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Conditioned Reinforcement and Locomotor Activating Effects of Caffeine and Ethanol Combinations in Mice

Megan L.T. Hilbert, Christina E. May, and William C. Griffin III

Center for Drug and Alcohol Programs, Department of Psychiatry and Behavioral Science, Medical University of South Carolina, Charleston, SC 29425

Abstract

A growing trend among ethanol drinkers, especially young adults, is to combine caffeinated energy drinks with ethanol during a drinking episode. The primary active ingredient of these mixers is caffeine, which may significantly interact with ethanol. We tested the two hypotheses that caffeine would enhance ethanol-conditioned place preference and also enhance ethanol-stimulated locomotor activity. The interactive pharmacology of ethanol and caffeine was examined in C57BL/6J (B6) mice in a conditioned place preference procedure with 1.75 g/kg ethanol and 3 mg/kg caffeine. Additionally, we used B6 mice to evaluate ethanol/caffeine combinations on locomotor activity using 3 doses of ethanol (1.75, 2.5 and 3.25 g/kg) and 2 two doses of caffeine (3 and 15 mg/kg). Both ethanol and caffeine administered alone increased preference for the drug paired side, though the effect of caffeine was more modest than that of ethanol. The drug combination produced significant place preference itself, but this was not greater than that for ethanol alone. Additionally, the combination of caffeine and ethanol significantly increased locomotion compared to giving either drug alone. The effect was strongest with a stimulatory dose of ethanol (1.75 g/kg) and waned with increasing doses of ethanol. Thus, combinations of caffeine and ethanol had significant conditioned reinforcing and locomotor activating effects in mice.

Keywords

alcohol; psychostimulant; place preference; drug interaction

INTRODUCTION

Heavy alcohol consumption continues to be a major health problem in the United States. One estimate indicates that one third of preventable deaths in the United States could be attributed to excessive alcohol (ethanol) consumption (Mokdad *et al.*, 2004). Further, among ethanol drinkers, a growing trend in recent years has been to combine caffeinated energy drinks with ethanol which may facilitate more drinking. Over the past decade, the growth of energy drink sales has been tremendous (Reissig *et al.*, 2009; Seifert *et al.*, 2011). In the United States, epidemiological studies (Arria *et al.*, 2010; Arria *et al.*, 2011), supported by field testing data (Thombs *et al.*, 2009) and frequent reports in the lay press indicate that consumption of ethanol mixed with energy drinks is common, particularly among young

Please Address Correspondence to: William C. Griffin III, PhD, Center for Drug and Alcohol Programs, Medical University of South Carolina, MSC861, 67 President St, Charleston, SC 29425, griffinw@musc.edu, 843-792-4324.

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people. Unfortunately, consuming these mixtures has been associated with increases in emergency room visits (SAMHSA, 2011), suggesting that consumption of these drinks can be dangerous. In 2010, the Food and Drug Administration banned the manufacture and sale of pre-mixed energy drink/ethanol combinations (e.g. Four-Loko), though drinkers are still free to mix their own. Although mixing caffeinated energy drinks with ethanol appears to be widespread, mixing caffeinated beverages and ethanol is not a new practice nor is it limited to energy drinks. Indeed, other caffeinated drinks like colas are also popular mixers with ethanol (Rossheim and Thombs, 2011; Thombs *et al.*, 2011). Regardless of whether it is an energy drink, cola or coffee, many investigators consider the primary pharmacologically active ingredient of these beverages to be caffeine. Therefore, it is important to examine the interactive pharmacology of these two widely used drugs, caffeine and ethanol, so that the interactive effects can be more clearly understood.

