Adolescent intermittent ethanol exposure induces sex-dependent divergent changes in ethanol drinking and motor activity in adulthood in C57BL/6J mice

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Abstract

With alcohol readily accessible to adolescents, its consumption leads to many adverse effects, including impaired learning, attention, and motor behavior. Adolescents report higher rates of binge drinking compared to adults. Accompanying increased binge drinking rates, adolescents are more prone to substance use disorder (SUD) during adulthood due to physiological changes during the adolescent developmental period. We used C57BL/6J male and female mice to investigate the long-lasting impact of binge ethanol exposure during adolescence on voluntary ethanol intake and open field behavior during later adolescence and in young adulthood. The present set of experiments were divided into four stages: (1) chronic intermittent vapor inhalation exposure, (2) abstinence, (3) voluntary ethanol intake, and (4) open field behavioral testing. During adolescence, male and female mice were exposed to air or ethanol using an intermittent vapor inhalation model with repeated binge pattern ethanol exposure from postnatal day (PND) 28-42. Following this exposure, mice underwent abstinence from PND 43-49 (Experiment 1) and PND 43-69 (Experiment 2). Beginning on PND 49-76 (Experiment 1) or PND 70-97 (Experiment 2), mice were assessed for intermittent voluntary ethanol consumption using a two-bottle drinking procedure over 28 days. Male mice that were exposed to ethanol during adolescence showed increased ethanol consumption during later adolescence (Experiment 1) and in adulthood (Experiment 2), while the females showed decreased ethanol consumption. These data demonstrate a sexually divergent shift in ethanol consumption following binge ethanol exposure during adolescence and differences in open field and anxiety-like behavior. These data highlight sex-dependent vulnerability to developing substance use disorders in adulthood.

1. Introduction

The adolescent developmental period across mammalian species has been associated with various age-related physiological, hormonal, neuronal, cognitive, and behavioral changes during the transition from childhood to adulthood (for review see (Spear, 2000)). Behaviorally, adolescents show increases in social behavioral motivations, risk-taking, and novelty-seeking, which may contribute to the initiation and escalation of alcohol use during this time (Spear, 2016; Patrick et al. 2011). On average, adults drink more often than adolescents, however, both humans and rodent models have shown that per occasion adolescent alcohol consumption is much higher than that of adults (Doremus, Brunell, Rajendran, & Spear, 2005; SAMHSA, 2007).

Although various factors contribute to alcohol abuse during adolescence, early and repeatedly heavy alcohol consumption has been associated with increased propensity for elevated intake of alcohol in later life in both humans and rodents (for review see (Spear, 2016)). Consequently, episodic heavy or "*binge"* drinking has proven to be a major point of concern in understanding adolescent alcohol drinking behaviors for the past several decades (Johnston et al., 2019). Further, binge drinking during adolescence leads to higher rates of substance use disorder symptoms by the age of 35, including dependence on alcohol, abuse, disease, and death (Patrick et al., 2011).

When studying the long-term effects of adolescent alcohol behaviors on adulthood alcohol consumption, animal models not only mitigate the ethical implications of using humans, but also control for genetic and environmental influences (Ehlers et al., 2013). In previous rodent studies, the impact of adolescent intermittent ethanol (AIE) exposure on adult drinking patterns and behaviors has varied. AIE has regularly been shown to increase alcohol drinking, social anxiety, and loss of behavioral flexibility in adulthood in rats (Crews et al. 2019; Gilpin,

Karanikas, and Richardson 2012; Maldonado-Devincci et al. 2010; Maldonado-Devincci, Badanich, and Kirstein 2010), while others have found no change in alcohol consumption following adolescent ethanol exposure (Nentwig et al., 2019; Slawecki and Betancourt, 2002). Other studies in rats have reported long-term negative effects on cognitive flexibility, including impairments in spatial learning and completing aversive tasks (Acheson et al., 2013; Miller et al., 2017). These differences in previous work are likely due to varied experimental exposure patterns, caging methods, and /or administration procedures of alcohol. Several models of AIE exposure have been used in both rats and mice, including voluntary consumption in genetically-bred mouse and rat strains, forced gavage exposure, intermittent intraperitoneal injections, forced ethanol consumption with only one bottle of ethanol presented, drinking in the dark, etc. (Acheson et al., 2013; Crews et al., 2019; Gilpin et al., 2012; Maldonado-Devincci et al., 2010a; Miller et al., 2017; Slawecki and Betancourt, 2002; Thiele and Navarro, 2014).

Another method that has recently been used for AIE exposure is intermittent vapor ethanol exposure (Barker et al., 2017; Centanni et al., 2017; Gass et al., 2014; Nentwig et al., 2019; Trantham-Davidson et al., 2017). This method exposes mice to two days of intermittent vapor inhalation to maintain high and elevated blood ethanol concentrations (BEC) (Gass et al., 2014). These studies in rats have found that AIE alters PFC-related decision making and behavioral control, but not ethanol-mediated behavior in adulthood (Centanni et al., 2017; Gass et al., 2014; Nentwig et al., 2019; Trantham-Davidson et al., 2017). Other studies using moderate to high chronic vapor ethanol exposure in male rats (e.g., BECs between 150-225 mg/dl) have shown hypoactivity in locomotion tests, increased anxiety-like behavior in the open field test, depressive-like behavior and lower body weight following exposure (Ehlers et al., 2013; Slawecki et al., 2004; Walker et al., 2010).

The present experiment was designed to use a model of binge ethanol exposure using the vapor inhalation exposure paradigm during adolescence to examine sex differences in the long-term consequences in voluntary unsweetened ethanol intake in adulthood in male and female mice. We exposed adolescent C57BL/6J mice to adolescent intermittent ethanol (AIE) vapor exposure and measured intermittent voluntary ethanol intake in adulthood using a two-bottle choice paradigm, followed by measuring open field behavior. We expected adolescent intermittent ethanol exposure to increase voluntary ethanol intake in adulthood in both males and females and for females to consume more ethanol compared to males.

2. Methods

2.1 Subjects

Animals: Adolescent male and female C57BL/6J mice (n=10-13/group) were obtained from Jackson Laboratories (Bar Harbor, ME) on PND 21. All mice were allowed to acclimate to the colony for one week (PND 21-27) prior to experimentation, where they were handled daily to allow for acclimation to experimenter manipulation. Animals were PND 28 of age at the beginning of the experiment. Animals were group-housed (4-5 per cage) with free access to food water throughout the AIE exposure. Beginning at PND 47 (Experiment 1) or PND 68 (Experiment 2), mice were individually housed for intermittent two-bottle choice voluntary ethanol intake (described below). Food and water were available ad libitum throughout the experiment. All mice were maintained in a temperature and humidity-controlled room with lights on from 0700-1900 hr. Body weights were recorded daily during adolescence and weekly during adulthood. Animal care followed National Institutes of Health Guidelines under North Carolina Agricultural and Technical State University Institutional Animal Care and Use Committee approved protocols.

