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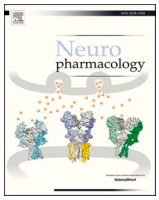

Adolescence alcohol exposure impairs fear extinction and alters medial prefrontal cortex plasticity

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ABSTRACT

After experiencing a traumatic event people often turn to alcohol to cope with symptoms. In those with post-traumatic stress disorder (PTSD) and a co-occurring alcohol use disorder (AUD), PTSD symptoms can worsen, suggesting that alcohol changes how traumatic memory is expressed. The objective of this series of experiments is to identify how alcohol drinking (EtOH), following cued fear conditioning and extinction, impacts fear expression in mice. Molecular (activity-regulated cytoskeleton-associated protein, Arc/arg3.1) and structural (dendrite and spine morphometry) markers of neuronal plasticity were measured following remote extinction retrieval. Mouse age (adolescent and adult) and sex were included as interacting variables in a full factorial design. Females drank more EtOH than males and adolescents drank more EtOH than adults. Adolescent females escalated EtOH intake across drinking days. Adolescent drinkers exhibited more conditioned freezing during extinction retrieval, an effect that persisted for at least 20 days. Heightened cued freezing in the adolescent group was associated with greater Arc/arg3.1 expression in layer (L) 2/3 prelimbic (PL) cortex, greater spine density, and reduced basal dendrite complexity. In adults, drinking was associated with reduced L2/3 infralimbic (IL) Arc expression but no behavioral differences. Few sex interactions were uncovered throughout. Overall, these data identify prolonged age-related differences in alcohol-induced fear extinction impairment and medial prefrontal cortex neuroadaptations.

1. Introduction

Post-traumatic stress disorder (PTSD) often co-occurs with an alcohol use disorder (AUD) (Hien et al., 2021; Kessler et al., 1995, 2005; Mills et al., 2006). Drinking tends to increase following a trauma (Jacobsen et al., 2001; Kline et al., 2014; Nickerson et al., 2014), and those with PTSD and AUD tend to show greater PTSD symptoms (Kessler et al., 2005). This suggests that alcohol contributes to PTSD symptomatology and that alcohol-induced neuroadaptations and the expression of traumatic fear may share common neuronal substrates. Because PTSD is often antecedent to AUD (Gilpin and Weiner, 2016), a critical mechanistic question is how alcohol may retroactively modify previously established fear memory. Rodent models are advantageous for addressing these questions because alcohol exposure can be precisely timed to circumscribe different learning and memory phases.

Pavlovian threat/aversive "fear" conditioning is a leading behavioral paradigm for studying brain circuitry underlying emotional learning and memory tied with PTSD (Bergstrom, 2016; Johnson et al., 2012). In fear conditioning, an initially innocuous sensory stimulus, such as an auditory tone, is paired with an aversive stimulus, such as a mild electric shock to the foot (unconditioned stimulus; US) that elicits an unconditioned response (UR). After pairing, the tone alone (conditioned stimulus; CS) can elicit a conditioned response (CR), such as freezing. When the CS is repeatedly presented without the US, the CR gradually reduces through a memory process termed extinction. Extinction does not necessarily overwrite the established fear memory (Sangha et al., 2020). Rather, it can generate a new inhibitory memory that competes with the original fear memory for behavioral control (Lacagnina et al., 2019). The study of fear extinction processes has considerable translational relevance since PTSD has been linked with impairments in the extinction of traumatic fear (Milad et al., 2009b). Further, exposure-based cognitive behavioral therapies for PTSD are based on principles of extinction

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(Rothbaum and Davis, 2003).

There are several lines of preclinical evidence linking alcohol (ethanol; EtOH) with impaired extinction (Broadwater and Spear, 2013; Cincotta et al., 2021; Holmes et al., 2012; Lattal, 2007; Ripley et al., 2003; Smiley et al., 2020, 2021). However, in all of these studies, EtOH was timed prior to extinction learning, making it impossible to parse the putative preferential impact of alcohol on the retrieval of an established extinction memory. In the only study to directly address the question of how EtOH might interact with the retrieval of an established extinction memory, daily systemic administration of alcohol timed after extinction learning impaired retrieval (Scarlata et al., 2019). Together, these data suggest that EtOH may not only impact extinction learning, but also retrieval. Considering the fragility of extinction memory and poor rate of extinction retention after exposure-based therapy, the study of long-term extinction retrieval processes is an important preclinical and clinical question. (Vervliet et al., 2013).

The medial prefrontal cortex (mPFC) mediates the formation and retrieval of fear, extinction, and reward (Peters et al., 2009). Alcohol targets mPFC structure and function (Jury et al., 2017; McCool, 2021; Salling et al., 2018; Varodayan et al., 2018), indicating the potential for alcohol-induced neuroadaptations in some of the same circuits and synapses recruited for the consolidation and expression of fear extinction. It is well established that maturational changes in various corticolimbic structures, including the mPFC, continue through adolescence. (Agoglia et al., 2017; Cunningham et al., 2002; Giedd et al., 1999; Klune et al., 2021). Consistent with this, there are numerous examples for differential sensitivity to the neurobehavioral effects of alcohol in adolescent rodent models (Brown et al., 2000; Dahl, 2004; Spear, 2018; Spear and Varlinskaya, 2005). Adolescents also differ on several behavioral measures related to emotional processing and regulation, including fear extinction (Baker et al., 2016; Koppensteiner et al., 2019a, 2019b; Pattwell et al., 2011, 2012, 2016). Finally, alcohol-induced neuroadaptations differ in the adolescent versus adult mPFC (Jury et al., 2017). Together, these findings suggest that continued maturational changes in the adolescent brain render adolescents uniquely sensitive to the neurobehavioral effects of *both* EtOH and traumatic stimuli.

While alcohol and trauma-related stimuli processing differs in the adolescent brain, the impact of alcohol consumption during adolescence on fear extinction performance and mPFC plasticity remains unknown. The primary objective of this study is to characterize the persisting age-dependent neurobehavioral effects of chronic EtOH on fear extinction performance and mPFC neuroadaptations. Mouse sex was included as an interacting variable in a full factorial design (Garcia-Sifuentes and Maney, 2021). We predict that adolescents will exhibit greater extinction impairments relative to adults, and females will show more extinction impairment relative to males. We hypothesize that EtOH-induced plasticity in the mPFC will be associated with behavioral differences in male and female adults and adolescents.

