Impact of Voluntary Alcohol Consumption During Early vs. Late Adolescence on Fear Acquisition, Extinction, and Renewal in Adult Long Evans Rats

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Abstract

Adolescence is a period of critical developmental windows that can be negatively impacted by external stressors leading to maladaptive responses. Previous studies illustrate that adolescent alcohol exposure can disrupt the structural organization of the limbic system and the circuits involved, specifically within the ventromedial prefrontal cortex (vmPFC), basolateral amygdala (BLA), and ventral hippocampus (vHipp), which are all involved in emotion regulation, learning, and decision making. These regions develop at different rates throughout adolescence depending on sex. Specifically, limbic regions such as the BLA develop in early adolescence, while cognitive processing regions such as the PFC develop later in adolescence. The present study analyzes a sex and timing of exposure dependent effect of adolescent alcohol consumption on fear learning and processing behaviors in adulthood. We hypothesized that, due to the differential rates of development between sexes, disruptions in fear learning would occur in females as a result of early exposure while disruptions in males would occur as a result of late exposure. Briefly, rats were trained to associate a mild shock with a predictive tone-light cue to acquire fear conditioning in Context A and then placed into a novel Context B and C one and two days later, respectively, wherein they were exposed to the tone-light cues alone. Freezing behavior was quantified using video-tracking approaches. Our results indicate a sex and timing of exposure dependent effect on fear learning behaviors, in that differences appeared in rate of acquisition of fear conditioning, extinction of contextual fear, and decreased fear renewal.

Introduction

Consumption of alcohol in humans can have lasting negative consequences on the brain and behavior. The neurotoxic effects of alcohol consumption are due to induced neuroinflammatory effects, such as activated glial cells and stimulation of intracellular signaling pathways that cause accumulation of inflammatory molecules, leading to cell death (Boerngen-Lacerda, 2016). Given that alcohol is a toxic substance, many neurocognitive deficits emerge after chronic, heavy (5 or more drinks in a row) alcohol use, including problems with attention, memory, visuospatial, verbal, and executive processing (Hanson et al., 2011). Indeed, alcohol use disorder is often associated with various mood and stress-related disorders that present with deficits in learning and emotion regulation, such as in fear processing and associative learning (Crews et al., 2007) which are typical of PTSD and other generalized fear disorders.

For this reason, it is particularly important to understand how drinking in this period may affect the brain that could set the conditions for persistent (adult) mental health susceptibility. Prior work indicates that alcohol-related changes in brain and mental health often persist into adulthood and include increased anxiety, impulsivity, risky behavior, and cognitive inflexibility (Bava and Tapert, 2010, Spear, 2018). Alcohol use typically starts in adolescence, with 74% of admissions to the Treatment Episode Data Set aged 18-30 stating they began consumption before age 17 (TEDS Report, 2014). Adolescent alcohol use corresponds to impaired stress resilience in adulthood, for instance in the emergence of depression and antisocial personality disorder (Rohde et al., 2001). Voluntary alcohol consumption in high school can lead to major depressive disorder (Grant and Harford, 1995) as well as anxiety-related and other stress-related disorders (Kyzar et al., 2016; Rohde et al., 1996). These relationships are sex-dependent with stronger

associations in females (Grant and Harford, 1995). The time-dependent interaction between voluntary alcohol use in adolescence and impaired stress resilience in adulthood is thought to be due to the development of regions such as the prefrontal cortex (PFC) and amygdala given that maturation of these learning and emotion regulating regions occurs primarily during early life, especially during adolescence (Bava and Tapert, 2010).

Alcohol tends to be the most widely abused substance during adolescence (Bava and Tapert, 2010), which is typically defined in humans as the period of development roughly between the ages 12 to 20-25 (Crews et al., 2007). As alluded to above, this period is marked by extensive growth and neuromaturation of the PFC and the limbic system, including the amygdala (Bava and Tapert, 2010). Due to rapid synaptogenic processes throughout the early stages of life, adolescence also marks a period of synaptic overproduction and subsequent synaptic loss (e.g. pruning, reorganization), which characterizes the inverted U-shape development of gray matter volume from early childhood to adulthood (Brenhouse and Andersen, 2011, Crews et al., 2007, Gass et al., 2014, Conrod, 2016). Specifically, gray matter (cell bodies and unmyelinated axons of neurons) in regions such as the PFC, caudate/putamen, and cerebellum peak during early adolescence (onset of puberty) (Conrod, 2016) and decrease as one ages (Brenhouse and Andersen, 2011). White matter tracts of fronto-parietal regions follow a more linear development across adolescence (Conrod, 2016). Regions such as the orbitofrontal cortex, basal ganglia, and various regions of the limbic system mature in early adolescence and are involved in emotion/fear regulation and sensation seeking (Conrod, 2016). In contrast, late adolescence (~ages 15-18) marks the development of frontal cortical regions involved in top-down control of cognitive and emotional behaviors (Conrod, 2016). The differential development of these regions is thought to heighten risk for substance abuse in adolescence (Kuhns et al., 2022, Spear, 2015)

as bottom-up sensation seeking outpaces top-down impulse control. In this manner, substance use in earlier phases of adolescence could also produce a heightened risk for substance use-induced impairments on brain and behavior in adulthood.

The consequences of alcohol use can vary based on sex and timing of consumption. Humans typically reach puberty in early adolescence: onset for females is between 9 and 14 years of age, for males it is between 10 and 15 (Dorn et al., 2006). This period encompasses hormonal, physical, and neurobiological changes (Dorn et al., 2006). The development of subcortical tracts differ based on sex and pubertal timing: As pubertal stage progresses in females, females lose amygdalar gray matter volume, although the opposite is seen for males (Vijayakumar et al., 2018). For both sexes, the hippocampus loses gray matter volume as the stage of puberty progresses, though findings in this area are inconsistent across studies (Vijayakumar et al., 2018). Reduction in cortical gray matter is linked to increased pubertal stage and testosterone levels (Vijayakumar et al., 2018). Gray matter development in the amygdala differs by sex; as opposed to the inverted U seen in other brain regions, some studies found that females reach maximum volume prepubertally, while male development continues into early adulthood (Brenhouse and Andersen, 2011). Subregion specific sex differences exist in the development of the amygdala as well, though the basolateral amygdala (BLA) follows an inverted U trajectory, with females losing neuronal count throughout adolescence (Hammerslag and Gulley, 2016). Rate of amygdalar volume development is higher in males than females, though the opposite is the case for the hippocampus, which, in terms of functionality, is speculated to impact neuronal processing and efficiency (Brenhouse and Andersen, 2011). Additionally, females undergo cell death and synaptic pruning throughout adolescence on a greater scale than males, thus contributing to greater medial PFC (mPFC) volume in males by

adulthood (Hammerslag and Gulley, 2016). With these sex-dependent differences in development of alcohol-sensitive regions in mind, more research is needed to understand whether and to what degree alcohol exposure at different developmental windows can influence resilience in adulthood in a sex-specific manner.