The interactive pharmacological effects of caffeine and ethanol have been studied in humans and to some extent in animal models. For example, research has shown that caffeine can antagonize, although not always completely, cognitive and psychomotor deficits induced by ethanol [e.g. (Hasenfratz *et al.*, 1993; Liguori and Robinson, 2001; Mackay *et al.*, 2002)]. There is also some work using animal models to study the interactive pharmacology of caffeine and ethanol. For example, caffeine injections can increase ethanol drinking in rats (Kunin *et al.*, 2000) and low doses (<10mg/kg) of caffeine promote significant ethanol-induced taste aversions when combined with a low dose of ethanol (Kunin *et al.*, 2001). Whereas the earlier studies in humans tended to focus on caffeine's ability to antagonize deficits caused by ethanol, the studies in rats indicate that caffeine can actually facilitate ethanol-related behaviors. In fact, recent research suggests that an energy drink combined with ethanol may actually enhance the desire to drink (Marczinski *et al.*, 2012b), which could be due to the interaction of the caffeine component of these drinks with ethanol. Therefore, in the context of the current trend to mix caffeinated beverages with ethanol, it is imperative to further develop our understanding of the facilitative effects of caffeine on ethanol-related behaviors. The use of animal models in this endeavor is crucial for establishing a strong foundation for additional work which cannot be conducted in humans; for example, examining the interactive effects of ethanol and caffeine in adolescents or at high dose levels.

Our laboratory has previously examined the interactive effects of the psychostimulant methylphenidate with ethanol in C57BL/6J (B6) mice. In our prior studies, the addition of methylphenidate significantly enhanced ethanol-stimulated locomotor activity, increased signs of ataxia during ethanol intoxication and augmented discrimination of low ethanol doses (Griffin *et al.*, 2012; Griffin *et al.*, 2010). Thus, low doses of a widely used psychostimulant co-administered with ethanol facilitated some behavioral effects of ethanol. Therefore, following from this earlier work, the present studies were designed to examine the interactive pharmacological effects of caffeine and ethanol in B6 mice. One common pharmacological target of ethanol and caffeine is the adenosinergic system. In particular, A₁ and A_{2A} adenosinergic receptors have been associated with arousal, locomotion and reinforcement (Hsu *et al.*, 2009; Kuzmin *et al.*, 2006; Lazarus *et al.*, 2011). The adenosinergic system has also been implicated in the reinforcing effects of ethanol (Arolfo *et al.*, 2004; Nam *et al.*, 2013). Although the adenosinergic system is complex, at the most basic level, ethanol administration increases adenosine levels (Dunwiddie and Masino, 2001; Nagy *et al.*, 1990) while caffeine non-selectively antagonizes adenosine receptors (Fredholm *et al.*, 2011) providing a potential mechanistic explanation for the interactive effects of these two drugs noted in humans and laboratory animals. In the present studies, we used two well characterized behaviors, ethanol-induced conditioned place preference and ethanol-induced alterations in locomotor activity to examine ethanol-caffeine interactions.

We hypothesized that the addition of caffeine to ethanol intoxication would enhance conditioned place preference and increase locomotor activity compared to either drug alone.

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. All procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee.

The mice used in the conditioned place preference experiment (Figure 1; n=48) were behaviorally naïve at the start of the experiment. The mice used in the locomotor activity experiments were not behaviorally naïve. The mice (n=12) used in the activity experiment shown in Figure 2 were previously part of a chronic treatment study in which they received vehicle gavage twice daily for 10 days. The mice used in Figure 3 (n=12) were previously vehicle injected mice and the mice used in Figure 4 (n=12) were previously ethanol treated mice, both groups from the present conditioned place preference experiment. Before beginning the locomotor activity experiment, the ethanol treated mice used in Figure 4 had a 3 week washout period after completing the place preference experiment.

Drugs

Drugs were administered intraperitoneally using 0.9% saline as the vehicle at a volume of 0.02ml/g of body weight in all experiments. Caffeine was used as the anhydrous base (Fluka, a subsidiary of Sigma-Aldrich, St Louis, MO) and 95% ethanol was obtained from AAPER (Shelbyville, KY). Before use, the ethanol was diluted with 0.9% saline [e.g. 12.2% (v/v) for a 1.75 g/kg dose]. When combined, caffeine and ethanol were administered in the same injection (*i.e.* simultaneously).

Conditioned Place Preference Apparatus and Procedures

The apparatus and procedures have been recently described (Griffin *et al.*, 2012). We used 6 Med Associates (St. Albans, VT, USA) activity chambers (ENV-510) each equipped with two compartment place preference inserts (ENV-512). The inserts were modified so that the floor was smooth on each side and the walls and floor were white on one side and black on the other, creating two distinct visual contexts [see (Griffin *et al.*, 2012) for complete details]. For this experiment, the floor modification consisted of either solid white or black plastic sheets (TAP Plastics, Inc) cut to fit exactly over the existing floors of the ENV-512 insert.