This series of experiments tests the long-term neurodevelopmental impact of voluntary alcohol drinking on Pavlovian auditory cued fear extinction retrieval and mPFC neuroplasticity in mice. A drinking-in-the-dark (DID) paradigm is incorporated to increase EtOH intake (Thiele et al., 2014), and modified to allow some degree of social contact during drinking sessions to minimize social isolation stress which may differentially impact adolescent brain and behavior (Skelly et al., 2015). This DID paradigm is dubbed the "social" DID (sDID). Following long-term extinction retrieval, several well-validated markers of neuronal plasticity were measured in the mPFC (Korb and Finkbeiner, 2011; Lamprecht and LeDoux, 2004): 1) activity-regulated cytoskeleton-associated protein (Arc/arg3.1), 2) pyramidal neuron dendritic morphology, and 3) pyramidal neuron dendritic spine density and morphology.

2. Materials and methods

2.1. Animals

Female and male C57BL/6NCrl (B6N) mice (N = 146 mice) were used in all experiments. At the start of EtOH drinking, adults ranged from 70 to 74 days old (female weight = 19.0–24.8 g, median weight = 21.9; male weight = 20.1–33.8 g, median weight = 28.6) and adolescents ranged from 38 to 41 days old (female weight = 14.1–22.63 g, median weight = 17.3 g; male weight = 17.2–25.3, median weight = 20.9 g). PND 34–47 is generally considered mid-adolescence (pubescent) and PND 60+ is considered adulthood in mouse (Brust et al., 2015). Mice were derived from a common stock (Charles River Laboratory, Kingston, NY) and bred on-site at Vassar College over multiple generations. Same sex mice were group-housed (2–5/cage) in standard cages. There were three types of environmental enrichment in each cage: 1) wood gnawing block, 2) nestlet, and 3) EnviroPAK. The vivarium temperature (23–25 °C), humidity (35–37%), and 12 h light/dark cycle (lights on 0600) were controlled throughout. Food and water were available *ad libitum* and cages changed 2 times/week. All experimental procedures were conducted in accordance with the National Institutes of Health guidelines on the Care and Use of Animals in Research and approved by the Vassar College Institutional Use and Animal Care Committee (IACUC). Disclosure of animal housing, husbandry, and experimental procedures follow principles for transparent reporting and reproducibility in behavioral neuroscience (Prager et al., 2011, 2018).

2.2. General behavioral procedures

All behavioral experiments were conducted during the light cycle (between 0800 and 1600). Mice were not handled prior to experimentation. All training and testing followed a 30 min habituation period in one of two adjacent holding rooms. To isolate the CS-elicited freezing response from contextual (background) freezing, various measures were taken to disguise the training context (hereafter referred to as "context A") from the extinction context (hereafter referred to as "context B″). Context A consisted of unmodified fear conditioning chambers (Coulbourn Instruments, Holliston, MA) and a 70% EtOH solution was used to clean the chambers between mice. For context B, (1) mice were transferred from the vivarium to the holding room using distinctive cages, carts, and covering, (2) the ambient lighting and background noise of the holding and testing room were changed using different illuminance and a fan for background noise, (3) a white plexiglass floorboard sprinkled with clean bedding was used to cover the shock bars, (4) the testing chamber walls were disguised with black- and white-striping, (5) the chambers were cleaned with a 1% acetic acid solution between mice (Bergstrom, 2020).

2.3. Fear conditioning and extinction

Fear conditioning procedures were identical across all experiments. Fear conditioning was conducted in commercial chambers (20 x 30 × 18 cm) located within sound-attenuating cabinets (58 x 61 × 45 cm) (Coulbourn Instruments, Holliston, MA). FreezeFrame 4 software was used for controlling and delivering the tone and foot shock stimuli (ActiMetrics, Wilmette, IL). Prior to all training and testing, the decibel level for the auditory tone frequency was measured in each chamber using a sound level meter (R8050, REED Instruments, Wilmington, NC) and calibrated to 72–75 dB. Mice were placed in the fear conditioning chamber for 180 s prior to three pairings of an auditory tone CS (20 s, 5-kHz, 72–75 dB) that co-terminated with a mild electric foot shock US (0.5 s, 0.6 mA). The CS/US pairings were separated by variable intertrial intervals (ITI) (20 and 80 s). Mice were removed from the chamber 60 s after the final CS/US pairing. The total training time was 400 s.

2.3.1. Context A extinction and context B pre-exposure

To reduce "background" contextual freezing (Jacobs et al., 2010), mice were placed into context A (context extinction) and context B (context pre-exposure and context generalization extinction) for 24 min and 45 s on consecutive days prior to cued fear extinction training.

2.3.2. Cued fear extinction training

The day following context B pre-exposure, mice underwent cued fear extinction training in context B. Following 180 s, mice were presented with 50 temporally massed (5 s fixed ITI) CS presentations (20 s, 5-kHz, 72–75 dB) (Cain et al., 2003). The total training time was 24 min and 45 s.

2.3.3. Extinction retention

Four days following the completion of sDID, mice were returned to context B and following 180 s, were given 10 presentations of the CS again with a 5 s fixed ITI.

2.3.4. ABA context renewal

The next day, mice were returned to context A (ABA renewal) and following 180 s, were given 10 presentations of the CS again with a 5 s ITI.

2.3.5. Remote fear extinction retrieval

15 days following the context renewal test (30 days following extinction), mice were returned to context B and given 10 presentations of the CS again with a 5 s ITI.

