Running title: Alcohol, Adolescence, Sex Differences, *Grin2B*

Adolescent Intermittent Ethanol Exposure Alters Exploratory and Affective Behaviors, and Cerebellar *Grin2B* Expression in Adulthood

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Highlights

- Adolescent intermittent ethanol (AIE) exposure decreased exploratory behavior in adulthood in male and female mice.
- In females, but not males, AIE increased anxiety-like behavior.
- In males, but not females, AIE reduced stress reactivity in adulthood.
- These findings indicate sex differences in the enduring effects of AIE
- Cerebellar *Grin2B mRNA* levels were increased in adulthood in both male and female AIE-exposed mice.
- These findings add to the small, but growing literature on behavioral AIE effects in mice, and establish cerebellar excitatory synaptic gene expression as an enduring effect of adolescent ethanol exposure.

Abstract

More than 90% of alcohol consumption by young people is in the pattern binge drinking. During adolescence, the brain undergoes maturational changes that influence alterations in behavior control, affective behaviors, and cerebellar brain volume and function in adulthood. We investigated long-term impacts of adolescent binge alcohol exposure on affective behaviors and cerebellar gene expression in male and female mice. We exposed C57BL/6J mice to adolescent intermittent ethanol (AIE) or air (control) vapor inhalation from postnatal day 28-42. After prolonged abstinence, in young adulthood we assessed behavior in the open field, light/dark, tail suspension, and forced swim stress tests to determine changes in affective behaviors including anxiety-like, depressive-like, and stress reactivity behavior. mRNA levels of *FMR1* and other interacting gene expressions (*Grin2a*, *Grin2B*, *Grm5*, PSD-95, and Eaat1) were measured in the cerebellum of control and adult AIE-exposed mice. In adult AIE-exposed mice, we show decreased movement in the open field in both sexes and modest changes in females in anxiety-like behavior (center zone activity). In the forced swim stress test, adult AIE-exposed male mice spent less time immobile compared to their same-sex controls, indicative of sex-specific changes in stress reactivity. Male and female AIE-exposed mice showed increased *Grin2B* mRNA expression in the adult cerebellum compared to their same-sex controls. Together, these data show that adolescent binge-like ethanol exposure altered affective behaviors and modified *Grin2B* expression in adulthood. This indicates the cerebellum may serve as an important brain region that is susceptible to long-term molecular changes with adolescent alcohol exposure.

1. Introduction

Adolescence is a critical period for the development and maturation of neural circuitry, a process required for the emergence of cognitive and affective characteristics in adulthood (see (Larsen and Luna, 2018). Adolescent intermittent ethanol (AIE) exposure alters brain morphology and function in both humans and animal models (Brown et al., 2015; Crews et al., 2019), which may render individuals vulnerable to affective and cognitive dysregulation in adulthood (Dawson et al., 2008; Deykin et al., 1987). In humans, chronic alcohol use during adolescence is associated with persistent cognitive and affective deficits (Spear, 2018), as well as increased risk of developing alcohol use disorder in adulthood (Dawson et al., 2008). Studies in animal models show persistent effects of AIE on some affective-like behaviors but not on others (Crews et al., 2019; Dannenhoffer et al., 2018; Healey et al., 2022; Pandey et al., 2015; Robinson et al., 2021). Thus, the data from clinical, and some basic research studies, support the conclusion that binge-like ethanol exposure during adolescence creates a propensity toward affective dysregulation (Dannenhoffer et al., 2018; Gilpin, 2012; Pandey et al., 2015; Varlinskaya et al., 2017). However, to better understand the neurobehavioral mechanisms underlying persistent AIE effects on affective behavior, a more fine-grained analysis of the specific nature of AIE-induced affective changes in animal models is needed.

Sex differences in the acute and long-term effects of adolescent alcohol exposure are increasingly found in clinical and preclinical research. In rats, AIE exposure sex-specifically alters cognitive flexibility (Macht et al., 2020), affective behaviors (Healey et al., 2022; Varlinskaya et al., 2020), fear conditioning (Chandler et al., 2022), and social drinking (Towner et al., 2022) in adulthood. We recently showed that male and female AIE-exposed mice showed differences in patterns of subsequent alcohol drinking and the time course for sex differences in affective behaviors. Specifically, females were more susceptible to shorter term changes in anxiety-like behaviors whereas males showed delayed changes in anxiety-like behavior following AIE and voluntary alcohol drinking (Maldonado-Devincci et al., 2022). Other studies found sex differences, with female

C57BL/6J mice showing greater AIE-induced changes in affective behaviors (e.g., novelty-induced hypophagia) compared to males (Kasten et al., 2020). Together, these data indicate that AIE alters later behavioral characteristics, some of which are sex-specific, but more work is needed to understand the underlying long-lasting neurobiological changes that mediate these behavioral alterations.

