Sex differences in adolescent ethanol drinking to behavioral intoxication

Sara R. Westbrook^a, Minsu Kang^a, Luke K. Sherrill^a, Dylan O'Hearn^a, Tanya Krishnamani^a, and Joshua M. Gulley^{a,b}

^aDepartment of Psychology and ^bNeuroscience Program
University of Illinois at Urbana-Champaign

Corresponding author: Joshua M. Gulley, Ph.D., Department of Psychology and Neuroscience Program, University of Illinois, Urbana-Champaign, 731 Psychology Bldg MC-716, 603 E Daniel St, Champaign IL 61820 USA. Tel: 001 (217) 265-6413; Fax: 001 (217) 244-5876; E-mail: jgulley@illinois.edu

Abstract

Rodent models have been especially useful for investigating adolescent ethanol exposure. However, there is a paucity of studies examining sex differences in behavioral intoxication from adolescent ethanol drinking. Here, we used an ethanol drinking model to investigate if adolescent rats of both sexes readily drink ethanol to measurable behavioral intoxication, indicated by increased impulsive action and motor incoordination. Beginning on postnatal day (P) 28, male and female Long Evans rats were given 30-min access to a solution of sucrose (20%) or sweetened ethanol (20% sucrose + 15% ethanol) every other day until P60 and once after two weeks of forced abstinence (on P75). On alternate (non-drinking) days, rats were reinforced with a food pellet for making a cued nosepoke response. Beginning on P56, rats were tested in this task after drinking sessions to assess ethanol-induced changes in impulsive action, defined as premature responding prior to cue presentation. Motor coordination was assessed before and after drinking sessions using an incline plane test. Adolescent male and female rats readily consumed ethanol to behavioral intoxication, measured as reduced motor coordination. Following forced abstinence, females displayed greater ethanol-induced impulsive action. These studies provide evidence for sex differences in behavioral intoxication following adolescent ethanol drinking.

Keywords: ethanol; consumption; impulsive behavior; nose poke; rat

Alcohol (ethanol) is one of the most commonly abused drugs worldwide. Ethanol use during adolescence, which is the transitional period between childhood and adulthood, is associated with an increased risk for developing alcohol use disorders (AUDs) later in life (Dawson et al., 2008; Grant & Dawson, 1997) possibly due to heightened sensitivity of the still developing adolescent brain to alcohol-induced changes in behavioral and neural function (Gulley & Juraska, 2013; Squeglia et al., 2009). In adulthood, males have a higher prevalence of alcohol use disorders (Hasin, Stinson, Ogburn, & Grant, 2007) and tend to exhibit greater behavioral impairments when intoxicated compared to females (Fillmore & Weafer, 2004; Weafer & Fillmore, 2012b). These sex differences in ethanol-induced behavioral intoxication have been hypothesized to emerge during adolescence (Witt, 2007), when ethanol use is typically initiated. Studies performed in laboratory rodents have been particularly important for addressing this hypothesis.

In rats, adolescence has been conservatively defined as beginning around postnatal day (P) 28 and extending as late as P60 (Spear, 2000; Brenhouse and Andersen, 2011). This definition is based partly on the rise of pubertal hormones, which leads to vaginal opening in female rats on or near P35 (range: P29-P37; Castellano et al, 2011) and preputial separation in male rats on or near P45 (range: P39-P47; Korenbrot, Huhtaniemi, & Weiner, 1977). Rodent adolescence coincides with gains in cognitive functioning, including impulse control (Andrzejewski et al., 2011; Hammerslag et al., 2017), and such gains are also seen in humans (Charles et al., 2016; Shulman, Harden, Chein, & Steinberg,

2015; Steinberg et al., 2008). When rats are exposed to alcohol during adolescence, there is evidence for significant damage in the prefrontal cortex (Pelicaio et al., 2016). This brain region is implicated in impulse control (Simon et al., 2013) and develops across adolescence in a sex-dependent fashion (Drzewiecki et al. 2014; Markham et al., 2007; Willing & Juraska, 2015).