2.4. Social drinking in dark (sDID)

Following extinction training, mice were randomly assigned to either the EtOH or control groups. For the sDID experiment, mice were first habituated to sipper tubes (Model SLX0534, Lixit Corp, Napa, CA) filled with water in the home cage for 1 week prior to the start of drinking. On the first day of drinking, mice were moved from their home cage into the "bar" cage. The "bar" cage was a standard sized cage separated into two equally sized halves (horizontal) with a perforated plexiglass divider that permitted olfactory, auditory, visual, and potentially tactile interactions. We incorporated this design to avoid social isolate stress which differentially impacts the developing adolescent brain and behavior (Skelly et al., 2015). At the start of EtOH drinking, mice were presented with tap water in sipper tube bottles for 45 min at exactly 2015 h (2 h and 15 min into the dark cycle). Following habituation to the sipper tubes and "bar" cage, EtOH mice had their bottles replaced with a bottle containing 20% v/v EtOH solution for 2 h (3 h into the dark cycle, 2100 h). Control mice had their bottles replaced with a bottle containing tap water. The 20% EtOH solution was made from a stock 100% EtOH solution. All mice had free access to food throughout the water or EtOH presentation. Mice were switched to the alternate side each drinking session. Following the 2 h EtOH drinking period, mice were moved back into their group-housed home cages. All mice received 5 days of the sDID paradigm. EtOH consumption was calculated in grams of EtOH consumed per kilogram of the animal's body weight.

2.5. Arc/arg3.1 immunohistochemistry

In a subset of mice across all groups (N = 58), exactly 90 min following the remote fear memory retrieval test, mice were injected with a ketamine/xylazine cocktail (100:10 mg/mL) and transcardially perfused with 1X PBS or saline, followed by 4% paraformaldehyde (PFA) in 1X PBS (7.4 pH). The time point for perfusion following the retrieval test was based on several previous reports (Maddox and Schafe, 2011; Ploski et al., 2008). Brains were extracted and placed into 4% PFA overnight at 4 °C then transferred to 1X PBS and kept at 4 °C until sectioning (no longer than 2 weeks). Coronal brains sections (40 μm thick) were cut using a vibratome (VT1200, Leica Biosystems Inc., Buffalo Grove, IL). Every other section (to avoid double-counting) was collected in a well plate containing 1X PBS (7.4 pH) for free-floating immunohistochemistry. Sections were rinsed in 1X PBS, and then blocked in a 1X PBS/1% bovine serum albumin (BSA)/0.2%Triton-X solution for 30 min. Sections were then incubated for 24 h on an orbital shaker in Arc (C-7) mouse monoclonal antibody (1:100) (Cat# sc-17839, RRID: AB_626696, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature. The next day, sections were rinsed in 1X PBS before a 1 h incubation on an orbital shaker in anti-mouse biotinylated IgG (1:200) (Vector Laboratories, Burlingame, CA) at room temperature. Sections were rinsed again in 1X PBS then incubated in an ABC kit (Vectastain, Vector Laboratories, Burlingame, CA) for 1 h on an orbital shaker at room temperature. Sections were rinsed again in 1X PBS, and then incubated in DAB peroxidase substrate (Vector Laboratories, Burlingame, CA) for exactly 2 min. Sections were then rinsed in 1X PBS and mounted onto gel-coated slides. Finally, sections were dehydrated, first in a graded series of EtOH concentrations, and then in xylenes, before cover-slipping with DPX. Immunohistochemical staining was counterbalanced across experimental conditions.

2.5.1. Arc/arg3.1 quantification in the mPFC

For quantification of Arc-immunopositive ($^+$) neurons in the mPFC, the researchers were blind to the experimental conditions throughout. All Arc$^+$ neurons were visualized using bright-field microscopy (Axio Imager.M2, Zeiss, Thornwood, NY). For each brain region, manual cell counts were conducted bilaterally in three sections (six hemispheres) per mouse using 250 × 250 μm counting frame with Neurolucida (MBF Bioscience, Williston, VT). Non-consecutive sections were sampled to avoid double-counting. Each brain region was first identified under 25x objective power (2.5x objective and 10x binoculars), and then the counting frame was positioned over the region of interest (ROI). Importantly, the counting frame was positioned at a consistent ROI location across sections using anatomical landmarks in conjunction with a mouse brain atlas (Paxinos and Franklin, 2004). The sections chosen for counting (Bregma 1.97–1.41) were spaced evenly and sampling was not conducted in the rostral- and caudal-most regions of the mPFC. All Arc$^+$ neuron quantification was conducted under a 20x (0.5 NA) objective lens (200x final magnification). Koehler illumination principles were applied throughout the quantification process. Arc$^+$ neuron quantification was conducted in the shallow (Layer (L) 2/3) and deep (L5/6) layers of the prelimbic (PL) and infralimbic (IL) cortex (4 total ROIs). Cells were averaged across the 3 sections (6 hemispheres) for each ROI. The distinct forceps minor of the corpus callosum was used as an anatomical landmark to locate the PL and IL. The center point of the counting frame was consistently positioned ~150–200 μm (shallow layers) and ~550–600 μm (deep layers) from midline. NeuroExplorer software (MBF Bioscience, Williston, VT) was used for all quantitative analyses.

2.6. Golgi-Cox stain

Following behavioral and EtOH drinking procedures identical to those described above in a separate group of male and female adolescent mice, 15–20 min following remote fear extinction retrieval, mice were anesthetized using a ketamine/xylazine cocktail (100:10 mg/mL) and intracardially perfused with 1X PBS. The whole brain was submerged in a Golgi-Cox solution composed of 5% w/v mercuric chloride, 5% w/v potassium chromate, and 5% w/v potassium dichromate. Brains were stored in the dark, at room temperature, for 24 h. The solution was refreshed after the second day and sat for another 96 h (Total: 5 Days in the Golgi-Cox solution). Prior to sectioning, brains were moved to a cryoprotectant and stored at 4 °C in the dark. Cryoprotectant was replaced 24 h after the first day and brains remained in the solution for 7 days. Coronal sections were cut on a vibratome (VT1200, Leica Biosystems Inc., Buffalo Grove, IL) at a thickness of 200 μm. Sections were mounted on a 3% gelatinized slide and left to dry for 24–48 h in the dark

at room temperature. For developing, sections were first rinsed in ddH20, dehydrated in 50% EtOH, and alkalinized in a 33% ammonia hydroxide solution. Following another rinse in dH20 and immersion in 5% sodium thiosulfate (in the dark), slides were dehydrated in a graded series of ethanol dilutions, cleared using xylenes, and cover slipped using a mounting medium (DPX). These procedures were adapted from several previous protocols (Bergstrom et al., 2010; Gibb and Kolb, 1998; Zaqout and Kaindl, 2016).