Memory and affective behaviors are co-regulated by multiple structures, including the cerebellum and the hippocampal formation. The hippocampus is known to regulate both memory and affective behaviors (Jimenez et al., 2018), and to be highly sensitive to the effects of AIE on both morphological (Mulholland et al., 2018; Risher et al., 2015a, 2015b; Swartzwelder et al., 2020) and physiological (Fleming et al., 2013, 2012; Li et al., 2013; Risher et al., 2015a, 2015b; Swartzwelder et al., 2017) endpoints. Affective regulation is also mediated through bidirectional connectivity between the hippocampus and the cerebellum (see (Yu and Krook-Magnuson, 2015) for review); as are spatial and temporal information processing (Fries, 2005; Onuki et al., 2015; Rochefort et al., 2011), behavioral conditioning (Takehara et al., 2003, 2002), and epileptogenesis (Krook-Magnuson et al., 2014). At a physiological level, there is a functional coupling of hippocampal and cerebellar neural network activity under certain behavioral circumstances that is manifested as a synchronization of theta oscillation activity across the two structures (Wikgren et al., 2010). This indicates ongoing synaptic co-regulation between the cerebellum and hippocampus that may be significant for both cognitive and affective functioning – both of which are altered by AIE (see (Crews et al., 2019; Robinson et al., 2021). The relationship between cerebellar and affective functioning is also well known in the clinical literature, as cerebellar dysfunction is the mechanism underlying the cerebellar cognitive affective syndrome (Schmahmann and Sherman, 1998). Finally, it is notable that the cerebellum is highly sensitive to the effects of acute and chronic ethanol exposure (Coleman et al., 2014; Dong et al., 2022; Meruelo, 2020; Sullivan et al., 2020) though the possible enduring impacts of

adolescent ethanol exposure on the cerebellum (Rapp et al., 2022) are only beginning to be addressed.

Recently, it was shown that expression of various genes in the cerebellum (e.g., Fragile X Messenger Ribonucleoprotein 1 (*FMR1*), postsynaptic density 95 (*Psd95*), excitatory amino acid transporter 1(*Eaat1*), subtype of metabotropic glutamate receptor (*Grm5*), and ionotropic glutamate N-methyl D-aspartate (NMDA) receptor subunits (*Grin2a, Grin2B)* are altered by adult acute and chronic ethanol exposure in rats (Dulman et al., 2021, 2019). However, the effects of adolescent ethanol exposure on synaptic genes in the cerebellum in adulthood are not known. To address these gaps, the present experiments were designed to explore the effects of AIE on affective behavior in adult male and female mice (which have been used far less than rats in rodent studies of AIE) more fully and to provide an initial assessment of AIE on cerebellar synaptic-relevant gene expression.

2. Methods

2.1 Subjects and Ethanol Exposure

Adolescent male and female C57BL/6J mice (n=8/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. Mice were PND 28 at the beginning of the experiment. Animals were group-housed (4-5 per cage) with free access to food and water. A visual depiction of the experimental procedure is shown in **Figure 1**. 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. Animal care followed National Institutes of Health Guidelines under North Carolina Agricultural and Technical State University Institutional Animal Care and Use Committee approved protocols.

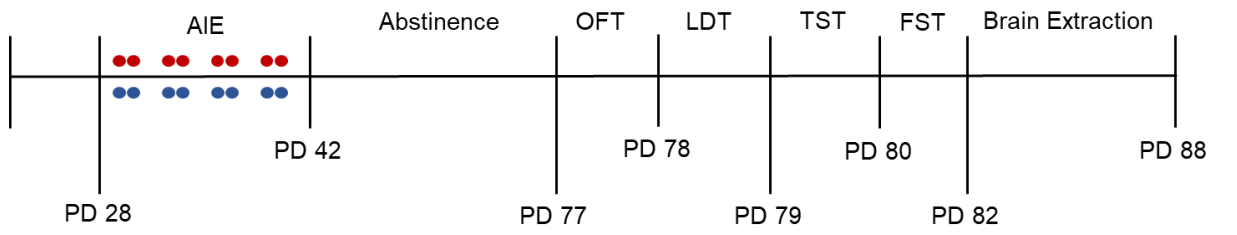

Figure 1: Experimental Timeline. Mice were exposed to AIE or air between PND 28-42 followed by abstinence until PND 76. Mice were then run on consecutive days to the open field test (OFT), light/dark test (LDT), tail suspension test (TST), and forced swim stress test (FST). Brains were extracted on PND 82.

Mice were exposed to repeated intermittent air or ethanol vapor using previously established protocols (Maldonado-Devincci et al., 2022). Briefly, mice were exposed to air (controls) or ethanol (experimental) vapor inhalation using four two-day ON/ two day OFF exposure cycles from PND 28-42. Blood ethanol concentrations were stabilized using the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg, as used in previous work (Becker and Lopez, 2004; Jury et al., 2017; Maldonado-Devincci et al., 2022, 2016, 2014b)). On each exposure day, mice were weighed and administered an intraperitoneal injection (0.02 ml/g) of pyrazole 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) 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. Ethanol (95%; Pharmco by Greenfield Global) was volatilized by passing air through an air stone (gas diffuser; Saint Gobain) submerged in ethanol.

On PND 29, 33, 37, and 41, at 0900 hr, mice were removed from the vapor inhalation chamber and 50 µL of blood was collected from the submandibular space for blood ethanol concentration (BEC) assessment and then returned to the home cage for 8 hr. On intervening days, mice were left undisturbed in the home cage. All blood samples were centrifuged at 5,000*g* and serum was collected and used to analyze BECs using the AM1 blood alcohol analyzer (Analox Instruments, Lunenburg,

MA). Mice were left undisturbed in the home cage, except for regular cage maintenance until behavioral testing began.