Rats are commonly used as a model for adolescent alcohol consumption, as implicated brain regions can be translated across the two species and variables such as sex and timing of exposure can be controlled (Kuhns et al., 2022). As in humans, adolescence is comprised of critical developmental periods in rats, with similar areas of the brain such as the PFC and amygdala undergoing sex-dependent neuromaturation (Crews et al., 2007). There is a noticeable reduction in impulse control and an increase in sensation seeking, as the PFC matures much later than limbic regions such as the amygdala and nucleus accumbens (Crews et al., 2007). Although there is some variability across strain and sex, adolescence in rats can be estimated to begin as early as the 25th day of life (post parturition 25, or P25; Crews et al., 2007, Spear, 2015). Adolescence can be further divided into early (P25-42), and late (P43-P55) adolescence, whereas P55-65 represents the emergence of adulthood (Spear, 2015).

Development of regions such as the PFC, amygdala, and hippocampus differ depending on the timing of adolescence. The PFC and BLA follow the same inverted U-shape development across adolescence as in humans (Hammerslag and Gulley, 2016). Regional specificity translates across species as well: the medial amygdala (MeA) follows a similar linear pattern of growth in males up until adulthood (Hammerslag and Gulley, 2016). An increase in substance-seeking neuronal changes is linked to early adolescence, with an increase in ventral tegmental area (VTA) dopaminergic neurons, dopamine receptor activation in the mPFC, and glutamatergic neurons to accumbens (Hammerslag and Gulley, 2016, Spear, 2015). Neurobiological

development is influenced by puberty-associated hormones. Gonadal hormones impact the organization of brain development during early adolescence, thus a stressor introduced at this point in development would have a greater impact on anxiety-like behaviors and resilience in adulthood (Spear, 2015). Indeed, early adolescence contains more extensive connections between the mPFC and BLA than in later adolescence or adulthood (Spear, 2015), two regions known to contribute to fear learning and extinction behaviors, a frequently used laboratory metric of emotion regulation and stress resilience in rodents.

These regional developmental differences produce some of the variable behavioral effects seen in adulthood based on alcohol exposure period. As reviewed by Spear (2015), research shows that early adolescent alcohol consumption leads to increased risky behaviors and social anxiety. Moreover, alcohol consumption at this early phase also decreased retention of contextual fear memories. On the other hand, consumption during late adolescence led to decreased attention and increased waiting impulsivity, context extinction deficits, and tolerance to ethanol sensitivity reminiscent of adolescence (Spear, 2015). Interestingly, only males displayed the late-adolescent dependent effect on social anxiety and adolescent-reminiscent ethanol sensitivity, which clashes with human studies illustrating a higher prevalence in females of ethanol-associated brain damage that impacted later cognitive task performance (Spear, 2015). Despite these differences, a trend in impaired resilience in adulthood appears after adolescent alcohol consumption, specifically in anxiety-like phenotypes. Sanchez-Marin et al., 2022 found increased anxiety-like behaviors and plasma corticosterone levels on elevated plus mazes in adulthood after binge alcohol exposure in adolescence. This evidence illustrates the concern adolescent ethanol exposure poses to anxiety and stress-related psychopathology in adulthood.

In this study, we aim to determine whether voluntary adolescent alcohol consumption impacts stress resilience in adulthood through altered fear processing behaviors, particularly when male and female rats are given access to alcohol at different periods of adolescence (early vs late). While classical cued conditioning involves plastic processes in the amygdala and mPFC, contextual fear conditioning involves these regions and the ventral hippocampus. During the initial learning of fear (i.e. acquisition), a one-neutral stimulus (e.g. light cue) is repeatedly paired with an unconditioned stimulus (e.g. footshock) to build an association. Once the association is acquired, the neutral stimulus becomes a conditioned stimulus (CS), which then can elicit a conditioned response (CR) that is linked to amygdalar activation, such as freezing behavior. The central amygdala (CeA) specifically is responsible for changes in freezing, as well as heart rate, respiration, and other fear responses due to direct (and indirect) pathways that innervate the hypothalamus, brainstem motor regions, and thus, sympathetic nervous system (Moustafa et al., 2013). Adolescent alcohol consumption also alters the sex differences in regions involved in adult fear learning processes. Specifically, adolescent ethanol consumption disrupted hippocampal-dependent learning processes (Bergstrom et al., 2006, Broadwater and Spear, 2013). As adolescence marks the development of regions and circuits involved in fear learning behaviors, and alcohol disrupts the development of those regions, we hypothesized that voluntary binge like alcohol consumption in adolescence would produce sex and age of exposure-dependent disruptions in contextual fear conditioning behaviors in adulthood.

Alcohol, a toxic substance, and its abuse in adolescence affects neuronal regions that later impact associative fear learning processes and manifest as PTSD or anxiety-like symptoms in non-threatening environments. Studying the precise behavioral impacts in a controlled environment on rat subjects provides the first step to understanding and treating disruptions in

fear learning due to adolescent alcohol exposure. The present study looks specifically at an age of adolescent exposure effect between males and females, due to differential development throughout adolescence. Since male and female BLAs develop at different stages of adolescence, we expected to find disruptions in contextual fear conditioning behaviors in adulthood based on timing of exposure. Specifically, we hypothesized that disruptions will occur in females as a result of early exposure, while they will occur in males as a result of late exposure.