2.2.1. Chronic Intermittent Vapor Inhalation Chamber Exposure

Mice were exposed to repeated intermittent ethanol or air vapor for four two-day cycles from PND 28-42 for both experiments. Mice were exposed to ethanol vapor or air using modified protocols from Gass and colleagues (2014). On PND 28-29, 32-33, 36-37, 40-41, at approximately 1630 hr, mice were weighed and administered an intraperitoneal injection (0.02 ml/g) of the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg) combined with saline for control mice or combined with 1.6 g/kg ethanol (8% w/v) for ethanol-exposed mice and immediately placed in the inhalation chambers (23 in x 23 in x 13 in; Plas Labs, Lansing, MI). Mice remained in the vapor inhalation chambers for 16 hr overnight with room air (control group) or volatilized ethanol (ethanol group) delivered to the chambers at a rate of 10 liters/min. These procedures were designed to maintain blood ethanol concentrations (BECs) at 150-275 mg/dl throughout the exposure period. Ethanol (95%) was volatilized by passing air through an air stone (gas diffuser) submerged in ethanol. The following morning (on PND 29, 33, 37, and 41), at 0900 hr, mice were removed from the vapor inhalation chamber 50 μL of blood was collected from the submandibular space for BEC assessment. Mice were then returned to the home cage for 8 hr. On the evening of PND 29, 33, 37, and 41, mice were administered pyrazole and placed in the chamber overnight for 16 hours. On the morning of PND 30, 34, 38, and 42, mice were removed from the chambers and returned to the home cage. Mice remained undisturbed for two days between each cycle on PND 30-31, 34-35, 38-39. All blood samples were centrifuged at 5,000*g* and serum was collected and used to analyze blood ethanol concentrations using the AM1 blood alcohol analyzer (Analox Instruments, Lunenburg, MA).

2.2.2 Abstinence

For Experiment 1, mice were left undisturbed from PND 42-46 in the home cage, except for regular cage maintenance. For Experiment 2, mice were left undisturbed from PND 42-67 in the home cage, except for regular cage maintenance. On PND 47 (Experiment 1) or PND 68 (Experiment 2), mice were transferred to individual cages to allow for acclimation to the intermittent two-bottle choice voluntary ethanol intake procedures.

2.2.3 Voluntary Ethanol Intake

Beginning on PND 49 through PND 76 (Experiment 1) or PND 70 through PND 97 (Experiment 2), all mice were assessed for intermittent 24 hr voluntary ethanol intake using a two-bottle choice paradigm. Fresh tubes were presented to all mice daily. On PND 49 or PND 70, mice were introduced with two graduated sipper tubes containing water. Volumetric measurements were collected when tubes were introduced to the cages and on the following morning when tubes were removed. On PND 50 (Experiment 1) or PND 71 (Experiment 2), one water sipper tube was replaced with a tube containing 20% v/v unsweetened ethanol. Each two-day cycle was repeated for a total of 28 days, with intermittent presentation of the ethanol solution. Fresh tubes of water and/or ethanol were presented to all animals daily. Data were recorded daily for water and ethanol intake across the 28 days of intermittent ethanol access (PND 49-76- Experiment 1 or PND 70-97- Experiment 2).

The side of presentation of the ethanol and water sipper tubes were alternated to avoid the development of a side preference. Volumes were recorded to the nearest 0.1 ml. The difference in volume indicated the amount of ethanol consumed, and data were presented as grams of ethanol consumed per kilogram of body weight (g/kg) for the 24 hr session. Spillage was accounted for by placing the ethanol and water tubes in a similar holding cage unoccupied by a mouse. The difference calculated between the presentation and removal of the tube from the

holding cage accounted for spillage and was subtracted from the daily difference calculated for each mouse. This procedure was repeated each day between 0900-1200 hr during the light cycle.

2.3 Open Field Test

Between PND 79-81 (Experiment 1) or PND 100-102 (Experiment 2), all mice were tested for a 60 min trial in the open field test. The animals were counterbalanced across groups for testing. Using a Plexiglas chamber (40.6 cm x 40.6 cm), mice were introduced to the open field testing arena (Kinder Scientific, Poway, CA) for 60 min to assess general exploratory behavior. Immediately upon removal from the arena, mice were returned to their home cage and returned to the colony. The open field arena was cleaned with 70% ethanol and allowed to completely dry before the next mouse was introduced. Data were quantified using Motor Monitoring Behavioral software (Kinder Scientific, Poway, CA).

2.4 Design and Analyses

Ethanol drinking and preference data were analyzed separately for males and females using a two-factor mixed-model design ANOVA with Exposure (2; Air, Control) as a between subjects factors and Day as a repeated measure. Given the innate sex difference in ethanol intake in the control animals, data were normalized as a percent of control with control animals set to zero and data for the ethanol-exposed mice expressed relative to zero. The Percent of Control data for the ethanol-exposed mice were analyzed using a two-factor mixed model ANOVA with Sex (2; Male, Female) as a between subjects variable and Day as a repeated measure. All open field data were analyzed using a two-factor mixed model ANOVA with Exposure (2; Air, Control) and Sex (2; Male, Female) as between subjects factors. In the presence of significant interactions, Tukey’s and Sidak’s multiple comparisons post hoc tests were used where appropriate.

3. Results

3.1 Blood Ethanol Concentrations and Weight

3.1.1 Experiment 1: Blood Ethanol Concentrations

There were no sex differences in blood ethanol concentrations (BECs) during any exposure cycle (Table 1). However, there were changes in BECs across cycles for both male and female ethanol-exposed mice as supported by a main effect of Cycle [$F(3, 43) = 21.39$, $p < 0.0001$] and a Cycle by Sex interaction [$F(3, 43) = 2.95$, $p < 0.05$]. For male ethanol-exposed mice, cycle 1 BECs were higher than cycle 3 ($p < 0.01$) and 4 ($p < 0.02$). Cycle 2 was higher than cycle 4 ($p < 0.05$). For female-exposed mice, cycle 1 BECs were higher than cycles, 3, and 4 (all $p < 0.05$). Cycle 3 was higher than cycle 4 ($p< 0.02$).

3.1.2 Experiment 1: Weight

When weight was analyzed as a three-factor mixed model ANOVA for Sex (2; Male, Female), Exposure (2; Air, Ethanol) and Day as a repeated measure, there was a significant Day by Sex interaction [$F(21, 798) = 9.24$, $p < 0.0001$] and Day by Exposure interaction [$F(21, 798) = 4.33$, $p < 0.0001$], and main effects of Sex [$F(1, 38) = 79.77$, $p < 0.0001$] and Day [$F(21, 798) = 783.3$, $p < 0.001$. Additionally, there was a trend for a main effect of Exposure [$F(1, 38) = 3.07$, $p = 0.08$]. Therefore, data were analyzed separately for each sex to assess ethanol-induced differences in weight across the experiment. When male mice were compared for weight gain across the experiment, there were differences in weight between air-exposed and ethanol-exposed mice as supported by a Day by Exposure interaction [$F(21, 378) = 4.15$, $p < 0.0001$] and a main effect of Day [$F(21, 378) = 643.1$, $p < 0.0001$]. However, post hoc analyses failed to reveal any statistically significant differences. When female mice were compared for weight gain across the experiment, there were differences in weight between air-exposed and ethanol-

exposed mice as supported by a Day by Exposure interaction [$F(21, 420) = 1.62$, $p < 0.05$] and a main effect of Day [$F(21, 420) = 261.7$, $p < 0.0001$]. On PND 33, 40, and 41, air-exposed mice weighed more than ethanol-exposed mice (all $p < 0.03$). There were no differences in weight between air-exposed and ethanol-exposed male or female mice when all mice were intermittently consuming ethanol.

