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Rewarding and aversive doses of caffeine alter activity but not conditioned place preference induced by ethanol in DBA/2J mice

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ABSTRACT

Increases in the consumption of ethanol and caffeine have been attributed to increased subjective feelings of intoxication and pleasure from the combination. Previous studies have shown that caffeine can be rewarding at low doses and aversive at high doses, although these findings are at times inconsistent between studies using comparable doses. Similarly, studies investigating the rewarding effects of ethanol and caffeine combinations have yielded mixed results. To address this issue, the present experiments were designed to investigate the rewarding effects of caffeine, as well as of caffeine + ethanol combinations. Male DBA/2J mice were exposed to an unbiased conditioned place preference (CPP) procedure with various doses of caffeine (1, 3, 10, 30 mg/kg) and ethanol (1, 2 g/kg), as well as various conditioning trial durations (5, 30, 60 min). Caffeine dose-dependently increased locomotor activity during conditioning, and produced a biphasic effect on place conditioning. Specifically, a low dose of caffeine (3 mg/kg) produced place preference, while a high dose (30 mg/kg) produced place aversion. When combined with alcohol, caffeine dose-dependently increased ethanol's stimulatory effect. However, the addition of caffeine had no effect on ethanol place preference, as there were no differences in the strength of place preference between mice conditioned with ethanol alone, and mice conditioned with any combination of ethanol and caffeine. These studies add evidence for caffeine's biphasic effects while also emphasizing the importance of considering temporal and methodological parameters when using Pavlovian conditioning procedures to study drug combinations.

1. Introduction

Over the last 20 years, a large increase has been observed in the amount of alcohol consumed in combination with caffeine, especially in adolescents and young adults (de Sanctis et al., 2017; Malinauskas et al., 2007; Reissig et al., 2009). This increase has had immediate health impacts, as it has been associated with increases in visits to the emergency room, among other things (Arria et al., 2011; Mattson, 2013). It has been suggested that among college students, alcohol is consumed in combination with caffeine to increase pleasure from intoxication, as well as to alter the intensity and subjective feelings of intoxication (Attwood et al., 2012; Azcona et al., 1995; Marczinski, 2014; Marczinski et al., 2016). Given this growing trend, it is imperative that we understand how these two substances interact to influence behavior.

The rewarding effects of alcohol have been well-studied using a variety of models, including Pavlovian conditioning, across rats, mice and other rodent species (Becker, 2013; Cunningham et al., 2000; Ryabinin and Walcott, 2018). To a lesser extent, the appetitive and aversive effects of caffeine have also been examined using Pavlovian conditioning procedures. In rats, caffeine has been shown to have a biphasic effect, with lower doses producing place preference, while higher doses produce place aversion (Bedingfield et al., 1998; Brockwell et al., 1991; Patkina and Zvartau, 1998; Steigerwald et al., 1988). Furthermore, high doses of caffeine have been shown to produce robust taste aversion in rats (Vishwanath et al., 2011; White and Mason, 1985). In mice, the few studies conducted to date have yielded inconsistent results. Indeed, while some groups have demonstrated that caffeine at the 3 mg/kg dose can produce place preference (Hilbert et al., 2013), others have found no place preference using similar doses (Sturgess et al., 2010). Additionally, although a moderate dose (10 mg/kg) has been shown to produce place preference (Hsu et al., 2009), Sturgess and colleagues found place aversion with this same dose, as well as with a higher dose (30 mg/kg). Little is known, however, about how the two drugs may enhance or counteract the subjective effects of each other.

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Self-administration studies have also produced mixed results. Some studies have shown that certain doses of caffeine have no effect (Robins et al., 2016a), or can decrease alcohol intake (Rezvani et al., 2013), while others can facilitate increases in alcohol intake (Fritz et al., 2016; Kunin et al., 2000). These differences in findings can likely be attributed to differences in procedural parameters, as well as differences in rodent species and strains; and illustrate the need to further understand the complex role played by caffeine and ethanol in motivated behaviors.

Across rodent species, even fewer studies have investigated the rewarding effects of caffeine and alcohol combinations using Pavlovian conditioning methods. Hilbert et al. (2013) found that the combination of caffeine and alcohol produced place preference in C57BL/6J mice, although it was not different than place preference to alcohol alone. Given the paucity of data in mice, the present studies were conducted in order to characterize the rewarding and aversive effects of caffeine, as well as of various caffeine and alcohol combinations using a mouse strain (DBA/2J) known to show robust place conditioning to alcohol (Cunningham, 2014). Furthermore, the present studies utilized a conditioning procedure that has been used successfully for conditioning with depressants (alcohol and morphine: Cunningham et al., 1992), as well as with stimulants (Cunningham et al., 1999; Dobbs and Cunningham, 2014) in order to assess the reinforcing effects of caffeine and caffeine-alcohol combinations. Based on previous studies, we hypothesized that caffeine would produce a bi-phasic effect, with lower doses producing place preference, and higher doses producing place aversion. Given that humans report a possible potentiating effect of the combination of ethanol and caffeine (Marczinski, 2014; Marczinski et al., 2016), we also hypothesized that a combination of a low dose of caffeine and alcohol would produce a stronger place preference than alcohol alone. Conversely, it is possible that a combining a high (aversive) dose of caffeine with alcohol would interfere with alcohol-induced place preference. Finally, we hypothesized that place preference for the combination of a low dose of caffeine and alcohol would differ in its rate of extinction, compared to alcohol alone.

2. Methods

2.1. General methods

2.1.1. Experimental animals

Upon arrival, seven-week old male DBA/2J mice (n = 384, 96 per experiment, Jackson Laboratories, Sacramento, CA, USA) were housed in groups of four and were allowed to habituate to the animal room for one week prior to the start of conditioning. Mice were kept on a 12:12 h light-dark cycle with lights on at 7:00 AM. Standard rodent chow and water were available ad libitum throughout the duration of the study. All procedures conducted were approved by the Oregon Health & Science University IACUC.

2.1.2. Drugs

Caffeine anhydrous (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% saline and was administered intraperitoneally (IP) at doses 3 or 30 mg/kg (0.3 and 3 mg/mL: 10 mL/kg). Ethanol (EtOH) (20% v/v) was prepared from a 95% stock solution in 0.9% sterile saline, and was injected IP at a dose of either 2 g/kg (12.5 mL/kg) in Experiment 2, or 1 g/kg (volume, 6.25 mL/kg), in Experiment 3. All IP injections of saline were administered at equivalent volumes to the drug volumes used in each experiment (i.e. 6.25, 10, or 12.5 mL/kg).