2.2 Behavioral Testing

Mice underwent four consecutive days (PND 77-86) of behavioral testing using the (1) open field test (OFT), (2) light/dark test (LDT), (3) tail suspension test (TST), and (4) forced swim stress test (FST). All mice were counterbalanced across days and across groups for testing. Mice were transported and acclimated to the behavioral testing room for at least 60 min before behavioral testing. For each behavioral test, the testing arena was cleaned with 70% ethanol and allowed to dry completely before the next mouse was introduced. All behavioral tests were video recorded for offline analysis with the experimenter blind to sex and exposure conditions. Experimental details are described below.

2.2.1 Open Field Test

Mice were tested for a 60 min trial in the open field test (Tatem et al., 2014). After acclimation, mice were introduced to the open field test under regular overhead lighting (400 lux). Mice were introduced to the testing arena (40.6 cm x 40.6 cm) to assess general exploratory behavior, rearing, and center zone activity. Immediately upon removal from the arena, mice were returned to their home cage and to the colony. Trials were video recorded and data were quantified offline using AnyMaze software (version 7.0).

2.2.2 Light/Dark Test

The Light/Dark Test is used to evaluate anxiolytic behavior in rodents as they have a natural aversion to brightly lit environments (Takao and Miyakawa, 2006). With this test, anxiety in mice can be measured through motor activity and time spent in the light compared to dark compartments of the apparatus (Himanshu et al., 2020). This test was conducted within the same chamber as the open field test with an opaque divider (20.3 cm x 20.3 cm) inserted to create the dark compartment. After acclimation mice were placed in the dark compartment for one min before the 10 min trial began. The

light side of the box was illuminated with a bright light (850 lux). Videos were scored by experimenters for latency to enter light side, duration of time on light side, and entries to light side.

2.2.3 Tail Suspension Test

The tail suspension test (TST) attempts to translate depressive-like behaviors in humans by observing initial escape-oriented movements made when the rodent is placed in an inescapable environment (Cryan et al., 2005). The tail suspension test was conducted using previously established procedures (Can et al., 2012). After acclimation mice were briefly restrained to pass the tail through a small plastic cylinder to prevent tail climbing and then attach an adhesive strip to the end of the tail. Mice were then suspended 60 cm above the ground for a duration of 6 min. Mice were then immediately returned to their home cage after the adhesive strip and plastic cylinder were removed. Sessions were recorded and scored offline by experimenters for latency to immobility and duration of immobility.

2.2.4 Forced Swim Stress Test

Mice were tested for response to the forced swim stress test using previously established protocols (Maldonado-Devincci et al., 2016, 2014a). Glass cylinders (Wholesale Flowers and Supplies; 20 cm diameter, 40 cm height) were used as the swim test apparatus. The glass cylinders were filled with water (23–25°C) to approximately 20 cm in depth and were separated by a visual barrier. After acclimation, mice were removed from the home cage and gently lowered into the apparatus for a 10 min swim session. Mice were removed, lightly dried, and returned to the home cage and placed on a warming pad for 15–20 min. Latency to immobility, time immobile, and struggling/climbing were assessed by experimenter-manual scoring after the session. Immobility was defined as the mouse remaining completely still or with minimal involuntary movements required to remain afloat. Struggling/climbing was defined as vigorous swimming, thrashing, and/or climbing in an attempt to escape, which usually subsided by the first three minutes after presentation to the inescapable swim tank; therefore, this behavior was only scored for the first three min of the session.

After each swim session, the cylinders were emptied, cleaned, and refilled with fresh water for the next subject.

2.3 Brain Tissue Collection

Forty-eight hours following the forced swim stress test, brains were collected. Mice were euthanized by rapid decapitation, brains were extracted, and tissue was harvested for various brain regions, including the cerebellum. Brains were snap frozen and stored at -80ºC until analysis.

2.4 RNA isolation and measurement of mRNA levels

Whole, mouse cerebellar tissue was crushed on dry ice with a mortar and pestle, and total RNA was extracted and purified from an aliquot of the tissue using the miRNeasy Mini Kit protocol and DNase Kit (Qiagen). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was amplified in duplicate on a CFX Connect qPCR system (Bio-Rad) using PowerUp SYBR master mix (Applied Biosystems). cDNA was amplified using primers for target genes (**Table 1**) with *hprt*, hypoxanthine–guanine phosphoribosyltransferase, used as a reference gene. Using the ΔΔCt method (Livak and Schmittgen, 2001), relative mRNA levels of target genes were normalized by subtracting the Ct value of *hprt* from the Ct value of the target gene, and delta Ct values for all samples were then normalized to the mean of the control group. Results are given as the fold change in mRNA levels of the ethanol treated group compared to the control group.

Table 1: Various Primers used to measure mRNA levels

Primer	Sequence (5' to 3')
FMR1 Forward	GGC GCT TTC TAC AAG GCA TTT G
FMR1 Reverse	ACC TAC AGG TGG TGG GAA TC
Slc1a3 (EAAT1) Forward	TCC TGG ATT TGA TCA GGA ACA T
Slc1a3 (EAAT1) Reverse	CGC CCA GAA GTG TTT CGT TG
Grm5 Forward	AAG GAC AGA TAA AGG TGA TCC G
Grm5 Reverse	TCA GGG TCA CCC CAT CGA A
Dlg4 (PSD-95) Forward	GGC TTC ATT CCC AGC AAA CG
Dlg4 (PSD-95) Reverse	CCG AGT CTT CTC GAC CCT GT
Grin2a Forward	TGA TAC GGA CCA AGA GGC TG
Grin2a Reverse	CGT GGA TGT CGG ATC CTT GTC

Grin2B Forward	CCA ACA TGC GCT CTC CCT TA
Grin2B Reverse	CAG CTC GTC GAC TCT CTT GG
Hprt Forward	GAG AGC GTT GGG CTT ACC TC
Hprt Reverse	ATC GCT AAT CAC GAC GCT GG

2.5 Statistical Analyses All behavioral data were analyzed using a two factor between subjects ANOVA with Exposure (2; Air, Ethanol) and Sex (2; Male, Female) as factors. All videos were quantified with the experimenter blind to sex and exposure. In the presence of significant interactions, Sidak's multiple comparisons post hoc tests were used where appropriate. The mRNA data were analyzed separately for each sex for each target using a student's t-test.