3.1.3 Experiment 2: Blood Ethanol Concentrations

There were no sex differences in blood ethanol concentrations (BECs) during any exposure cycle (Table 1). However, there were changes in BECs across cycles for both male and female ethanol-exposed mice as supported by a main effect of Cycle [$F(3, 68) = 51.9$, $p < 0.0001$]. For male ethanol-exposed mice, cycle 1 BECs were lower than cycle 2 ($p < 0.0001$) and higher than cycle 3 ($p < 0.02$). Cycle 2 was higher than cycles 3 ($p < 0.0001$) and 4 ($p < 0.005$). Cycle 3 was lower than cycle 4 ($p< 0.005$). For female-exposed mice, cycle 1 BECs were lower than cycle 2 ($p < 0.001$), but not different from cycles 3 and 4. Cycle 2 BECs were higher than cycles 3 ($p < 0.0001$) and 4 ($p < 0.005$). Cycle 3 was lower than cycle 4 ($p< 0.0001$).

3.1.4 Experiment 2: Weight

When weight was analyzed as a three-factor mixed model ANOVA for Sex (2; Male, Female), Exposure (2; Air, Ethanol) and Day as a repeated measure, there was a significant Sex by Exposure by Day interaction [$F(15, 793) = 2.63$, $p < 0.005$], a Day by Exposure interaction [$F(15, 793) = 3.64$, $p < 0.0001$], Day by Sex interaction [$F(15, 793) = 28.87$, $p < 0.0001$], and main effects of Exposure [$F(1, 56) = 4.59$, $p < 0.05$], Sex [$F(1, 56) = 150.2$, $p < 0.0001$], and Day [$F(15, 793) = 1069$, $p < 0.0001$]. Therefore, data were analyzed separately for each sex to assess ethanol-induced differences in weight across the experiment. When male mice were compared for weight gain across the experiment, there were differences in weight between air-

exposed and ethanol-exposed mice on PND 33, 36, 37, and 41 (all $p < 0.02$) as supported by a Day by Exposure interaction [$F (15, 390) = 4.42$, $p < 0.0001$] and a main effect of Day [$F (15, 390) = 592.5$, $p < 0.0001$]. There were no differences in weight between male air-exposed and ethanol-exposed mice in adulthood when all mice were intermittently consuming ethanol. When air-exposed and ethanol-exposed female mice were compared for changes in weight, there were no differences between groups, but they did gain weight across the experiment as supported by a main effect of Day [$F (15, 403) = 475.3$, $p < 0.0001$].

Table 1: Blood Ethanol Concentrations

	Experiment 1		**Experiment 2**	
	Male	**Female**	**Male**	**Female**
Cycle 1	278.9 ± 11.6	299.8 ± 11.6	238.5 ± 19.5	230.8 ± 15.3
Cycle 2	252.7 ± 28.9	225.7 ± 12.9	346.8 ± 25.7	330 ± 14.7
Cycle 3	214.1 ± 20.7	260.4 ± 11.9	165.6 ± 11.9	188.5 ± 8.6
Cycle 4	153.2 ± 22.9	159.3 ± 19.5	233.7 ± 19.2	272.0 ± 10.7

Data are presented as mean BECs (mg/dl) ± SEM across AIE-exposure cycles for each experiment. Data are presented separately for each experiment.

3.2 Ethanol Consumption and Preference Data

3.2.1 Experiment 1: Ethanol Consumption

Overall, there were changes in voluntary ethanol consumption during adolescence following one week since the last vapor exposure to ethanol. There were sex differences in ethanol consumption that varied as a function of exposure and day as supported by a Sex by Exposure interaction [$F (1, 36) = 4.34$, $p < 0.05$], and main effects of Sex $F (1, 36) = 18.11$, $p < 0.0001$] and Day [$F (13, 468) = 6.73$, $p < 0.0001$]. Given these sex differences in ethanol

consumption, data were analyzed separately or male and female mice. As shown in Figure 1A, adolescent ethanol-exposed male mice showed an overall increase in voluntary ethanol consumption compared adolescent air-exposed male mice as supported by a main effect of Exposure [F (1, 17) = 5.14, $p < 0.05$) and a main effect of Day [F (13, 221) = 2.96, $p < 0.05$). However, the Day by Exposure interaction failed to reach statistical significance. In adolescent female mice, AIE exposure did not alter voluntary ethanol consumption (Figure 1B). However, there was an increase in ethanol consumption among all groups across time as supported by a main effect of Day [F (13, 247 = 5.07, $p < 0.001$).

Figure 1.

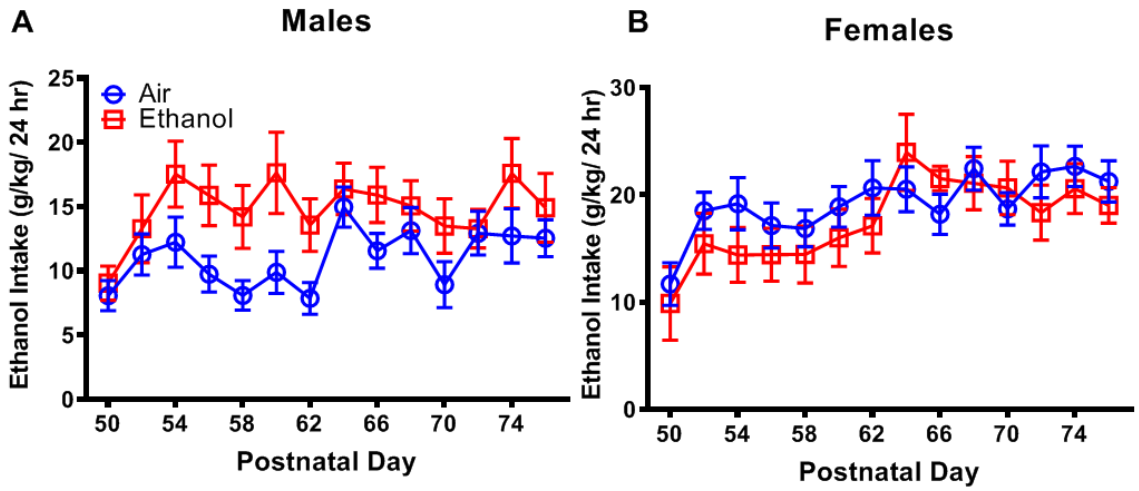

Figure 1: Intermittent voluntary ethanol consumption expressed as grams of ethanol consumed per kilogram of body weight (g/kg) across days (24 hr) from PND 50-74 in male (panel A) and female (Panel B) mice. Data are presented as mean +/- SEM. Male Air-Exposed n= 10, Male Ethanol-Exposed n=9, Female: Air-Exposed n=12, Female: Ethanol-Exposed n=10.