Methods

Subjects:

70 male and female Long Evans rats were ordered from Envigo and housed in a temperature controlled vivarium on a 12/12 h light/dark cycle (lights on at 0700). All rats were group-housed (2-3/cage) upon arrival at P21 and left undisturbed until experiments began (P25 or P45, as below). Except where noted, all animals received *ad libitum* access to food and water and all animal experimentation followed guidelines for the National institute of Health and were approved by the University of Colorado, Boulder Institutional Animal Care and Use Committee.

Apparatuses:

Drinking Context: Standard home cage that lacked food and water. The sipper tube replaced the standard water bag and was measured between drinking sessions to ensure no leakiness.

Behavioral boxes: All fear behavior took place in a rectangular apparatus (43 cm × 43 cm × 53 cm; Med Associates) with three stainless steel walls and a plexiglas front hinge door. The shock grid floor comprised stainless steel rods placed 2 mm apart (Bercum et al., 2021).

Differing contexts: A three-context ABC renewal procedure was used as described by Jin and Maren (2015) that included three different behavioral contexts for each conditioning session as well as three different transport contexts.

Ethanol Exposure (adolescence):

Males and females were randomly assigned H2O or 15% ethanol using the voluntary, binge-like, Drinking-in-the-Dark (DID) protocol (Thiele and Navarro, 2014). Here, 15% ethanol in drinking water was provided during the first four hours of the dark cycle and was repeated daily for the duration of the assigned period, determined based on the assigned treatment group. Treatment group 1 comprised early adolescent (P25-45) animals while treatment group 2 comprised late adolescent (P45-55) animals. Upon lights out, animals were placed into separate cages provided solely water (controls) or ethanol from drip-resistant bottles. Water bottles were weighed at the start and end of each drinking period, and consumption was calculated based on the reduction in weight of the bottle each day. Rats were also weighed daily to account for any ethanol-induced changes in growth. At the end of the assigned period (i.e. P45 for early adolescent, P55 for late adolescent), animals were left in their home cages to age to P75, where they started fear conditioning behaviors. The experimental timeline is depicted in Figure 1.

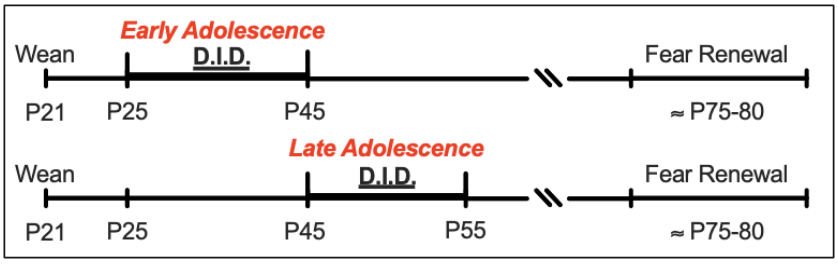

Figure 1. Timeline of ethanol exposure and fear renewal testing for both cohorts.

Fear Conditioning (adulthood):

Fear Acquisition (Day 1): Upon adulthood (P75), water- and ethanol-drinking rats were transported in a contextually significant bucket from their home cage to the fear conditioning

apparatus (“Context A”), where they were exposed to a compound auditory tone (2kHz; 10s; 80dB) and visual (white LED light) stimulus (Cue). This stimuli was administered in conjunction with mild shocks (1mA; 2s; n=5; 60s inter-trial intervals, [ITI]).

Fear Extinction (Day 2): The following day (24hrs later), animals were transported in a contextually different bucket from their home cages to the fear conditioning apparatus (“Context B”) and received 40 of the same light+tone cues with 30s ITIs. However, they received no shocks.

Fear Renewal (Day 3): The following day (24 hours later), animals were once again transported in a contextually different bucket to the fear conditioning apparatus (“Context C”) and received 5 of the same light+tone cues with 30s ITIs but no shock.

Data Quantification/Analysis:

Freezing behavior is a robust measure of fear in rodents. Freezing was quantified using computer-based tracking software (Video Freeze). Statistical analyses include T-test and repeated measures, two-factor ANOVAs were followed with *a priori* post-hoc tests. For all analyses of adult behaviors, we report first an omnibus ANOVA that includes all subjects used, including factors of Sex (M/F), Alcohol (Water, Early-EtOH, Late-EtOH), and the repeated measure of interest (i.e., either Fear Acquisition, Fear Extinction, or Fear Renewal). The purpose of this first analysis is to specifically identify any potential sex differences in our study. Following that, analyses of the effect of Alcohol are then conducted specifically within Sex (i.e., males and females separately) to better isolate and understand the effects of Alcohol on these groups.

Results

Ethanol Consumption

Ethanol consumption during the assigned adolescence period is shown in Figure 2. No difference is seen between sexes or between early and late drinkers. Early adolescents consumed ethanol steadily during the first 12 days, before their consumption increased and peaked at day 18. Late adolescents consumed ethanol daily consistently throughout the ten day period.