The dosing groups for this experiment were as follows (all n=12): vehicle; caffeine 3mg/kg; ethanol 1.75 g/kg; and the combination of caffeine 3 mg/kg + ethanol 1.75 g/kg. The dose of ethanol was chosen based on previous work (Griffin *et al.*, 2012; Nocjar *et al.*, 1999) indicating that it produces conditioned place preference in B6 mice using well-established procedures in our laboratory. A low dose of caffeine (3mg/kg) was chosen because the literature indicated it would be borderline in terms of stimulating locomotion (Buckholtz and Middaugh, 1987; Hsu *et al.*, 2009) and as well as producing significant place preference (Bedingfield *et al.*, 1998; Patkina and Zvartau, 1998; Steigerwald *et al.*, 1988) presumably making it easier to detect interactive effects with ethanol in this behavioral assay.

To begin the experiment, mice were randomly assigned to the groups and first exposed to the apparatus during the Pretest in which they had access to both sides. Conditioning sessions commenced 24hrs later with 8 sessions occurring prior to Test 1 and then an additional 8 sessions occurring prior to Test 2 for a total of 16 conditioning sessions. During conditioning, mice were injected with their assigned drug and consistently placed on one side of the apparatus throughout the experiment so that the drug injection was paired with that context. On alternating days, mice were placed into the non-drug paired side after receiving a vehicle injection. Mice in the vehicle conditioned group received vehicle on both sides. For mice assigned to active drug treatments, drug treatment was paired with the white side while vehicle was paired to the black side. All sessions, both conditioning and testing, were 10 min in duration. Injections were given 5 min prior to entry into the chamber with mice waiting in their home cage during this time. Prior to Pretest, Test 1 and Test 2 all mice received vehicle injections regardless of group assignment so each test session was conducted under drug-free conditions similarly to the conditioning sessions. Two tests were used in these experiments because of the possibility that the addition of caffeine to ethanol reinforcement could reduce the number of sessions needed to produce significant place preference compared to the 16 sessions needed for ethanol administered alone as previously reported in B6 mice (Griffin *et al.*, 2012; Nocjar *et al.*, 1999).

Locomotor activity apparatus and procedures

We used 6 Med Associates (St. Albans, VT, USA) activity chambers (ENV-510) with a light-dark testing insert (ENV-511). The ENV-511 inserts were installed so as to restrict the size of the activity chamber by placing the entry door of the insert toward the outside wall of the chamber. Thus, the size of activity chamber used for testing in these experiments became 13.5 cm wide $\times$ 27.5 cm long $\times$ 20.5 cm deep with the use of the insert. The area was restricted because unpublished observations in our laboratory have indicated that the size of the open field arena can influence the magnitude of the ethanol-stimulated locomotor activity response, with larger arenas tending to produce a smaller effect.

The basic procedure using a Latin-square design to test locomotor activity using vehicle, caffeine, ethanol and ethanol/caffeine combinations has been described (Griffin *et al.*, 2010). Before each session, mice were injected with the assigned treatment and returned to their home cage for 5 min. After 5 min, the mice were placed into the activity chamber for 20 min and then removed. The activity chambers were wiped down with water and dried before the next session. All mice were habituated to the activity chambers with vehicle injections for two sessions before beginning any active drug treatments. For the experiments described here, there was a 48 hour washout period between sessions for each mouse. These procedures have previously been used to show locomotor stimulation with ethanol in this strain of mouse as well as an enhanced locomotor activity when ethanol was combined with methylphenidate (Griffin *et al.*, 2012; Griffin *et al.*, 2010).

Data analysis

The primary data for the conditioned place preference experiment were the amount of time on the drug paired side (sec) and the primary data for the locomotor activity experiments were total distances (cm) traveled over 20 min. The data were analyzed using Analysis of Variance (ANOVA) with repeated measures (RM) when necessary. Post-hoc analysis was conducted using pairwise comparisons with Bonferroni's correction. Alpha was set to 0.05 for all analyses.