2.1.3. Apparatus

The conditioning apparatus used here is described in detail by Cunningham and colleagues (Cunningham et al., 2006). Briefly, conditioning boxes (30 × 15 × 15 cm) were individually enclosed in larger, well-ventilated chambers (Coulbourn Instruments, Model E10–20, dimensions: 56.1 × 46 × 39.4 cm), in which both light, and sound were attenuated. Activity and the position of the animal were detected using six sets of infrared photodetectors (5 cm intervals, 2.2 cm above the floor) mounted along the sides of each conditioning box.

Conditioning floors consisted of two interchangeable halves that were either a *grid* or a *hole* pattern. *Grid* floors were made up of 2.3 mm stainless steel rods, mounted to acrylic sides in 6.4 mm intervals. *Hole* floors were made from stainless steel sheets perforated with 6.4 mm holes in a staggered manner (9.5 mm apart). All floors, boxes, and chambers were cleaned and wiped between animals using a damp sponge.

2.2. Conditioning procedure

All mice were exposed to an unbiased one-compartment place-conditioning procedure with sessions conducted once per day. Within each experiment mice were divided into subgroups so as to fully counterbalance tactile stimulus (grid/hole) and drug assignment (caffeine, caffeine & ethanol, or saline). That is, mice were assigned to either the G+ subgroup, (in which the grid floor was paired with drug, and the hole floor was paired with saline) or the G− subgroup (in which the hole floor was paired with drug, and the grid floor was paired with saline). A significant difference in time spent on the grid floor between conditioning subgroups during the preference test was used as the primary dependent variable (Cunningham et al., 2003; Cunningham et al., 2006). Across all experiments, mice received one trial per day. The order in which mice were exposed to each trial was also counterbalanced (drug trial first; CS+, vehicle trial first; CS−). Finally, the position of each floor during testing (left vs. right) was also counterbalanced.

2.3. Experiment 1: caffeine place conditioning with various doses

Upon arrival mice were assigned to one of four groups based on the dose of caffeine used during conditioning (1, 3, 10, or 30 mg/kg). As described above, mice were then assigned to either the G+ or G− subgroup. Conditioning consisted of a single habituation session followed by eight conditioning sessions of each type (CS+ vs. CS−). During habituation mice were weighed and injected with saline (10 mL/kg) and immediately placed on a smooth paper floor in the conditioning apparatus for 5 min. During conditioning mice were weighed and injected with caffeine (1, 3, 10, or 30 mg/kg) on CS+ days or saline (10 mL/kg) on CS− days immediately before being placed in the apparatus for 30 min. Twenty-four hours after the fourth, sixth, and eighth conditioning trial, mice were weighed and injected with saline immediately before being placed onto a split-cue floor for a 30-min preference test.

2.4. Experiment 2: caffeine place conditioning with various trial durations

Mice were separated into groups depending on the caffeine dose used (3 or 30 mg/kg), as well as the conditioning trial duration (5, 30, or 60 min). Within each dose by trial-duration group, mice were assigned to one of two conditioning subgroups described above (G+, G−) Place conditioning consisted of one habituation day, followed by four conditioning trials of each type (CS+ vs. CS−) and a single 30-min preference test. Habituation consisted of one, 5-min session in which mice were weighed and injected with saline (10 mL/kg) immediately before being placed in the conditioning apparatus. During the conditioning phase, animals were weighed and injected with either 3 or 30 mg/kg caffeine on CS+ days and saline on CS− days immediately before being placed in the apparatus for either 5, 30, or 60 min. Forty-eight hours after the last conditioning day, mice were weighed and injected with saline immediately prior to being placed in the apparatus for a 30-min test.

2.5. Experiment 3: caffeine and 2 g/kg EtOH place conditioning

Mice were assigned to one of three groups based on the drug combination used during conditioning (2 g/kg EtOH + 10 mL/kg saline, 2 g/kg EtOH + 3 mg/kg caffeine, 2 g/kg EtOH + 30 mg/kg caffeine), as well as by conditioning subgroup (G+, G−). Here, place conditioning consisted of a single habituation day, followed by six conditioning trials of each type and three, 30-min preference tests that occurred 24 h after mice had received two, four, and six conditioning trials of each type. On CS+ days, mice received two IP injections of EtOH and 10 mL/kg saline, EtOH and 3 mg/kg caffeine or EtOH and 30 mg/kg caffeine immediately prior to being placed in the apparatus for a 5-min conditioning session. During CS− trials, mice received two saline injections (12.5 mL/kg, 10 mL/kg) before being placed in the apparatus. During preference testing, mice again received two saline injections immediately before being placed in the apparatus with split-floors for 30 min.

2.6. Experiment 4: caffeine and 1 g/kg EtOH place conditioning and extinction

As with Experiment 3, mice were assigned to three groups depending on the drug combination used during conditioning, and then again by conditioning subgroup. Here however, a lower dose of ethanol was used (1 g/kg) in combination with the two doses of caffeine previously used in Experiment 2 and Experiment 3 (3 and 30 mg/kg). The place conditioning procedure consisted of one, 5-min habituation day, six conditioning trials of each type and three, 30-min preference tests that occurred 24 hours after mice had received two, four, and six conditioning trials of each type, and 10, 30-min choice-extinction sessions. On CS+ days, mice received two IP injections of 1 g/kg EtOH and 10 mL/kg saline, 1 mg/kg EtOH and 3 mg/kg caffeine or 1 mg/kg EtOH and 30 mg/kg caffeine immediately prior to being placed in the apparatus for a 5-min conditioning session. During CS− trials, mice received two saline injections (6.25 mL/kg, 10 mL/kg) before being placed in the apparatus. During testing, mice again received two saline injections prior to being placed in the apparatus with both floor types for 30 min. Twenty-four hours following the 3rd preference test mice were exposed to 10, 30-min choice extinction sessions separated by 24 h between each session. Choice extinction sessions consisted of mice receiving two saline injections immediately before being placed in the apparatus with the same two-floor configuration used during the three preference tests.

