4 summarizes the results of the second footslip experiment conducted 2 days after completing rota-rod testing (see below). These data were also analyzed using one way ANOVA, which found a significant effect of Group [$F(3,44) = 81, p < 0.0001$], replicating the previous findings. The post-hoc analysis found that the drug combination of increased footslip errors more than either drug alone and vehicle (all $p < 0.05$). Together, the results of these two experiments indicate that the MPH + EtOH combination significantly reduces motor coordination ability.

Rota-Rod performance

The additional examination of the motor coordination in the rota-rod experiment, used mice tested 2 days after completing the second CPP experiment that were maintained in the same drug treatment group used for CPP. In this experiment, the mean latencies to fall (sec $\pm$ S.E.M.) for the 4 groups were as follows: 121 ± 18 vehicle; 97 ± 13 MPH; 20 ± 3 EtOH and 28 ± 10 for the drug combination. The final rotation speeds (RPM $\pm$ S.E.M.) were as follows: 25 ± 3 VEH; 21 ± 2 MPH; 8 ± 0.5 EtOH and 10 ± 2 for the drug combination. One way

ANOVAs on these data found significant effects of Group on Time [$F(3,44) = 16.9$, $p < 0.0001$] and RPM [$F(3,44) = 17.4$, $p < 0.0001$]. Post-hoc analyses with Bonferroni's corrected T-test showed that for groups treated with EtOH, there were significant reductions in Time and RPM compared to vehicle and MPH treatment. Additionally, there appeared to be a slight reduction in both Time and RPM for the MPH treated mice in rota-rod performance, but this was not statistically supported. In this study, we found that the conditions of the rota-rod test were quite challenging since mice could not stay on the apparatus for more than 2 min and 25 RPMs under vehicle treatment conditions. Under these challenging conditions, there was no evidence of a pharmacological interaction between MPH and EtOH.

Brain MPH and EPH Concentrations after MPH-EtOH co-administration

We investigated the unique stereo-specific interaction in brain tissue from mice treated with either 1.25 mg/kg d,l-MPH or this dose of MPH combined with 1.75 g/kg EtOH (Figure 5). The co-administration of EtOH significantly increased the *d*-isomer of MPH and there was no effect on brain concentrations of the *l*-isomer. This was supported by a significant interaction in the 2 way ANOVA [$F(1,44) = 9.79$, $p = 0.003$]. Post-hoc analyses with Bonferroni's corrected multiple T-test found that overall, concentrations of *l*-MPH were lower ($p < 0.05$) and only *d*-MPH concentrations increased in the presence of EtOH. Further, as shown in panel **5B**, we detected the metabolite EPH nearly exclusively as the *l*-isomer. In fact, *d*-EPH only reached detectable concentrations in 4 out of 12 mice. A Student's T-test confirmed the larger concentration of *l*-EPH ($t = 5.311$, $df = 22$, $p < 0.0001$). Therefore, the combination of *d,l*-MPH and EtOH enantioselectively increased *d*-MPH and produced the transesterification metabolite, *l*-EPH.

Brain EtOH concentrations after MPH-EtOH co-administration

We measured tissue concentrations of EtOH in the same brains used for MPH and EPH determinations. The mean sample tissue weight was 14.5 ± 0.5 mg across all mice. As expected, EtOH was undetectable in tissue from mice treated only with vehicle and MPH (1.25 mg/kg). For mice administered EtOH (1.75 g/kg) or the MPH + EtOH combination the tissue concentrations were 19.56 ± 1.51 and 19.65 ± 1.59 $\mu\text{g EtOH/mg cortex}$, respectively. Student's T-test confirmed the lack of a difference ($t = 0.04$, $df = 22$, $p = 0.96$). Therefore, co-administration of EtOH and MPH did not alter EtOH concentrations in the cortex at a time point relevant to the behavioral tests used in the present studies.

Discussion

In these studies, we found that EtOH and MPH interacted when simultaneously injected to enhance locomotor stimulation, motor incoordination (ataxia) and discrimination of MPH in MPH-trained mice, but not EtOH-trained mice. A very important feature of these enhanced behavioral effects of the MPH-EtOH combination is that they were observed at relatively low doses of each drug. Further, the increased ataxia found with the drug combination may well have important clinical implications. These specific drug interactive effects on behavior are very likely due to the selective increase in the *d*-isomer of MPH that we observed soon after co-administration of the two drugs at a time point relevant for all the behaviors. Interestingly, we did not find evidence of enhanced reinforcing effects of the drug combination, at least as measured by conditioned place preference. Accordingly, these new data extend our understanding of the behavioral effects of a clinically relevant drug interaction.