RESULTS

Conditioned Place Preference Results

These data are summarized in Figure 1. One mouse in the drug combination group was excluded from the analyses because during the Pre-Test it spent 528 secs out of 600 total secs on one side of the apparatus. The data show that repeated conditioning sessions will increase time spent on the drug-paired side for mice conditioned with caffeine (3mg/kg), ethanol (1.75 g/kg) and a combination of both drugs at the same doses. This observation was supported by a 4(Group) $\times$ 3(Time) repeated measures ANOVA that detected a significant factor interaction ($F_{6,86} = 5.161$, $p < 0.0001$). Post-hoc analysis of this interaction was conducted using pairwise comparisons with Bonferroni's correction. Importantly, this analysis showed that ethanol produced significant place preference by Test 2 ($p < 0.05$), consistent with our earlier work (Griffin *et al.*, 2012; Nocjar *et al.*, 1999). Interestingly, the significant increase in time on the drug-paired side noted for ethanol during Test 2 was also significantly greater than that for caffeine ($p < 0.05$). Caffeine also produced significant place preference, as indicated by increased time on the drug paired side, although this effect was only significant during Test 1 ($p < 0.05$), not during Test 2. Similar to ethanol conditioning, the drug combination group demonstrated significant place preference by Test 2 ($p < 0.05$), but this effect was similar in magnitude to that obtained for ethanol alone. Together, these data indicate that ethanol produces significant place preference in B6 mice and also show that caffeine is a relatively weak conditioned reinforcer in comparison to ethanol. Lastly, the addition of caffeine to ethanol reinforcement did not augment the development of place preference, either in terms of the magnitude of the preference response or by reducing the number of sessions needed to acquire significant place preference.

Locomotor Activity Results: Ethanol 1.75 g/kg + Caffeine

These data are summarized in Figure 2. These data indicate the combination of a dose of ethanol that stimulates locomotor activity (Griffin *et al.*, 2010) and stimulatory doses of caffeine (Buckholtz and Middaugh, 1987), produced a greater locomotor activity response than either drug given alone. This was supported by a one-way RM ANOVA that found a significant drug treatment effect ($F_{5,55} = 34.6$, $p < 0.0001$). Post-hoc analysis showed that ethanol 1.75 g/kg produced a significant increase in total distance traveled as did both doses of caffeine (all $p < 0.05$). The low dose of caffeine did not increase activity relative to the ethanol dose while the higher dose of caffeine (15mg/kg) fell short of significance ($p = 0.056$) when compared to ethanol alone. However, when combined with this stimulatory dose of ethanol, both doses of caffeine increased total distance compared to either ethanol or caffeine alone. Caffeine 15 mg/kg + ethanol produced the largest stimulatory effect which was significantly greater than ethanol alone or either dose of caffeine alone (all $p < 0.05$), although it was not statistically greater than the caffeine 3mg/kg + ethanol condition ($p > 0.5$). The combination of the low dose of caffeine (3mg/kg) + ethanol also significantly increased total distance traveled compared to ethanol and the low dose of caffeine administered alone (both $p < 0.05$). These data indicate that stimulatory doses of ethanol and caffeine, when combined, increase activity more than either drug given alone.

Locomotor Activity Results: Ethanol 2.5 g/kg + Caffeine

These data are summarized in Figure 3. Procedural errors forced the elimination of 3 mice entirely from the data set, leaving $n = 9$ for the final analysis. Similar to the data shown in Figure 2, these data also indicate that the combination of ethanol and caffeine interact to increase total distance traveled more than either drug given alone. This observation was supported by a one-way RM ANOVA demonstrating a significant drug treatment effect ($F_{5,40} = 14.2$, $p < 0.0001$). Ethanol 2.5 mg/kg as well as caffeine 3m/kg both appeared to increase total activity compared to vehicle, however, the post-hoc analysis did not indicate

that this effect was statistically significant (both $p > 0.1$). However, the effect of caffeine 15 mg/kg on total distance traveled was significant compared to vehicle as well as the lower dose of caffeine (both $p < 0.05$). Importantly, results of this experiment confirmed that the combination of caffeine and ethanol increased activity more than either drug alone. Again, the combination of caffeine 15 mg/kg + ethanol produced the largest stimulatory effect which was significantly greater than ethanol alone, the low dose of caffeine alone (both $p < 0.05$), although not the high dose of caffeine alone ($p = 0.2$). Additionally, the combination of caffeine 3 mg/kg + ethanol increased total distance significantly more than ethanol or caffeine (3mg/kg) alone (both $p < 0.05$). Thus, the interactive effects of these 2 drugs extends to higher doses of ethanol.