Sex differences are evident in adolescent ethanol drinking with females consuming more ethanol than males (Morales et al., 2014; Tambour et al., 2008). These differences in consumption are diminished under conditions that increase ethanol intake, such as alcohol deprivation during forced abstinence (Tambour et al., 2008). It is currently unclear whether these sex differences in consumption are associated with sex differences in behavioral measures of intoxication, including impulsive action and coordination. Furthermore, as alcohol use disorder is a chronic relapsing disorder, the behaviorally intoxicating effects of alcohol may change following periods of abstinence. Sex differences in these effects after abstinence may contribute to the sex differences in prevalence of AUDs (Hasin et al., 2007). However, analyses of adolescent sex differences in behavioral intoxication following forced abstinence from sweetened ethanol drinking have not been reported.

The goal of the current study was to investigate sex differences in adolescent ethanol consumption using a paradigm that leads to measurable behavioral intoxication. Every other day for the duration of the adolescent period, male and female Long-Evans rats were given limited access to sipper tubes that contained solutions of sucrose or sucrose plus ethanol. At the end of the

adolescent period, tests of impulsive action and motor coordination immediately followed drinking sessions in order to assess behavioral intoxication.

Furthermore, ethanol consumption and behavioral intoxication were measured following two weeks of forced abstinence to determine sex differences in the alcohol deprivation effect (Heyser, Schulteis, & Koob, 1997). We hypothesized that adolescent rats would readily drink ethanol to intoxication, in line with previous reports (Morales et al., 2014; Vetter-O'Hagen et al., 2009), and that this intoxication would be measurable as increased impulsive action and decreased motor coordination. In addition, we expected these effects to be more pronounced in females compared to males given studies showing females consume more ethanol than males (Morales et al., 2014), with alcohol deprivation attenuating these sex differences (Tambour et al., 2008).

Materials and Methods

Subjects

The present experiments used a total of 52 male and female Long-Evans rats, which were the offspring of unrelated males and females purchased from Harlan Labs (Indianapolis, IN) and bred in our animal facility. Rats were weaned on P22 and housed in same-sex groups of 2-3 littermates/cage. They were maintained in a temperature controlled room on a 12 hr light/dark cycle (lights on at 0800 h). Our rats were not food- or water-deprived, but rather had food and water available *ad libitum* throughout the experiment. We have previously used a similar approach in studies using adolescent rats, which have high caloric needs

during this period of rapid growth, and have found they are sufficiently motivated for performing food-reinforced instrumental behavior tasks (Hammerslag et al., 2017; Westbrook et al., *in press*). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana-Champaign, and were consistent with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Ethanol drinking sessions

Drinking sessions began on P28 and continued every other day until P60. After two weeks of forced abstinence, a final drinking session occurred on P75. For each of the drinking sessions, rats were moved from their home colony to a testing room in groups of 8-12 animals. After a brief acclimation period (<10 min), they were placed individually into clear plastic rectangular containers (43 cm x 22 cm x 20 cm) that were similar to their home cages except the wire lid did not contain their normal lab chow and water bottles. Instead, three sipper tubes, each containing 5 ml of fluid, were attached to the lid. Rats in the control groups (n = 15 males and 12 females) were given 30 min of access to 20% sucrose and the ethanol groups (n = 13 males and 12 females) were given 30 min of access to 20% sucrose + 15% (w/v) ethanol.

Impulsive action

On non-drinking days beginning P29, rats were trained in standard operant chambers (Coulbourn Instruments; Allentown, PA, USA) on a cued response task to measure impulsive action. For the first three sessions animals were given magazine training wherein 45 mg food pellets (Bioserv, #F0021;

Frenchtown, NJ) were delivered at a rate of 2.5 pellets/min. Each magazine training session was 40 min in duration. For the next 11 sessions, animals were trained to nosepoke into a recessed port at the rear panel of the chamber on a continuous reinforcement schedule. Each session began with a 5-sec intertrial interval (ITI), followed by a 20-sec illumination of the nosepoke port. A nosepoke during the port illumination period extinguished the port light and led to delivery of a food pellet into the illuminated food bin. The next trial did not begin until rats retrieved the food pellet indicated by a photobeam break inside the food bin. The light in the food bin remained illuminated for up to 10 sec. Nosepoke responses into the rear port during the ITI were counted as premature responses and restarted the 5-sec ITI. Failure to make a nosepoke within the 20-sec illumination period was scored as an omission. Each session was terminated upon the completion of 100 trials or after 30 min.