2.7. Statistical analyses

2.7.1. Conditioning activity

Conditioning activity data for all experiments were converted to activity rates (counts/min) and averaged across trials. For Exp. 1, conditioning activity was analyzed via two-way repeated-measures (RM) ANOVA with Dose (1 vs. 3 vs. 10 vs. 30 mg/kg) as a between-group factor and Trial Type (CS+ vs. CS−) as a within-group factor. Only the first 5 min was analyzed for Exp. 2 because mice were exposed to varying trial durations during conditioning. More specifically, activity rates during the first 5 min were averaged over trials and analyzed via a three-way RM ANOVA with Dose (3 vs. 30 mg/kg) and Trial Duration (5 vs. 30 vs. 60 min) as the between-group factors, while Trial Type (CS+ vs. CS−) was used as a within-group factor. Conditioning activity data for Exp. 3 and 4 were analyzed via two-way RM ANOVAs with Drug Combination as a between-group factor and Trial Type (CS+ vs. CS−) as a within-group factor.

2.7.2. Grid times

As described above, a significant difference in time spent on the grid floor between conditioning subgroups during the preference test was used as the primary dependent variable (Cunningham et al., 2003; Cunningham et al., 2006). For Exp. 1, time spent on the grid floor was analyzed using a three-way RM ANOVA with Dose (1 vs. 3 vs. 10 vs. 30 mg/kg) and Conditioning Subgroup (G+ vs. G−) as between-group factors, and Test (Test 1 vs. Test 3) as the within group factor. Grid time data for Exp. 2 was analyzed using a three-way ANOVA with Dose (3 vs. 30 mg/kg), Trial Duration (5 vs. 30 vs. 60 min) and Conditioning Subgroup (G+ vs. G−) as between-group factors. For Exp. 3 and 4, grid times were analyzed via a three-way RM ANOVA with Drug Combination and Conditioning Subgroup (G+ vs. G−) as between-group factors, and Test (Test 1 vs. Test 3) as the within group factor. Lastly, Grid Times during the choice extinction procedure in Exp. 4 were converted to mean percent times spent on the drug-paired floor for ease of presentation. These data were then analyzed with a two-way RM ANOVA with Drug Combination as a between group factor and Test as a within group factor.

2.7.3. Test activity

Locomotor activity data during the 30-min preference tests were converted to activity rate (counts/min). For Exp 1, test activity was analyzed via a two-way RM ANOVA with Dose (1 vs. 3 vs. 10 vs. 30 mg/kg) as between-group factor, and Test (1 vs. 3) as the within group factor. Activity data in Exp. 2 was analyzed using a two-way ANOVA with Dose (3 vs. 30 mg/kg) and Trial Duration (5 vs. 30 vs. 60 min) as between groups factors. For Exp. 3 and 4, test activity data were analyzed via a two-way RM ANOVA with Drug Combination as a between-group factor, and Test (1 vs. 3) as the within group factor.

3. Results

3.1. Subject attrition

Two subjects were removed from statistical analysis in Experiment 1 due to procedural error. Similarly, data from one mouse in Experiment 4 was not analyzed due to a procedural error. Lastly, six mice were removed from Experiment 2 due to health issues or procedural errors. Final numbers per experiment are listed in the figure captions.

3.2. Experiment 1: caffeine place conditioning with various doses

3.2.1. Conditioning activity

Mean activity rates collapsed across the eight conditioning trials of each type are presented in Fig. 1. Overall, caffeine produced a dose-dependent increase in activity during conditioning. Conditioning activity was significantly higher on caffeine (CS+) trials than on saline (CS−) trials, and the difference in activity between trial types was more pronounced in animals conditioned with higher doses of caffeine. In

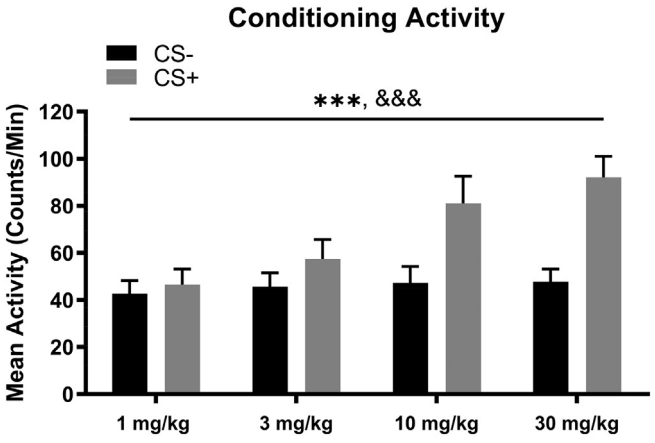

Fig. 1. Mean activity rates (activity counts/min ± SEM) during CS− (saline) and CS+ (caffeine) conditioning trials in male DBA/2J mice. Each bar depicts data from 23 to 24 mice. ***; significant main effect of Trial Type ($p < 0.001$), &&&; significant Trial Type × Dose interaction ($p < 0.001$).

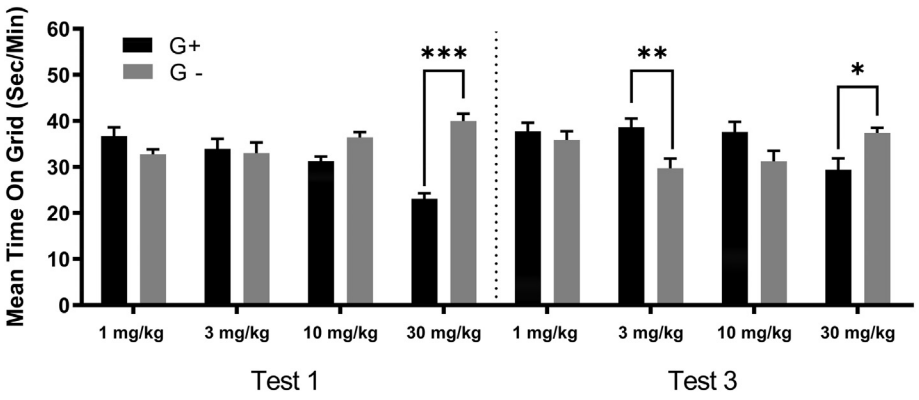

Fig. 2. Mean time spent on the grid floor (s/min ± SEM) after four (test 1) and eight (test 3) conditioning trials in Experiment 1. The grid floor was paired with drug injections in the G+ subgroups whereas the grid floor was paired with vehicle injections in the G− subgroups. Each bar represents data from 11 to 12 mice. *; $p < 0.05$, **, $p < 0.01$, ***; $p < 0.001$.

support of this, a two-way ANOVA revealed main effects of Trial Type [$F(1,91) = 1613.1$, $p < 0.001$] and Dose [$F(3,91) = 64.23$, $p < 0.001$], as well as a Trial Type × Dose interaction [$F(3,91) = 261.7$, $p < 0.001$].