2.6.1. Dendrite and spine imaging, 3D reconstruction, and quantification

Golgi-Cox-stained L2/3 PL pyramidal neurons were identified based on location relative to major landmarks (e.g., genu of the corpus callosum and cortical midline) and cortical depth (200–300 μm). Neurons with a prominent, single apical tree extending from the apex of the soma toward the pial surface of the cortex and two or more basal dendritic trees were chosen for reconstruction (Bergstrom et al., 2008). Neurons were visualized using brightfield microscopy (Axio Imager M2, Zeiss, Thornwood, NY) under a 63x (0.75 N.A.) air objective lens (630x final magnification) and manually reconstructed in 3D using Neurolucida software (MBF Biosciences, Williston, VT). The tracer was blind to all experimental conditions. Neurons were sampled randomly and from both hemispheres evenly. Neurons chosen for reconstruction were well-stained (fully impregnated dendritic trees) and with unobstructed dendritic arbors that could be followed from soma to terminal tip without interruption. Dendrite diameter tapering was also visually inspected. Dendritic trees without sufficient dendrite diameter tapering (chopped or incompletely stained dendrites) were not reconstructed. Morphometric analysis was restricted to cells located between bregma +1.97 mm and +1.41 mm. Neurons chosen for reconstruction averaged 273.3 ± 5.7 μm (range = 210–353 μm) soma-to-pial surface distance. To rule out potential artifactual morphological differences resulting from differential sampling across layers, soma-to-pial differences were compared between groups using one-way ANOVA (ns).

Dendritic spines were visualized under an oil immersion 100x (N.A. 1.2) objective lens (final objective power = 1000x). Spines were characterized on a randomly chosen basilar and apical dendritic tree from a reconstructed neuron. Spines were counted from soma to terminal tip. Spines were defined as small (<3 μm in length) protrusions emanating from the dendrite. Spines were characterized as one of four types: stubby (protrusions that lack a neck), mushroom (neck with a larger head), thin (long, skinny neck and small, bulbous head), or unknown (Yuste, 2010).

2.7. Statistics

2.7.1. Behavior

A camera positioned above the fear conditioning chambers recorded digital video footage. Videos were analyzed using FreezeFrame 4 (ActiMetrics, Wilmette, IL). Freezing was defined by a lack of movement, except for respiration, that lasted >1 s. Freezing data were calculated by scoring freezing during the CS presentations (20 s) and during the pre-CS period (habituation). For context renewal and spontaneous recovery, the last 3 CSs of the extinction retention test were averaged and compared with the mean of the first 3 CSs of contextual renewal or spontaneous recovery test. Data were first checked for normality to determine data distribution using Levine's test. For data with a normal Gaussian distribution, ANOVA was applied following by Scheffe post hoc tests when appropriate. The Welsch test was used when variances were unequal. Freezing was the dependent variable for all behavioral analyses. The between group independent variables were "Age," (adolescent versus adult) "Sex," (male versus female) and, "EtOH" (EtOH versus control). For extinction data, mixed ANOVA was used. Data were first checked for normality using Mauchley's test of sphericity, and if significant, a Greenhouse-Geisser correction was applied. The within-subject variable was "Time" (CS bins). Any data values 1.5 steps outside the interquartile range were designated outliers and removed from the analysis. Cohen's d values are reported for all univariate ANOVA tests. Statistical significance was set at $p < 0.05$ and all tests were two-tailed. Statistics were run on SPSS (IBM, Armonk, NY v. 26).

2.7.2. Arc/arg3.1 immunochemistry

Multivariate ANOVA (MANOVA) was used to analyze the pattern of Arc expression across the 4 ROIs in the mPFC. For MANOVA, Box's test was used to test the equality of variance-covariance matrices. In the case of this violation, a non-parametric alternative to MANOVA (ranked scores) was used. Follow-up ANOVAs were checked for the assumption of equality of covariance matrices using Levine's test. The Welsch test was used in the case of a significant Levine's test.

2.7.3. Dendrites and spines

For dendrite analyses, cells were averaged within each subject and compared. The dendrite complexity index (DCI) was calculated as a central measure of dendrite morphology (Pillai et al., 2012). DCI is defined as: (sum of branch tip orders + number of branch tips) * (total dendritic length/number of primary dendrites). To characterize the pattern of branching and length along the extent of the dendritic tree, a 3D Sholl analysis was applied (20 μm concentric increments starting at 10 μm), with parameters dendrite length and intersections with Sholl radii. For comparisons of overall dendrite material, ANOVA was used. For the Sholl analysis, mixed ANOVA was used with Sholl radii the within-subjects variable. Any significant Sholl radii interactions were followed up by one-way ANOVAs. All ANOVA assumptions were checked and adjusted using methods described above.

For all analyses of dendritic spines, the density of spines on each neuron was averaged within-subject and compared between groups. Spines were analyzed by branch order using Bonferroni corrected ANOVAs.

3. Results

3.1. Fear conditioning

All mice showed comparable fear acquisition (Fig. 1), with no group differences (Sex or Age) in freezing at the final CS/US pairing (Age; $p = 0.238$ and Sex; $p = 0.067$). There were also no differences in freezing between groups during the 180 s habituation period prior to CS/US presentation (Age; $p = 0.111$ and Sex; $p = 0.218$). These data indicate that adult and adolescent male and female B6N mice acquired a similar conditioned freezing response. These data also indicate no differences in freezing prior to the CS/US presentations.

3.2. Context fear extinction

Mice were placed into context A and context B on consecutive days prior to cued fear extinction to reduce "background" contextual freezing (Bergstrom, 2020; Jacobs et al., 2010). Results showed greater freezing in the adult group during context extinction in both context A ($p < 0.001$) and context B ($p < 0.001$) environments (Fig. S1).