The present drug discrimination experiments build on our previous studies examining the discriminative stimulus of MPH by adding EtOH to our test procedures. Previous studies

indicated that MPH doses of >4 mg/kg were required for C57 mice to acquire discrimination and, further, that the discriminative stimulus was dependent upon the pharmacologically active d-isomer, not the inactive l-isomer of MPH (McGovern *et al.* 2011). In the present study, once discrimination was established, mice demonstrated partial generalization to MPH doses lower than the training dose as expected (Stolerman *et al.* 2011). The partial generalization provided a sensitive test for examining whether EtOH would augment MPH discrimination. Essentially, our data show that EtOH enhanced the ability of mice to discriminate doses of MPH smaller (1-2 mg/kg) than the training dose. On the other hand no such shift in the generalization curve occurred in the EtOH-trained mice when MPH and EtOH were combined. Accordingly, the results of these two experiments are consistent with our observations that the combination of MPH and EtOH increases d-MPH concentrations in B6 mice (Bell *et al.* 2011a; Bell *et al.* 2011b). Thus, only mice trained to discriminate MPH would be expected to demonstrate a leftward shift in their MPH generalization curve, as shown by our data.

The discrimination experiment with MPH-trained mice indicated that MPH and EtOH could interact to affect behavior in B6 mice at lower doses than previously demonstrated (Bell *et al.* 2011a; Bell *et al.* 2011b; Griffin *et al.* 2010). This was examined further using locomotor activity. We found that a non-stimulatory dose of MPH (1.25 mg/kg) combined with a non-stimulatory dose of EtOH (1 g/kg) significantly interacted to increase total activity of B6 mice. Therefore, both the discrimination and locomotor activity experiments indicate that significant pharmacological interactions between MPH and EtOH can occur at low doses (e.g. 1-2 mg/kg MPH vs 1 g/kg EtOH). Demonstrating a lower threshold for this drug-drug interaction in our preclinical studies has important implications for clinical scenarios. Indeed, it has been recently pointed out that MPH doses used in the preclinical literature have historically translated into doses that are much higher than used clinically to treat ADHD (Kuczenski *et al.* 2005). In addition to our findings, low MPH doses (<2 mg/kg) have been shown to cause sensitization (Kuczenski *et al.* 2001) and differentially affect dopamine levels in several brain regions in other preclinical models (Berridge *et al.* 2006; Kuczenski *et al.* 1997). In humans, the combination of a therapeutic dose of MPH and a standard EtOH drink elicited interoceptive cues (positive subjective effects) that the study participants endorsed more than MPH alone (Patrick *et al.* 2007). Thus, our findings of significant pharmacological interactions in the B6 mouse model using lower doses of MPH and EtOH than previously reported, coupled with our human laboratory study (Patrick *et al.* 2007) indicate the distinct possibility of clinically relevant interactions for social drinkers being treated for ADHD with d-MPH (Biederman *et al.* 2005; Biederman *et al.* 2002).

Reports in humans indicate that the combination of MPH and EtOH might have pleasurable effects (Barrett *et al.* 2002; Patrick *et al.* 2007), implying enhanced reinforcing effects of the drug combination compared to MPH or EtOH alone. Therefore, we hypothesized that the combination of EtOH and MPH would demonstrate enhanced reinforcing value compared to either drug alone. However, our data do not support this hypothesis, at least as measured by conditioned place preference under two different conditions. Nevertheless, the data from CPP experiment 2 do indicate that the drug combination has at least a similar reinforcing value of either drug alone. Conditioned place preference testing is conducted in the drug-free state and therefore evaluates the secondary reinforcing effects of drugs. Since the literature suggests that the primary reinforcing value of the drug combination when co-ingested is salient, evaluating MPH/EtOH combinations using oral self-administration procedures in rodent models represents an intriguing future area of investigation.

An important finding from the CPP studies is that the 1.25 mg/kg dose of MPH supported significant place preference, consistent with other recent reports investigating smaller doses of MPH (3 mg/kg or less) in the context of reward (dela Peña *et al.* 2012; dela Peña *et al.*

2011; Trezza *et al.* 2009; Wooters *et al.* 2011). Interestingly, another recent report found that 0.75 mg/kg MPH did not support place preference in contrast to a 10-fold higher dose (Zhu *et al.* 2011b), suggesting a lower limit to the reinforcing effects of MPH. The 1.25 mg/kg dose of MPH we used is close to the range reported to be relevant for clinical use (Kuczenski *et al.* 2005). This finding was evident in CPP experiment 1 in which there was a clear bias by the mice away from the parallel rod side of the apparatus (see methods), but showed significant place preference to this side after only 4 conditioning sessions. The two groups that were conditioned with EtOH and EtOH/MPH combination in CPP experiment 1 did not show significant place preference at all. Thus, under these conditions, MPH alone appears to be more rewarding than either EtOH or the drug combination. In contrast, using the modified apparatus with smooth flooring, the MPH treated mice demonstrated place preference within the same number of conditioning sessions and to the same magnitude as the other active drug groups.