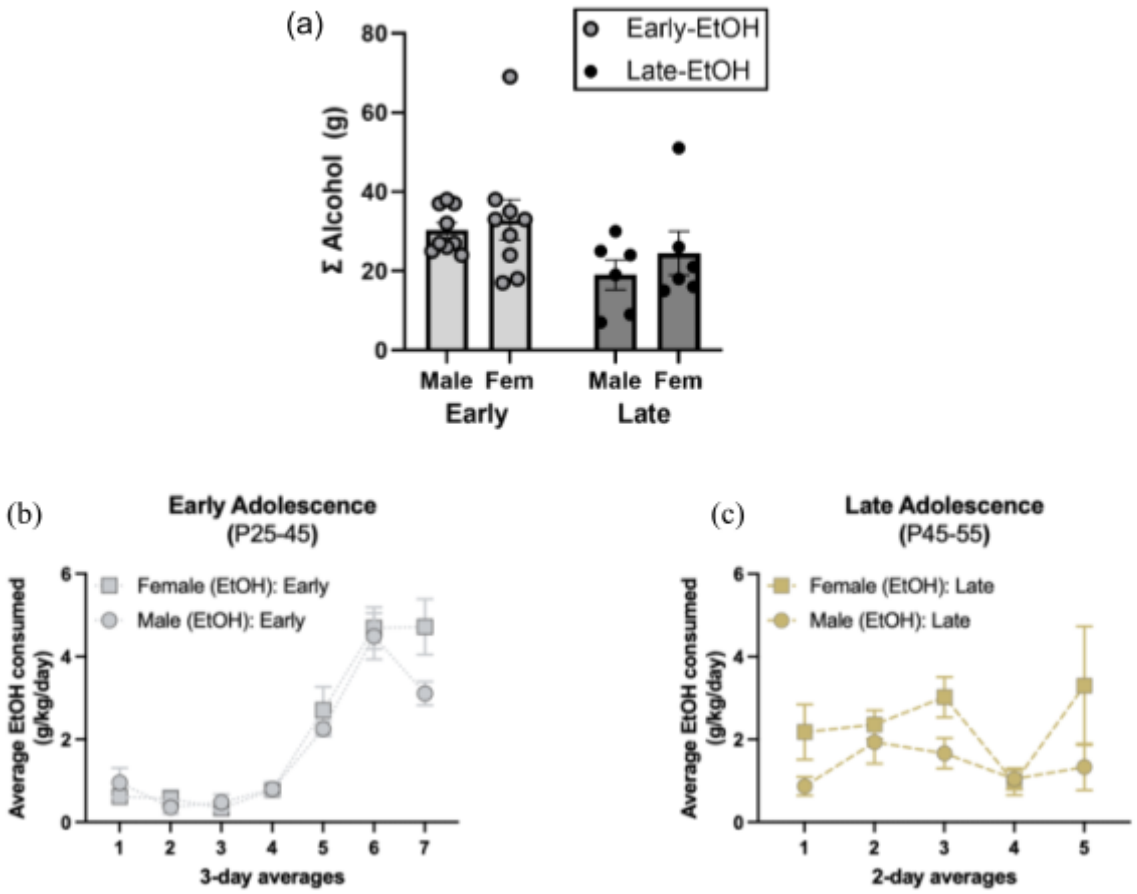

Figure 2. Qualitative assessment of ethanol consumption. (a) No differences in consumption across sex or age of ethanol exposure. (b) No difference in consumption rates between sexes in early adolescence. Consumption picks up in the third week. (c) No difference in consumption rates between sexes in late adolescence. Consumption was steady across the assigned period.

Fear Acquisition: Freezing During Cue Presentation

After consumption periods, both cohorts were left undisturbed in the vivarium until P75 . All animals then underwent fear acquisition, with exposure to five tones paired with shocks. Freezing behavior was measured in Context A to determine an impact of sex and age of ethanol exposure. Overall, there was no main effect of cue-elicited freezing due to Sex ($F(1,49) = 1.80$, $p = 0.19$) or an interaction of Sex X Alcohol ($F(2, 48) = 0.52$, $p = 0.60$). However, an interaction between the repeated measure, Cue (i.e., the pre-cue baseline period and the five presentations of the cue), and Sex was significant, ($F(5, 240) = 5.62$, $p < 0.0001$). Specifically, male rats showed higher levels of freezing compared to females during the second CS presentation (Tukey: $p < 0.0001$). Therefore, we looked more closely at the impact of ethanol exposure on freezing within sexes. First, in females, we found a main effect of Alcohol, ($F(2, 24) =3.82$, $p = 0.036$). There was a main effect of the repeated measure Cue ($F(5, 120) = 151.01$, $p < 0.0001$), indicating that cued fear was significantly different across the acquisition session. However, this effect differs significantly depending on exposure period to ethanol, as there was an interaction between Cue X Alcohol, ($F(10, 120) = 3.09$, $p = 0.002$). No differences were seen between groups during pre-cue baseline (BL) period and all CS presentations, except CS2, where it is shown that early ethanol exposure results in greater freezing than either the Water exposure (Tukey: $p = 0.002$) or the late ethanol exposure (Tukey: $p = 0.0004$). As for the males, a similar pattern was observed with a main effect of Alcohol, ($F(2, 24) = 3.43$, $p = 0.049$) due to differences between Early-EtOH and Late-EtOH groups (Tukey: $p = 0.039$). However, there was no interaction between Cue X Alcohol, ($F(10, 120) = 1.29$, $p = 0.24$).