Locomotor Activity Results: Ethanol 3.25 g/kg + Caffeine

These data are shown in Figure 4. Using a higher dose of ethanol that does not stimulate locomotor activity, these data indicate that caffeine is still capable of interacting with ethanol to increase locomotor activity. However, these doses of caffeine were less effective against the higher dose of ethanol. The analysis using one-way RM ANOVA detected a significant drug treatment effect ($F_{5,55} = 2.9$, $p = 0.021$). Ethanol given at a dose of 3.25 mg/kg clearly did not stimulate activity ($p = 1$) compared to vehicle challenge and despite the increase in total distance noted for caffeine 3mg/kg, this also was not significant ($p > 0.3$) compared to vehicle. However, caffeine 15 mg/kg did produce a significant increase in activity compared to vehicle as well as the low dose of caffeine (both $p < 0.05$). Despite a general increase in total distance traveled with the drug combinations, neither dose of caffeine was able to completely antagonize the effects of ethanol, although the effect of the combination of caffeine 3 mg/kg + ethanol versus vehicle nearly reached significance ($p = 0.052$). Thus, these data indicate an upper dose limit of ethanol that reduces the effectiveness of caffeine to increase locomotion.

DISCUSSION

In this series of experiments, ethanol and caffeine interacted pharmacologically to affect behavior. We found that the combination of ethanol and caffeine increased locomotor activity more than either drug given alone. The enhanced stimulatory effect on locomotor activity was most robust when a stimulatory dose of ethanol (1.75 g/kg) was used in combination with caffeine. However, this enhanced stimulatory effect of the drug combination waned as the dose of ethanol increased and was considerably diminished when the 3.25 g/kg ethanol dose was combined with caffeine. Interestingly, this interactive effect on locomotor activity of ethanol (1.75 g/kg) with the low dose of caffeine (3 mg/kg) did not extend to the conditioned place preference procedure. The place preference experiment demonstrated that this specific combination of ethanol and caffeine exhibited conditioned reinforcing effects, as indicated by a significant increase in time on the drug-paired side. However, the effect of the drug combination to increase time on the drug paired side was not greater than that produced by ethanol alone. Additionally, we noted that caffeine (3mg/kg) itself was a weak conditioned reinforcer in this procedure, producing a only slight increase in preference that was smaller than ethanol.

Because of the recent trend in caffeinated-energy drink consumption in combination with ethanol (Arria *et al.*, 2010; Arria *et al.*, 2011; Attwood, 2012), the focus of the current work was on the interaction of the primary active ingredient of these beverages, caffeine, with ethanol. Extrapolating the present results with caffeine and ethanol to caffeinated-energy drinks and ethanol must be done with caution, however, since many of these energy drinks can contain other additives that may have under-appreciated pharmacological activity. One example of this kind of additive is taurine, which appears to be capable of affecting behavior in humans and animals (Giles *et al.*, 2012; Olive, 2002). Of course, effort is being made to

address caffeinated-energy drink/ethanol combinations using human laboratory paradigms. Recent evidence provides support for the idea that caffeinated-energy drinks consumed with ethanol have different effects than ethanol alone (Ferreira *et al.*, 2006; Marczinski *et al.*, 2012a, b) although other studies offer less support for this interaction (Alford *et al.*, 2012). Additionally, there is at least one report using energy drinks in a mouse model indicating that an energy drink can antagonize the depressant effects of ethanol (Ferreira *et al.*, 2004). These important observations notwithstanding, it is clear that the interactive effects of caffeine and ethanol are an important consideration in the practice of mixing caffeinated energy drinks and ethanol. Thus, it is vital to consider the interaction of these two drugs separately, without the potential complications that use of energy drinks may encompass.