3. Results

3.1 Blood ethanol concentrations

Blood ethanol concentrations (BECs; **Table 2**) varied across cycles ($F_{(3,42)} = 38.92$, $p<0.0001$). Post hoc analyses for BECs collapsed across sex indicate that cycles 1 and 3 were lower than cycles 2 and 4, and cycle 3 was lower than cycle 1. Although the main effect of Sex achieved statistical significance ($F_{(1,14)} = 5.29$, $p=0.0374$), post hoc analyses did not reveal any statistically significant differences between males and females at any given cycle.

Table 2: Blood Ethanol Concentrations

	1	2	3	4
Male	235.6±6.7	328.8±17.2	213.9±9.9	294.7±18.7
Female	276.5±8.5	341.5±7.9	235.0±15.2	328.9±14.0

3.2 Behavioral Testing

All mice underwent a series of behavioral tests over four consecutive days. This included the open field, light/dark, tail suspension, and forced swim stress tests to determine changes in exploratory

behavior, anxiety-like behavior, depressive-like behavior, and stress reactivity, respectively. All statistical analyses are presented in **Supplemental Table 1**.

3.2.1 Open Field Test

To assess overall activity in the open field, we measured the total distance moved in the apparatus during the trial. AIE reduced the total distance moved ($F_{(1,28)} = 31.49$, *p<0.0001*), an effect that was observed in both male (*p=0.01*) and female (*p=0.0001*) mice (**Fig. 2A**). In addition, AIE reduced the frequency of exploratory rearing in the open field ($F_{(1,28)} = 8.98$, *p=0.0058*) (**Fig. 2B**). This effect appeared to be driven by an AIE effect in females (*p=0.0017*), as there was a significant AIE x Sex interaction ($F_{(1,28)} = 4.71$, *p=0.0385*), but no significant effect in male animals (*p=0.77*).

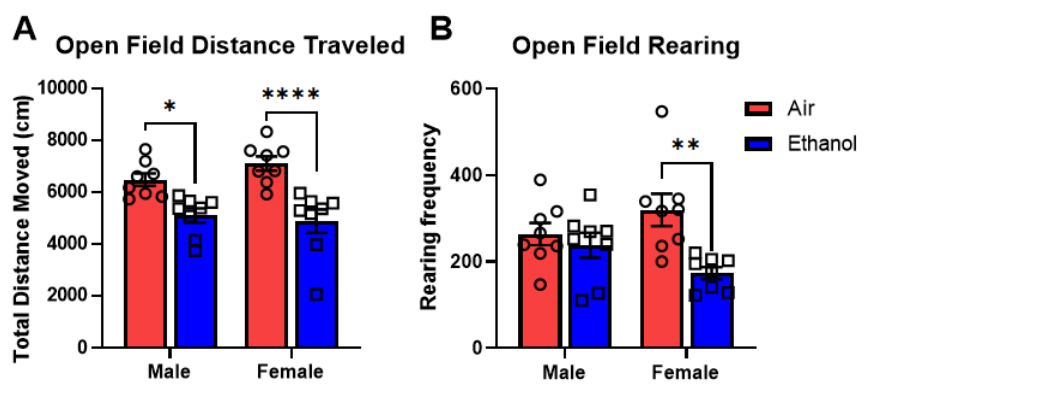

Figure 2: Open Field Data. A) Total distance traveled (cm) in the entire arena, B) Rearing Frequency. Data are presented as mean +/- SEM. * p<0.05, ** p<0.002, **** p<0.0001

Entries into the center region of the open field are often interpreted to reflect risk-taking or disinhibition (Colorado et al., 2006). AIE reduced the number of entries into the center portion of the open field ($F_{(1,28)} = 14.64$, *p=0.0007*), an effect that appears to have been driven largely by the effect of AIE in female mice (*p=0.0009*) because post-hoc analysis of male mice indicated no significant effect of AIE (*p=0.29*)(**Fig. 3A**). Although AIE diminished the number of entries into the center portion of the arena, it did not significantly alter the amount of time spent (**Fig. 3B)**, nor the total distance moved within the center portion of the field (**Fig. 3C)**.