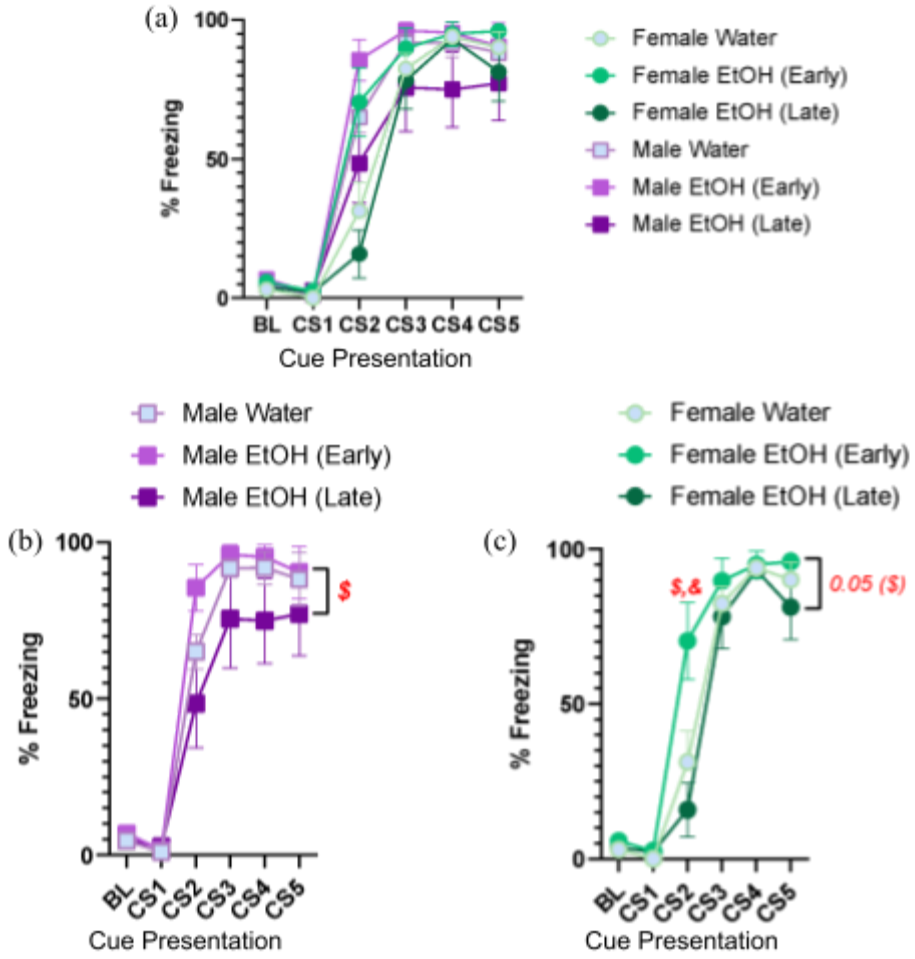

Figure 3: Measure of freezing behavior during fear acquisition. (a) Little freezing during the pre-cue period and first cue presentation. Variability is seen upon presentation of the second cue dependent on sex and age of ethanol exposure. (b) Within males, early ethanol drinkers froze significantly more than late drinkers during 2nd-5th cue presentations. (c) Within females, early ethanol drinkers froze significantly more than controls and late drinkers during the second cue presentation, and more than late drinkers during final cue presentation. $p < 0.003, &p < 0.003

Fear Recall and Extinction

Twenty four hours after fear acquisition, we tested fear recall and extinction in a novel context B. In females, there was no main effect of Alcohol, ($F(2, 24) = 0.97$, $p = 0.39$), but there was a significant interaction between Alcohol X Cue, ($F(18, 216) = 1.71$, $p = 0.039$). This interaction appeared in the pre-cue BL region, where early drinkers froze significantly less than both the water controls (LSD: $p = 0.004$) and the late drinkers (LSD: $p = 0.001$). Freezing during

fear recall (first three bins) and fear extinction (middle three bins) did not differ between groups (LSD: all pairwise comparisons, $p < 0.17$). In males, there were no significant alcohol-related effects for freezing behavior (main effect Alcohol, ($F(2, 24) = 0.76$, $p = 0.48$); interaction Alcohol X Cue, ($F(18, 216) = 1.35$, $p = 0.16$). Freezing behavior was also measured between cue presentations for all groups. We found a main effect of Sex, ($F(1, 48) = 0.045$), with males freezing more than females. There was also an effect of ethanol exposure in that Early-EtOH females froze less than males from PostCue bins P2-P5 (LSD: all $p < 0.05$). In females, an interaction between Alcohol X PostCue exists due to significantly less freezing in the first PostCue bin for the early ethanol group compared to the Water controls (LSD: $p = 0.0002$) and to Late-EtOH (LDS: $p = 0.0003$). Males demonstrate this same interaction with early ethanol freezing significantly less than Water controls (LSD: $p < 0.0001$) and Late-EtOH (LSD: $p < 0.0001$) in the first PostCue bin. However, the late ethanol group continued to freeze less than Water Controls (LSD: P4, $p = 0.042$; P5, $p = 0.012$) and the early ethanol group (LSD: P5, $p = 0.043$) in subsequent PostCue bins.