The interaction of ethanol and caffeine during acute intoxication to increase locomotor activity is an important observation. Ethanol is widely recognized as a depressant and it does depress behavior at high doses. However, ethanol also has stimulatory properties at low doses which have been objectively described in mice [present study; (Griffin *et al.*, 2010; Jerlhag, 2008; Middaugh *et al.*, 1992)] as well as subjectively in human laboratory studies (King *et al.*, 2002; Thomas *et al.*, 2004). Our data clearly indicate that the stimulatory effect of ethanol is significantly enhanced with caffeine. Further, caffeine increased locomotion with doses of ethanol that were not stimulatory. Therefore, the combination of ethanol and caffeine is capable of increasing behavior more than ethanol alone, even over a relatively wide range of ethanol doses. The implication of these findings for humans is that acute co-intoxication with these drugs may support continued drinking activity at a greater risk of personal injury to the drinker since caffeine does not appear to completely antagonize the effects of ethanol in a driving task (Liguori and Robinson, 2001) or other psychomotor tasks (Attwood *et al.*, 2012; Marczinski and Fillmore, 2003). In fact, in previous work using our mouse model, we found that the combination of ethanol and methylphenidate actually increased signs of ataxia, likely because of the increased locomotion with the drug combination (Griffin *et al.*, 2012). Whether this effect occurs with caffeine and ethanol in our animal model remains to be tested. Of course, the relationship of our findings to the human drinking condition is speculative, but the present findings are evocative of reports describing people intoxicated with caffeinated-energy drinks and ethanol who do not appear to succumb to the depressant effects of ethanol.

Caffeine is widely consumed. The 3 mg/kg dose used in the present studies is similar to the amount of caffeine that humans have been reported to consume daily (Barone and Roberts, 1996) and within the range used in recent human laboratory studies investigating ethanol and caffeine interactions (Attwood *et al.*, 2012; Fillmore, 2003; Liguori and Robinson, 2001; Marczinski and Fillmore, 2003), adding relevance to this choice of dose. Additionally, based on human pharmacological responses, caffeine is often characterized as a mild psychostimulant. The results in our study with caffeine alone are consistent with this characterization, especially at the low dose used in the present studies (3mg/kg). Caffeine dose dependently increased locomotor activity, with the high dose reliably increasing activity more than the low dose. Nevertheless, even though the 3 mg/kg dose increased total distance traveled in all the locomotor activity experiments, this only reached statistical significance in one experiment, suggesting this low dose is near the threshold for stimulating activity in mice. This interpretation is supported by earlier work in mice showing dose-dependent increases in locomotion over a range of doses in which doses that are 5 mg/kg or less modestly stimulated activity (Buckholtz and Middaugh, 1987; Hsu *et al.*, 2009).

The rewarding effects of ethanol are generally considered milder than other drugs of abuse such as cocaine or heroin. Still, several decades of research using a variety of model systems provide unequivocal evidence that ethanol has rewarding effects in rodents [*e.g.* (Middaugh *et al.*, 1999)] non-human primates [*e.g.* (Grant *et al.*, 2008)] and humans [*e.g.* (Mendelson

and Mello, 1966)]. Therefore, considering the continuum of reinforcing potency, our results indicate that caffeine is a less potent conditioned reinforcer than ethanol. Although 3 mg/kg caffeine increased time on the drug paired side in both Test 1 and Test 2, this was only statistically significant during Test 1 and generally the effect was of a smaller magnitude than ethanol. This finding is generally consistent with other published work showing that low doses of caffeine (5 mg/kg or less) may support place conditioning in some cases (Bedingfield *et al.*, 1998; Patkina and Zvartau, 1998) but not in others (Hsu *et al.*, 2009; Steigerwald *et al.*, 1988).