3.2.2. Grid times

Fig. 2 depicts the mean times spent on the grid floor during the 30-min preference tests. After four trials (Test 1), and eight trials (Test 3) the magnitude of place conditioning varied depending on caffeine dose and the number of conditioning trials. More specifically, the high dose of caffeine produced place aversion during both preference tests. Conversely, a place preference was seen in the 3 mg/kg caffeine group, but only after eight conditioning trials. A three-way ANOVA (Conditioning Subgroup × Dose × Test) confirmed this observation, as there were significant Conditioning Subgroup × Dose [$F(3,86) = 14.1$, $p < 0.001$] and Test × Conditioning Subgroup interactions [$F(1,86) = 17.4$, $p < 0.001$], as well as a Conditioning Subgroup × Dose × Test three-way interaction [$F(3,86) = 3.7$, $p < 0.05$]. However, no main effects were detected. In order to better understand this three-way interaction, separate two-way ANOVAs (Conditioning Subgroup × Dose) were conducted for tests 1 and 3. The two-way ANOVA for test 1 revealed a main effect of Conditioning Subgroup [$F(1,86) = 13.6$, $p < 0.001$], as well as a Conditioning Subgroup × Dose interaction [$F(3,86) = 16.1$, $p < 0.001$]. A Bonferroni-adjusted post-hoc analysis for the Conditioning Subgroup × Dose interaction revealed a significant place aversion at the 30 mg/kg dose, as mice in the G+ conditioning subgroups spent significantly less time on the Grid floor than those in the G− subgroup ($p < 0.001$). The two-way ANOVA for test 3 revealed a significant Conditioning Subgroup × Dose interaction [$F(3,87) = 6.9$, $p < 0.001$], but no main effect of conditioning. Through a Bonferroni-adjusted post-hoc analysis, place aversion was again observed at the 30 mg/kg dose ($p = 0.02$), while significant place preference was seen at the 3 mg/kg dose, as indicated by G+ mice spending significantly more time on the grid floor than G− mice ($p = 0.009$).

3.2.3. Test activity

Mean locomotor activity rates during the two preference tests are presented in Supplementary Table 1. As can be seen, mice conditioned with the higher doses of caffeine displayed greater locomotor activity during the preference tests. In addition, mice were generally more active during the third test, compared to the first test. In support of this, a RM ANOVA conducted for the two tests revealed a significant main effect of Test [$F(1,90) = 8.54$, $p < 0.01$] and of Dose [$F(3, 90) = 3.28$, $p < 0.05$], but no Test × Dose interaction [$F(3,90) = 1.89$, $p = 0.13$].

3.3. Experiment 2: caffeine place conditioning with various trial durations

3.3.1. Conditioning activity

Fig. 3 depicts mean conditioning activity rates during the first 5 min

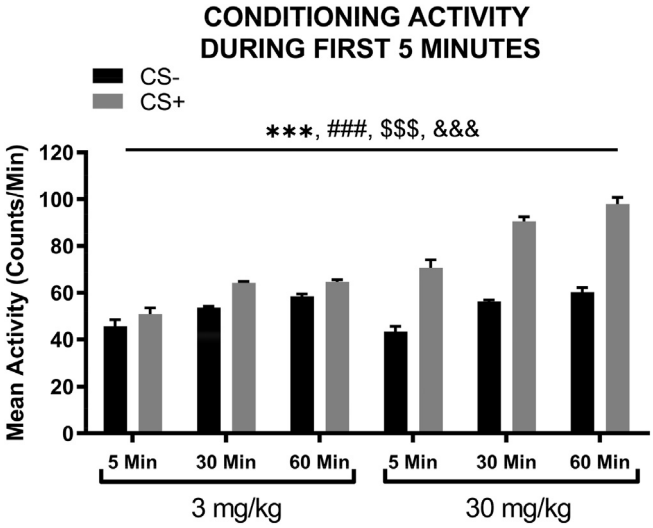

Fig. 3. Mean activity rates (activity counts/min ± SEM) during the first 5 min of CS− (saline) and CS+ (caffeine) conditioning trials in male DBA/2J mice conditioned with 5, 30, or 60-min conditioning trials. Each bar represents data from 14 to 16 mice. ***; significant main effect of Trial Type ($p < 0.001$), ###; significant main effect of Trial Duration ($p < 0.001$), $$$; significant main effect of Dose ($p < 0.001$), &&&; significant Trial Type × Dose interaction ($p < 0.001$).

of each trial, collapsed over the four conditioning trials of each type. Activity was higher on caffeine (CS+) trials than on saline (CS−) trials. This difference in activity between trial types was more pronounced in mice that were conditioned with the large dose of caffeine. Additionally, this difference in activity between CS+ and CS− trials was greater in mice exposed to the 30-min and 60-min trial duration, when compared to that of mice exposed to 5-min durations. A three-way ANOVA (Trial Type × Trial Duration × Dose) revealed significant main effects of Trial Type [$F(1,84) = 394.8$, $p < 0.001$], Trial Duration [$F(2,84) = 22.4$, $p < 0.001$], and Dose [$F(1,84) = 36.9$, $p < 0.001$]. Additionally, significant Trial Type × Trial Duration [$F(2,84) = 3.9$, $p = 0.02$] and Trial Type × Dose [$F(1,84) = 161.9$, $p < 0.001$] interactions were observed.