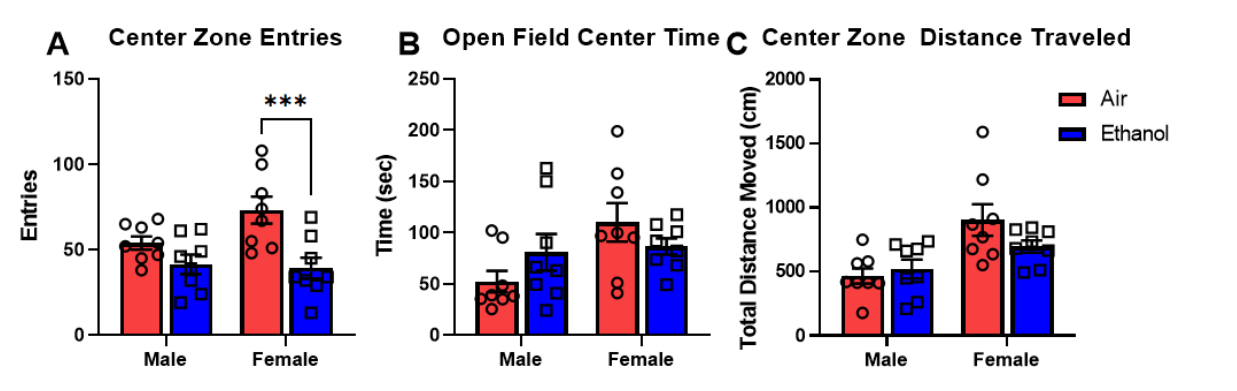

Figure 3: Center Zone Data. A) Entries into Center Zone, B) Time spent in Center Zone (sec), C) Distance traveled in center zone (cm). Data are presented as mean +/- SEM. *** p<0.001

3.2.2 Light-Dark Test

The light-dark test is used as a measure of anxiety and stress reactivity. Neither AIE nor sex had significant effects on the latency to enter the lighted area **(Fig. 4A)** [Sex ($F_{(1, 28)}$ = 0.4649, *p=0.5010*); Exposure ($F_{(1, 28)}$ = 0.6670, *p=0.4210*), Sex by Exposure (F(1, 28) = 0.4282, p=0.5182)], the average time spent in the lighted area (**Fig. 4B)** [Sex ($F_{(1, 28)}$ = 0.0212, *p=0.8851*); Exposure ($F_{(1, 28)}$ = 0.8020, *p=0.3781*); Sex by Exposure ($F_{(1, 28)}$ = 0.0056, *p=0.9411*)], nor the average number of entries into the lighted area (**Fig. 4C)** [Sex ($F_{(1, 28)}$ = 3.308, *p=0.0755*); Exposure ($F_{(1, 28)}$ = 2.897, *p=0.0999*); Sex by Exposure ($F_{(1, 28)}$ = 0.1631, *p=0.6894*)].

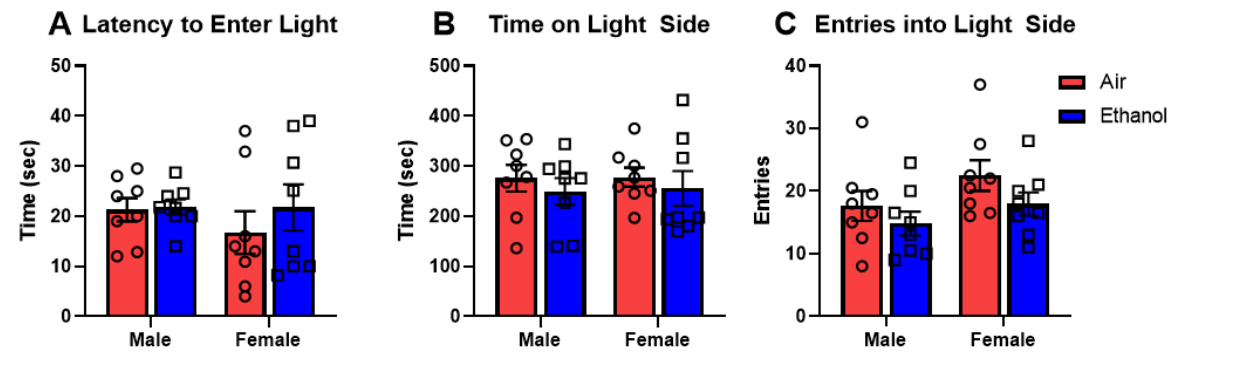

Figure 4: Light/Dark Test Data A) Latency to enter light side (sec), B) Time on the light side (sec), C) Entries into the light side. Data are presented as mean +/- SEM.

3.2.3 Tail Suspension Test

The tail suspension test is used as a measure of depression-like behavior and learned helplessness in mice (Cryan et al., 2005). Neither AIE ($F_{(1, 28)} = 0.2250$, *p=0.6390*) nor sex ($F_{(1, 28)} = 2.215$, *p=0.1478*) had a significant effect on the latency to immobility in the test (**Fig. 5A)**. However, there was a significant interaction of AIE and sex on the amount of time spent immobile within the test session ($F_{(1,28)} = 4.59$, *p=0.0408*) (**Fig. 5B**). *Post-hoc* analysis showed no significant effect of AIE on immobility time in males (*p=0.25*), but there was a trend for immobility times to increase among females that received AIE (*p=0.07*).

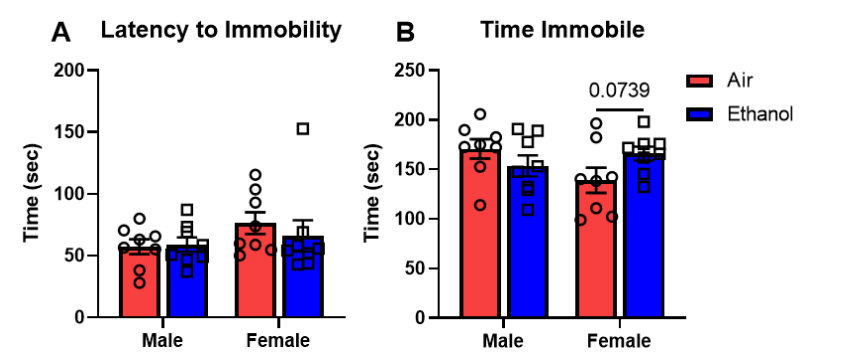

Figure 5: Tail Suspension Test Data A) Latency to become immobile (sec), B) Time spent immobile (sec). Data are presented as mean +/- SEM.