With regards to the interactive effects of ethanol and caffeine on place preference, the mice conditioned with the ethanol-caffeine combination did not show significant preference of any greater magnitude compared to ethanol by Test 2 (*i.e.* more time on the drug paired side) and did not they demonstrate significant place preference any sooner than ethanol (*e.g.* by Test 1). It has been previously acknowledged that it is difficult to measure graded dose response functions in terms of the magnitude of place preference (Bedingfield *et al.*, 1998; Groblewski *et al.*, 2008). Therefore, it may not be surprising that the ethanol-caffeine combination did not increase the magnitude of the conditioned response any further by Test 2 and it is unclear if using higher doses of caffeine (*e.g.* 15 mg/kg) with ethanol will provide clarity on this issue. There is the additional observation from an earlier study that, in general, as caffeine doses in rats increased above 3 mg/kg, place preferences tended to shift to place aversions (Patkina and Zvartau, 1998). Our study design also provided the ability to examine whether the drug combination produced place preference with fewer conditioning sessions. In this regard, ethanol (1.75 g/kg) did not produce a significant place preference in the mice by Test 1, consistent with our previous study (Griffin *et al.*, 2012). This indicates that the number of conditioning sessions with ethanol by Test 1 is not optimal for demonstrating preference. Moreover, because time on the ethanol-paired side increases significantly by Test 2, this indicates that ethanol conditioned mice should be amenable to further increases in preference and not subject to a “ceiling” effect at Test 1. Similarly, although mice treated with 3mg/kg caffeine did show a modest preference at Test 1, the magnitude of this effect was only slightly greater than the other groups. Nevertheless, despite small effects on preference during Test 1 for the two drugs given alone, the ethanol-caffeine combination group did not evidence a significant place preference at the Test 1 time point. These findings indicate that ethanol and caffeine do not interact to potentiate the development of place preference under the conditions of our experiment.

Our study design using two test sessions was similar to a previous study using a combination of very low caffeine and cocaine doses, both 0.32 mg/kg (Bedingfield *et al.*, 1998). In this previous study, the caffeine-cocaine combination produced significant place preference by the first test which remained of a similar magnitude by the second test (Bedingfield *et al.*, 1998). Interestingly, relatively recent data also indicate that very low caffeine concentrations (<0.25 mg/ml) support conditioned flavor preferences (Myers and Izbic, 2006). Considering our current data and previously published data (Bedingfield *et al.*, 1998; Buckholtz and Middaugh, 1987; Hsu *et al.*, 2009), it is unlikely that such low caffeine doses (*e.g.* 0.32 mg/kg) will increase locomotor activity or produce place preference in our model. However, it is an intriguing possibility that these very low doses of caffeine could potentiate the development of ethanol-induced place preference with fewer conditioning sessions and this remains of interest for future experiments.

The neurobiological mechanism driving the interaction between ethanol and caffeine to affect behavior was not addressed in the current studies, but the interaction of several neurotransmitter systems deserves consideration in future experiments. Ethanol's stimulant effects may be related to increased dopaminergic neurotransmission in the nucleus accumbens (Ramachandra *et al.*, 2007; Tang *et al.*, 2003) and caffeine's antagonism of

adenosine receptors which also enhances dopaminergic transmission in this region (Quarta *et al.*, 2004) may contribute to the interactive effect on behavior. This possibility is further supported by the observation that the stimulating effects of caffeine have been linked to it's blockade of the both A₁ and A₂ adenosine receptors (Kuzmin *et al.*, 2006), although recent a recent study using KO mice more strongly implicates A_{2a} receptors in the activating effects of caffeine, at least in the accumbens (Lazarus *et al.*, 2011). Antagonism of adenosine receptors by caffeine may be a particularly important mechanism to consider because the depressant and ataxic effects of ethanol can be blocked using adenosine receptor antagonists (Carmichael *et al.*, 1991; Dar and Clark, 1992; Israel *et al.*, 1994). On the other hand, both ethanol and caffeine interact with the glutamatergic system. For example, ethanol can increase glutamate release in the accumbens (Szumlinski *et al.*, 2008) which may be initiated by adenosine reuptake inhibition (Nam *et al.*, 2012). Caffeine appears to increase glutamate release via A₁ receptor mechanisms which can be blocked by A₂ receptor mechanisms (Quarta *et al.*, 2004). Thus, several different possibilities exist to explain the enhanced activating effects of ethanol and caffeine combinations.

