Sensitization and Tolerance Following Repeated Exposure to Caffeine and Alcohol in Mice

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Abstract

Introduction—Energy drinks are popular mixers with alcohol. While energy drinks contain many ingredients, caffeine is an important pharmacologically active component and is generally present in larger amounts than in other caffeinated beverages. In these studies, we investigated the hypothesis that caffeine would influence the effects of alcohol (ethanol) on conditioned taste aversion, ataxia and locomotor activity after repeated exposure.

Methods—Four groups of mice were exposed by oral gavage twice daily to vehicle, ethanol (4 g/kg), caffeine (15 mg/kg), or the ethanol/caffeine combination. Conditioned taste aversion to saccharin and ataxia in the parallel rod task were evaluated after 8 or 16 gavages, respectively, using ethanol (1–3 g/kg) or ethanol/caffeine (3mg/kg + 2 g/kg) challenges. In addition, locomotor activity was evaluated initially and after repeated exposure to oral gavage of these drugs and doses.

Results—Repeated oral gavage of ethanol produced significant locomotor sensitization, with those mice increasing total distance traveled by 2-fold. The locomotor response to caffeine, while significantly greater than vehicle gavage, did not change with repeated exposure. On the other hand, repeated gavage of caffeine/ethanol combination produced a substantial increase in total distance traveled after repeated exposure (~4-fold increase). After repeated ethanol exposure, there was significant tolerance to ethanol in the conditioned taste aversion and parallel rod tests. However, neither a history of caffeine exposure nor including caffeine influenced ethanol-induced conditioned taste aversion. Interestingly, a history of caffeine exposure increased the ataxic response to the caffeine/ethanol combination and appeared to reduce the ataxic response to high doses of ethanol.

Conclusion—The data support the general hypothesis that repeated exposure to caffeine influences the response to ethanol. Together with previously published work, these data indicate that caffeine influences some ethanol-related behaviors, notably locomotion and ataxia, but appears not to influence the expression of conditioned behaviors.

Keywords

ethanol; psychostimulant; place preference; ataxia; drug interaction

INTRODUCTION

A growing trend in recent years has been to combine caffeinated energy drinks with alcohol, due in part to the tremendous growth in energy drink sales (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 (Pennay *et al.*, 2014; Thombs *et al.*, 2009), indicate that consumption of alcohol mixed with energy drinks is common. Unfortunately, consuming these mixtures has been associated with increases in emergency room visits (SAMHSA, 2011; SAMHSA, 2013), suggesting that consumption of these drinks can be dangerous. Mixing caffeinated beverages and alcohol is not a new practice nor is it limited to energy drinks since other caffeinated drinks like colas are also popular mixers with alcohol (Rossheim and Thombs, 2011; Thombs *et al.*, 2011). However, many energy drinks typically have more caffeine per serving than other caffeinated beverages, ranging widely from 50 to 500 mg total caffeine per package (Heckman *et al.*, 2010; Reissig *et al.*, 2009). While the higher amount of caffeine can be comparable to brewed coffee, it far exceeds that of a typical cola (~30 mg/serving; see Heckman *et al.* 2010). Additionally, unlike coffee, some manufacturers of energy drinks promote rapid consumption of their product (Reissig *et al.*, 2009). Thus, energy drinks provide quick access to relatively large amounts of caffeine, and in combination with alcohol, increase the risk of clinically significant interactions between caffeine and alcohol.

Pharmacological interactions of caffeine and alcohol (ethanol) have been studied in humans and animal models. Studies in humans have focused on caffeine's ability to antagonize deficits caused by ethanol and indicate that caffeine antagonizes some of the cognitive and psychomotor deficits caused by ethanol (Attwood *et al.*, 2012; Hasenfratz *et al.*, 1993; Liguori and Robinson, 2001; Mackay *et al.*, 2002; Marczinski and Fillmore, 2003). Other research using human subjects has focused on energy drinks as the source of caffeine in laboratory tests and report that these combinations can mildly influence subjective intoxication (Ferreira *et al.*, 2006; Peacock *et al.*, 2013), perceived stimulation (Peacock *et al.*, 2012; Peacock *et al.*, 2013, 2014) and can enhance the desire to drink (Marczinski *et al.*, 2012; McKetin and Coen, 2014). With regards to animal models, a recent review nicely summarizes the growing research with caffeine/ethanol combinations (Lopez-Cruz *et al.*, 2013). Previous work has shown that caffeine can increase ethanol drinking in rats (Kunin *et al.*, 2000; Rezvani *et al.*, 2013) and can promote significant ethanol-induced taste aversions (Kunin *et al.*, 2001). Additionally, it has been reported that combinations of caffeine and ethanol, compared to either drug alone, increase locomotor activity after acute injection (Hilbert *et al.*, 2013) or while consuming the drug mixtures in a drinking paradigm (Fritz *et al.*, 2014). Some research has also incorporated energy drinks into animal models and shown that they can antagonize locomotor deficits with higher doses of ethanol (Ferreira *et al.*, 2004) similar to administering only caffeine (Hilbert *et al.*, 2013). Further, when given acutely energy drinks combined ethanol can significantly enhance activity when there is a history of ethanol exposure (Ferreira *et al.*, 2013).