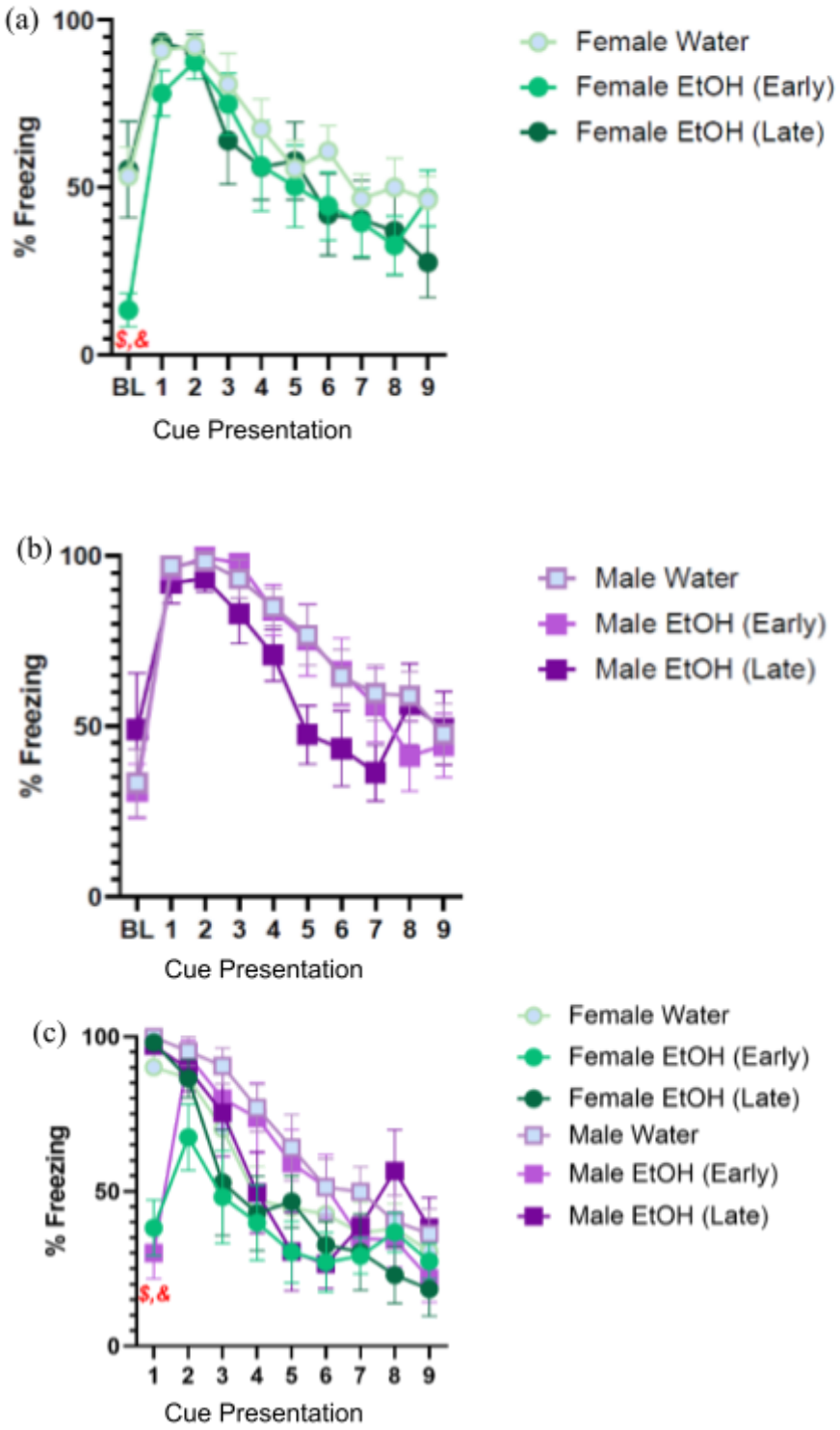

Figure 4: Measure of freezing during extinction cue presentations and post-cue intervals. (a) Within females, early ethanol drinkers froze significantly more than controls and late drinkers during baseline period. Upon first cue presentation, all groups show high levels of freezing that decreases steadily across remaining cue presentations. (b) Within males, all groups show some initial freezing during the baseline period. Upon first cue presentation, all groups show high levels of freezing that decreases steadily across

remaining cue presentations. (c) In the post-cue period after the first cue presentation, early drinkers in both sexes show decreased freezing compared to their respective controls and late drinkers. In subsequent post-cue periods, there is no difference between female groups. Late drinking males demonstrated decreased freezing in the subsequent post-cue periods, with the exception of P8 where their freezing is increased, compared to controls and early drinkers. $p < 0.003, &p < 0.003

Fear Renewal: Freezing During Cue Presentation

Twenty four hours after fear extinction, rats were placed in a novel context C and received five cues without shocks. Freezing was measured within sexes to determine the strength of extinction memory outside the extinction context. Posthoc analysis indicated decreased overall freezing in females compared to males (Tukey: $p = 0.044$). In females specifically, the early ethanol group froze less compared to the Water controls (Tukey: $p = 0.042$) and to the Late-EtOH group (Tukey: $p = 0.036$). A similar result was seen in males, with Early-EtOH displaying less freezing than the water controls (Tukey: $p = 0.026$), though not compared to Late-EtOH ($p = 0.244$).

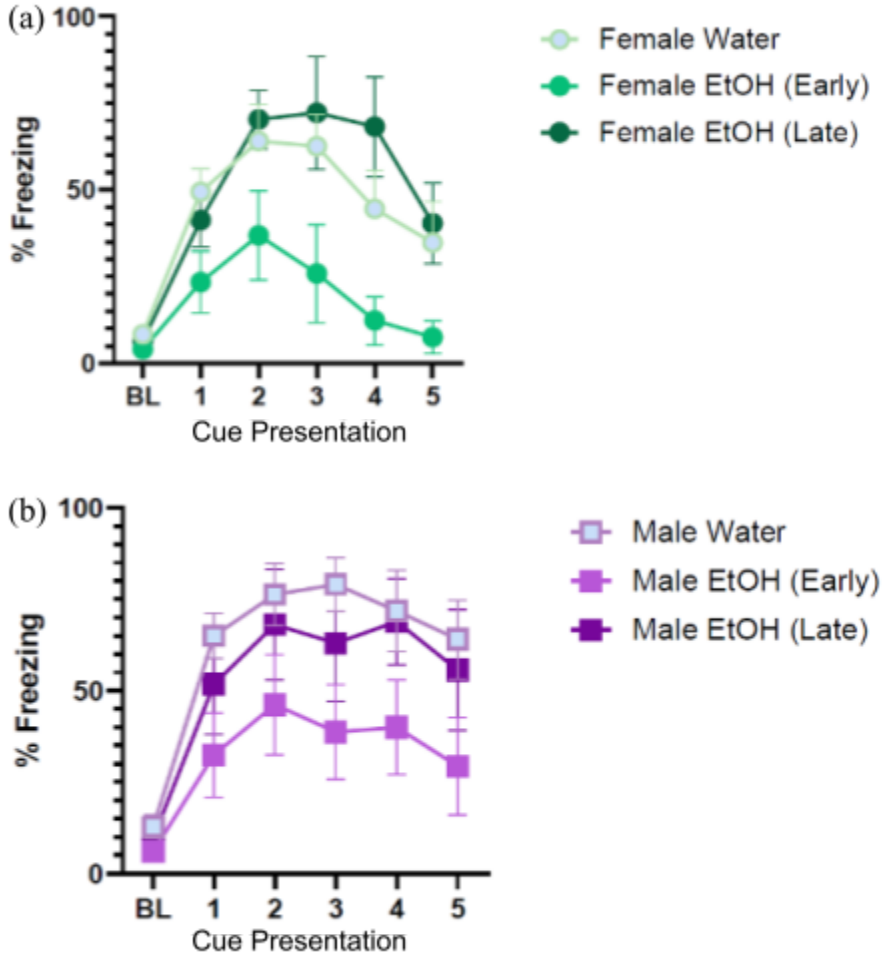

Figure 5: Measure of freezing behavior during fear renewal. (a) In females, early ethanol drinkers froze significantly less than late ethanol drinkers and controls. (b) In males, early ethanol drinkers froze significantly less than water controls.

Relationship between ethanol consumed and freezing

A linear regression analysis was run to determine the effects of the amount consumed on freezing behavior during each fear conditioning session. No significant trends were found for fear acquisition or fear renewal, but an interesting sex difference appears during fear extinction. While not significant, a trend towards negative association ($R^2 = 0.15$; $p = 0.16$) was seen in males between average ethanol consumption in adolescence and later freezing during fear extinction in adulthood. On the other hand, females displayed a significant positive relationship

($R^2 = 0.48$; $p = 0.004$) between ethanol consumption and freezing during extinction; i.e. higher consumption = more freezing.