3.2.4 Forced Swim Stress Test

As used in this study, the forced swim stress test is used as an index of stress reactivity in rodents (Commons et al., 2017; Maldonado-Devincci et al., 2014a), with time to immobility used as the dependent variable of interest. AIE produced a reduction of immobility time (**Fig. 6A)** ($F_{(1,28)} = 4.52$, *p=0.04*) and there was a significant AIE x sex interaction ($F_{(1,28)} = 4.67$, *p=0.039*). *Post-hoc* analyses indicated a significant AIE-induced reduction of immobility time among male mice (*p=0.01*), but no AIE effect on immobility time among females (*p=0.99*) (**Fig. 6)**. There were no differences in time spent struggling (**Fig. 6B**) as a function of Sex ($F_{(1, 28)} = 0.3465$, *p=0.5608*) or Exposure ($F_{(1, 28)} = 1.098$, *p=0.3036*).

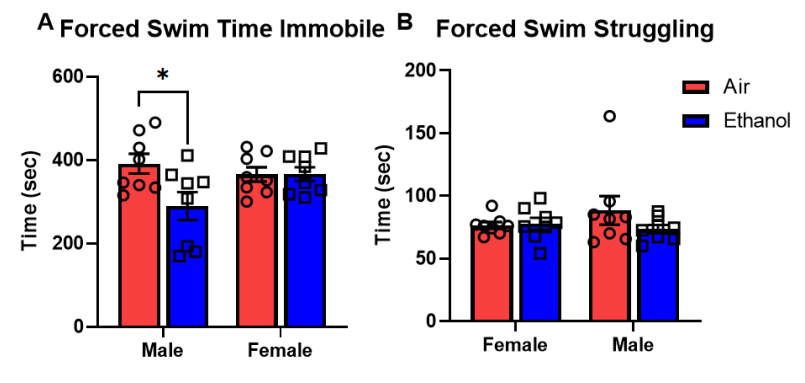

Figure 6: Forced Swim Stress Test Data A) Time spent immobile (sec), B) Time spent struggling (sec). Data are presented as mean +/- SEM. * p<0.05

3.3 Gene Expression

We measured mRNA levels of *FMR1* and other interacting gene expressions such as *Grin2a*, *Grin2B*, *Grm5*, *PSD-95*, and *Eaat1* in the cerebellum of control and adult AIE-exposed male and female mice. We observed that mRNA levels of all these genes are not altered by AIE except *Grin2B* mRNA levels (**Fig. 7**). Interestingly, *Grin2B* mRNA expression was significantly increased by AIE in whole cerebellar tissue from both male (*p=0.031*, t-test) and female *(p=0.039*, t-test) mice. All statistical analyses for each target are shown in **Supplemental Table 2**.

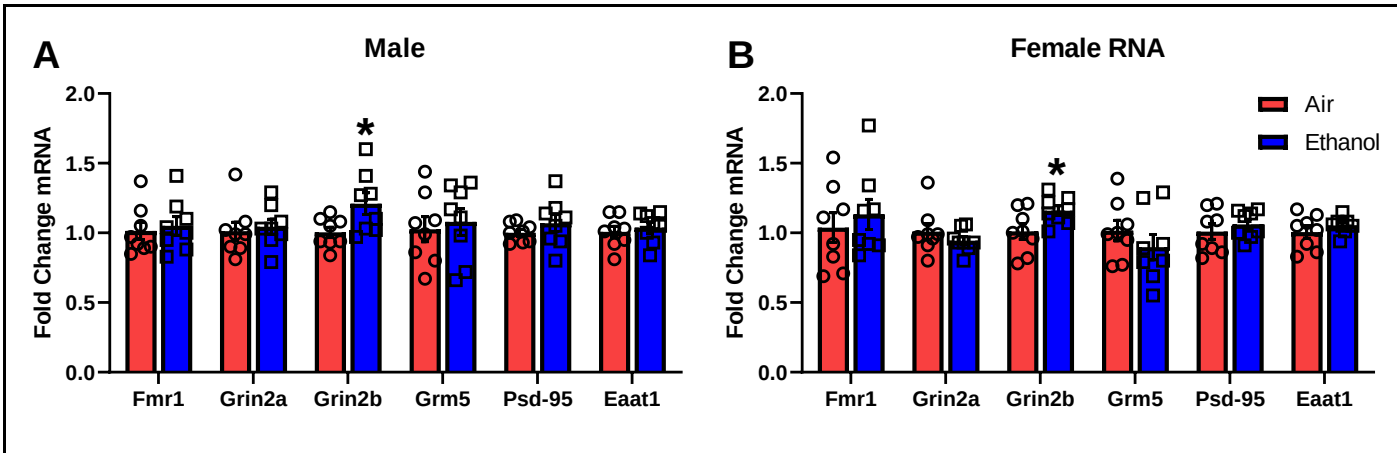

Figure 7: Fold change in mRNA expression A) Male, B) Female cerebellar mRNA expression of *FMR1* and other interacting genes. Data are presented as mean +/- SEM. * p<0.05