Energy drinks sold by different manufacturers contain a variety of ingredients, but caffeine is common to most of them and many studies have defined caffeine's pharmacological effects in humans and animal models. Therefore, the present studies focus on the interactive

effects of caffeine and ethanol. Since there is relatively little information regarding chronic exposure to the combination of caffeine and ethanol, the primary goal of these studies was to examine how repeated oral exposure to these drugs influences the development of tolerance or sensitization using two established behavioral procedures that are known to be influenced by chronic ethanol exposure. To do this, we used a conditioned taste aversion procedure (Lopez *et al.*, 2012) and another procedure to measure ataxia (Griffin *et al.*, 2013). Prior work by our laboratory and others, indicated that chronic ethanol exposure would produce tolerance to an ethanol challenge (*i.e.* less aversion and less ataxia) and we hypothesized that caffeine combined with ethanol might augment the development of tolerance in these procedures. Additionally, to extend our earlier work looking at the effects of acute administration of these drugs on locomotion (Hilbert *et al.*, 2013), we measured locomotor activity in an open field after the first oral gavage and then again after repeated oral gavage. Following from our earlier work (Griffin *et al.*, 2013; Griffin *et al.*, 2010; Hilbert *et al.*, 2013; McGovern *et al.*, 2014), our general hypothesis was that caffeine would increase the stimulatory effects of ethanol when administered acutely and that these effects would increase with repeated exposure.

MATERIALS AND METHODS

General study design

These studies examined conditioned taste aversion (CTA), ataxia and locomotor activity in C57BL/6J mice with histories of repeated exposure to ethanol (ethanol; 4 g/kg), caffeine (15 mg/kg), or the combination of these two drugs and doses. The choice of the ethanol dose for the repeated exposure procedure was influenced by earlier work showing that the oral gavage route of administration can achieve high blood ethanol levels (Griffin *et al.*, 2009), but also that it would not completely impair locomotion. We chose to use 15 mg/kg caffeine based on previous work showing that administration of this dose would significantly increase locomotor activity (Hilbert *et al.*, 2013; Kaplan *et al.*, 1989). For the exposure procedure, drugs were administered by oral gavage twice per day (at 0730h and 1530h). After 4 days (i.e. 8 gavages), the exposure procedure was suspended for 3 days while mice were evaluated for CTA induced by ethanol or a caffeine-ethanol combination. After completing the CTA procedure, mice resumed twice daily gavage for 4 additional days and the following day, were evaluated for the ataxic effects of ethanol, caffeine or a caffeine-ethanol combination. These studies were conducted over 3 experiments with the third conducted in 2 replicates (details in Table 1). Additionally, the third experiment included measurement of locomotor activity after the 1st and 15th gavages while conducting the repeated exposure procedure, with CTA and ataxia still evaluated at the same time points as indicated for experiments 1 and 2.

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; lights off 1400hr) in an 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.

Conditioned Taste Aversion (CTA) procedures

This procedure was conducted similarly to that described by Lopez and colleagues (Lopez *et al.*, 2012). For this procedure, saccharin (1%) was presented on two consecutive days for 30 minutes at the beginning of the dark phase in the circadian cycle. Immediately at the conclusion of the second session, mice were intraperitoneally injected with vehicle (0.9% NaCl), ethanol (1 or 2 g/kg) or a caffeine-ethanol combination (3 mg/kg caffeine + 2 g/kg ethanol). The next day, mice were presented with the saccharin solution for 30 minutes (Test day). Each day saccharin intake was recorded to the nearest 0.1 mL and the amount consumed was corrected for spillage by using bottles placed on empty cages. An important methodological difference for the experiment reported in Figure 1C compared to other two experiments was that only one conditioning session was used, rather than two as for the other experiments This resulted in some mice across the 4 exposure groups consuming <0.2 ml saccharin during the 30 minute conditioning period and being excluded from the CTA analysis. Because of this, an additional replicate of experiment 3 was conducted to increase the number of mice available for CTA analysis and mice in this replicate were treated exactly as described experiment 3.

Ataxia apparatus and procedures

The apparatus and procedures have been described previously (Griffin *et al.*, 2013) and were adapted from previous work (Kamens and Crabbe, 2007). In brief, footslips were counted as mice ambulated within a small arena on a floor constructed of parallel rods. Footslips were defined as any hindfoot or forefoot slip between the parallel rods. Additionally, vertical lines on the rear of the arena spaced 5 cm apart allowed the footslip counts to be normalized to the number of lines crossed to control for drug effects on general activity (using the base of the tail as the reference point). For the test, mice were intraperitoneally injected with vehicle (0.9% NaCl), ethanol (2 or 3 g/kg) or a caffeine-ethanol combination (3 mg/kg caffeine + 2 g/kg ethanol) and returned to their home cage for 5 minutes before being placed into the arena. The session was 2 minutes and video recorded for later visual quantification.

Locomotor activity (LA) apparatus and procedures

Modified Med Associates (St. Albans, VT, USA) activity chambers (ENV-510) were used for these experiments. As previously described, the open field arenas were restricted in size to 13.5 cm wide × 27.5 cm long × 20.5 cm deep (Hilbert *et al.*, 2013). In this experiment, the mice were placed immediately into the locomotor activity apparatus after the 1st and 15th gavages and distance traveled (cm) was measured for 45 minutes. Both of these activity measurement sessions occurred during light phase of the circadian cycle.