3.2.2 Experiment 2: Ethanol Consumption

Overall, there were long-term changes in ethanol consumption in adulthood following adolescent intermittent ethanol exposure in both male and female mice. There were sex differences in ethanol consumption that varied as a function of exposure and day as supported by a Day by Sex by Exposure interaction [$F(13, 637) = 3.38$, $p < 0.0001$], Sex by Exposure interaction [$F(1, 49) = 17.35$, $p < 0.0001$], Day by Sex interaction [$F(13, 637) = 3.94$, $p < 0.0001$], and main effects of Sex [$F(1, 49) = 53.18$, $p < 0.0001$] and Day [$F(13, 637) = 19.86$, $p < 0.0001$]. Given these sex differences in ethanol consumption, data were analyzed separately or male and female mice. As shown in Figure 2A, male mice that were exposed to ethanol during adolescence showed increased voluntary ethanol intake in adulthood on PND 73-77 ($p < 0.005$) and PND 83 ($p < 0.01$) compared to air-exposed controls, as supported by a significant Day by Exposure interaction [$F(13, 312) = 2.95$, $p < 0.005$] and a significant main effect of Exposure [$F(1, 24) = 10.10$, $p < 0.005$] and Day [$F(13, 312) = 11.05$, $p < 0.0001$]. In contrast, as shown in Figure 2B, female mice that were exposed to ethanol during adolescence showed less voluntary ethanol intake in adulthood on PND 71-75 ($p < 0.05$) compared to air-exposed controls, as supported by a significant Day by Exposure interaction [$F(13, 325) = 2.00$, $p < 0.05$] and a significant main effect of Exposure [$F(1, 25) = 7.77$, $p < 0.05$] and Day [$F(13, 325) = 12.5$, $p < 0.0001$].

Figure 2:

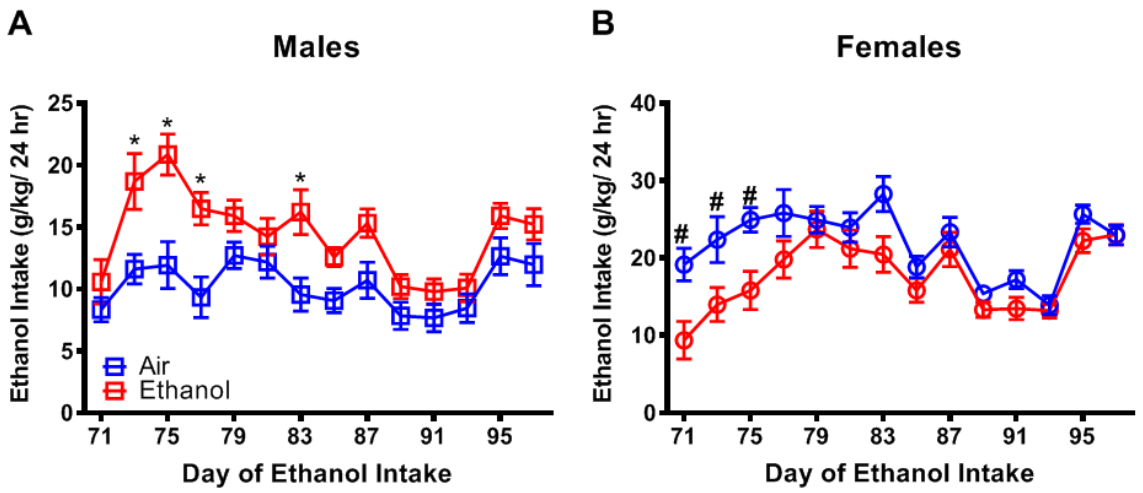

Figure 2: Intermittent voluntary ethanol consumption expressed as grams of ethanol consumed per kilogram of body weight (g/kg) across days (24 hr) from PND 71-97 in male (panel A) and female (Panel B) mice. Data are presented as mean +/- SEM. * indicates male ethanol-exposed mice drank more ethanol than air-exposed mice. # indicates female air-exposed mice drank more ethanol than ethanol-exposed mice. Male Air-Exposed n= 13, Male Ethanol-Exposed n=13, Female: Air-Exposed n=13, Female: Ethanol-Exposed n=14.

3.2.3 Experiment 1: Ethanol Preference

When data were analyzed using a three-way ANOVA for Exposure (2; Air, Ethanol) and Sex (2; Male, Female) as between subjects factors and Day as a repeated measure, there were differences in overall preference for ethanol across days and between sexes, with a main effect of Day [$F(13, 466) = 10.42$, $p < 0.0001$] and Sex [$F(1, 36) = 4.38$, $p < 0.05$] and a trend for a Sex by Day interaction [$F(1, 36) = 3.77$, $p = 0.06$]. Therefore, data were analyzed separately for each sex. As shown in Figure 3A, for male mice, there was a main effect of Day [$F(13, 221) = 3.92$, $p < 0.005$], but no effect of Exposure. Similarly, for female mice (Figure 3B), there was a main

effect of Day [F (13, 247) = 8.20, p < 0.0001]. However, there were no differences in preference for ethanol between air-exposed and ethanol-exposed mice.

Figure 3:

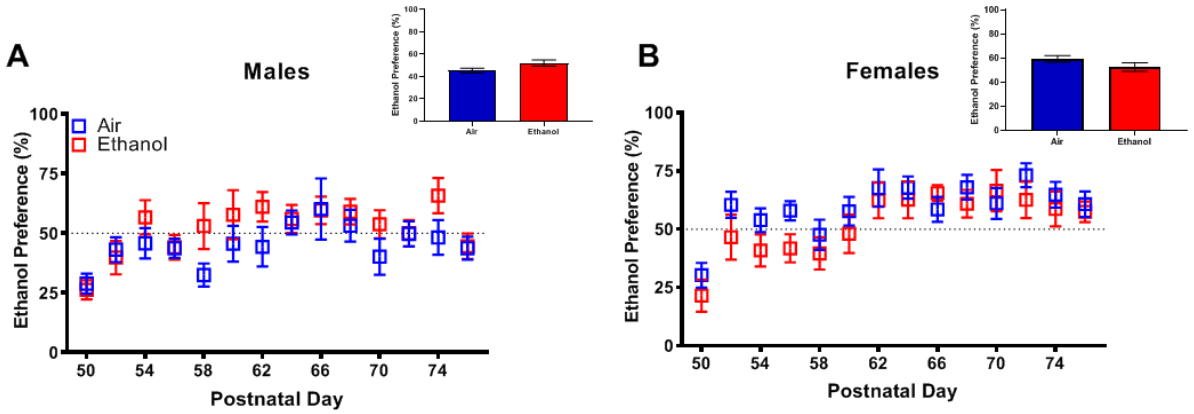

Figure 3: Preference for ethanol expressed as preference (%) computed as {(EtOH (ml)/ [EtOH (ml) + Water (ml))*100]} across days (24 hr) from PND 50-74 in male (panel A) and female (Panel B) mice. Data are presented as mean +/- SEM. Male Air-Exposed n= 10, Male Ethanol-Exposed n=9, Female: Air-Exposed n=12, Female: Ethanol-Exposed n=10.