4. Discussion

The results of this study support the growing understanding that AIE alters exploratory and affective-like behaviors in adult rodents, and that some of those changes are sex-mediated. Specifically, we found that AIE reduced open field activity in adult mice of both sexes. It also reduced exploratory rearing and entries into the center portion of the open field, though those effects were driven largely by AIE effects in females. In contrast, AIE had no main effect on behavior in the light-dark or tail-suspension tests. However, results from the forced swim stress test showed a significant interaction of AIE and sex, indicating altered immobility among males and suggesting that AIE exposure may lead to differential stress responsiveness between the sexes in adulthood. This interpretation is consistent with our recent finding that AIE elevated the stress responsivity of male (but not female) rats to environmental stress (Healey et al., 2023). Thus, it may be that AIE-exposed females show an altered anxiety-like phenotype and AIE-exposed males show an altered stress-reactivity phenotype in adulthood.

It is notable that most of the work on AIE in rodent models has been done in rats (see (Crews et al., 2019; Robinson et al., 2021; Spear and Swartzwelder, 2014), though studies in mice have been proliferating of late (Carzoli et al., 2019; Kasten et al., 2020; Khan et al., 2023; Maldonado-Devincci et al., 2022; Piekarski et al., 2022). Studies of AIE using mice are needed because mouse models readily enable studies of genetic influence via transgenic models and inbred strains not available in rats, and because mice consume ethanol in free-choice paradigms at levels that significantly exceed free-choice self-administration in rats (Griffin, 2014; Maldonado-Devincci et al., 2022). Thus, mice provide an AIE model that has advantages for both genetic and self-administration studies. The present results also demonstrate that there are behavioral differences between the effects of AIE on mice and rats that should be understood as the models develop. For example, although in the open field test we found no effect of AIE in male mice on the number of center entries, time spent in the center portion, or the distance traveled in the center portion; Desikan and colleagues (Desikan et al.,

2014) found that AIE decreased the latency to enter the center portion, and increased the time spent in the center of an open field by male rats in an open field conflict test. Although the two studies used the open field differently, the species differences in AIE sensitivity within an open field framework are notable. Similarly, we found no AIE effects on the behavior of mice in the light-dark test, while Desikan and colleagues (Desikan et al., 2014) reported that AIE-exposed male rats returned to the light portion of the testing apparatus sooner than did water-exposed male rats, and engaged in more rearing in the lighted chamber. In addition, whereas we found that AIE decreased immobility time in the forced swim stress test among male mice, the previous study reported that AIE caused an increase in forced swim immobility in male rats (Desikan et al., 2014). The forced swim parameters were different between the two studies, but the observed behavioral differences suggest possible species-specific differences in stress responsivity in adulthood after AIE. Similarly, the open field and light-dark test data suggest an AIE-induced increase in risk tolerance in rats, but not in mice. These differences have methodological implications for future studies, and the species differences in enduring AIE responsivity may also lead to new mechanistic hypotheses. However, other studies have shown consistent findings of AIE effects of anxiety-like behaviors (elevated plus maze and light-dark box exploration tests) in male rats (Bohnsack et al., 2022; Kyzar et al., 2019; Pandey et al., 2015). In these studies animals were subjected to only one behavior test after AIE in adulthood.

The present study was not designed to directly assess the impact of cerebellar mechanisms on AIE-induced behavioral changes. However, the well-known relationship of cerebellar function to affective behavior (see (Schmahmann and Sherman, 1998), and the reciprocal and behaviorally relevant interactions of the cerebellum with the hippocampal formation, which is markedly modified by AIE (see (Crews et al., 2019; Robinson et al., 2021; Spear and Swartzwelder, 2014) for reviews), indicate the need to begin to address the effects of AIE on the cerebellum. In addition, previous work in humans showed that both male and female adolescent binge drinkers had decreased cerebellar volume (Lisdahl et al., 2013), and subregion-specific changes in cerebellar volume (Kekkonen et al.,

2021). In female rats, heavy chronic ethanol exposure impaired motor behavior and induced cerebellar neuronal loss (da Silva et al., 2018). In mice, AIE increased cerebellar brain volume in adulthood in males (Coleman et al., 2014). The present findings demonstrate an AIE-induced increase of cerebellar *Grin2B*-associated mRNA expression in both sexes, in the absence of changes in a number of other markers for excitatory synapse-related gene expression. This provides a specific gene expression focus for future studies of AIE on cerebellar function and a possible mechanism underlying those changes. For example, *Grin2B* hyperexpression after AIE could relate to the previously reported cerebellar cell loss after chronic ethanol exposure (da Silva et al., 2018) through an excitotoxic mechanism because increased GluN2b synaptic drive has been shown in hippocampal circuits after AIE (Swartzwelder et al., 2017) and is associated with potentially neurotoxic extrasynaptic glutamatergic drive (Zhou et al., 2013). Thus, the present findings indicate that excitatory synaptic mechanisms in both the hippocampus and cerebellum are altered by AIE and support the hypothesis that AIE may alter cerebellum-hippocampal interactions through those mechanisms. It is also notable that AIE did not alter the expression of *FMR1* in the cerebellum in this study, though *FMR1* expression was increased in the hippocampus after AIE (Mulholland et al., 2018), suggesting regional specificity with respect to the effects of AIE on synapse-related gene expression.