3.3.2. Grid times

Mean times spent on the grid floor during the 30-min preference test are shown in Fig. 4. Overall, the high dose of caffeine (30 mg/kg) produced place aversion, but only with longer trial durations (30 or 60 min). No significant place conditioning was observed in mice conditioned with the low dose of caffeine (3 mg/kg). In support of this, a three-way ANOVA (Conditioning Subgroup × Dose × Trial Duration) revealed significant Conditioning Subgroup × Trial Duration [$F(2,78) = 4.3$, $p = 0.02$] and Conditioning Subgroup × Dose [F

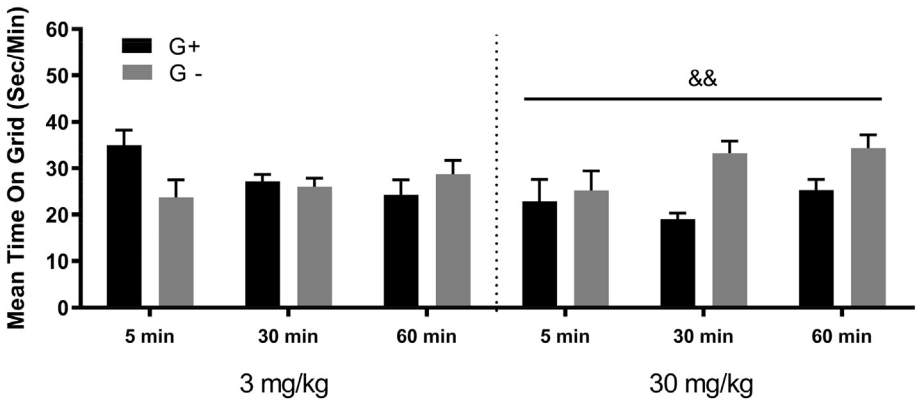

Fig. 4. Mean time spent on the grid floor (s/min ± SEM) after four conditioning trials of each type in Experiment 2. The grid floor was paired with drug injections in the G+ subgroups whereas the grid floor was paired with vehicle injections in the G− subgroups Each bar represents data from 7 to 8 mice. &&; significant Conditioning Subgroup × Dose interaction ($p < 0.01$).

(1,78) = 10.3, $p = 0.002$] interactions, while no main effects were detected. These two interactions were both analyzed further, and the Bonferroni-adjusted post-hoc analysis for the Conditioning Subgroup × Dose interaction showed that the 30 mg/kg dose of caffeine produced significant place aversion ($p = 0.002$), as indicated by mice in the G+ subgroup spending significantly less time on the grid floor than mice in the G− subgroup. Additionally, when the Conditioning Subgroup × Trial Duration interaction was examined, differences in the amount of time spent on the grid floors between G+ and G− subgroups were observed in the 30 min and the 60 min groups, although these differences did not reach significance (30 min: $p = 0.11$, 60 min: $p = 0.08$). No differences between G+ and G− subgroups were observed in the 5 min group.

3.3.3. Test activity

Mean locomotor activity rates during the 30-min preference test are presented in Supplementary Table 1. In line with Experiment 1, mice conditioned with the higher dose of caffeine were more active during the preference test. Additionally, mice exposed to longer conditioning trials were generally more active than those exposed to the 5-min conditioning sessions. A two-way ANOVA (Dose × Trial Duration) confirmed these observations, as main effects of Dose [F(1,84) = 5.65, $p < 0.05$] and Trial Duration [F(2,84) = 11.00, $p < 0.001$] were observed, while a significant interaction was not detected.

3.4. Experiment 3: caffeine and 2 g/kg EtOH place conditioning

3.4.1. Conditioning activity

Mean conditioning activity collapsed across the six conditioning trials of each type is depicted in Fig. 5. Mice were significantly more active on CS+ trials, compared to CS− trials. Additionally, animals conditioned with caffeine and EtOH were dose-dependently more active than those conditioned with EtOH alone. Interestingly, the difference between trial types was more pronounced in animals conditioned with the EtOH and caffeine combination, compared to those conditioned with EtOH by itself. A two-way ANOVA confirmed these observations, yielding main effects of Trial Type [F(1,93) = 3765, $p < 0.001$], Drug [F(2,93) = 29.15, $p < 0.001$], and a Trial Type × Drug interaction [F(2,93) = 49.58, $p < 0.001$].

3.4.2. Grid times

Fig. 6 depicts the mean times spent on the grid floor during the preference tests. As can be seen, place preference was observed in all three groups, although mice conditioned with the combination of 2 g/kg EtOH +30 mg/kg caffeine developed slightly weaker place preference after two conditioning trials, relative to the other groups suggesting that the high dose of caffeine may have interfered with ethanol place conditioning. A three-way ANOVA (Conditioning Subgroup × Drug Combination × Test) revealed a main effect of Conditioning Subgroup [F(1,90) = 44.4, $p < 0.001$], indicating that across

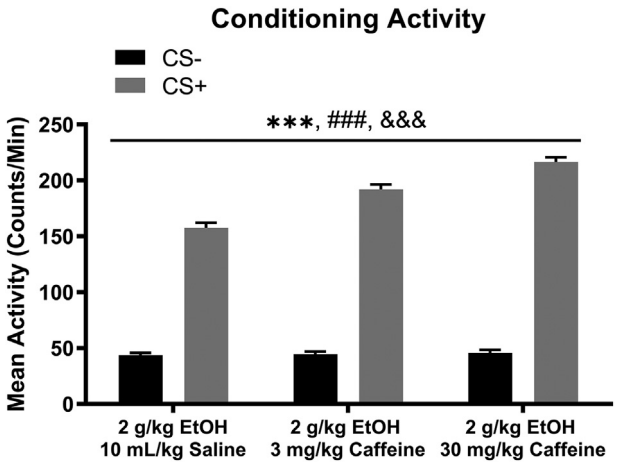

Fig. 5. Mean activity rates (activity counts/min ± SEM) on CS− (saline) and CS+ (EtOH or EtOH + Caffeine) conditioning trials. Each bar represents data from 32 mice. ***; significant main effect of Trial Type ($p < 0.001$), ###; significant main effect of Drug Combination ($p < 0.001$), &&&; significant Trial Type × Drug Combination interaction ($p < 0.001$).

both tests there was significant place preference, as the difference in the amount of time spent on the grid floor between G+ and G− subgroups was significantly different, regardless of the drug combination used during conditioning. Additionally, place preference was stronger in Test 3 than in Test 1, as indicated by a Conditioning Subgroup × Test [F(1,90) = 6.4, $p < 0.05$] interaction. The three-way ANOVA also revealed a Conditioning Subgroup × Drug Combination × Test three-way interaction [F(2,90) = 3.5, $p < 0.05$]. This interaction was followed up by individual two-way ANOVAs (Test × Conditioning Subgroup) for each of the three drug combinations (2 g/kg EtOH + 10 mL/kg saline, 2 g/kg EtOH +3 mg/kg caffeine, 2 g/kg EtOH + 30 mg/kg caffeine). In agreement with the initial ANOVA, all three of these two-way ANOVAs revealed main effects of Conditioning Subgroup (2 g/kg EtOH + 10 mL/kg saline: $p = 0.004$, 2 g/kg EtOH + 3 mg/kg caffeine: $p < 0.001$, 2 g/kg EtOH + 30 mg/kg caffeine $p < 0.001$). Additionally, only the 2 g/kg EtOH + 30 mg/kg caffeine group revealed a significant Test × Conditioning Subgroup interaction [F(1,30) = 10.8, $p < 0.01$]. This interaction was followed up with a Bonferroni-adjusted post-hoc analysis, which revealed that the difference in time spent on the grid floor between G+ and G− subgroups was only significant during Test 3, suggesting that the addition of 30 mg/kg caffeine delayed the development of CPP ($p < 0.001$).

