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Caffeine Alters Diurnal Variation in Ethanol-Induced Ataxia in Mice

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Abstract

It is common among American youth to mix alcohol (ethanol) with caffeine, which is associated with high dose/ high frequency drinking patterns and much greater health risks than alcohol alone. However, few studies to date have investigated the neurobiology of drugs used in combination. Growing evidence suggests that disruption of circadian (daily, 24 h) rhythms is key to the pathophysiology of addiction, yet the underlying mechanisms are poorly understood. Several key determinants of vulnerability to alcohol addiction, such as sensitivity to intoxication, reward responsiveness, and even drinking itself, vary diurnally. Here, we investigated the effect of caffeine on ethanol-induced ataxia in mice at six 4 h intervals over the 24 h photocycle. We observed a distinct diurnal variation in ataxia caused by ethanol (1.5 g/kg), with a nadir in sensitivity occurring early in the daytime (rest-phase). Co-administration of caffeine (7.5 mg/kg) advanced this diurnal pattern and surprisingly, abolished the nadir (e.g. increased sensitivity to ethanol early in the day). Our data support the notion that there may be a circadian rhythm in ethanol-induced motor incoordination and demonstrate that the effect of caffeine on this measure is dependent upon the time of day the two drugs are administered.

Keywords: Alcohol; Ethanol; Caffeine; Circadian; Diurnal; Ataxia; Motor incoordination

Introduction

Alcohol use disorders are among the most burdensome categories of neuropsychiatric diseases in the world, second only to unipolar depression [1]. However, development of effective therapies is complicated by the fact that many individuals are polydrug users. It is especially common among youth in the U.S. to mix alcohol (ethanol) with caffeine. O'Brien et al. [2] estimate that ~25% of college student drinkers mix alcohol and energy drinks, and the prevalence of caffeinated alcoholic beverage (CAB) consumption among 13-20 year-olds is 52.4% [3]. Compared to alcohol alone, CAB drinking is associated with increases in alcohol consumption, binge drinking, risky behavior, injury and hospitalization, withdrawal, and severity of alcohol dependence [4-8]. Moreover, it is well established that these high dose/high frequency drinking patterns, as well as early exposure, are risk factors for long-term dependence [9].

There is now much evidence to suggest that disruption of circadian (daily, ~24 h) rhythms is central to the pathophysiology of addiction. Circadian rhythms are regulated by the suprachiasmatic nucleus (SCN), a neural clock whose timing (phase) is determined by oscillatory gene expression and synchronized daily to the environment by photic (light) and nonphotic (behavioral) stimuli. Ethanol blunts circadian expression of Period genes in the brain and periphery [10-12], and nearly all clock genes have been associated with alcohol use disorders or animal models thereof [13]. Ethanol also shortens free-running (endogenous) circadian period [14], inhibits circadian behavioral responses (phase-resetting) to photic and nonphotic cues [15-18], and disrupts sleep [19]. Ethanol removal after chronic drinking does not normalize behavior. Rather, persistent chronodisruption in abstinent alcoholics often precipitates self-medication with ethanol for its sedative properties and leads to relapse [20]. Ethanol deprivation in rodent models has also been shown to induce maladaptive hypersensitivity to the phase-resetting effects of light [17]. Caffeine also has adverse effects on circadian rhythms and sleep. Caffeine lengthens circadian period [21] and attenuates nonphotic phase-resetting in rodents [22]. Habitual caffeine use in humans impairs sleep and causes rebound sleepiness that reinforces caffeine intake for its alerting properties [23].

It is well accepted that sensitivity to the subjective effects of acute ethanol influences ethanol consumption. Combining caffeine or other stimulants with ethanol is thought to reflect an attempt to increase sensitivity to the pleasurable effects (e.g. reward) and/or reduce sensitivity to the unwanted effects (e.g., sedation) of ethanol intoxication. Interestingly, many sensitivity measures vary diurnally, including ethanol-induced loss of righting reflex [24], natural- and drug-reward-seeking behavior [25], and even drinking itself [15,17]. Thus, it is possible that caffeine may not only alter ethanol sensitivity at a single time point, but may also alter the circadian pattern in such behaviors as well. In the present study, we sought to determine whether diurnal variation in ethanol-induced ataxia exists in mice, and if so, whether caffeine alters this rhythm.