Drugs

Drugs were administered by either oral gavage using water as the vehicle for the repeated exposure or given intraperitoneally (IP) with normal saline as the vehicle (0.9% NaCl). The volume was 0.02ml/g of body weight for all dosing. When the drug combination is

indicated, caffeine and ethanol were administered simultaneously in the same solution. Caffeine used was the anhydrous base (Fluka, a subsidiary of Sigma-Aldrich, St Louis, MO). Ethanol (ethanol; 95%) was obtained from AAPER (Shelbyville, KY) and diluted as needed.

Statistical analyses

The primary dependent variables for these studies were saccharin intake (mL), footslip counts per line cross, and distance traveled (cm). For saccharin intake, the Test day data are presented as a percent of the saccharin intake occurring during the conditioning session 24 hours earlier. Note that for the data shown in Figures 1 and 2, the data in the light shaded bar (0 g/kg) represents approximately equal numbers of mice (n=3–5) from each exposure history that were combined into a single group to represent a baseline comparison for the challenge doses of ethanol and ethanol plus caffeine used for the CTA or the Footslip procedures. Additionally, for the CTA experiments (Figure 1), the data were normalized to the saccharin intake of vehicle conditioning group (0 g/kg) on test day.

All data in these experiments were analyzed using Analysis of Variance (ANOVA) with repeated measures (RM) when necessary and significant factor interactions were followed up using Pairwise Multiple comparisons. Additionally, to facilitate comparisons between exposure histories across the different experiments shown in Figure 1 and Figure 2, we calculated Cohen's d values for each possible combination of groups (Nakagawa and Cuthill, 2007). The Cohen's d values were compared across relevant pairs using Chi square analyses. Alpha was set to 0.05 for all analyses.

RESULTS

Conditioned Taste Aversion (CTA)

The influence of repeated exposure of ethanol, caffeine or the combination on the conditioned taste aversion was examined using saccharin as a conditioned stimulus and either ethanol or an ethanol-caffeine combination as the unconditioned stimulus (Figure 1).

For the experiment shown Figure 1A, the one way ANOVA was significant ($F(4, 42) = 8.513$, $p < 0.001$) indicating that the exposure history of the mice contributed to different intake responses during the CTA test. As can be seen, relative to the 0 g/kg conditioning dose, the 2 g/kg ethanol dose produced a significant aversion (~80% decrease, *p<0.05) in saccharin consumption during the test in mice with a history of vehicle exposure consistent with previous work (Lopez *et al.*, 2012). Also as expected, mice with a history of ethanol exposure (EtOH group) demonstrated tolerance to the aversive effects of 2 g/kg ethanol because there was no significant decrease in saccharin consumption during the CTA test. Further, it was noted that the saccharin consumption was significantly greater in the EtOH group compared to the VEH mice (^p<0.05). Mice with a history of caffeine exposure demonstrated a similar response to 2 g/kg ethanol when compared with the VEH history mice, indicating that a history of CAFF exposure did not alter the conditioned aversive effects of ethanol in this paradigm. Saccharin intake for those mice with a history of exposure to ethanol plus caffeine (BOTH group) was significantly reduced indicating a

significant aversion (*p<0.05) although there was not a significant difference compared to the EtOH group response. Thus, the history of combined ethanol and caffeine exposure did not alter the capacity of the mice to develop aversion to an ethanol challenge compared to only a history of ethanol exposure.

In another group of mice, the influence of these same exposure histories on aversive conditioning was examined using a lower dose of ethanol (1 g/kg; Figure 1B). The one way ANOVA did not reveal a significant effect of exposure (F(4, 36) = 2, p > 0.05). The lack of conditioned aversion using 1 g/kg ethanol in both the VEH exposure group and the EtOH exposure group was expected (Lopez *et al.*, 2012). However, this analysis also indicates that a history of caffeine or caffeine in combination with ethanol did not facilitate aversive conditioning with a low dose of ethanol.

The possibility that a combination of ethanol and caffeine would produce CTA to saccharin was also examined (Figure 1C). The one way ANOVA was significant (F(4, 45) = 5.153, p < 0.002), again indicating that the exposure history altered the conditioned response to the drug combination in the CTA paradigm. Post-hoc analysis found that the mice with a history of VEH exposure and CAFF exposure demonstrated significant aversion to the drug combination (both *p<0.05), consistent with a previous report (Kunin *et al.*, 2001).. On the other hand, there was clear evidence of ethanol tolerance since the saccharin intake was not different in the EtOH exposure group relative to the 0 g/kg group and the EtOH group's intake was also significantly greater than the VEH exposure group (^p<0.05). The mice in the BOTH exposure group demonstrated an intermediate response to conditioning with the drug combination. This group did not have a significant reduction in saccharin intake compared to the 0 g/kg group (p>0.05), indicative that some tolerance developed to the aversive effects of the ethanol plus caffeine combination. However, mice in the BOTH group did not show a significant increase in saccharin intake relative to the VEH exposure group indicating that the tolerance was less robust than that observed in the EtOH exposure group.