3.2.4 Experiment 2: Ethanol Preference

When data were analyzed using a three-way ANOVA for Exposure (2; Air, Ethanol) and Sex (2; Male, Female) as between subjects factors and Day as a repeated measure, there were differences in overall preference for ethanol across days and between sexes, with a main effect of Day [F (7.959, 381.4) = 4.704, p < 0.0001], a Sex by Exposure [F (1, 48) = 7.505, p < 0.01] and a Sex by Day by Exposure interaction [F (13, 623) = 1.920, p < 0.05]. Therefore, data were analyzed separated for each sex. For male mice, there was a trend for increased preference for ethanol in adulthood in the ethanol-exposed mice compared to control mice as shown in Figure

4A [Exposure ($F(1, 24) = 4.05$, $p = 0.055$)]. Additionally, there was a main effect of Day [$F(13, 312) = 2.08$, $p < 0.05$]. For female mice exposed to alcohol during adolescence, there was an overall decrease in preference for ethanol in adulthood (Figure 4B) as supported by a main effect of Exposure [$F(1, 25) = 4.42$, $p < 0.05$] and a main effect of Day [$F(13, 325) = 4.29$, $p < 0.0001$] and a trend for Day by Exposure interaction [$F(13, 325) = 1.687$, $p = 0.06$].

Figure 4:

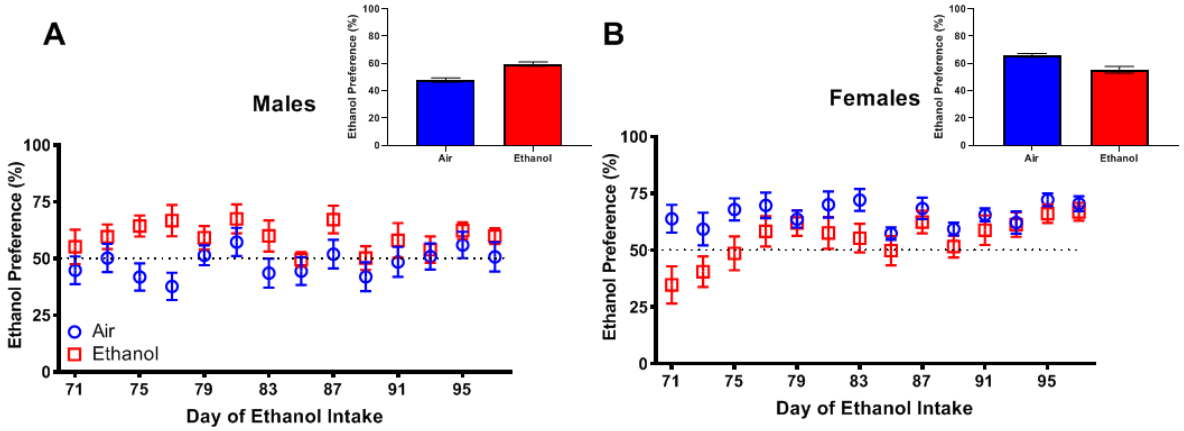

Figure 4: Preference for ethanol expressed as preference (%) computed as {(EtOH (ml)/ [EtOH (ml) + Water (ml))*100]} across days (24 hr) from PND 71-97 in male (panel A) and female (Panel B) mice. Data are presented as mean +/- SEM. Male Air-Exposed n= 13, Male Ethanol-Exposed n=13, Female: Air-Exposed n=13, Female: Ethanol-Exposed n=14.

3.3 Percent of Control Data

3.3.1 Experiment 1

When sex differences in the control mice were examined, female control mice consumed approximately twice as much ethanol as compared to male control mice when data were expressed as g/kg as supported by a main effect of Sex [$F(1, 19) = 26.22$, $p < 0.0001$] and a

main effect of Day [F (13, 247) = 1.05, p < 0.0001]. Therefore, data were transformed and expressed as a percent of their respective sex control, with control mice set to 0 [((ethanol-exposed / air-exposed mean)*100)-100] to allow us to compare sex-differences in ethanol consumption for the ethanol-exposed mice. Overall, adolescent ethanol-exposed male mice consumed more ethanol than adolescent ethanol-exposed female mice (Figure 5) as supported by a Day by Sex interaction [F (13, 221) = 2.31, p < 0.01] and a main effect of Sex [F (1, 17) = 8.76, p < 0.01]. Specifically, adolescent ethanol-exposed male mice consumed between 73.3-91.3% more ethanol compared to ethanol-exposed female mice on PND 54-62 (all p < 0.03). On all other days, there were no differences in ethanol consumption between males and females.

Figure 5:

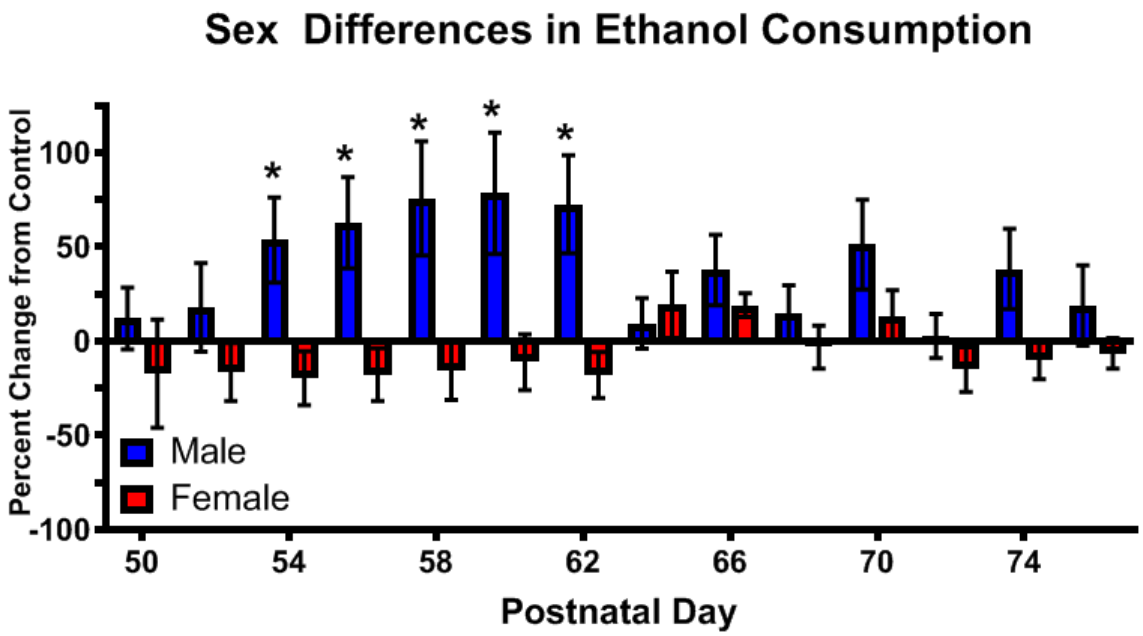

Figure 5: Voluntary ethanol consumption expressed as relative percent of control in ethanol-exposed mice across days (24 hr) from PND 50-74 in male (panel A) and female (Panel B) mice. Control mice were set to 0 and ethanol intake was expressed relative to control, with higher scores indicating an increase in ethanol consumption relative to air-

exposed mice and lower scores indicating a decrease in ethanol consumption relative to air-exposed mice. Data are presented as mean +/- SEM. * indicates males are significantly greater than females. Male Air-Exposed n= 10, Male Ethanol-Exposed n=9, Female: Air-Exposed n=12, Female: Ethanol-Exposed n=10.