3.4.3. Test activity

Mean locomotor activity is depicted in Supplementary Table 1. As can be seen in both tests, regardless of the dose of caffeine, mice conditioned with the combination of 2 g/kg EtOH and caffeine did not

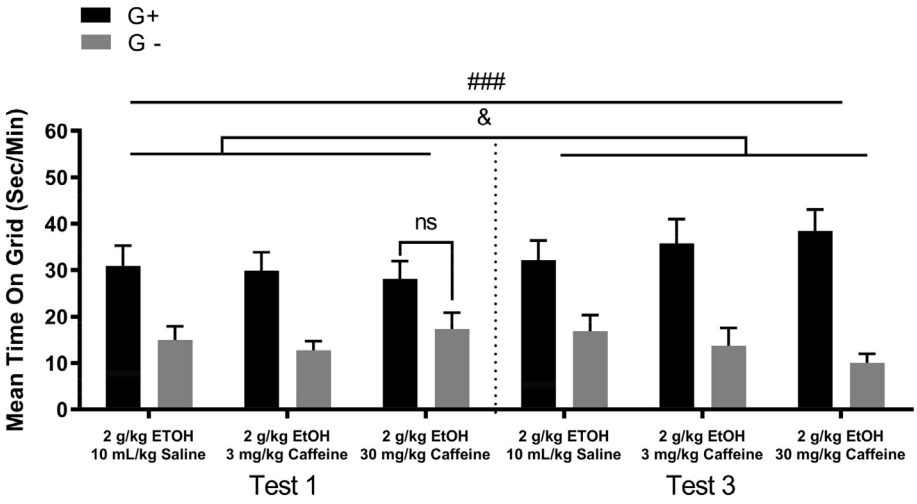

Fig. 6. Mean time spent on the grid floor (s/min ± SEM) after two (test 1) and six (test 3) conditioning trials. The grid floor was paired with drug injections in the G+ subgroups whereas the grid floor was paired with vehicle injections in the G− subgroups. Each bar represents data from 16 mice. ###; significant main effect of Conditioning Subgroup ($p < 0.001$), &; significant Conditioning Subgroup × Test interaction ($p < 0.05$), ns; not significant.

display locomotor activity that was different from that of animals conditioned with EtOH alone. Indeed, a repeated-measures ANOVA (Drug Combination × Test) revealed no significant main effects of either Drug Combination or Test. Additionally, no significant Drug Combination × Test interaction was detected.

3.5. Experiment 4: caffeine and 1 g/kg EtOH place conditioning

3.5.1. Conditioning activity

Fig. 7 depicts mean conditioning activity rates, collapsed across the six conditioning trials of each type. Conditioning activity was significantly higher in mice conditioned with the caffeine and EtOH, compared to those conditioned with EtOH alone. Furthermore, mice were more active on CS+ trials, compared to CS− trials. When compared to EtOH alone, the difference in activity between trial types was greater in mice conditioned with the drug combination. A two-way ANOVA confirmed these observations, with main effects of Drug Combination [$F(2,92) = 42.720$, $p < 0.001$], Trial Type [$F(1,92) = 1705.4$, $p < 0.001$], and a Drug Combination × Trial Type interaction [$F(2,92) = 82.912$, $p < 0.001$] all being detected.

3.5.2. Grid times

Mean times spent on the grid floor during the 30-min preference tests are depicted in Fig. 8. Regardless of the caffeine dose, the combination of caffeine and 1 g/kg ethanol did not produce place preference that was different than ethanol alone. As expected, however, place conditioning strengthened over conditioning trials. Indeed, a three-way ANOVA (Conditioning Subgroup × Drug Combination × Test) revealed a significant main effect of Conditioning Subgroup [$F(1,89) = 71.9$, $p < 0.001$], demonstrating that mice in the G+ subgroups spent significantly more time on the grid floor than G− mice, regardless of the drug combination used. Additionally, a Conditioning Subgroup × Test interaction [$F(1,89) = 14.1$, $p < 0.001$] was detected, indicating that conditioning was stronger in Test 3 than in Test 1. No significant main effects of Drug Combination or Test and no other interactions were detected.

Throughout extinction, mean times spent on the grid floor were converted into mean percent times spent on the drug-paired floor for ease of presentation. As can be seen in Fig. 9, over the course of 11 choice-extinction trials, preference for the drug-paired floor decreased. There were no differences in extinction between groups, suggesting that the addition of either dose of caffeine during acquisition had no impact on the rate of extinction to ethanol-paired cues. In support of this, a two-way repeated measures (Drug Combination × Test) ANOVA revealed a main effect of Test [$F(10,840) = 12.0$, $p < 0.001$]. Neither the interaction nor main effect of Drug Combination was detected. In addition, a separate two-way repeated-measures (Drug Combination × Test) ANOVA was conducted for tests 3 and 13, so as to confirm the significant decrease in place preference following extinction. Indeed, a main effect of Test [$F(1,92) = 27.2$, $p < 0.001$] was detected, while no main effect of Drug Combination or interaction were detected.

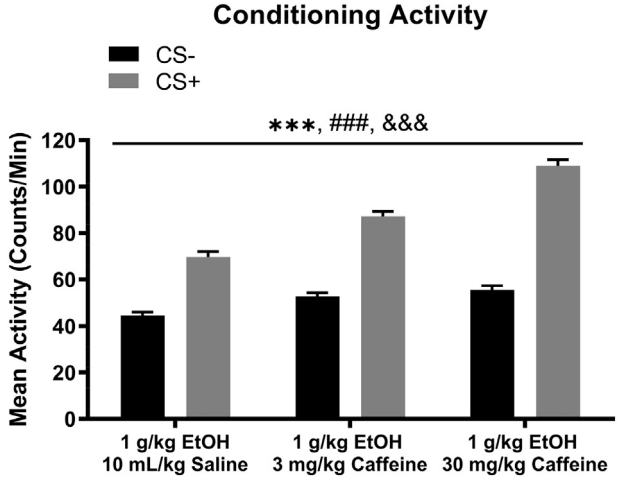

Fig. 7. Mean activity rates (activity counts/min ± SEM) during CS− (saline) and CS+ (EtOH or EtOH + Caffeine) conditioning trials in Experiment 4. Each bar represents data from 31 to 32 mice. ***; significant main effect of Trial Type ($p < 0.001$), ###; significant main effect of Drug Combination ($p < 0.001$), &&&; significant Drug Combination × Trial Type interaction ($p < 0.001$).