3.3. Cued fear extinction

Following context fear extinction in the original training environment (context A) and pre-exposure to the test context (context B), all mice (N = 126, n = 26–38/sex/age) were returned to context B for cued fear extinction (Fig. 1). Mixed ANOVA revealed a significant interaction of Age x Time (Greenhouse-Geisser; $F[15.41, 1880.85] = 5.6$; $p < 0.001$) and main effect of Age ($F[1, 122] = 35.73$; $p < 0.001$). Adolescent mice exhibited dramatically diminished freezing over the course of extinction relative to adults. There was also a smaller, but significant, change in freezing over CS presentations with regards to sex (Greenhouse-Geisser; $F[15.33, 1900.85] = 1.9$; $p = 0.014$). In adults, mixed ANOVA revealed a significant decrease in freezing over 50 CS presentations ($F[49, 2548] =$

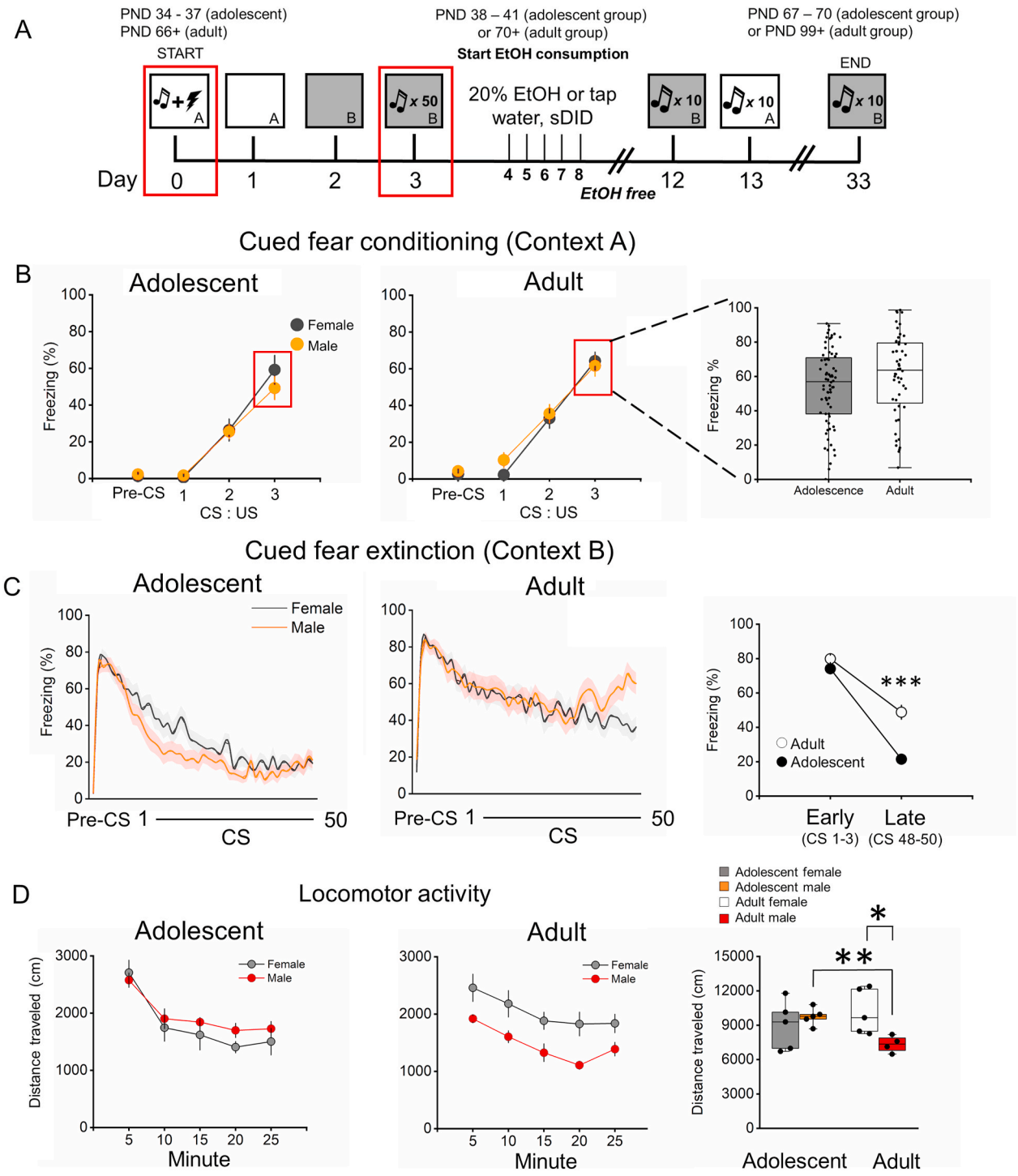

Fig. 1. A characterization of conditioned freezing (panels B–C) and locomotor activity (panel D) in male and female adolescent and adult C57BL/6NCrl mice. (A) Schematic depicting the experimental design for the extinction retention experiment. Open boxes depict "context A" and shaded boxes "context B." The Red box indicates Day 0 Fear conditioning and Day 3 fear extinction training indicated in Panels B–C. (B) All mice acquired an equivalent freezing response across CS/US presentation. The red box indicates mean freezing at the final CS:US pairing. (C) Freezing declined more rapidly in adolescent versus adult mice across 50 massed CS presentations (Left panel). A comparison of freezing during CS presentation 1–3 (mean) versus CS 48–50 (mean) indicating greater extinction in the adolescent group than adults (right panel). There were no sex interactions throughout. (D) Locomotor activity in the novel open field (25 min test). In males, adolescents traveled more distance than adults and in adults, females traveled more than males. Postnatal day (PND), 180 s habituation (Pre-CS), Conditioned stimulus (CS). Error bars and shaded error bars (Panel C, extinction) represent ± standard error of the mean. (N = 126, n = 26–38/sex/age). * denotes $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