3.3.2 Experiment 2

Sex differences in ethanol consumption were observed in control mice (main effect of Sex [$F(1, 24) = 99.85$, $p < 0.0001$]; main effect of Day [$F(24, 312) = 7.83$, $p < 0.001$] and Day by Sex interaction [$F(13, 312) = 2.61$, $p < 0.0002$]. Therefore, as described above for Experiment 1, data were transformed and expressed as a percent of their respective sex control, with control mice set to 0 to allow us to compare sex-differences in ethanol consumption for the ethanol-exposed mice (Figure 6). Overall, among the ethanol-exposed mice, males consumed more ethanol than females over the 28 days of ethanol consumption as supported by a significant main effect of Sex [$F(1, 25) = 26.06$, $p < 0.0001$] and Day [$F(13, 325) = 2.19$, $p < 0.01$], and a Sex by Day interaction [$F(13, 325) = 5.61$, $p < 0.0001$]. Specifically, on PND 71-77, males consumed between 67.1-100% more ethanol than females (all $p < 0.0005$) and on PND 83-87, males consumed between 45.9-87.8% more ethanol than females (all $p < 0.05$). On all other days, there were no differences in ethanol consumption between males and females.

Figure 6:

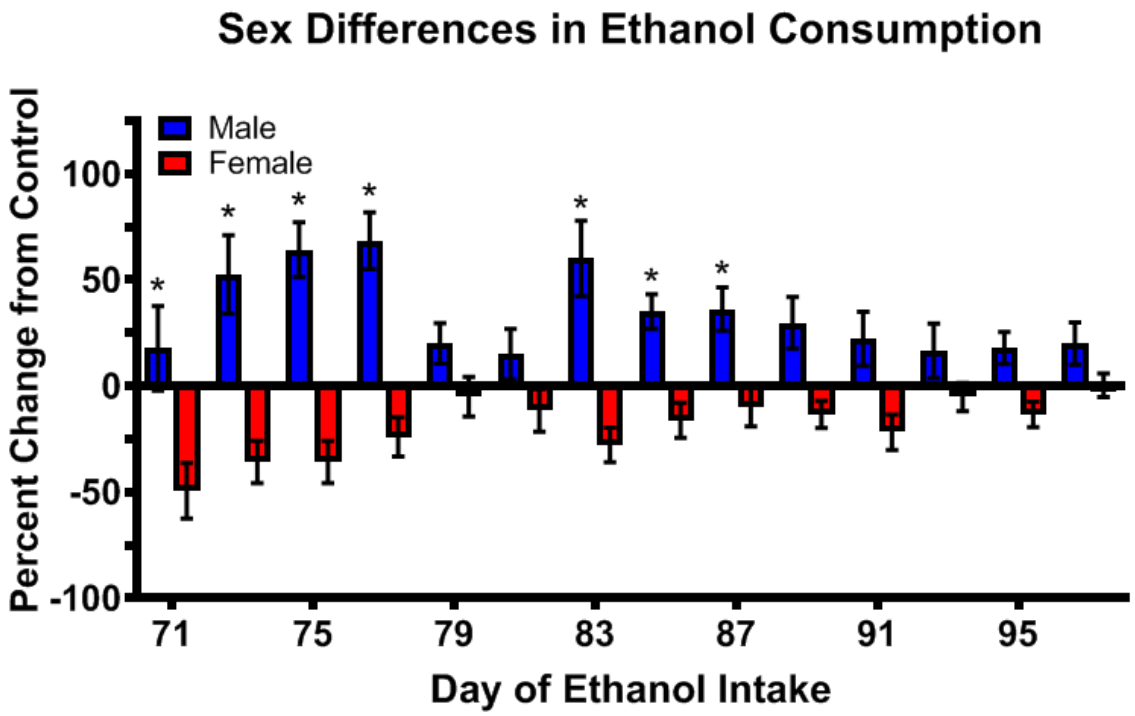

Figure 6: Voluntary ethanol consumption expressed as relative percent of control in ethanol-exposed mice across days (24 hr) from PND 71-97 in male (panel A) and female (Panel B) mice. Control mice were set to 0 and ethanol intake was expressed relative to control, with higher scores indicating an increase in ethanol consumption relative to air-exposed mice and lower scores indicating a decrease in ethanol consumption relative to air-exposed mice. Data are presented as mean +/- SEM. * indicates males are significantly greater than females. Male Air-Exposed n= 13, Male Ethanol-Exposed n=13, Female: Air-Exposed n=13, Female: Ethanol-Exposed n=14.

3.4 Open Field Test

3.4.1 Experiment 1: Open Field

Data for the entire 60 min trial for the open first test were analyzed for total distance traveled and rearing. Overall, ethanol exposure decreased distance traveled compared to air-exposure during adolescence (Figure 7A) as supported by a main effect of Exposure [F (1, 38) =

4.37, $p < 0.05$] and a trend for a Sex by Exposure interaction [$F (1, 38) = 3.66$, $p = 0.06$]. There were no differences in total rearing frequency between any of the groups (Figure 7B).

Figure 7:

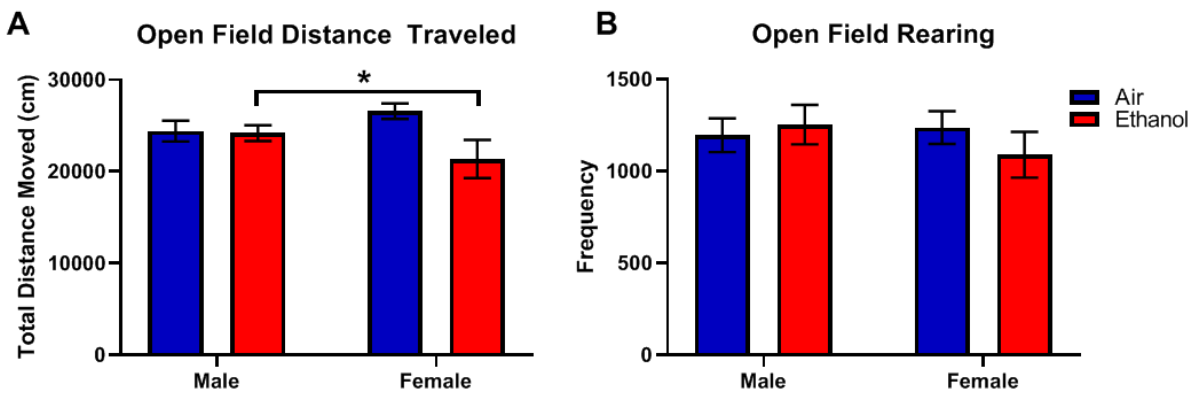

Figure 7: Behavior during the open field test for the entire arena following the 28 day voluntary ethanol drinking paradigm between PND 79-81. Total distance traveled in centimeters (Panel A) and frequency of rearing (Panel B) for the entire 60 min trial. Data are presented as mean +/- SEM. * indicates ethanol-exposed mice are significantly greater than air-exposed mice. Male Air-Exposed n= 10, Male Ethanol-Exposed n=9, Female: Air-Exposed n=12, Female: Ethanol-Exposed n=10.