Materials and Methods

Animals

C57BL6/J mice were purchased from The Jackson Laboratory and used for experiments between the ages of 8-24 weeks. Mice were housed in standard Plexiglas cages with food and water available ad libitum. The chamber was maintained on 12 h light and 12 h dark cycle (LD). Animal care/handling and experimental procedures were approved by the Indiana University of Pennsylvania IACUC according to NIH guidelines.

Ataxia

Ethanol-induced ataxia (motor incoordination) was evaluated using a constant velocity rotarod treadmill (UGO Basile, Varese, Italy) at a fixed speed of 20 rpm. Mice were preselected by their ability to remain on the rotarod for 180 s. On the day of the experiment, mice received either caffeine (7.5 mg/kg, i.p.) or ethanol (1.5 g/kg, i.p.) in a single injection (experimental drug) or ethanol alone (1.5 g/kg, i.p.; control drug). The doses of each drug were chosen based upon their translational relevance as moderate doses [26]. Rotarod performance was evaluated by measuring their latency to fall beginning 15 min after the injection and during sequential 15 min intervals for 1 h. To examine diurnal variation of ethanol-induced ataxia (control), as well as caffeine's effects on diurnal variability in this measure (experimental), experiments took place at 6 different time points at 4 h intervals throughout the 24 h cycle (referred to as Zeitgeber Time, or ZT, 2, 6, 10, 14, 18, and 22, where ZT12 is lights-off and ZT0 is lights-on). Experiments for time points that fell during the night took place under dim red light.

Statistical analysis

Data were analyzed using two-way ANOVA with factors of treatment and ZT (time of day), and appropriate post-hoc tests were used where interactions were found. Results were considered significant when $P < 0.05$. All data are expressed as mean ± SEM.

Results

Caffeine alters diurnal variation in ethanol-induced ataxia in mice

To determine whether diurnal variation in ethanol-induced ataxia was evident in mice and whether caffeine affected this measure, rotarod perfomance at each 15 min testing time after injection was compared between the two treatments at different times of day (ZT). At 15 min post-injection, there was a main effect of ZT ($F_{11,88}=2.756$, p=0.0041) and an interaction between ZT and treatment ($F_{11,88}=2.444$, p=0.0104). Posthoc testing showed that mice receiving caffeine + ethanol performed better on the rotarod at 15 min post-injection at ZT22 (p<0.05; Figure 1A), late in the night (active-phase for nocturnal rodents). At 30 min post-injection, an effect of ZT was also evident ($F_{11,88}=3.704$, p=0.0002), as was an interaction between ZT and treatment ($F_{11,88}=3.693$, p=0.0002). Posthoc testing confirmed that mice receiving ethanol alone were able to stay on the rotarod significantly longer than those receiving caffeine co-administration at ZT2 (p<0.01; Figure 1B), early in the day (rest-phase for nocturnal rodents). At 45 min post-injection, the same pattern was seen as for the 30 min testing time. There remained an effect of ZT ($F_{11,88}=2.057$, p=0.0320) and an interaction between ZT and treatment ($F_{11,88}=3.507$, p=0.0004) that was due to better performance among mice receiving ethanol alone compared to those receiving caffeine + ethanol at ZT2 (p<0.05; Figure 1C). Finally, there was a trend (p=0.0721) for a continued effect of ZT on rotarod performance at 60 min post-injection, and an interaction between ZT and treatment ($F_{11,88}=2.194$, p=0.0216) that was once again explained by differential performance between treatment groups at ZT2 (p<0.01; Figure 1D).
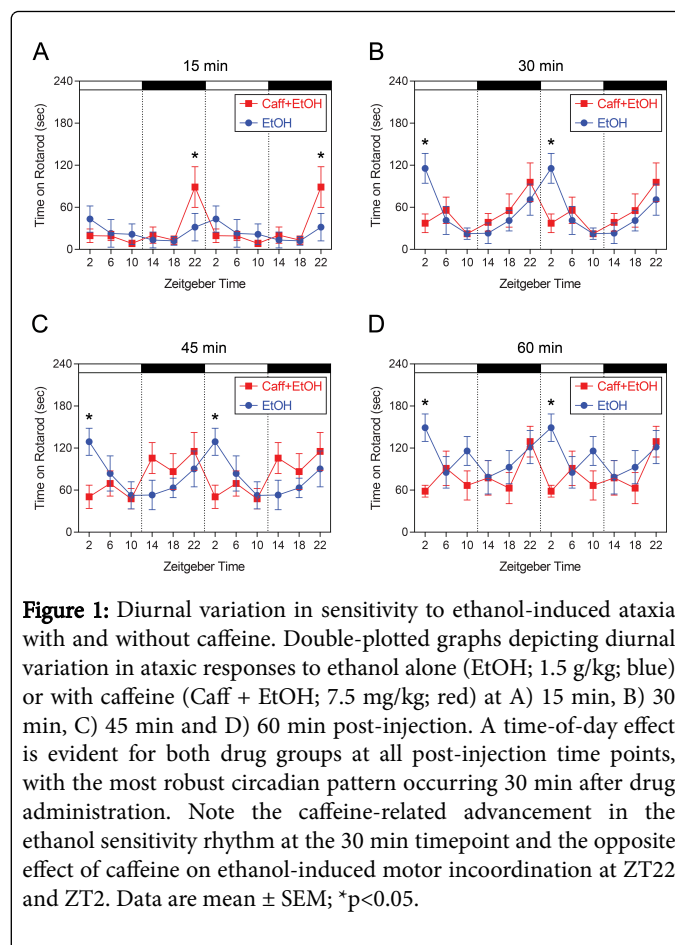
Figure 1: Diurnal variation in sensitivity to ethanol-induced ataxia with and without caffeine. Double-plotted graphs depicting diurnal variation in ataxic responses to ethanol alone (EtOH; 1.5 g/kg; blue) or with caffeine (Caff + EtOH; 7.5 mg/kg; red) at A) 15 min, B) 30 min, C) 45 min and D) 60 min post-injection. A time-of-day effect is evident for both drug groups at all post-injection time points, with the most robust circadian pattern occurring 30 min after drug administration. Note the caffeine-related advancement in the ethanol sensitivity rhythm at the 30 min timepoint and the opposite effect of caffeine on ethanol-induced motor incoordination at ZT22 and ZT2. Data are mean ± SEM; *p<0.05.