In summary, combinations of ethanol and caffeine interacted to increase locomotor activity more than either drug given alone. Evidence of this was present over a range of ethanol and caffeine doses. In a place preference test, we demonstrated that ethanol produced significant place preference but that preference produced by caffeine was modest in comparison. The significant interactive effects of ethanol and caffeine found on locomotor activity did not extend to the place preference procedure because under the conditions of our conditioning procedure, we did not find that caffeine augmented ethanol's effects. It was previously reported that injected caffeine increases ethanol consumption in a rat model (Kunin *et al.*, 2000), which appears consistent with recent data indicating that caffeine enhances self-administration of oral reinforcers such as sucrose solutions (Sheppard *et al.*, 2012) or to condition flavor preferences (Myers and Izbic, 2006). Thus, the interaction of caffeine with ethanol's reinforcing effects during self-administration sessions is an important consideration for future experiments.

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Highlights

- Ethanol, caffeine and the combination increased place preference.
- Ethanol, caffeine and the combination significantly increased distance traveled.
- The drug combination yielded the largest increase in distance traveled.

Figure 1.

Conditioned place preference with vehicle (VEH) caffeine (CAFF; 3 mg/kg), ethanol (EtOH; 1.75g/kg) and the combination of caffeine and ethanol (COMBO) at those same doses. All three active drug groups demonstrated significant place preference, although the effect for caffeine alone was generally weak compared to ethanol and the drug combination. The drug combination did not demonstrate enhanced place preference either in the magnitude of time spent on the drug paired side or in the number of sessions needed to acquire significant place preference. Values are means $\pm$ S.E.M. (# $p < 0.05$ within group compared to Pretest; * $p < 0.05$ within Test compared to vehicle; ^ $p < 0.05$ within Test compared to caffeine)

Figure 2.

Locomotor activity after challenge with ethanol 1.75 g/kg, caffeine 3 and 15 mg/kg and the combination of ethanol and caffeine. Ethanol at a known stimulatory dose (1.75 g/kg) increased total distance as did both doses of caffeine when given alone. The combination of caffeine at either dose with ethanol (1.75 g/kg) significantly increased total distance more than either caffeine or ethanol alone. Values are means $\pm$ S.E.M. Key: VEH=vehicle, EtOH=ethanol at the specified dose, 3C=caffeine 3mg/kg, 15C=caffeine 15mg/kg, 3C+E=caffeine 3mg/kg + ethanol at the specified dose, 15C+E=caffeine 15mg/kg + ethanol at the specified dose (* p <0.05 compared to vehicle; ^ p <0.05 caffeine 15 mg/kg vs caffeine 3 mg/kg; # p <0.05 compared to ethanol and caffeine 3 mg/kg alone; + p <0.05 compared to ethanol and caffeine 15 mg/kg alone).

Figure 3.

Locomotor activity with ethanol 2.5 g/kg, caffeine 3 and 15 mg/kg and the combination of ethanol and caffeine. Using a higher dose of ethanol, the combination of ethanol and caffeine again increased total distance more than either drug given alone, although the effect at the 15 mg/kg caffeine + ethanol dose was not as robust as that shown in Figure 2. Values are means $\times$ S.E.M. See Figure 2 for key. (* $p < 0.05$ compared to vehicle; ^ $p < 0.05$ caffeine 15 mg/kg vs caffeine 3 mg/kg; # $p < 0.05$ compared to ethanol or caffeine 3 mg/kg alone; + $p < 0.05$ only compared to ethanol alone).

Figure 4.

Locomotor activity with ethanol 3.25 g/kg, caffeine 3 and 15 mg/kg and the combination of ethanol and caffeine. The highest dose of ethanol tested (3.25 g/kg) did not increase total distance traveled. Further, the effect of caffeine 3 mg/kg to increase activity when co-administered with a non-stimulatory dose of ethanol nearly reached significance (@p=0.052) and was not significant with the higher caffeine dose, suggesting an upper limit for the interactive effects of these two drugs. Values are means $\pm$ S.E.M. See Figure 2 for key. (*p<0.05 compared to vehicle; ^p<0.05 caffeine 15 mg/kg vs caffeine 3 mg/kg).