We postulate that the increased difficulty ambulating on the parallel rods while intoxicated with EtOH, evidenced as increased footslips, provided an aversive quality to the apparatus that could not be overcome with 16 conditioning sessions to produce significant place preference in the first CPP experiment. In part, this interpretation is supported by a previous experiment showing that EtOH (1.75 g/kg) produces significant place preference in B6 mice (Nocjar *et al.* 1999). Moreover, current results from the modified apparatus to remove the ambulation challenge demonstrated significant place preference in both groups of mice conditioned with EtOH. The observation that parallel rods can challenge ambulation under the influence of EtOH intoxication, leading to increased footslip errors, has been reported earlier (Kamens *et al.* 2007; Kamens *et al.* 2005). Interestingly, the addition of MPH to EtOH intoxication exacerbated the motor incoordination, shown by even greater numbers of footslips in the MPH+EtOH condition. This observation may have clinical implications since people often combine MPH (or other psychostimulants) with EtOH to antagonize the depressant effects of EtOH. Our results in the mouse model clearly indicate that MPH does not antagonize the ataxic effects of EtOH but rather, does quite the opposite.

The lack of potentiated ataxia with the combination of EtOH and MPH in the rota-rod test was not intuitive. We intentionally designed the rota-rod test to include a rapid increase in rotation speed in a short period of time to make the test challenging. However, this came at the expense of the sensitivity needed to detect differences in ataxia between the EtOH alone and the drug combination groups (see results). Therefore, further testing will be required with the rota-rod to determine if differences in ataxia can be detected between drug treatments using this method. Notwithstanding, the rota-rod test and footslip test offer interesting contrasts in probing motor coordination. The rota-rod test forces the mice to move because of the turning rod and the threat of a fall from the elevated position provides incentive for the mice to maintain their balance. On the other hand, in the footslip test, errors in fine motor skill are completely the result of the mouse's own ambulation around the interior of the chamber. It should be noted that EtOH (1.75 g/kg) stimulates locomotor activity (Griffin *et al.* 2010; Jerlhag 2008; Middaugh *et al.* 1992; Middaugh *et al.* 1987); moreover, the combination of this EtOH dose and MPH (1.25 mg/kg), which is non-stimulatory by itself, produces greater activity than either drug given alone (Griffin *et al.* 2010). Thus, a plausible explanation for the increase in footslips with the drug combination in the current study is that mice ambulate more while intoxicated with the drug combination, providing more opportunities for footslip errors.

The potentiated pharmacological effects revealed in the present report, and those previously published in mice (Bell *et al.* 2011a; Griffin *et al.* 2010) and humans (Patrick *et al.* 2007), are likely due to the selective increase in the d-isomer of MPH when dl-MPH and EtOH are combined. In our previous preclinical work using oral dosing (Bell *et al.* 2011a), the 3-h

time point after drug challenge that brain tissue was collected did not reveal a significance difference in *d*- and *l*-MPH concentrations, though there was a trend. In the present study, we collected brain tissue at an earlier time point relevant the current behavioral studies and found a significant increase in the *d*-isomer of MPH. The significant increase of the *d*-isomer of MPH with the *dl*-MPH-EtOH combination was first noted in a human laboratory trial (Patrick *et al.* 2007) and postulated to be part of the mechanism behind the increased self-reports of increased pleasurable feelings noted in that study. A great deal of evidence indicates that the *d*-isomer of MPH, rather than the *l*-isomer, is pharmacologically active (Markowitz *et al.* 2008; Patrick *et al.* 1987; Patrick *et al.* 1981). However, the *l*-isomer of MPH is not necessarily biologically inert. Mounting evidence suggests that *l*-MPH inhibits carboxylesterase 1 (Zhu *et al.* 2008), the enzyme primarily involved in the metabolic clearance of MPH. This effectively reduces *d*-MPH metabolism, producing an increase in systemic *d*-MPH concentrations. Thus, an important contribution of the present results is confirmation that *d*-MPH concentrations are increased in B6 mice soon after co-administration of EtOH and *dl*-MPH, as noted in humans (Patrick *et al.* 2007). Collectively, these data indicate that the combination of EtOH and MPH produces a significant increase in *d*-MPH concentrations, the pharmacologically active isomer of MPH.