Rats next underwent three test sessions on P56, P58, and P60 that were identical to the training sessions except they occurred approximately 10 min after rats finished their drinking sessions (following assessment of motor coordination described below). On P75 following a 14-day forced abstinence period (P61-74) during which rats remained in their homecages, a final test session was performed.

Motor coordination

Motor coordination was assessed in an incline plane test using procedures adapted from White et al. (2002) and originally described by Arvola et al. (1958). Briefly, each test consisted of three pre-drinking and three post-drinking trials.

For each trial, rats were placed individually on the upper part of a custom built clear plastic box (60 cm x 20 cm x 45 cm) that had a hinged floor. The plane was raised slowly (approximately 10 degrees/sec), from a 20 degree starting point, to a maximum angle of 70 degrees. The mean slip angle for the three trials, which were administered in quick succession, was measured for each rat immediately before and after drinking sessions on P58, P60, and P75.

Data analysis

Fluid intake was calculated by weighing the drinking tubes before and after each 30-min drinking session. Ethanol (g/kg) and 20% sucrose (g/kg) consumption was analyzed using separate two-way repeated measures ANOVAs with sex (male or female) as the between-subjects factors and age (P28-P60) as the repeated measure. Pre-planned comparison of consumption on P75, which followed two weeks of forced abstinence, was analyzed using independent samples Satterthwaite t-tests for unequal variances.

Changes in motor coordination following voluntary drinking sessions were assessed using the mean slip angles for each rat during the pre-drinking and post-drinking trials. The percent change in slip angle after drinking was analyzed using separate two-way repeated measures ANOVAs for males and females with group (control or ethanol drinking) as the between-subjects factors and session (P58, P60 or P75) as the repeated measure. Additionally, two-way ANCOVA was conducted to examine the relationship between ethanol consumption and percent change in slip angle for each test day (P58, 60, and 75), with ethanol consumption as the continuous variable and sex as the between-subjects factor.

Linear regressions using the false discovery rate (FDR) controlling procedures (less than 5%; Benjamini & Hochberg, 1995) were used to analyze the relationship between ethanol consumption and percent change in slip angle on each test day. Data for one female in the control group and one female in the ethanol drinking group were removed from this analysis because the rats would not remain in the apparatus long enough to complete testing.

Premature responding during the impulsive action task was analyzed using three-way repeated measures ANOVA with treatment and sex as between subject factors and session as the repeated measure. Planned separate two-way (treatment x session) repeated measures ANOVAs for males and females were also conducted. Baseline performance was calculated as mean responses on the last three days of training (P51, 53, and 55). Separate two-way ANCOVAs were conducted on premature responding on the test days (P58, 60, and 75) with ethanol consumption on these days included as the covariate and sex as the between-subjects factor. Linear regressions using FDR were used to examine the association between ethanol consumption and premature responding on these test days. Data for one female in the ethanol drinking group were excluded in these analyses because the rat failed to acquire the task.

Results

Rats' intake during intermittent drinking sessions is shown in Figure 1. Consistent with previous studies, a sex difference in ethanol consumption was observed. Females drank significantly more ethanol per body weight than males

on P28-60 (Fig. 1A). Two-way ANOVA of ethanol consumption data revealed a main effect of sex [$F_{1,23} = 4.74$, $p = 0.040$] and session [$F_{16,368} = 2.06$, $p = 0.0094$] but no significant interaction [sex x session, $p > 0.81$]. Tukey post-hoc analyses of the main effect of session revealed no statistically significant differences (all p 's > 0.09). The sex difference in ethanol consumption was still evident in the drinking session on P75, following two-weeks of forced abstinence [Satterthwaite $t_{15.5} = 2.88$, $p = 0.011$], with a much wider range of ethanol intake in females (0.1-3.5 g/kg) compared to males (0.4-1.9 g/kg). In fact, during this drinking session the sex difference was more pronounced compared to the sessions occurring prior to abstinence. In contrast to animals given ethanol access, there were no significant sex differences in consumption of 20% sucrose solution in the control groups from P28-60 (Fig. 1B) or after two weeks of forced abstinence on P75 [Satterthwaite $t_{16.2} = 1.75$, $p > 0.09$]. Consumption of sucrose solution was significantly less during the first session on P28 than all subsequent sessions [session: $F_{16,400} = 4.64$, $p < 0.0001$, post-hoc p 's < 0.05].