Caffeine increases sensitivity to ethanol-induced ataxia early in the rest-phase

Two-way ANOVA revealed main effects of time after injection ($F_{4,32}=41.50$, p<0.0001), treatment ($F_{1,8}=23.32$, p=0.0013) and an interaction between the two variables ($F_{4,32}=9.309$, p<0.0001) on ataxia in mice at ZT2, which is 2 h after lights-on (the beginning of the rest-phase for nocturnal rodents). Posthoc analysis confirmed that mice receiving ethanol and caffeine performed significantly worse on the rotarod compared to those receiving ethanol alone at 30, 45 and 60 min after receiving the injection (p<0.0001; Figure 2A). There was a significant main effect of time after injection at ZT6 ($F_{4,32}=24.83$, p<0.0001; Figure 2B), ZT10 ($F_{4,32}=69.51$, p<0.0001; Figure 2C), ZT14 ($F_{4,32}=45.53$, p<0.0001; Figure 2D), ZT18 ($F_{4,32}=31.84$, p<0.0001; Figure 2E), and ZT22 ($F_{4,32}=13.12$, p<0.0001; Figure 2F), indicating that mice were able to recover motor coordination over the course of the experiment. However, no treatment-related differences or interactions between variables were noted at these times of day.

Discussion

To our knowledge, the present study is the first to report diurnal variation in ethanol-induced ataxia in mice. Moreover, in contrast to the few other studies that have examined diurnal variability in ethanol sensitivity at 2-4 times of day (typically 1-2 in daytime and 1-2 at night), we assayed ethanol responses in sufficient resolution (4 h intervals over the 24 h photocycle) to show a detailed circadian