As described in the methods, the experiments shown in each panel of Figure 1 were conducted independently. To facilitate comparison across these experiments, we calculated bias corrected Cohen's D values to serve as a measure of effect size for each possible pair of groups in the experimental design. The Cohen's D values from relevant pairs of data from the 3 experiments were compared using Chi Square analyses (see Methods). Of interest were comparisons of the response differences between various groups across the 3 experiments mice to determine if caffeine influenced the effects of ethanol-induced CTA. For example, the difference in behavioral responses between the EtOH and BOTH groups resulted in effect sizes (*i.e.* Cohen's d) of 0.647, 0.842 and 0.517 for Experiments 1, 2 and 3, respectively. Given the relatively similar effect sizes, the Chi Square analysis did not find a significant effect (X^2 = 0.084, p>0.05). Further, the 95% confidence intervals (CI) for the calculated effect sizes overlapped substantially. Other comparisons from these 3 experiments also did not reveal any significant differences in effect sizes between any of the other groups (all X^2<1.7, p>0.05; See Supplementary Table 1 for the complete list of comparisons) and there was typically significant overlap in the 95%CI. It is noteworthy that as part of these analyses, Student's T-tests were conducted for each pair of comparisons and

those findings replicate those already described above that used ANOVA and post-hoc tests (Supplementary Table 1).

Ataxia

Next, we examined the influence of repeated exposure of ethanol, caffeine or the combination on ataxia caused by acute ethanol or caffeine-ethanol challenge (Figure 2). The data are reported as footslips normalized according to their activity in the arena (*i.e.* line crosses) and compared to mice challenged with 0 g/kg.

As shown in Figure 2A, a 2 g/kg ethanol dose increased footslips in all mice relative to the 0 g/kg challenge. A one way ANOVA on these data was significant ($F(4, 35) = 7.193$, $p < 0.01$) and confirmed that the history of exposure modified the amount of ataxia demonstrated by the mice. Post-hoc analysis indicated that all mice challenged with 2 g/kg ethanol had increased footslips/line cross compared to the 0 g/kg challenge (*p<0.05). The VEH and CAFF exposure history mice exhibited the most footslips. Compared to the VEH exposure group, EtOH and BOTH exposure histories had reduced ataxia (^p<0.05), consistent of the development of tolerance to the ataxic effects of ethanol as reported by others (Linsenbardt *et al.*, 2011) when ethanol was part of the exposure history. However, in this study, previous CAFF exposure did not influence the ataxic effects of acute ethanol challenge.

An additional group of mice with the same exposure histories was challenged with a higher dose of ethanol (3 g/kg; Figure 2B). The one way ANOVA on these data was significant ($F(4, 43) = 4.914$, $p < 0.05$), indicating that the exposure history influenced the ataxic response to ethanol. Post-hoc analysis found that only mice in the VEH, EtOH and CAFF groups exhibited footslips greater than 0 g/kg (*p<0.05) while mice in the BOTH group did not (#p>0.05). The reduced number of footslips in the BOTH group suggests that the exposure to caffeine and ethanol influenced the development of tolerance to the ataxic effects of the high dose of ethanol (3 g/kg).

Lastly, we examined the acute effects of ethanol plus caffeine on ataxia in mice with a history of exposure to VEH, EtOH, CAFF or BOTH (Figure 2C). The one way ANOVA on these data was significant ($F(4, 67) = 26.42$, $p < 0.05$), indicating that exposure history influenced ataxia in response to a challenge with 2 g/kg ethanol plus 3 mg/kg caffeine. However, the analysis revealed that the response to the drug combination in this experiment were similar to that for 2 g/kg ethanol alone (Figure 2A) with the exception that the history of CAFF exposure increased the footslips recorded compared to the VEH group. This small, but significant (^p<0.05), increase in footslips following a history of CAFF exposure suggests that sensitization developed to the ataxic effects of caffeine.

Similar to the CTA studies above, Cohen's d values for the footslip data were also calculated to facilitate comparison across the three experiments. Interestingly, these analyses found a significant difference in effect sizes for the response differences between the EtOH and BOTH groups across the three experiments ($X^2 = 10.687$, p<0.01). The Cohen's d values were 0.19, 1.364 and 0.079 for Experiments 1, 2 and 3, respectively. In this case, the significant X^2 statistic was driven by the relatively large effect size for the difference

between footslips for the EtOH and BOTH mice from Experiment 2 using the high dose of ethanol (3 g/kg), compared to the relatively small differences between those same groups for Experiments 1 and 2. The 95% confidence intervals for the Cohen's d values diverged to a larger extent than for other comparisons. This analysis corroborates the findings in Figure 2B described above that combined exposure to ethanol and caffeine appears to increase tolerance to heavy doses of ethanol. The same analyses for the other footslip data sets did not reveal any other significant effects (all $X^2 < 2.5$, $p > 0.05$; See Supplementary Table 2).

Locomotor Activity

We measured locomotor activity immediately after the 1st and 15th oral gavages (see Table 1). As described in the methods, these gavages were with VEH, EtOH (4 g/kg), CAFF (15mg/kg) and BOTH (EtOH 4g/kg + CAFF 15 mg/kg).