4.1 Limitations

Some of the limitations in this study include the age of AIE exposure was limited to only early adolescence and did not include adult comparisons. It is well known that adolescence can be divided into different developmental periods and exposure during early compared to late adolescence can induce different phenotypes including anxiety-like behavior, reward devaluation, conditioned taste aversion, peptide signaling, dopamine transmission, and ethanol sensitivity (Desikan et al., 2014; Healey et al., 2020; Saalfield and Spear, 2015; Spodnick et al., 2020; Towner and Spear, 2021; Varlinskaya et al., 2020, 2014). Testing order was not counterbalanced across animals and may have

influenced behavioral outcomes, especially in the light/dark test compared to the open field test. Only one withdrawal time point was used and shorter or longer withdrawal time periods may alter behavioral and cerebellar findings (Maldonado-Devincci et al., 2022). All of these limitations can be addressed in future work.

It is also possible that the behavioral testing sequence could have diminished the likelihood of observing AIE-induced changes in the expression of some of the genes assessed. Still, our goal was to assess gene expression in behaviorally tested animals, so we anticipated that limitation. Either way, it underscores the significance of the AIE effect on *Grin2B* expression in both males and females. Similarly, it is possible that previous behavioral testing could have influenced the outcomes on the latter behavioral tests, though the tests were intentionally ordered so that the most stressful tests occurred last in the behavioral testing sequence, and we did observe AIE effects on the last test given (FST). Still, the lack of effect we observed on the light/dark box and tail suspension tests should be interpreted cautiously.

4.2 Final Conclusions

These findings indicate that adolescent ethanol exposure induces long-lasting behavioral and cerebellar alterations in both male and female mice. These findings suggest that female mice may manifest post-AIE behavior change in the domain of anxiety whereas males may manifest post-AIE behavioral change more in the domain of stress reactivity. In addition, the findings show, for the first time, that AIE elevates the expression of *Grin2B*-associated mRNA.

5. References

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Supplemental Tables:

Supplemental Table 1: Behavior Testing Statistics

	F(interaction)	n	p	F(Sex)	n	p	F(AIE)	n	p
Open Field Test									
Total Distance Moved	F(1,28) = 1.77	n = 24	p = 0.194	F(1,28) = 0.38	n = 24	p = 0.542	F(1,28) = 31.49	n = 24	p < 0.001
Rearing	F(1,28) = 4.72	n = 24	p = 0.039	F(1,28) = 0.02	n = 24	p = 0.876	F(1,28) = 9.62	n = 24	p = 0.004
Center Time	F(1,28) = 3.20	n = 24	p = 0.843	F(1,28) = 4.74	n = 24	p = 0.038	F(1,28) = 0.03	n = 24	p = 0.866
Center Entries	F(1,28) = 3.13	n = 24	p = 0.088	F(1,28) = 1.96	n = 24	p = 0.173	F(1,28) = 14.64	n = 24	p < 0.001
Center Total Distance Moved	F(1,28) = 2.61	n = 24	p = 0.117	F(1,28) = 14.32	n = 24	p < 0.001	F(1,28) = 0.888	n = 24	p = 0.354
Light-Dark Box									
Latency to Enter Light Side	F(1,28) = 0.52	n = 24	p = 0.58	F(1,28) = 0.46	n = 24	p = 0.501	F(1,28) = 0.667	n = 24	p = 0.421
Avg Time in Light Side	F(1,28) = 0.01	n = 24	p = 0.941	F(1,28) = 0.02	n = 24	p = 0.885	F(1,28) = 0.80	n = 24	p = 0.378
Avg Entries in Light Side	F(1,28) = 0.16	n = 24	p = 0.689	F(1,28) = 3.41	n = 24	p = 0.076	F(1,28) = 2.90	n = 24	p = 0.100
Tail Suspension Test									
Latency to Immobility	F(1,28) = 0.47	n = 24	p = 0.500	F(1,28) = 2.22	n = 24	p = 0.148	F(1,28) = 0.23	n = 24	p = 0.639
Time Immobile	F(1,28) = 4.60	n = 24	p = 0.041	F(1,28) = 0.91	n = 24	p = 0.348	F(1,28) = 0.23	n = 24	p = 0.634
Forced Swim Test									
Time Immobile	F(1,28) = 4.67	n = 24	p = 0.039	F(1,28) = 1.63	n = 24	p = 0.290	F(1,28) = 4.52	n = 24	p = 0.042
Struggle Behavior	F(1,28) = 1.54	n = 24	p = 0.226	F(1,28) = 0.347	n = 24	p = 0.561	F(1,28) = 1.10	n = 24	p = 0.304

Supplemental Table 2: mRNA Statistics

Target	Male			Female		
	t(AIE)	n	p	t(AIE)	n	p
Fmr1	t(14) = 0.40	16	p = 0.694	t(14) = 0.62	16	p = 0.545
Grin2a	t(14) = 0.38	16	p = 0.707	t(14) = 0.99	16	p = 0.335
Grin2b	t(14) = 2.39	16	p = 0.031	t(14) = 2.27	16	p = 0.039
Grm5	t(14) = 0.40	16	p = 0.696	t(14) = 1.01	16	p = 0.330
pSD-95	t(14) = 1.03	16	p = 0.322	t(14) = 0.79	16	p = 0.444
Eaat1	t(14) = 0.54	16	p = 0.600	t(14) = 0.95	16	p = 0.360