Ethanol-induced motor impairment was assessed on the incline plane by measuring the percent change in slip angle after drinking relative to the slip angle measured prior to drinking. Three-way ANOVA revealed significant main effects of sex [$F_{1,46} = 4.66$, $p = 0.036$], treatment [$F_{1,46} = 42.40$, $p < 0.0001$], and session [$F_{2,46} = 4.20$, $p = 0.021$]. Males and ethanol rats displayed greater impairments than their respective counterparts, with the effects being most pronounced on P60 (Fig. 2). Analysis of the percent change in slip angle using separate two-way ANOVAs for each sex revealed a significant session x treatment interaction in

males [$F_{2,25} = 3.51$, $p = 0.045$]. The interaction was largely driven by the deficit observed in ethanol rats on P60. Females in the ethanol group also showed a lower slip angle after drinking sessions compared to control females [main effect of treatment: $F_{1,21} = 13.16$, $p = 0.0016$], but this effect did not differ significantly across testing. Thus, both males and females displayed deficits in motor control following ethanol drinking and this impairment tended not to vary robustly with repeated exposure or forced abstinence. ANCOVA analyses of percent change in slip angle on P58, P60 and P75 revealed no significant effects of sex, ethanol consumption, or their interaction. In addition, there were no significant associations between ethanol consumption and percent change in slip angle on P58, P60 and P75 for either sex alone or combined (data not shown).

Rats' performance on the impulsive action task during baseline (P51, 53, and 55) and following ethanol drinking sessions (P56, 58, 60, and 75) is shown in Figure 3. Three-way ANOVA of premature responses revealed a significant treatment by session interaction [$F_{4,47} = 5.08$, $p = 0.0017$], indicating that control rats reduced their premature responses on P60 relative to baseline. Planned two-way ANOVAs within each sex indicated that the number of premature responses did not change significantly across sessions in males. In contrast, females displayed a significant increase in premature responses following the forced abstinence period during the P75 session [session x treatment interaction: $F_{4,88} = 4.25$, $p = 0.0034$]. The ANCOVA of P60 premature responding revealed no significant effects of sex, ethanol consumption, or their interaction (Fig. 3B). Two-way ANCOVA of P75 premature responding revealed a significant main effect of

dose consumed [$F_{1,21} = 9.08$, $p = 0.0066$], such that the greater the ethanol dose consumed, the greater the premature responding. However, the ANCOVA revealed no significant effect of sex or its interaction with ethanol consumption. The significant linear regression (adjusted $r^2 = 0.41$, FDR adjusted $p = 0.009$) collapsed across sex is shown in Fig. 3C. There were no discernible effects of ethanol drinking on the latencies to nosepoke or retrieve food pellets on the opposite side of the chamber (data not shown; all p 's > 0.05). Instances of pellet retrieval after more than 10 s reduced rapidly across training [session: $F_{14,48} = 38.76$, $p < 0.0001$], such that delayed pellet retrieval was very uncommon during the testing period (data not shown).

Discussion

The goal of this study was to examine sex differences in adolescent ethanol drinking to behavioral intoxication. We found that both male and female adolescent Long-Evans rats readily consumed a sweetened ethanol solution, with females drinking significantly more than males. Although a previous report indicated that ethanol solutions sweetened with sucrose led to lower blood ethanol concentrations than unsweetened ethanol (Roberts et al., 1999), we were still able to detect behavioral evidence of intoxication following sweetened ethanol consumption in both males and females, with the latter exhibiting impairments in both the impulsive action and inclined plane tasks. This report provides evidence for sex-dependent differences in adolescent ethanol drinking and behavioral intoxication.