9.646; $p < 0.001$), confirming within-session extinction in adults. Next, we tested whether adolescent mice showed an equivalent cued fear response as adults. ANOVA on the mean of the first 3 CS presentations showed slightly reduced freezing in adolescents compared with adults (F[1, 120] = 7.9, $p = 0.006$, $d = 0.80$). Pre-CS freezing was also lower in adolescent mice (F[1, 120] = 34.09, $p < 0.001$, $d = 1.0$; Fig. S2) Together, these results indicate that adolescent B6N mice show a reduced generalized context response, a decreased cued response, and faster extinction relative to adult B6N mice (Fig. S1)

This led us to speculate whether adolescent B6N mice might exhibit

increased locomotor activity levels relative to adults, which may influence conditioned freezing. To address this question, we ran a separate group of male and female adult and adolescence B6N mice (N = 20, n = 5/age/sex) in the novel open field (58 L x 58 W × 36 H cm; 22 Lux). Mixed ANOVA revealed an Age × Time interaction ($F[4, 60] = 3.09$, $p = 0.022$). Follow-up comparisons (ANOVA) at each timepoint indicated greater locomotor activity in adolescents compared to adults in the first 5 min of the test ($p < 0.05$) (Fig. 1). There was also a Sex × Age interaction ($F[1, 15] = 6.06$, $p = 0.026$). In males, adolescents traveled more distance than adults ($p = 0.002$) and in adults, females traveled more than males ($p = 0.031$) (Fig. 1). There were no differences in the percentage of time spent in the center of the arena.

3.4. EtOH drinking

After characterizing fear acquisition and extinction, we assessed EtOH drinking over the five-day social drinking in the dark (sDID) protocol. Mixed ANOVA revealed a significant Sex × Day interaction (Greenhouse-Geisser; $F[3.4, 179.2] = 2.8$; $p = 0.03$) and main effects of Sex (Welch; $F[1, 64] = 5.38$; $p = 0.024$, $d = 0.66$) and Age (Welch, $F[1, 64] = 5.16$; $p = 0.027$, $d = 0.64$). Females drank more than males ($3.03 \pm 0.24 > 2.35 \pm 0.17$ g/kg/2h) and adolescents drank more than adults ($2.96 \pm 0.21 > 2.29 \pm 0.20$ g/kg/2h). With regards to the Sex × Day interaction, drinking in adolescent females escalated over drinking days ($F[4, 68] = 4.75$; $p = 0.002$). No other groups exhibited escalated drinking (Fig. 2). (N = 66, n = 14–20/sex/age). An analysis of water consumption during the 2 h sDID timeframe in the control group revelated no difference in water consumption between sex, but greater water consumption in the adolescent relative to adult group ($F[1, 56] = 12.35$; $p = 0.001$).

3.5. Extinction retention

Four days following sDID, mice (N = 118, n = 12–20/sex/age/drug) were tested for cued fear memory retention in context B. Six mice were removed due to video recording error and 2 mice were dropped as outliers. Mixed ANOVA revealed a significant Age X Drug × Time interaction (Greenhouse-Geisser; $F[6.6, 769.1] = 2.45$; $p = 0.009$). In the adolescent group, EtOH-exposed mice froze more than controls ($F[1, 59] = 10.80$, $p = 0.002$, $d = 0.90$). A Bonferroni corrected post hoc test indicated greater freezing in the EtOH group relative to control at the first CS ($p < 0.001$). There were no differences detected in adults ($p = 0.387$, $d = 0.137$). There were no sex effects and no pre-CS drug effects (Fig. 3).

Following these initial findings, K-means cluster analysis was applied to Days 1–5 EtOH drinking levels (g/kg) to determine how the amount of EtOH consumed ("Drinking Level") may relate to freezing performance. The optimal number of clusters was determined to be 2 using the "elbow method", where increasing the number of clusters did not considerably improve within-cluster variability. K-means cluster analysis revealed 2 clusters that contained "High" drinkers (mean = 4.19 g/kg, range = 3.11–5.71 g/kg, n = 22) and "Low" drinkers (mean = 1.9, range = 0.90–2.96 g/kg, n = 44) (Fig. 3).

Mixed ANOVA with "Drinking Level" an independent variable revealed a significant Age X Drinking Level × Time interaction (Greenhouse-Geisser; $F[13.06, 737.69] = 2.03$; $p = 0.016$). In the adolescent group (Control n = 29, High n = 16, Low n = 22), mixed ANOVA revealed a main effect of Drinking Level on freezing ($F[2, 65] =$