pattern. We are not, however, the first to suggest the existence of a circadian rhythm in ethanol responsivity. Deimling and Schnell investigated this possibility in Swiss-Webster mice and reported significant diurnal variation in ethanol-induced locomotor activity changes, hypothermic responses, and toxicity. These authors saw no daily fluctuations in hepatic alcohol dehydrogenase levels and an inverse correlation between sensitivity and ethanol levels in blood and brain at different times of day, suggesting that central sensitivity to ethanol, rather than temporal changes in ethanol metabolism or clearance, explain their findings [27]. Since we did not measure blood or brain ethanol levels in the present study, we cannot completely rule out circadian changes in ethanol metabolism. However, given that this possibility has been refuted in other studies [23,25], it seems highly unlikely to explain our results. It is noteworthy that our results are consistent with those of Deimling and Schnell, who showed that Swiss-Webster mice were most sensitive to ethanol during the dark phase, and those of Perreau-Lenz et al. [24], who showed that the highest sensitivity to ethanol-induced sedation occurred at ZT11 (just prior to the dark phase), while lower sensitivity was observed during the night-to-day transition (ZT23-ZT5) in wild-type mice [24]. Whether these observations reflect true, endogenous circadian rhythms in central ethanol sensitivity would require testing in constant darkness and/or constant light. The present study adds compelling data to support this as an important future direction for inquiry.

Although we saw a time-of-day effect at all post-injection time points, the diurnal ethanol sensitivity pattern was most pronounced (highest amplitude) at 30- and 45 min post-injection compared with either the 15- or 60 min time points. This likely reflects a differential influence of individual variability in ethanol metabolism on the 15- and 60 min time points. For example, there is little variability in rotarod performance at 15 min post-injection regardless of time of day, when ethanol would be at its highest levels compared to later testing times. On the other hand, there is more variability at 60 min post-injection, when variation in ethanol clearance among individuals would be most evident. So, while it is unlikely that ethanol metabolism is dependent upon time-of-day [24,27], individual differences in the rate of clearance may influence the diurnal pattern of rotarod performance to a lesser extent soon after ethanol administration and to a greater extent later. Our data indicate that 30 min post-injection may be optimal to observe a circadian rhythm and factors that may affect that rhythm in mice (e.g. other drugs).

The other important set of findings from the present study concerns the influence of caffeine on ethanol-induced ataxia. It is informative to consider these results from two different points of view, corresponding to the two ways in which the data are presented (Figures 1 and 2). The most consistent time-of-day related finding is that caffeine abolished the normal nadir in ethanol-induced ataxia (i.e., longest duration on the rotarod) at ZT2, which is clearly shown in both Figures. It is also evident from Figure 1 that caffeine disrupts the pattern of diurnal variability in ethanol response, appearing to advance this putative circadian rhythm by approximately 4 h. Furthermore, Figure 1 shows that caffeine co-administration had the opposite effect on rotarod performance at night, generally enhancing the ability of mice to remain on the rotarod longer (although this enhancement was only statistically significant at 15 min post-injection at ZT22). In this respect, our data expose the flaw in investigating pharmacological and other influences on ethanol sensitivity measures at only one time of day. If we had only performed this experiment at ZT2 (Figure 2A), we might have concluded that caffeine simply prevents recovery from ethanol's motor incoordinating effect. If we had only examined this