3.5.3. Test activity

Locomotor activity means during tests 1 and 3 are depicted in Supplementary Table 1. As can be seen, the addition of the high dose of caffeine increased locomotor activity, as mice conditioned with 1 g/kg EtOH +30 mg/kg caffeine were more active than those conditioned with 1 g/kg EtOH alone. In support of this, a repeated-measures ANOVA revealed a significant main effect of Drug Combination [$F(3,90) = 3.28$, $p < 0.05$] but not of Test. No significant Test × Drug Combination was detected. When test activity was collapsed across tests, a one-way ANOVA again revealed a main effect of Drug Combination [$F(2,187) = 7.0$, $p < 0.01$]. A follow-up Bonferroni-adjusted post-hoc analysis revealed a significant difference between the 1 g/kg EtOH and 10 mL/kg saline and the 1 g/kg EtOH + 30 mg/kg Caffeine groups ($p < 0.01$).

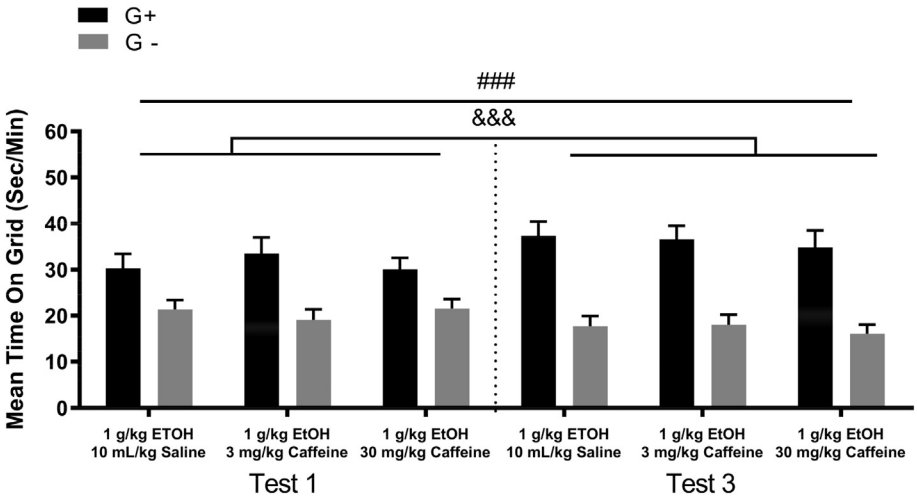

Fig. 8. Mean time spent on the grid floor (s/min ± SEM) after two (test 1) and six (test 3) conditioning trials of each type. The grid floor was paired with drug injections in the G+ subgroups whereas the grid floor was paired with vehicle injections in the G− subgroups. Each bar represents data from 15 to 16 mice. ###; significant main effect of Conditioning Subgroup ($p < 0.001$), &&&; significant Conditioning Subgroup × Test interaction ($p < 0.001$).

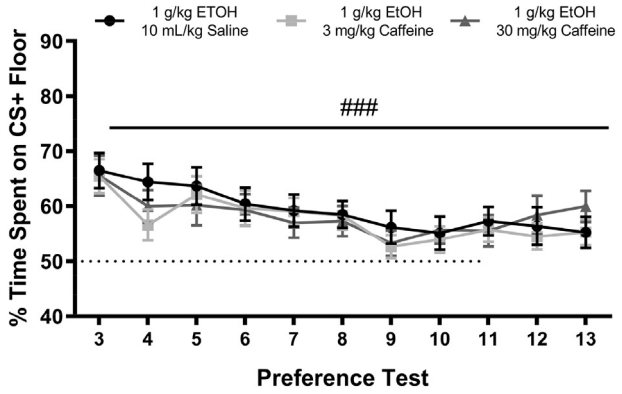

Fig. 9. Mean percent time (± SEM) spent on the drug-paired floor over the course of eleven 30-min choice extinction trials in Experiment 4. Each line represents data from 31 to 32 mice. ###; significant main effect of Test ($p < 0.001$).

4. Discussion

The studies presented here demonstrate that caffeine dose-dependently increases locomotor activity and has a biphasic effect on place conditioning in male DBA/2J mice. More specifically, we found that the low dose of caffeine (3 mg/kg) produced place preference, while the high dose of 30 mg/kg caffeine produced place aversion. Additionally, these studies show that the combination of caffeine and alcohol does not produce place preference that is different from that of alcohol alone. Using the two doses of caffeine that produced place conditioning (3 and 30 mg/kg), in combination with either 1 or 2 g/kg ethanol, we found that strength of place conditioning did not differ between animals conditioned with ethanol alone and those conditioned with caffeine-ethanol combinations. Caffeine and ethanol combinations did, however, produce greater increases in conditioning activity during conditioning, suggesting an additive effect of the two drugs on locomotor activity.

The finding that caffeine dose-dependently increases locomotor activity in DBA/2J mice is in line with previous studies that have shown similar effects in this same strain of mouse (Buckholtz and Middaugh, 1987), as well as in other strains (Kuzmin et al., 2000; Phillips et al., 1992). Importantly, similar results have also been found in studies that specifically used Pavlovian conditioning procedures in both rats (Bedingfield et al., 1998) and mice (Hilbert et al., 2013). In addition, we also found that during CS+ trials, mice conditioned with longer trial durations (30 and 60 min) were more active during the first 5 min of conditioning than those conditioned with trials lasting only 5 min. These two longer trial durations were chosen based on previous findings in our lab showing that longer trial durations are better for producing CPP with a different stimulant (cocaine; Cunningham et al., 1999). In their 1999 paper, however, Cunningham and colleagues did not find an effect of trial duration on conditioning activity. One possible explanation for the increase in activity observed here is based on the pharmacokinetics of caffeine and the ability of caffeine to produce a conditioned response (CR). In mice, plasma and brain concentrations of caffeine peak 30–60 min after mice have been given an IP injection of caffeine (Kaplan et al., 1990). Thus, only mice conditioned with longer trials would have been exposed to the maximum concentrations of caffeine. Given the well-established positive relationship between CR magnitude and unconditioned stimulus (US) intensity (e.g., Kehoe and Macrae, 2002), it is possible that only mice that were exposed to the maximum concentrations of caffeine while being exposed to the CS were able to show this conditioned increase in initial locomotor activity.