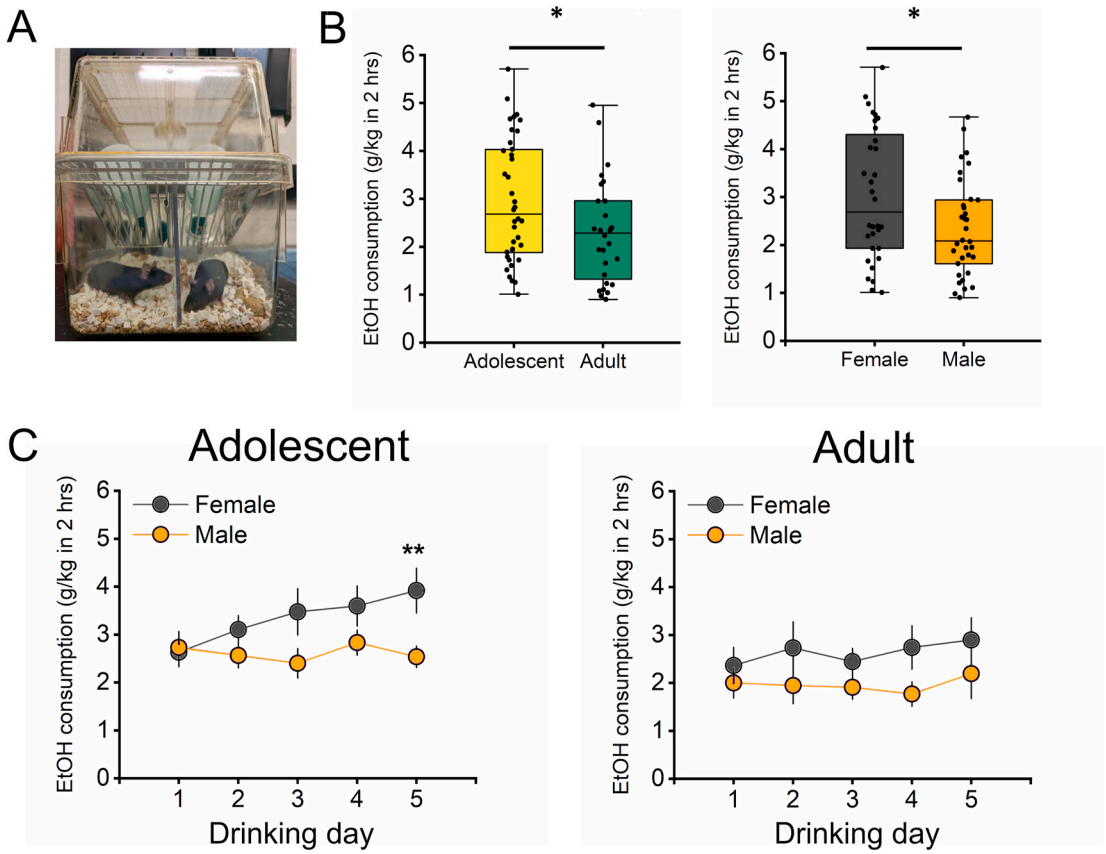

Fig. 2. (A) photo depicting the social drinking-in-the-dark (sDID) "bar" cage set-up. (B) Adolescents consumed more EtOH than adults (left panel) and females consumed more EtOH than males (right panel). (C) Female adolescent mice (left panel) exhibited greater drinking over 5 days. Error bars represent ± standard error of the mean. For the box plots, the top and bottom of the box represent the 75% (upper) and 25% (lower) quartiles, respectively, the center lines represent the median, and the whiskers represent the minimum and maximum values. (N = 66, n = 14–20/sex/age). * denotes $p < 0.05$ and **$p < 0.01$.