3.4.2 Experiment 2: Open Field

Overall, females showed greater distance traveled in the open field test compared to males (Figure 8A) as supported by a main effect of Sex [$F (1, 49) = 6.02$, $p < 0.05$]. Additionally, there was a trend for mice that were exposed to alcohol to show lower distance traveled compared to air-exposed control [Exposure ($F (1, 49) = 2.96$, $p = 0.09$)]. There were no changes in rearing behavior across the 60 minute trial (Figure 8B).

Figure 8:

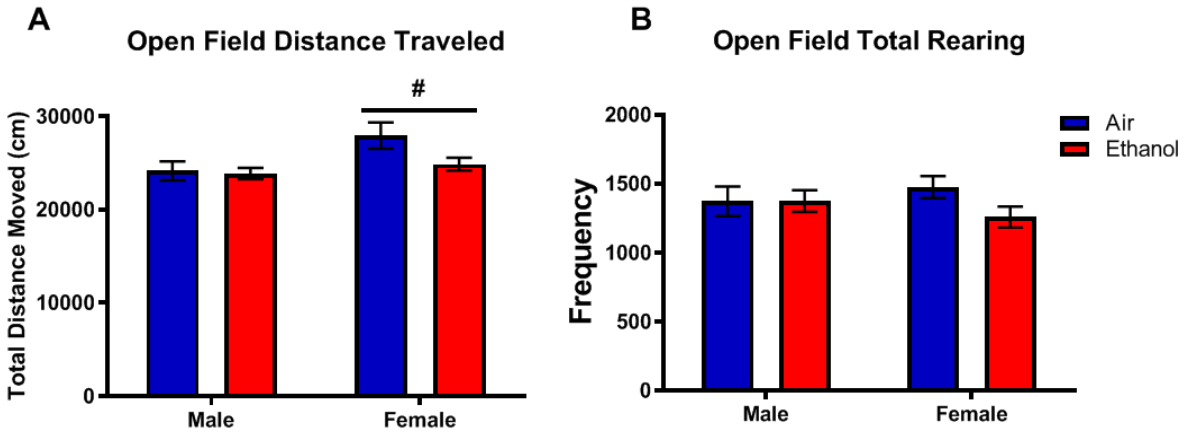

Figure 8: Behavior during the open field test for the entire arena following the 28 day voluntary ethanol drinking paradigm between PND 100-102. Total distance traveled in centimeters (Panel A) and frequency of rearing (Panel B) for the entire 60 min trial. Data are presented as mean +/- SEM. # indicates female mice are significantly greater than male mice. Male Air-Exposed n= 13, Male Ethanol-Exposed n=13, Female: Air-Exposed n=13, Female: Ethanol-Exposed n=14.

3.4.3 Experiment 1: Open Field Center Zone

As shown in Figure 9A, female mice that were exposed to ethanol during adolescence traveled less in the center zone compared to female mice that were exposed to air during adolescence as supported by a significant Sex by Exposure interaction [$F(1, 38) = 3.99$, $p = 0.05$]. There were no differences in time spent in the center zone between any of the groups (Figure 9B). Female mice exposed to ethanol during adolescence entered the center zone fewer times than those exposed to air during adolescence (Figure 9C) as supported by a significant Sex by Exposure interaction [$F(1, 38) = 4.67$, $p < 0.05$].

Figure 9:

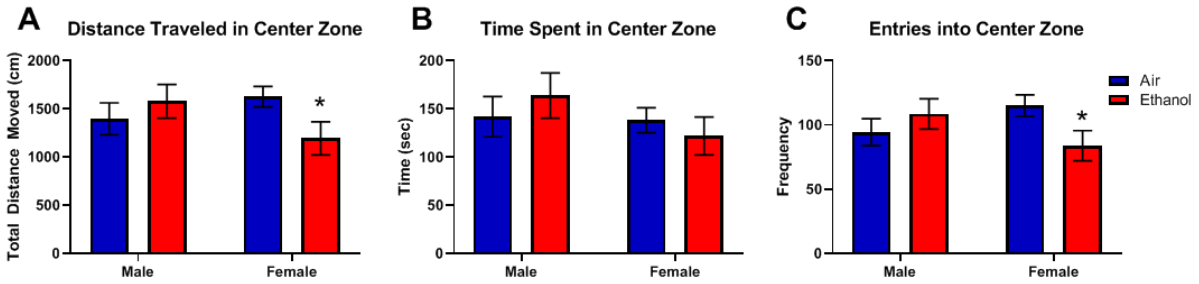

Figure 9: Behavior during the open field test for the center zone following the 28 day voluntary ethanol drinking paradigm between PND 79-81. Total distance traveled in centimeters for the center zone (Panel A), time spent (seconds) in the center zone (Panel B), and frequency of entries into the center zone (Panel C) for the entire 60 min trial. Data are presented as mean +/- SEM. * indicates female ethanol-exposed mice are significantly lower than female air-exposed mice. Male Air-Exposed n= 10, Male Ethanol-Exposed n=9, Female: Air-Exposed n=12, Female: Ethanol-Exposed n=10.

3.4.4 Experiment 2: Open Field Center Zone

In general, AIE exposure decreased distance traveled and entries into the center zone, but did not change the amount of time spent in the center zone. Specifically, mice exposed to ethanol during adolescence showed decreased movement in the center zone compared to mice exposed to air during adolescence (Figure 10A), as supported by a main effect of Exposure [$F(1, 49) = 4.99$, $p < 0.05$]. There was a trend for males to show higher distance traveled in the center zone compared to females as supported by a trend for Sex [$F(1, 49) = 3.03$, $p = 0.08$]. As shown in Figure 10B, male mice spent more time in the center zone compared to female mice, regardless of exposure, as supported by a main effect of Sex [$F(1, 49) = 14.63$, $p < 0.0005$]. Figure 10C shows that AIE exposure decreased the number of entries into the center zone as supported by a main effect of Exposure [$F(1, 49) = 5.64$, $p < 0.03$].

Figure 10:

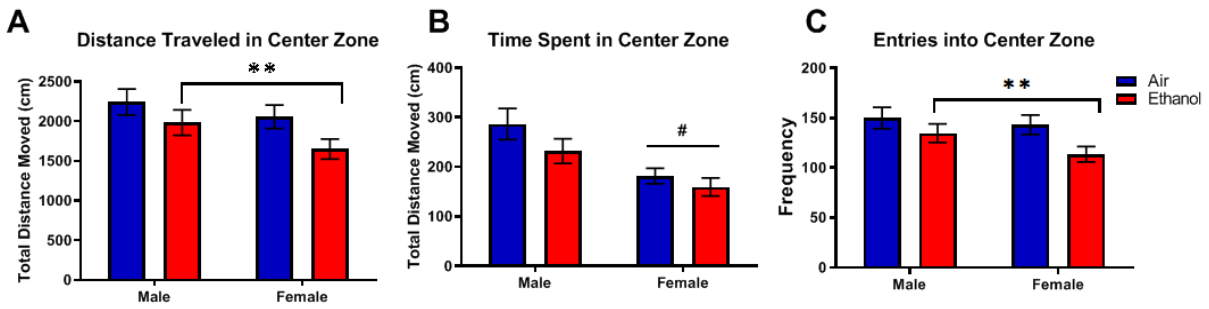

Figure 10: Behavior during the open field test for the center zone following the 28 day voluntary ethanol drinking paradigm between PND 100-102. Total distance traveled in centimeters for the center zone (Panel A), time spent (seconds) in the center zone (Panel B), and frequency of entries into the center zone (Panel C) for the entire 60 min trial. Data are presented as mean +/- SEM. ** indicates ethanol-exposed mice are significantly lower than air-exposed mice. # indicates female mice are significantly lower than male mice. Male Air-Exposed n= 13, Male Ethanol-Exposed n=13, Female: Air-Exposed n=13, Female: Ethanol-Exposed n=14.