The additive effects of caffeine and ethanol on locomotor activity presented here add to previous work demonstrating similar findings with this same combination (Hilbert et al., 2013; May et al., 2015; Robins et al., 2016b), as well as with combinations of ethanol and other stimulants (Hamida et al., 2008; Masur et al., 1989). Both caffeine- and ethanol-mediated increases in locomotor activity have been attributed to increased dopamine levels in the striatum (Garrett and Griffiths, 1997; Pierce and Kumaresan, 2006), which have in turn been correlated with locomotor activity (Do et al., 2012). The additive effects of caffeine and ethanol may then be due to additive increases in dopamine levels in the striatum. Indeed, greater increases in deltaFosB in the nucleus accumbens have been reported when mice were treated with a combination of caffeine and ethanol, compared to either drug alone (Robins et al., 2016a, 2016b). The doses of ethanol used here have been shown to produce robust increases in locomotor activity over the course of short trial durations (Cunningham and Prather, 1992), while the sedative effects of ethanol are not apparent until after a trial has ended. Even so, another possible explanation for the increased locomotor activity seen in mice conditioned with the combination of caffeine and alcohol is that caffeine further enhanced the stimulatory effects of ethanol by dampening its sedative effects.

Our finding that the dose of 3 mg/kg caffeine produced place preference is in line with previous work in the C57BL/6 strain (Hilbert et al., 2013) and it extends the generalizability of that effect to another inbred strain (DBA/2J). Relatively low doses of caffeine have been shown to produce either weak place preference (Bedingfield et al., 1998; Brockwell et al., 1991) or no place preference (Sturgess et al., 2010). One possible explanation for this variation in results is the difference in conditioning parameters between studies. Here we found that

the 3 mg/kg dose only produced place preference after eight 30-min conditioning trials (Fig. 2). Shorter conditioning trials (5 min) did not produce significant place preference with this dose (Fig. 4), although it is possible that this lack of preference is due to the fact that mice in Exp. 2 were only exposed to 4 conditioning trials, and not due to the shorter trial duration. Experiments 1 (Fig. 2) and 2 (Fig. 4) add to the various studies showing that a high dose of caffeine can produce robust place (Brockwell et al., 1991; Sturgess et al., 2010) and taste aversion (Myers and Izbicki, 2006; Steigerwald et al., 1988) in rodents. Additionally, Myers and Izbicki (2006) demonstrated caffeine's biphasic effect using a taste conditioning procedure, providing additional evidence for caffeine's appetitive effects at low doses. One possible explanation for caffeine's biphasic effect on conditioning is that at higher doses caffeine's aversive effects outweigh its rewarding effects. Similar biphasic effects have also been seen in nicotine (Risinger and Oakes, 1995), and as in the present studies, intermediate doses of nicotine did not produce place preference, due to a potential summation of the drug's rewarding and aversive effects. As it is known that caffeine produces dose-dependent discriminative stimulus effects in rats (Mumford and Holtzman, 1991), future studies that allow mice to titrate their caffeine intake could provide additional insight into the preferred concentration of caffeine. Our studies also demonstrate that longer trial durations may facilitate place aversion. Mice conditioned with 30- and 60-min conditioning sessions appeared to develop place aversion, although both groups only displayed modest place aversion that did not reach significance. As previous cocaine place preference studies using similar trial durations did not find place aversion (Cunningham et al., 1999), it is unlikely that this effect is caused by prolonged exposure to the apparatus. Instead it is more likely that the longer trial durations were necessary for animals to experience peak concentrations of caffeine while being exposed to the CS.

Although the present studies did not find any difference in the magnitude of place preference between mice conditioned with a combination of a rewarding dose of caffeine and ethanol, and ethanol alone, we did find that an aversive dose of caffeine did not dampen the rewarding effects of ethanol (Figs. 6 & 8). The simplest explanation for this latter finding would be that the trial duration (5 min) in these experiments is more conducive to the development of place preference to ethanol, as opposed to the aversive effects of caffeine. Indeed, as we did not see place aversion in mice conditioned with 30 mg/kg alone using 5-min trials (Fig. 4), it is most likely that such a short trial was not sufficient for the aversive effects of caffeine to interfere with ethanol's rewarding effects while mice were being exposed to the CS. The lack of a summation effect in our combination groups is not entirely surprising, as similar results were found by Hilbert et al. (2013). Using our procedure, several possible explanations exist for this lack of a stronger place preference with the combination of caffeine and ethanol. For example, it is possible that a “ceiling effect” interfered with our ability to see increases in place preference with the addition of caffeine. This explanation seems rather unlikely however, given that Exp. 4 used a dose that produces pre-asymptotic place preference (Groblewski et al., 2008). As noted above, it is likely that the 5-min trials were too short for caffeine concentrations to be at their highest, and thus the rewarding effects of caffeine would not have summed with those of ethanol. Although increasing the trial duration could result in the weakened ethanol-place preference (Cunningham and Prather, 1992), future studies using various trial durations might be used to further investigate the rewarding effects of caffeine and alcohol combinations. In an attempt to observe differences in place preference strength not visible during the acquisition or expression phases, mice in Exp. 4 were exposed to a choice-extinction procedure. Following 10 extinction trials we found that the addition of either dose of caffeine during conditioning had no effect on the rate of place preference extinction (Fig. 9). One assumption of Pavlovian conditioning is that the time course of extinction will be affected by the strength of the initial conditioning (Cunningham et al., 2011).Thus, our finding that groups did not differ in their rates of extinction further suggests that the low and high doses of caffeine did not strengthen or weaken ethanol's rewarding effects, respectively.

The studies presented here are the first to characterize the rewarding and aversive effects of caffeine in male DBA/2J mice. Caffeine produced dose-dependent increases in locomotor activity, that were additive to those of ethanol when the two drugs were combined. When combined with ethanol, neither rewarding nor aversive doses of caffeine produced place preference that was different from ethanol alone. Future studies investigating the neurobiological basis for these findings will be essential to understanding how these two drugs influence behavior.

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