These data were initially analyzed using a 4(History) $\times$ 2(Gavage) $\times$ 9(Bin) ANOVA with Gavage and Bin as repeated measures. The 3-way interaction was significant (F (24, 536) =12.069, p<0.0001) as were all of the 2-way interactions (all F's >28, p<0.0001) and factors (all F's >11, p<0.0001). This analysis confirmed what can be seen in Figure 3, that locomotor activity was increased with repeated gavage, particularly in the ethanol plus caffeine group.

To determine how exposure history interacted with repeated gavage challenge, the 4(History) $\times$ 2(Gavage) repeated measures ANOVA, which is part of the overall 3-way ANOVA, was examined further and the analysis is summarized in Figure 3A. The factor interaction was significant (F (3, 67) = 32, p<0.0001) as were effects of History (F (3,67) = 64, p<0.0001) and Gavage (F (1,67) = 40, p<0.0001). Post-hoc analysis of the data after the 1st gavage found that only the CAFF challenge increased distance traveled more than VEH (*p<0.05) and EtOH (^p<0.05). By the 15th gavage, the distances traveled increased for the EtOH, CAFF and BOTH groups compared to VEH during that gavage (*p<0.05). In particular, there was a dramatic increase in distance traveled for the BOTH group that was significant compared to all other groups (+p<0.05). Additionally, when compared with activity following Gavage 1, the activity of the EtOH and BOTH groups increased significantly (#p<0.05) but this was not found for the VEH and CAFF groups. These data indicate the development of sensitization following repeated gavage to ethanol and the combination of ethanol plus caffeine. Repeated exposure to ethanol resulted in a ~2-fold increase in activity from Gavage 1 to Gavage 15 while the same comparison for the BOTH group showed a ~4-fold increase in activity.

Given the large increase in distance traveled during the 15th gavage in the drug combination group, we examined how locomotor activity changed within session for all 4 groups. These data are summarized in Figure 3B. The analysis used separate 4(History) $\times$ 9(Bin) ANOVAs with Bin as a repeated measure for both Gavage 1 and Gavage 15. For Gavage 1, the factor interaction was significant (F (24, 544)= 2.991, p<0.0001) as were History (F (3,68)= 6.6, p=0.001) and Bin (F (8, 544)= 32, p<0.0001). Due to the number of comparisons being made, Bonferroni's correction was used in the post-hoc analysis. The post-hoc analysis for Gavage 1 did not reveal very many specific differences between the four groups during any given Bin and therefore are not shown. However, the analysis did support the general

observation that activity declined over time, though this was not evident for the CAFF group (for example, Bin 1 vs Bin 9, $p > 0.05$).

For Gavage 15, the factor interaction for the 2 way ANOVA was significant ($F_{(24, 536)} = 14$, $p < 0.0001$) as were the History ($F_{(3,67)} = 50$, $p < 0.0001$) and Bin ($F_{(8, 544)} = 74$, $p < 0.0001$) factors. Again, Bonferroni's correction was applied to the post-hoc comparisons for this analysis. The post-hoc analysis revealed that the BOTH group traveled significantly more distance during Bins 1 through 9 compared to VEH and EtOH groups as well as Bins 1 through 6 for the CAFF group (all these comparisons denoted as $+p < 0.05$). Additionally, the EtOH group showed increased activity during Bins 1 and 2 ($*p < 0.05$) compared to the VEH group and Bin 1 versus the CAFF group ($*p < 0.05$). These findings support the analysis described for Figure 3A and further indicate that the large increases in distance traveled for the BOTH and EtOH groups occurred at the beginning of the session and declined with time.

DISCUSSION

Our results demonstrate that caffeine alters the behavioral effects of ethanol, in particular, when measured immediately after drug administration but there is evidence that a history of caffeine exposure influences the response to ethanol as well. The most substantial finding was that the combination of caffeine and ethanol produced a robust locomotor sensitization. After repeated exposure, the effect of the drug combination on activity was approximately 2.5 times greater than either that produced by ethanol or caffeine alone, suggesting that caffeine/ethanol co-intoxication has a synergistic effect on the development of locomotor sensitization. Although ethanol tolerance was established after repeated exposure in both the conditioned aversion and ataxia procedures, only the ataxic effects of acutely administered ethanol or the ethanol/caffeine combination were influenced by the presence of caffeine in the exposure history.

When given by oral gavage for the first time, the combination of caffeine and ethanol tended to antagonize the slight (non-significant) locomotor depression caused by ethanol given alone and by the 15th gavage there was a striking locomotor sensitization effect with the drug combination group compared to the other three groups after either the 1st or 15th gavage. Although this large effect declined with time during the session, the large increase in distance traveled was sustained for most of the 45 minute session compared to the other three groups. These results indicate that repeated co-exposure to ethanol and caffeine produces a much larger effect than repeated exposure to either drug alone. A recent report by Fritz and colleagues using a voluntary consumption model in the home cage hinted at the possibility that repeated exposure to a combination of ethanol and caffeine could produce a sensitized locomotor response (Fritz *et al.*, 2014), though the effect was not reported as significant. Another report indicated that an ethanol sensitization effect could be enhanced in mice when acutely challenged with a mixture of an energy drink and ethanol, particularly in those mice in which the ethanol sensitization effect is small (Ferreira *et al.*, 2013). Taken together, these data indicate that a history of exposure to the drug combination influences the locomotor response when subjects are acutely challenged.