Lastly, we observed that the transesterification metabolite EPH was also detected in brain tissue of B6 mice consistent with previous work in mice (Bell *et al.* 2011a; Bell *et al.* 2011b; Williard *et al.* 2007) and in human plasma (Markowitz *et al.* 2000; Markowitz *et al.* 1999; Patrick *et al.* 2007). The majority of EPH produced was the *l*-isomer and previous behavioral experiments indicate that this isomer is pharmacologically inactive (Patrick *et al.* 2005; Williard *et al.* 2007). The *d*-isomer of EPH is pharmacologically active (Williard *et al.* 2007) but is only generated in small amounts in mice (Bell *et al.* 2011a; Bell *et al.* 2011b; Williard *et al.* 2007) and humans under controlled laboratory conditions (Patrick *et al.* 2007), thus it is not likely to contribute to the potentiated behavioral effects reported for the drug combination. However, the production of EPH appears to be unique to the combination of MPH and EtOH and therefore offers the potential as a biomarker of concomitant MPH-EtOH, as reported in overdoses [e.g. (Markowitz 1999)] for identifying causative toxicity.

In conclusion, the present studies as well as our recent published work (Bell *et al.* 2011a; Bell *et al.* 2011b; Griffin *et al.* 2010; Williard *et al.* 2007) are an example of reverse translation because the hypotheses we tested in mice were originally generated by findings observed in humans (Markowitz *et al.* 2000; Markowitz *et al.* 1999; Patrick *et al.* 2007). The preclinical studies presented here demonstrate significant pharmacological interactions of MPH and EtOH at doses extrapolated to be clinically relevant on drug discrimination, locomotor activity and confirmed the unique stereospecific increases in *d*-MPH and production of *l*-EPH. Additionally, we found that the combination of EtOH and MPH increases ataxia, as measured by footslips in a parallel rod test. Taken together, these results indicate that MPH and EtOH interact in clinically important ways. The widespread use of these two drugs and interactive pharmacology demands careful attention from prescribers treating ADHD as well as a greater awareness from the consuming public about the risks of co-ingestion of EtOH and MPH.

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Figure 1.

EtOH enhances the discriminative stimulus of *d,l*-MPH in mice trained to discriminate *d,l*-MPH from vehicle injections. **A)** Including 1 g/kg EtOH with the *d,l*-MPH injection produced a significant shift to the left (* $p < 0.05$; closed circles) versus administration of *d,l*-MPH alone (open circles) for the *d,l*-MPH generalization curve. **B)** For the 3 lowest doses of MPH in combination with EtOH, the response rates of the drug combination were lower than for *d,l*-MPH administered alone. All values are means $\pm$ S.E.M.

Figure 2. Non-stimulatory doses of EtOH (1 g/kg) and *d,l*-MPH (1.25 mg/kg) interact to increase locomotor activity when co-administered. All values are means $\pm$ S.E.M.

Figure 3.

EtOH (1.75 g/kg) and *d,l*-MPH (1.25 mg/kg) do not interact to enhance conditioned place preference. Two different experiments were conducted, see Methods for details on the side-bias. **A)** Only *d,l*-MPH produced significant place preference the biased apparatus. The side-bias of the mice is shown by the time spent on the drug-paired side (~300 sec), which was the parallel rod side of the apparatus. The *d,l*-MPH effect was significant at Test 1 and Test 2 (* $p < 0.05$). In post-hoc testing, EtOH (1.75 g/kg) did not yield significant place preference in this apparatus ($p > 0.07$ for Test 1 & 2 versus vehicle). **B)** In the non-biased apparatus, all three dosing conditions produced significant place preference at Test 2 versus vehicle (* $p < 0.05$). In this apparatus, the side-bias was nearly eliminated (~420secs, drug paired

side) by using smooth flooring and contrasting colors on the walls. All values are means $\pm$ S.E.M.

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Figure 4.

EtOH and *d,l*-MPH interact to increase motor incoordination. When challenged with ambulating on parallel rods after drug injection, mice exhibited more footslip errors with the combination of EtOH (1.75 g/kg) and *d,l*-MPH (1.25 mg/kg) than either drug alone ($+p<0.05$) and vehicle ($*p<0.05$). All values are means $\pm$ S.E.M.

Figure 5.

EtOH and *d,l*-MPH interact to significantly increase brain tissue concentrations of the active isomer *d*-MPH (* $p < 0.05$) and the pharmacologically inactive transesterification metabolite, *l*-EPH (* $p < 0.05$). Concentrations of the pharmacological inactive isomer *l*-MPH were not altered. Brain tissue concentrations of EtOH were not altered either (See Results). Although the *d*-isomer of EPH is pharmacologically active, very little was produced, and was only detectable in 4 of 12 mice. *n.d.* indicates not detected. All values are means $\pm$ S.E.M.

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Table 1

Experimental Use of C57BL/6J Mice

Group	N	Procedures
1	16	MPH Discrimination
2	24	EtOH Discrimination
3	40	Locomotor Activity
4	40	Place Preference 1 Footslip 1
5	48	Place Preference 2 Rota-Rod Footslip 2 Drug Concentrations