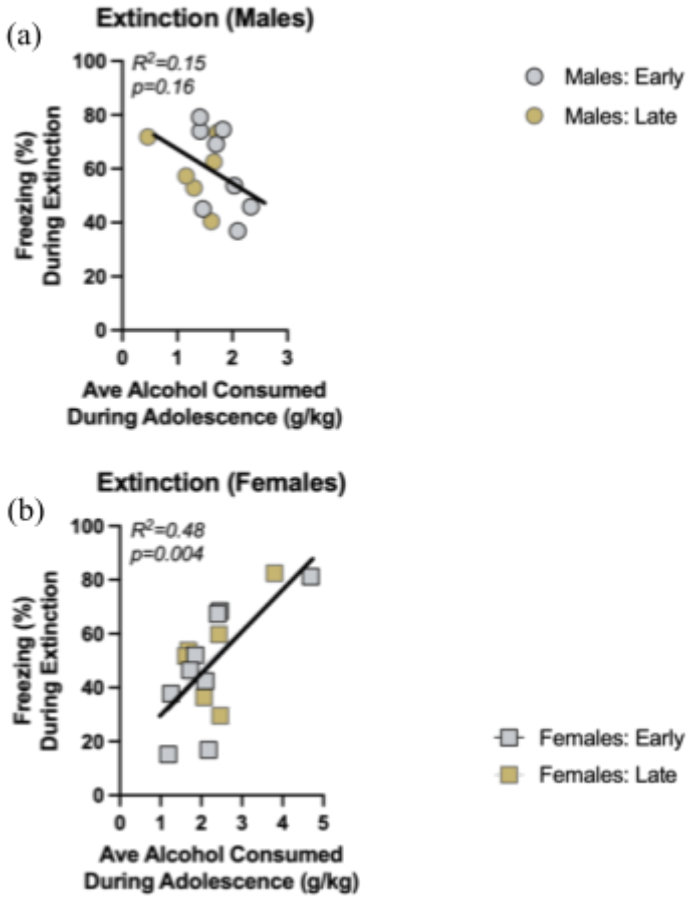

Figure 5. The association between ethanol consumption in adolescence and subsequent freezing behavior during fear extinction in adulthood. (a) In males, a trend exists towards a negative relationship between consumption and freezing during extinction. (b) In females, a significant positive association exists between consumption and freezing during extinction.

Discussion

The present study focused on the effects of adolescent voluntary binge ethanol consumption on fear behaviors in adulthood. This "fear renewal" procedure (Jin & Maren, 2015) utilized an ABC contextual design, so that contextual testing could occur where there was no association with prior footshocks. No clear sex differences in alcohol consumption were seen at either stage of adolescence, however differences emerged in fear learning. We showed that in

adult rats with a history of repeated ethanol consumption in adolescence, there is (1) earlier acquisition of fear conditioning, (2) accelerated contextual extinction, (3) reduced post-cue freezing (but not cued freezing) in early extinction, and (4) decreased fear renewal in a novel context following extinction. These differences depended on both sex and timing of ethanol exposure. In fear acquisition (1), there is a large variation in freezing upon presentation of the second cue, indicating that some groups made the association between cue and shock earlier than others. Specifically, there is more freezing for males than females, and more freezing for early ethanol rats compared to controls and late ethanol rats in females. It is surprising that there is a delay for females compared to males considering the activation of prelimbic cortical neurons of the mPFC (PL) consistent with increased cued fear conditioning is higher in females (Bauer, 2023). However, it was found that chemogenetic inhibition of the PL did not affect freezing behaviors, though it did impact conditioned suppression in females only (Bauer, 2023), suggesting that the PL is preferentially engaged depending on the task. Seeing as the late drinkers did not differ from controls, the increased freezing brought on by drinking in early adolescence could be due to the development of the BLA occurring during early adolescence (Conrod, 2016). It is interesting that the impact seems to promote fear learning, as opposed to the impairment we originally hypothesized. In addition, these results differ from previous studies reporting no effect of adolescent consumption on freezing behavior during acquisition (Bergstom et al., 2006, Broadwater and Spear, 2013). Taken together, it appears as though alcohol consumption during early adolescence specifically seems to interact with the development of the BLA such that their freezing response is sensitized. This suggests that they have either learned the pairing quickly or are increasingly sensitized and are freezing in response to a noise. Future

studies could dissect whether acquisition of fear learning or sensitization of acoustic/visual stimuli are differentially impacted by alcohol consumption during early adolescence.

Fear extinction creates a new memory that competes with the fear acquisition memory for behavioral control, i.e. overriding the association between CR and CS. In response to cue presentations throughout the extinction protocol, there is no difference in freezing between groups. However, differences appear during the pre-cue baseline period (2), during which no cues have been presented. The females who consumed alcohol during early adolescence showed significantly less freezing during the baseline period of the fear extinction experiment. On the other hand, water-drinking and late adolescence alcohol-drinking females exhibited greater freezing during this time, which suggests that some context recognition could have elicited freezing in the latter groups, but that these context cues could have been undetected by early adolescent drinkers. During contextual fear acquisition, males tend to show increased activation of the hippocampus, while females show increased activation of the BLA (Bauer, 2023), which may explain why this effect of ethanol exposure is seen in females, but not in males. It is plausible that this effect is context specific, suggesting the decrease in freezing in our early drinkers is likely due to the development of the hippocampus. Hippocampal development largely takes place in rats after P8 and occurs in a linear fashion throughout adolescence (Chin et al., 2010), with the majority of synaptic pruning occurring around P30 (early adolescence) (Spear, 2000). In fact, hippocampal slices from early adolescent rats were more sensitive to hippocampal defects, specifically in long term potentiation, compared to adult slices (Spear, 2000). Taken together, our results suggest a disruption in hippocampal development due to early ethanol exposure, and this expression is more evident in the females due to the intrinsic preferential activation of the BLA, instead of the hippocampus during contextual fear recall.