The experimental design of the locomotor activity measurements also afforded the opportunity to examine the effects of repeated ethanol and caffeine exposure, separately, on locomotor activity in the mice. The initial activity response to ethanol oral gavage was reduced compared to vehicle gavage (albeit non-significantly) but then increased significantly more than the vehicle response, indicative of sensitization. Locomotor sensitization following repeated ethanol exposure has been reported in mice in a variety of studies (Carrara-Nascimento *et al.*, 2011; Fish *et al.*, 2002; Lessov *et al.*, 2001; Lessov and Phillips, 1998; Phillips *et al.*, 1997). With regards to caffeine, although it acutely increased activity consistent with previous reports (Buckholtz and Middaugh, 1987; Hilbert *et al.*, 2013; Kaplan *et al.*, 1989), repeated exposure to caffeine did not produce locomotor sensitization in our model. While several other reports indicate that caffeine can induce a small, albeit significant, locomotor sensitization effect (Ball and Poplawsky, 2011; Hsu *et al.*, 2009; Hsu *et al.*, 2010; Zancheta *et al.*, 2012), the reason for the lack of caffeine locomotor sensitization in our model is unclear but may be related to methodological differences such as dose, route of administration and frequency of administration.

When given acutely to the mice with a history of vehicle exposure (VEH group), ethanol and the ethanol/caffeine combination both produced significant conditioned taste aversion and increased footslips (i.e. ataxia). For the conditioned taste aversion experiments, the effect of ethanol was dose-dependent, with the 2 g/kg dose of ethanol producing significant aversion while the 1 g/kg dose did not, as expected (Lopez *et al.* 2012). The addition of caffeine to ethanol during conditioning did not further enhance aversion. Perhaps future testing with the smaller ethanol dose (1g/kg) in combination with caffeine would yield significant aversion as previously reported (Kunin *et al.*, 2001). Alternatively, examining conditioned taste aversion after 16 gavage exposures (as done for the parallel rod test) could also reveal an influence of caffeine on this behavior. Similarly, in the ataxia measurements, acutely administered ethanol significantly increased footslips as expected but the addition of caffeine did not further augment the increase in VEH exposed mice. Interestingly, the higher dose of ethanol (3 g/kg) produced slightly less footslips than the 2 g/kg dose (Figure 2), which reflects similar numbers of footslips but less overall activity due to sedation from the high dose. Earlier, we reported that adding methylphenidate to ethanol intoxication further increased footslips (Griffin *et al.*, 2013) and another laboratory indicated this possibility using caffeine in rats (Dar *et al.*, 1987), though at higher doses. However the present data do not indicate that this occurred with the combination of ethanol and caffeine given acutely in this experiment.

Repeated exposure to ethanol leads to tolerance in several behavioral paradigms, resulting in diminished effects to ethanol during a subsequent challenge, including measures of ataxia (Linsenbardt *et al.*, 2011) and conditioned taste aversion (Lopez *et al.*, 2012). Indeed, as expected, significant ethanol tolerance was found in both the ataxia and the aversive conditioning procedures after repeated oral gavage of ethanol. Of interest in the present studies was whether caffeine increased or decreased the development or expression of ethanol tolerance. In our conditioned taste aversion procedure, we found that a history of caffeine or caffeine/ethanol did not significantly increase or decrease either the development or expression of ethanol tolerance. However, in contrast, a history of caffeine exposure did

significantly affect ethanol-induced ataxia in the parallel rod test. First, for mice having a history of ethanol/caffeine exposure and then challenged with a high dose of ethanol (3 g/kg) there was not a significant increase in footslips compared to the 0 g/kg challenge as with the other groups (Figure 2B). This effect was not found using a lower ethanol dose, consistent with other work (Linsenbardt *et al.*, 2011), and suggests the development of tolerance to the ataxic effects of a high ethanol dose when caffeine is included as part of the ethanol exposure history, though more experimental work is needed for confirmation. Secondly, mice with a history of only caffeine exposure showed increased footslips when challenged with the ethanol/caffeine combination (Figure 2C). There was a trend for this same behavioral effect when mice were challenged only with ethanol (Figure 2B), although it did not reach statistical significance. This finding indicates that a history of caffeine exposure negatively influences fine motor coordination after a ethanol challenge, consistent with an earlier study using 10 days of caffeine exposure and a 1.5 g/kg ethanol challenge dose (Dar and Wooles, 1986).