measure at any other time of day (Figures 2B-E), we would have erroneously concluded that caffeine is without effect, or may tend to improve rotarod performance during intoxication (ZT22; Figure 2F).
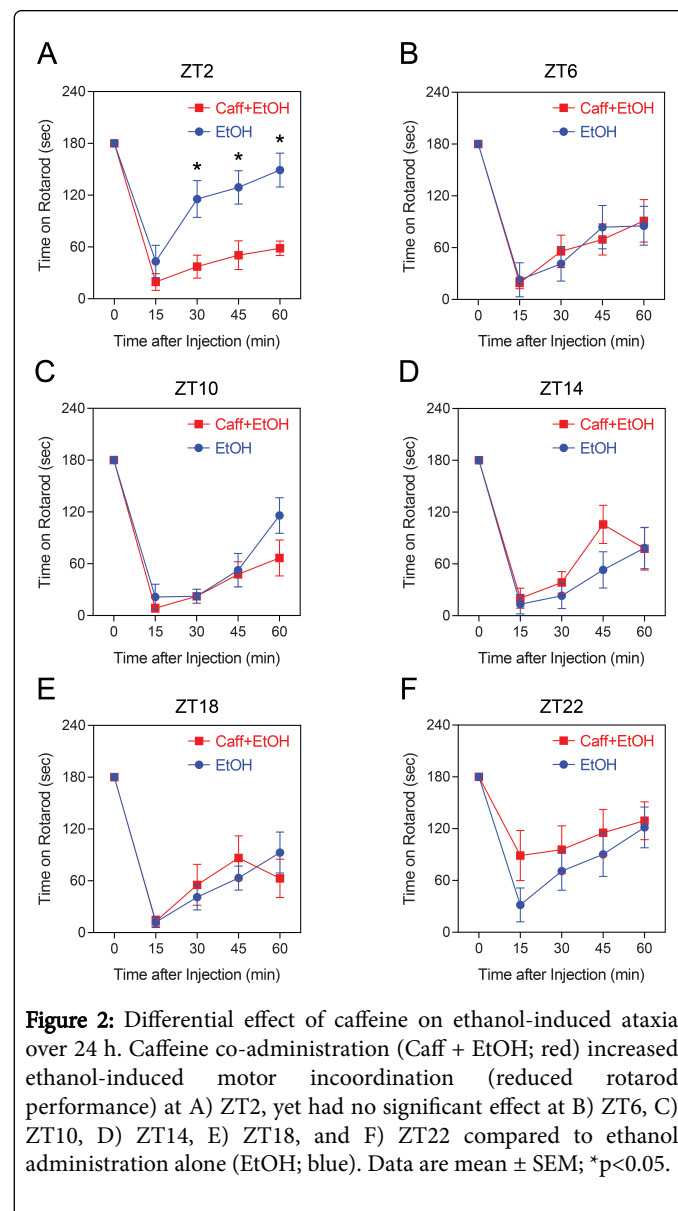
Figure 2: Differential effect of caffeine on ethanol-induced ataxia over 24 h. Caffeine co-administration (Caff + EtOH; red) increased ethanol-induced motor incoordination (reduced rotarod performance) at A) ZT2, yet had no significant effect at B) ZT6, C) ZT10, D) ZT14, E) ZT18, and F) ZT22 compared to ethanol administration alone (EtOH; blue). Data are mean ± SEM; *$p<0.05$.

Another interesting point to consider is the valence of change in ethanol sensitivity with co-administration of caffeine early in the day. Consumption of caffeinated alcoholic beverages is typically associated with increased drinking [5,28], driven in part by reductions in perceived, but not actual, intoxication [29]. Given that adenosine mediates ethanol-induced motor incoordination [30,31], and that caffeine is an adenosine A1/A2A receptor antagonist [32], we expected that caffeine would decrease sensitivity to ethanol-induced ataxia. However, caffeine did not change ethanol sensitivity most of the time, as reported in humans [29], and when it did, caffeine increased this measure. These counterintuitive results may involve the reciprocal relationship that appears to exist between adenosine signaling and circadian timing. Circadian variation has been reported in astrocytic ATP release (a source of adenosine), extracellular adenosine concentrations, and expression of A1 receptors and adenosine

transporter equilibrative nucleoside transporter 1 (ENT1) in wake-promoting areas of the brain [33-36]. Conversely, adenosine modulates cellular and behavioral circadian timing [22,31,37,38], and has a well-established role in promoting sleep. Thus, it is conceivable that daily fluctuations in adenosine levels, receptor numbers and/or activity may underlie time-of-day changes in ethanol sensitivity and caffeine's effects. Again, we cannot rule out an effect of caffeine on ethanol metabolism or diurnal variation therein, though other studies (e.g. [29]) suggest this is implausible. We plan to address this limitation and test the effect of additional doses of caffeine on ethanol-induced ataxia in future studies.

In summary, the present study reveals a marked diurnal variation in ethanol-induced ataxia and a pronounced effect of caffeine on this putative circadian rhythm. Our data expose the potential pitfall of relying upon ethanol sensitivity measures at only one time of day, and may explain some of the disparities between studies investigating caffeine and alcohol. Indeed, we provide evidence that differences in ethanol measures may be influenced more by time of day than by pharmacological manipulation, at least in the case of caffeine. These results may inform the selection of an appropriate time(s) to test ethanol-related motor incoordination in future studies. Finally, if caffeinated alcohol affects motor coordination with an analogous diurnal pattern in humans, it may be most dangerous to drink such beverages early in the night, at the time of day they are most likely to be consumed.

Acknowledgement

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