During the 30-min drinking sessions, rats consumed approximately 1.5-2 g/kg ethanol on average, and females consumed significantly more ethanol per body weight than males during adolescence (P28-60) as well as on P75 following a two-week forced abstinence. An alcohol deprivation effect, wherein ethanol intake increases following forced abstinence (Heyser, Schulteis, & Koob, 1997), was not apparent in our adolescent rats who drank sweetened ethanol. This lack of an alcohol deprivation effect is consistent with a previous study where adolescent rats drank unadulterated ethanol (Schramm-Sapota, Francis, MacDonald, Keistler, O'Neill, & Kuhn, 2014), which suggests that this phenomenon is only robust in adult-onset animals. However, the range of the individual adolescent female ethanol consumption on P75 may suggest that some adolescent females are sensitive to the alcohol deprivation effect. Unfortunately, examination of high and low drinkers within sex (Schramm-Sapota et al., 2014) in the present study would result in sample sizes too low for meaningful analyses. Future studies are needed to examine individual differences in the alcohol deprivation effect in adolescent females. Our results regarding sex differences are consistent with previous studies reporting a sex difference in ethanol consumption in adolescent rats (Morales et al., 2014) and mice (Tambour et al., 2008). On the other hand, there was no sex difference in the control groups that were given access to 20% sucrose solution.

One factor that may contribute to sex differences in ethanol intake is the increase in testosterone levels in males relative to females. Testosterone has been shown to decrease voluntary ethanol consumption in rats. For instance,

male rats that received a gonadectomy before puberty showed increased ethanol consumption during adulthood (Sherrill et al., 2011; Vetter-O'Hagen & Spear, 2011), while females that received ovariectomy did not significantly change ethanol consumption (Vetter-O'Hagen & Spear, 2011). In addition, gonadectomized male rats that received testosterone replacement showed a significant reduction in voluntary ethanol consumption to similar levels as sham-gonadectomized rats (Vetter-O'Hagen, Sanders, & Spear, 2011). These findings suggest that testosterone may be a contributing factor to sex differences in voluntary ethanol consumption by reducing ethanol consumption in male rats.

The sex differences we found in ethanol intake and subsequent behavioral intoxication may not be fully explained by the level of ethanol in the blood, as some studies have reported a dissociation between sex differences in ethanol consumption and measurements of blood ethanol concentrations (BECs) achieved (Vetter-O'Hagen & Spear, 2011; Vetter-O'Hagen, Varlinskaya, & Spear, 2009). Given recent evidence of opposing effects of restraint stress on subsequent ethanol intake in male and female adolescent rats (Wille-Bille et al., 2017), and the fact that blood collection for BEC analysis requires restraint, we chose not to measure BECs in the current study. Instead, we assessed sex differences in ethanol intake and intoxication by examining ethanol-induced alterations in motor behaviors, a presumably less stressful procedure.

To assess behavioral intoxication following ethanol drinking sessions, animals were trained in an impulsive action task. Whereas male rats did not show any significant changes in the number of premature responses across

testing, females showed a significant increase in premature responses only following resumption of ethanol drinking after 2 weeks of forced abstinence. However, the positive relationship between ethanol consumption and premature responding on P75 did not depend on sex, suggesting that the difference in impulsive action on P75 was not due to sex, per se, but rather the level of consumption. It is likely that if males drank ethanol to levels comparable to females on P75, they would also show increased impulsive action. Nonetheless, our results show that following abstinence females more readily drank ethanol to levels that led to increased impulsive action.

The increase in impulsive action was only evident following abstinence, which may suggest that adaptations occur during abstinence that may lead to this change in the behavioral response to ethanol. Abstinence from chronic intermittent ethanol exposure in adult male rats leads to neural (Irimia, Buczynski, Natividad, Laredo, Avalos, & Parsons, 2017) and behavioral adaptations (Irimia, Tuong, Quach, & Parsons, 2014; Irimia et al., 2017), including increased premature responding, without re-exposure to ethanol. In the present study, we measured premature responding after resumption of ethanol drinking following abstinence, but did not measure premature responding during abstinence. Further investigations are needed to determine the contribution of behavioral adaptations during abstinence and behavioral adaptations in response to subsequent ethanol re-exposure to the increased premature responding found in the present study.