4. Discussion

In the present work, we show that ethanol, but not air, exposure during adolescence increased voluntary ethanol consumption during adolescence and adulthood in male mice. However, in female mice there was no effect of ethanol exposure on voluntary alcohol consumption during adolescence, and a transient decrease in voluntary ethanol consumption was seen in adulthood. Furthermore, when voluntary consumption data was analyzed in terms of preference, male mice exposed to AIE show no preference for alcohol consumption during adolescence and a trend for increased preference for ethanol in adulthood. For female mice that were exposed to ethanol during adolescence there was no change in preference for ethanol during

adolescence (Experiment 1) and decreased preference for ethanol in adulthood (Experiment 2), when compared to control air-exposed mice. To account for the fact that female control mice innately consume more ethanol than male control mice, we transformed data as a percent of control to allow us to compare relative differences in ethanol consumption between male and female mice. When data were expressed relative to control mice, we observed that adolescent ethanol-exposed male mice consumed more ethanol than adolescent ethanol-exposed female mice during adolescence (Experiment 1) and young adulthood (Experiment 2,) primarily during the first half of the 28 day ethanol drinking paradigm and this effect dissipated across the second half of the ethanol drinking in both experiments.

During adolescence, there is lower sensitivity to alcohol induced motor impairments (Day et al., 2013), greater sensation seeking (Spear, 2016), and lower risk aversion (Casey and Jones, 2010). To assess potential consequences on the motor system from ethanol exposure during adolescence, we tested the mice in an open field test during adolescence (Experiment 1) and adulthood (Experiment 2) following 28 days of the intermittent ethanol drinking paradigm. When tested during adolescence in the open field test, ethanol-exposed mice showed a modest decrease in overall distance traveled over the 60 min trial [Figure 7]. This effect appeared to be driven by the decrease in distance traveled in the ethanol-exposed female mice, with little difference between groups in the male mice. When the mice were tested in adulthood following the 28 days of ethanol drinking, females showed greater distance traveled compared to males, regardless of ethanol exposure during adolescence [Figure 8]. However, there were no differences in rearing behavior during the 60 min trial in either experiment. Overall, ethanol-exposed female mice show a modest decrease in distance traveled in the open field following

adolescent AIE exposure and 28 days of intermittent ethanol drinking using a two-bottle choice paradigm.

The open field test is one of the most commonly used measures of anxiety-like behavior by using the center zone as a measure of thigmotaxis (Bolivar et al., 2000; Crawley and Bailey, 2008; Lipkind et al., 2004; Simon et al., 1994; Walz et al., 2016). Mice naturally have a phobia of bright open spaces, therefore a more anxious mouse would spend less time in the center zone of the open field. In this test, more movement, entries, and time spent in the center zone are considered characteristics of less anxiety (Kulikov et al., 2008). In Experiment 1, ethanol-exposed female mice entered the center zone and moved less in the center zone less than air-exposed female mice, an effect that was absent in the adolescent male mice. However, there were no differences in time spent in the center zone between any of the groups. In Experiment 2, when mice were tested in young adulthood after the 28 day drinking period, we found that both male and female ethanol-exposed mice showed decreased distance traveled and entries into the center zone compared to air-exposed mice. Regardless of exposure during adolescence, female mice spent less time in the center zone compared to male mice. Overall, in experiment 1, ethanol-exposed female mice showed a motor-associated (center zone distance traveled and entries) anxiety-like phenotype. In Experiment 2, during adulthood both male and female ethanol-exposed showed this motor behavior anxiety-like phenotype. Therefore, the open field anxiety-like behavioral phenotype appears earlier in female mice following AIE and takes longer to manifest in the ethanol-exposed male mice.

Stress, including deprivation from ethanol, has been shown as an important variable in long-term consequences of AIE in animal models (Patchev and Patchev, 2006). Research shows repeated cycles of adolescent ethanol exposure and stress have been associated with increased

susceptibility to stress-induced alcohol consumption in both adult male and female rats (Füllgrabe et al., 2007; Siegmund et al., 2005). This suggests the initiation of alcohol use during adolescence may lead to elevated consumption in adulthood following exposure to stress. The interaction between stress and repeated exposure to alcohol is still unclear, but may contribute to progressive loss of behavioral control, increased impulsivity, anxiety-behavior, craving, and reward-seeking, which are characteristic of stages leading to alcohol use disorders (Crews et al., 2017; Volkow et al., 2016). Repeated withdrawal from ethanol exposure may also be a stressor and could alter subsequent ethanol consumption (Becker, 2017). In the present work, female ethanol-exposed mice showed decreased weight gain during the AIE exposure in Experiment 1, an effect that was absent in male mice. This should be addressed in future work. However, the opposite pattern was present in Experiment 2. This may be due to differences in blood ethanol concentrations during AIE exposure between experiments (Table 1). Cycles that showed higher BECs would likely induce more severe withdrawal from ethanol during AIE cycles. There were no differences in weight between exposure groups during the intermittent ethanol drinking when all mice were exposed to ethanol.

There have been conflicting results regarding binge ethanol exposure during adolescence and ethanol consumption in adulthood in rodent models. In the present set of experiments, male mice seem to be more sensitive to the effects of early alcohol exposure on voluntary alcohol consumption later in life. In previous work, when AIE vapor exposure occurred during adolescence, mice were shown to have alterations in PFC-related decision making and behavioral control, hypoactivity in locomotion tests, increased anxiety-like behavior in the open field test, depressive-like behavior and lower body weight following exposure, and sex differences in negative affect (Centanni et al., 2017; Ehlers et al., 2013; Gass et al., 2014; Kasten

et al., 2020; Nentwig et al., 2019; Slawecki et al., 2004; Walker et al., 2010). It is important to note that in the present work, the increased drinking behavioral phenotype in male mice began to emerge after the 7 day abstinence period during adolescence (Experiment 1) and was more robust after the 27 day abstinence period (Experiment 2). Together, these data show sex-dependent persistent changes in alcohol drinking, anxiety-like behavior, and general exploratory behavior well into adulthood following AIE exposure. These findings suggest that males show persistent changes manifested as a change in ethanol-drinking phenotype based on their increased voluntary consumption in adulthood. Females appear to be more affected in locomotion and anxiety-like behavior based on their reduced distance traveled and center-zone behavioral changes in the open field test. These data demonstrate a sexually divergent shift in ethanol consumption following binge ethanol exposure during adolescence and highlight sex-dependent vulnerability to developing substance use disorder in adulthood.

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