As previously reviewed (Ferre and O'Brien, 2011), the adenosinergic system is likely to be an important mediator in the behavioral effects reported here since both caffeine and ethanol interact with this neurotransmitter system. It is well established that caffeine antagonizes central A1 and A2a adenosine receptors (Fredholm *et al.*, 2011) and it was reported that these pharmacological actions underlie the locomotor activating effects of caffeine (Kuzmin *et al.*, 2006). Additionally, ethanol increases adenosine concentrations by inhibiting transporters and increases in adenosine may underlie the sedative and ataxic effects of ethanol (Dunwiddie and Masino, 2001; Nam *et al.*, 2012; Ruby *et al.*, 2014). Further, chronic exposure to either ethanol or caffeine results in adaptations of the adenosine receptors (Butler and Prendergast, 2012; Fredholm *et al.*, 2011). In fact, evidence suggests that chronic antagonism of the A2a receptor contributes to caffeine sensitization (Hsu *et al.*, 2009; Hsu *et al.*, 2010). While it is clear that caffeine and ethanol can interact at the level of adenosine transmission, the downstream effects of A1 and A2a receptor blockade likely involve alterations in glutamate and dopamine neurotransmission (Quarta *et al.*, 2004) to affect behavior. Thus, adaptations in the adenosinergic system and its subsequent role in modulating glutamate and dopamine transmission may underlie the behavioral changes that reported here.

The per capita consumption of caffeine appears to be increasing and many investigators have expressed concerns regarding caffeine consumption in general (Attwood, 2012; Higgins *et al.*, 2010; Reissig *et al.*, 2009; Wolk *et al.*, 2012). The half-life of caffeine in humans varies with the dose with higher doses having a longer half-life (Kaplan *et al.*, 1997). A single 500 mg dose of caffeine yields peak blood concentrations of ~18 μg/ml that, after 4 hours, declines to 7–8 μg/ml, a concentration range still associated with significant behavioral effects when lower doses are administered (Kaplan *et al.*, 1997). Interestingly, a 15 mg/kg dose in mice yields nearly similar blood levels of caffeine (~15 μg/ml) as the 500 mg dose in humans (Kaplan *et al.*, 1989), providing some relevance to our choice of dose in this set of experiments. Moreover, ethanol appears to increase caffeine's half-life in humans (George *et al.*, 1986) though this may not be true in mice (Fritz *et al.*, 2014). Therefore, consideration of higher caffeine doses is important in the context of energy drink

consumption, not only because of the higher concentration of caffeine in some of these drinks, but also that many people could already have significant amounts of caffeine in their body from other sources. With caffeine already on board at the beginning of a drinking episode that includes energy drinks, caffeine levels increase even further. However, examining higher doses of caffeine (and ethanol) in human laboratory experiments is obviously problematic due to the development of pleasant side effects and safety concerns once doses rise above 200mg caffeine (Wolk *et al.*, 2012), underscoring the crucial role of animal models for examining the interactive effects of caffeine and ethanol over a wide range of doses (Griffin, 2013).

In conclusion, our results support the general hypothesis that caffeine influences the effects of ethanol, particularly locomotion and ataxia. We found that pre-exposure to the caffeine/ethanol combination tempered the acute ataxic effects of a high ethanol dose and, similar to an earlier study (Dar and Wooles, 1986), that the drug combination itself slightly increased ataxia when administered to mice with a history of caffeine exposure. A history of caffeine exposure (with or without ethanol) did not influence conditioned aversion to ethanol or the caffeine/ethanol combination. This finding recalls results from our recent place-preference experiment (Hilbert *et al.*, 2013), suggesting that caffeine does not influence conditioned effects of ethanol, though more work is necessary to draw a firm conclusion. When given by oral gavage, caffeine also tended to acutely antagonize the locomotor depressant effects of a high ethanol dose, consistent with our earlier work (Hilbert *et al.*, 2013) and other reports (Ferreira *et al.*, 2004; Fritz *et al.*, 2014). Moreover, we found that with repeated exposure, caffeine co-exposure with ethanol leads to large increases in activity compared to either drug given alone. Though speculative, the implication of these findings is that the pro-stimulant effects of this drug combination give people more time to drink and to engage in risky behaviors before ethanol-induced sedation curtails activity. Indeed, emerging data appears to support the idea that drinkers who mix energy drinks and alcohol have longer drinking episodes (Pennay *et al.*, 2014). Therefore, caffeine's influence on ethanol-related behaviors may have important implications both acutely as well as over the long term.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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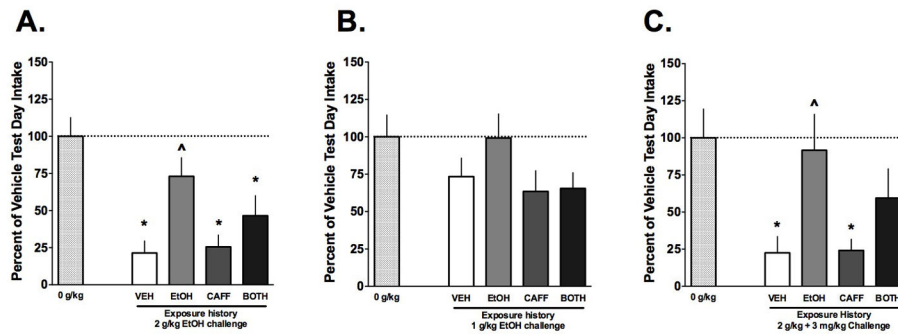
Figure 1. Caffeine exposure history did not influence conditioning to the aversive effects of ethanol or ethanol plus caffeine