In fear extinction, the BLA and CeA are inhibited by intercalated interneurons (ITC) that are innervated by neurons projecting from the infralimbic cortex (IL) of the mPFC, preventing the fear responses seen during fear acquisition (Gruene et al., 2015, Moustafa et al., 2013). Interactions between the IL and BLA during fear extinction differ between males and females (Bauer, 2023). Though there were no sex differences in freezing and rates of extinction during cue presentation and PostCue, linear regression analyses of individual animals' consumption, regardless of the developmental period of consumption, suggest that the amount of alcohol consumed differentially impacts freezing during the extinction period in a sex-dependent manner. Extrapolating from this, our results allow for a possible inference to be made about a sex-dependent interaction between the IL and BLA. We also found an age of ethanol dependent effect on freezing during the first PostCue period (3), with early adolescent drinkers freezing less than late adolescent drinkers and controls, regardless of sex. This suggests that early adolescent drinkers are significantly more tuned into the cues themselves as opposed to the context. Retrieval of an extinguished CS is demonstrated to induce Fos expression in areas of the VH projecting to the mPFC and BLA (Jin and Maren, 2015). Following the understanding that early adolescent consumption impacts hippocampal development, our results suggest decreased hippocampal intervention in the PFC -> BLA pathway, thus heightening the activation of that pathway in response to cue, while producing little response based on context.

Previous studies also found a latency to freezing in extinction after adolescent alcohol consumption, suggesting that impacts of adolescent consumption on adult fear behaviors are age of exposure dependent (Bergstom et al., 2006, Broadwater and Spear, 2013). This trend appears to be context dependent, as a study on tone extinction did not find the same latency to freezing in ethanol exposed rats (Broadwater and Spear, 2013). Together, these results suggest that early

adolescence is a time of hippocampal development, and that alcohol consumption impairs this development that impacts fear learning behaviors in adulthood. Additionally, alcohol exposure during mid-late adolescence and adulthood invoked greater freezing compared to controls during context extinction (Broadwater and Spear, 2013), a result not present in this study. The mPFC is known to be a structure that develops later in adolescence (Brenhouse and Andersen, 2011, Spear, 2015) and therefore impairments in fear extinction due to late adolescent exposure would be expected. Broadwater and Spear, 2013 utilized male Sprague Dawleys that began consumption in mid adolescence (P35). Our late drinkers began consumption at P45, and our early drinkers drank for double the time, which might account for the difference.

Fear renewal relies heavily on the ventral hippocampus (VH) (Jin and Maren, 2015) and preferentially activates regions depending on sex, with more activation of the hippocampus seen in males and more activation of the BLA seen in females (Bauer, 2023). Lesions in the VH prevented context specificity in fear renewal, impairing fear extinction in new or same contexts through disruption of firing in the amygdala and the mPFC, indicating that fear renewal involves projections of the VH to both the BLA and mPFC (Jin and Maren, 2015, Moustafa et al., 2013). Specifically, the VH projects to the PL region of the mPFC, activating the BLA, CeA, and thus subsequent fear responses seen in fear renewal (Knapska et al., 2012). The present study found an age of ethanol exposure effect in fear renewal, regardless of sex (4). Early ethanol consumers displayed less freezing compared to the other two groups, suggesting once more an impact on later contextual fear behaviors due to effects on hippocampal development, following the pattern displayed during fear extinction, where cue elicits an effect in fear response, but context has significantly less of an effect. These results indicate a difference in adolescent development that

were not indicative of learning impairments, but rather later impacted hippocampal dependent fear behaviors.

Some studies have noted an effect of the estrous cycle on fear responses, which lends to sex differences. In rodents, there are four different phases of the estrous cycle, based on fluctuation of estrogen 17β estradiol (E2), a particular hormone implicated in fear behaviors (Bauer, 2023). E2 has a dose and treatment duration dependent effect on fear learning; in naturally cycling rats, high levels of E2 correlate with low levels of context fear conditioning (Matsumoto et al., 2018). E2 also impacts dendritic spine density in the hippocampus, amygdala, and prefrontal cortex, increasing long term potentiation within the hippocampus (Bauer, 2023). Hormonal differences (estrogen/phase of estrous cycle) contribute to regional and behavioral sex differences. For example, E2 leads to stronger excitatory inputs and synaptic plasticity in the lateral amygdala, leading to enhanced cued fear conditioning in females (Blume et al., 2017). These hormones act in conjunction with inhibitory interneurons, specifically GABAergic interneurons, which are found to impact cued fear conditioning as well (Bauer, 2023). Specifically, calcium binding protein parvalbumin is the main source of inhibition on the BLA, and is expressed more during periods of low E2 in rats (Bauer, 2023). The present study did not account for estrous phase, and therefore future studies are necessary to analyze phases of the estrous cycle of female rats throughout the drinking period and fear conditioning in order to determine any patterns based on hormones, especially since some studies found no correlation between estrous cycle and context fear conditioning (Keiser et al., 2017).

It is important to note that differences in fear response behavior have been noted in males and females, with males demonstrating more passive responses such as freezing, while females demonstrate more active responses such as darting (Bauer, 2023). This study accounted for this

limitation by looking at motion index differences across sexes. These were not included in the results as the pattern followed that of freezing behavior and no differences occurred within sexes or age of ethanol exposure groups. Unaccounted for is the difference in duration of drinking, in that the early cohort drank for three weeks while the late cohort drank for two. This was due to the fact that consumption did not start picking up until the end of week two for the early cohort. In addition to looking at estrous phase and including specific circuitry measures/changes, future studies should control for duration of drinking between early and late adolescents.

Concluding Remarks

This is the first investigation of the different ways in which adolescent ethanol exposure impacts fear behaviors in adulthood depending on sex and timing of ethanol exposure. The present study found both a sex and timing of exposure difference in the rate of acquired fear and contextual extinction as well as freezing during post-cue fear extinction and fear renewal. These findings provide insight into the possible neurocircuitry involved in adolescent ethanol consumption at different points throughout adolescence in males and females. Moreover, the results provide insight into the mechanisms by which adolescent ethanol consumption alters stress resilience and associative learning in adulthood.

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