Ethanol did not seem to affect motivation to respond as there was no significant effect of ethanol on nosepoke latency or pellet retrieval latency in the impulsive action task. Previous findings regarding the effect of ethanol on reaction time in rats have shown the changes tend to be in the range of 100-200 ms from baseline (Slawecki, 2006). Since the time window available for nosepoke responses used here was 20 sec, our rats were not required to make a rapid response as they were in other studies (Slawecki, 2006). Furthermore, animals used here were not food or water-deprived and thus may have been relatively less motivated to fully engage in the task compared to studies where deprivation was utilized (Givens, 1997).

In addition to the impulsive action task, we assessed ethanol-induced impairment in motor coordination with an inclined plane test. All rats that consumed ethanol, regardless of sex, exhibited impaired motor coordination on the inclined plane test on all test days, shown as sliding at a lower inclination compared to the control animals that were given access to 20% sucrose solution. Both male and female rats were affected by ethanol, with males exhibiting a relatively greater degree of impairment. This finding is consistent with a previous study demonstrating that males were more sensitive to ethanol-induced ataxia than females in a rotarod test (Tayyabkhan et al., 2002). Interestingly, a recent report highlighted an age-dependent effect of ethanol on motor coordination where aged rats were more sensitive to the motor-impairing effect of injected ethanol (1-3 g/kg, i.p.) compared to adolescents and young adults (Ornelas et al., 2015). Although our study did not include adult or aged animals, it would be

interesting to investigate age differences in sensitivity to the motor deficits of behavioral intoxication following ethanol drinking.

Overall, the present study demonstrates that adolescent male and female rats readily drink sweetened ethanol solution to levels that produce measurable behavioral intoxication. In our paradigm, this intoxication was evident and robust in our measures of motor coordination across testing. Behavioral intoxication was not evident, however, in our impulsive action task except following abstinence. Furthermore, males exhibited greater motor incoordination than females while females displayed more impulsive action following abstinence. Collectively, our findings indicate task- and sex-specific effects of ethanol on behavioral intoxication. Future studies should consider these factors and others in their investigations of ethanol's behavioral effects. Additionally, it is important to note that our intoxication measurements likely occurred during the rising phase of the blood-alcohol function. Future investigations should examine these behavioral intoxication measurements during the descending limb, as humans have shown acute tolerance to the motor incoordinating effects of alcohol but persistent impairment in inhibitory control (Weafer & Fillmore, 2012a). Although there appears to be opposing, species-specific sex differences – adult men consume more ethanol than women (Erol & Karpyak, 2015) but female rodents display greater ethanol intake than males – our results suggest that sex differences in ethanol drinking and subsequent behavioral intoxication emerge as early as adolescence.

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Figure legends.

Figure 1. Oral consumption of sweetened ethanol (**A**) or sucrose (**B**) solutions in males and females (n= 12-15/group). Data are expressed as mean ($\pm$ SEM) consumption of ethanol (EtOH; in g/kg) or sucrose (in g/kg) during 30-min drinking sessions. On the test following two weeks forced abstinence (P75), the bar indicates the mean ($\pm$ SEM) and symbols represent individual animal data. $^{\#}p < 0.05$, males vs. females collapsed across sessions; $^{*}p < 0.05$, vs. males on P75

Figure 2. Percent change ($\pm$ SEM) in slip angle after 30-min sessions of drinking 20% sucrose or 15% ethanol (EtOH) in 20% sucrose solution. Data were normalized for each rat to its mean slip angle measured before each drinking session. $^{\#}p < 0.05$, males vs. females collapsed across treatment and sessions; $^{*}p < 0.05$, EtOH vs. sucrose collapsed across sex and sessions; $^{**}p < 0.01$, vs. P75 within the male EtOH group

Figure 3. Premature responses on the impulsive action task in males and females (n= 12-15/group). (**A**) The mean number ($\pm$ SEM) of premature responses during the three baseline sessions (BL; P51, P53 and P55) and the four test sessions (between P56 and P75). Test sessions occurred 10 min after the conclusion of drinking sessions where rats in the control groups ingested 20% sucrose and those in the ethanol (EtOH) group ingested 15% (w/v) EtOH in 20% sucrose. $^{**}p < 0.01$, vs. control females within session (**B-C**) Ethanol

consumption did not predict subsequent premature responses on P60, but was positively correlated with subsequent premature responses on P75.

Figure 1

A

B

Figure 2

Figure 3