A) Compared with 0 g/kg, a strong aversion was produced using 2 g/kg ethanol as indicated by the significant reduction in saccharin intake in the mice with VEH exposure history. Further, the repeated exposure to ethanol (EtOH group) produced tolerance because no significant reduction occurred and the intake was greater than the VEH group. However, caffeine pre-exposure did not influence the development of aversion or tolerance to ethanol (n=7–10/grp). **B)** A lower dose of ethanol (1 g/kg) did not produce aversion and a history of caffeine exposure did not influence this (n=9–12/grp). **C)** The combination of ethanol and caffeine produced aversion in the VEH and CAFF groups and there was tolerance to this effect in the EtOH and BOTH (*i.e.* EtOH + CAFF) groups, though the EtOH and BOTH groups did not differ in the magnitude of the response (n=8–12/grp). Data are means ± S.E.M. (*=p<0.05 *vs.* 0 g/kg challenge, ^=p<0.05 *vs.* vehicle history).
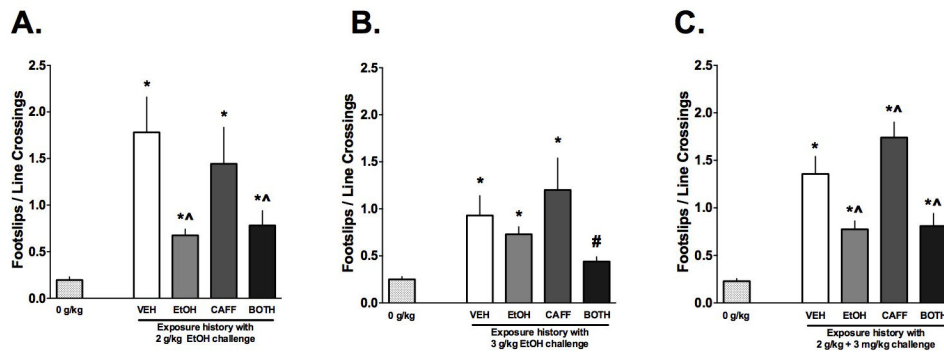
Figure 2. Caffeine exposure history mildly influences ethanol-induced or ethanol plus caffeine-induced ataxia

A) Acute injection of 2 g/kg increased footslips in all groups compared to 0 g/kg, however the EtOH and BOTH (*i.e.* EtOH + CAFF) had smaller increases, indicative of tolerance to the ataxic effects of ethanol (n=7–10/grp. **B)** A higher dose of ethanol (3 g/kg) increased footslips in the VEH, EtOH and CAFF groups. Footslips recorded in the BOTH group were similar to those of the 0 g/kg group indicating that the history of ethanol/caffeine exposure produced tolerance to high doses of ethanol (n=9–12/grp). **C)** Acute challenge with ethanol and caffeine also produced ataxia similarly to the results shown in Panel A. The CAFF mice showed an increase in footslips compared to the VEH mice, indicating that the history of caffeine increased the ataxic response to the drug combination (n=12–20/grp). Data are means ± S.E.M. (*=p<0.05 *vs.* vehicle challenge, ^=p<0.05 *vs.* vehicle history, #=p>0.05 *vs.* 0 g/kg).
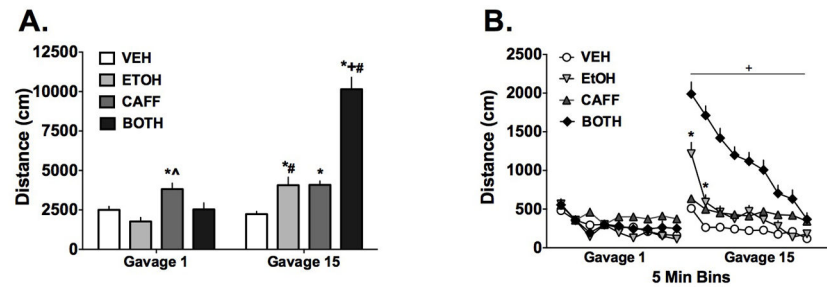
Figure 3. Repeated gavage of ethanol or ethanol plus caffeine produces locomotor sensitization
A) Total distances traveled following the 1st gavage were only increased in the CAFF group compared to vehicle but by the 15th gavage, the EtOH, CAFF and BOTH (*i.e.* EtOH + CAFF) groups showed increased activity compared to VEH. Further, the EtOH and BOTH groups demonstrated significantly increased activity by the last gavage compared to the first with the increase in activity for the BOTH group being particularly robust (n=12–20/grp). **B)** The same data shown as a within-session time course and demonstrate that for the EtOH and BOTH groups, the increased activity after the 15th gavage was greatest at the beginning of the session and declined with time. Data are means ± S.E.M. (*=p<0.05 compared to vehicle group, ^=p<0.05 compared to ethanol group, #=p<0.05 compared to same exposure group gavage 1)

TABLE 1

Experiment	Figure	CTA Doses	Ataxia Doses	Activity?
1	1A, 2A	2 g/kg	2 g/kg	No
2	1B, 2B	1 g/kg	3 g/kg	No
3 & 4	1C, 2C, 3A, 3B	2 g/kg + 3 mg/kg	2 g/kg + 3 mg/kg	Yes

Ethanol doses in g/kg; caffeine doses in mg/kg