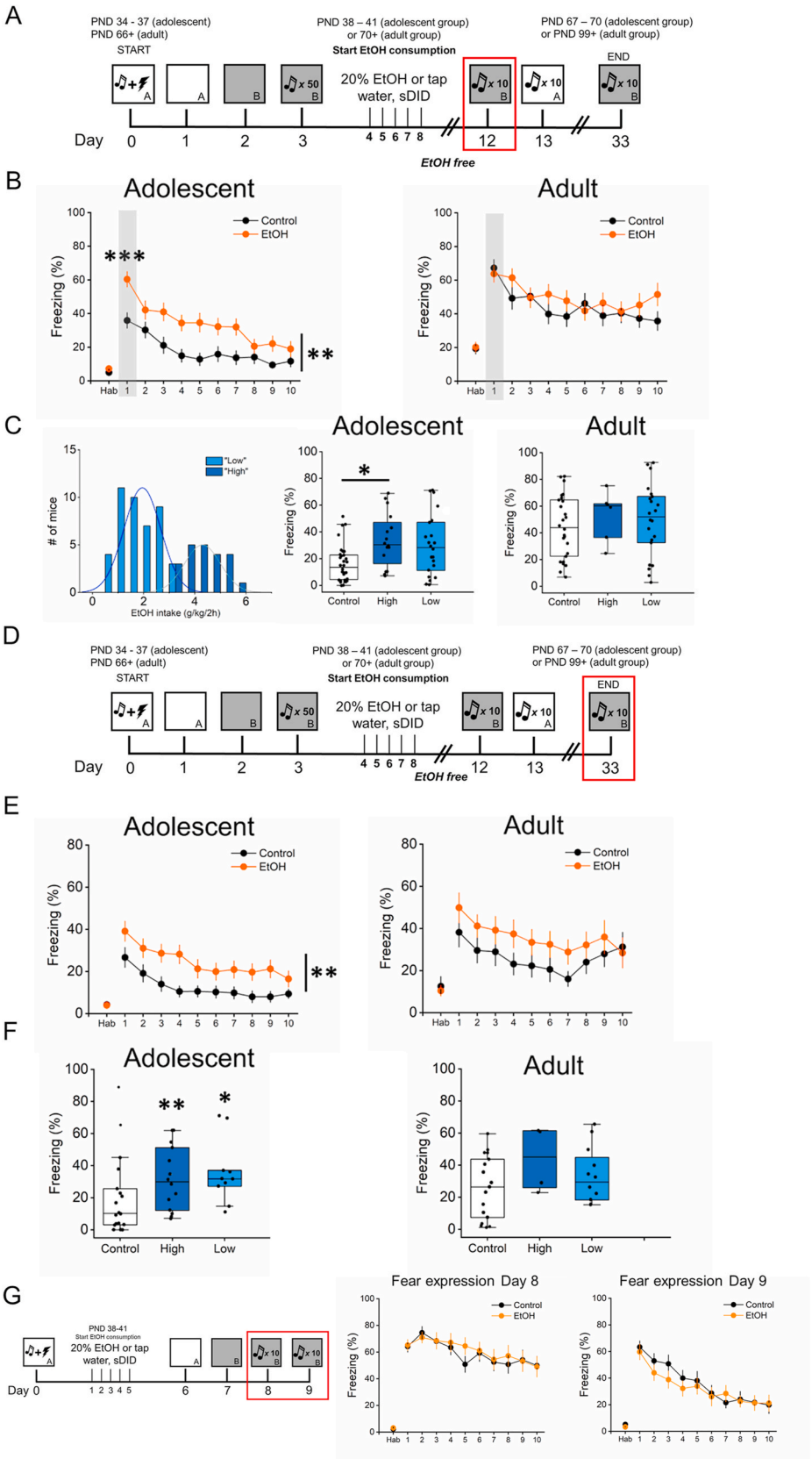

Fig. 3. (A) Schematic depicting the experimental timeline for the cued fear extinction test. Open boxes represent "context A", and shaded boxes represent "context B." The red box highlights the day 12 cued fear extinction test data depicted in Panel B. (B) Mice that drank EtOH during adolescence exhibited greater freezing, especially during the first CS presentation (left panel, gray shaded CS1 for emphasis). No differences in freezing were detected in adults. (C) K-means cluster analysis revealed 2 clusters of "low" and "high" EtOH intake (left panel). Freezing by consumption level ANOVA showed greater freezing in mice with "high" consumption levels (middle panel). No differences were detected in adults (right panel) (N = 118, n = 12–20/sex/age/drug). (D) Schematic depicting the experimental timeline for the remote cued fear extinction test (indicated by the red box). (E) At the remote time point, mice in the adolescent group exhibited greater freezing relative to controls (left panel). There were no differences in adults (right panel) (N = 85, n = 7–14/sex/age/drug). (F) An analysis of consumption levels by freezing at the remote time point revealed greater freezing after both "high" and "low" intake relative to controls (left panel). There were no differences in adults. (G) Schematic depicting the experimental design for the cued fear expression test. There were no differences in freezing between adolescent EtOH and control groups when EtOH drinking was timed following fear conditioning and tested 3 days later: days 8 and 9 (right panels) (N = 38, n = 9–10/group). ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ compared with control. Error bars represent ± standard error of the mean. For box plots, top and bottom of the box represent 75% (upper) and 25% (lower) quartiles, respectively, the center line represents median, and whiskers show outliers.

5.48, $p = 0.006$, $d = 0.88$). Post hoc testing showed greater freezing in the "High" group relative to controls (Scheffe, $p = 0.03$) with no differences in freezing in the "Low" group relative to control ($p = 0.077$) or between the "High" and "Low" groups. There was also no correlation detected between EtOH consumption levels and freezing levels, or any sex effects. In the adult group (Control n = 26, High n = 6, Low n = 22), mixed ANOVA revealed a Drinking Level × Time interaction on freezing (Greenhouse-Geisser; F[12.82, 326.97] = 2.09; $p = 0.015$). Subsequent ANOVAs on each CS bin revealed no significant differences. There were also no sex effects. To address the important question of whether group differences in freezing may have pre-existed (prior to EtOH exposure), we ran a retrospective analysis on the fear conditioning and fear extinction data sets with "Drinking Level" as an additional independent variable. We found no effects, indicating that EtOH-induced freezing differences were more likely related to the pharmacological action of EtOH, rather than a result of unintended non-random assignment, or a non-specific effect not related to EtOH action.

3.6. Context renewal

The day following the extinction retention test in context B, a random subset of mice (N = 85; n = 7–14/sex/age/drug) were returned to context A for a contextual renewal test. Mixed ANOVA on the within-session data revealed no effects. There were also no effects when "Drinking Level" was included as a variable. A comparison of extinction retention (last 3 CSs) with context renewal (first 3 CSs) revealed a Time × Age interaction (Greenhouse-Geisser; F[1, 85] = 17.18; $p < 0.001$). Mice in the adolescent group showed a 3.5-fold increase in freezing from context B to context A relative to adults (1.4-fold increase) (Fig. S5).

3.7. Remote extinction retention and spontaneous recovery

PTSD can only be diagnosed when symptoms last longer than 1-month, making the study of fear memory recall at remote time points following acquisition an important variable of preclinical study (Bergstrom, 2016). Here, we examined memory recall at a remote time point (30 days following extinction) in the same subset of mice (N = 85, n = 7–14/sex/age/drug). Two mice were designated outliers and removed. Mixed ANOVA revealed a main effect of Drug (F[1, 97] = 7.34, $p = 0.008$, $d = 0.77$). Because the strongest EtOH-induced effects were previously found in the adolescent group only, we analyzed the age groups separately. In the adolescent group, mixed ANOVA revealed a main effect of Drug (F[1, 68] = 7.92; $p = 0.006$, $d = 0.79$), with greater freezing in the EtOH group relative to controls. No effects were uncovered in the adult group ($p = 0.19$, $d = 0.26$). Subsequent analysis incorporated drinking levels. Mixed ANOVA showed a Time x Drinking Level × Age interaction (Greenhouse Geisser; F[12.3, 375.27] = 2.86; $p < 0.001$). In the adolescent group, both "High" (Scheffe; $p = 0.01$) and "Low "($p = 0.03$) EtOH drinkers exhibited greater freezing relative to controls. No effects were detected in adults. There were also no sex differences (Fig. 3).

The 30-day time point we chose for remote extinction retrieval is sufficient for spontaneous recovery (Wotjak, 2019). To assess the degree of spontaneous recovery, we compared the last 3 CSs of the extinction retention test with the first 3 CS of the remote test. Mixed ANOVA showed a Time × Age interaction (Greenhouse-Geisser; F[1, 71] = 13.99; $p < 0.001$). Mice in the adolescent group showed a high degree of spontaneous recovery (2.3-fold change), while mice in the adult group showed no spontaneous recovery (Fig. S5). Overall, several age-dependent extinction related effects were uncovered in B6N mice, including within- and between-sessions extinction, context renewal, and spontaneous recovery (Fig. S5).

3.8. Fear expression

To this point, we speculated that EtOH-induced exaggerated freezing during the extinction test reflected either a degraded extinction memory or an extinction retrieval deficit. An alternative hypothesis is that increased freezing may have resulted from a strengthened cued response related to sensitization in the original fear memory response or a change in retrieval mechanisms. To address the question of whether alcohol may have strengthened conditioned freezing outside of extinction processes, we tested the cued fear memory after EtOH in a separate adolescent male and female cohort (N = 38, n = 9–10/group). An adult comparison group was not included because EtOH-induced effect on extinction were not uncovered in adults. Results revealed no effects of alcohol on the conditioned response (CS 1–3) (Fig. 3), supporting the notion that EtOH may preferentially impact extinction processes. To test whether alcohol may proactively modify extinction, we ran extinction training (10 CSs) in the same experiment. Results revealed no effects of EtOH. The next day, we ran extinction training again (10 CSs), and again found no effect of EtOH (Fig. 3). Next, we questioned whether EtOH drinking immediately following fear conditioning may have led to more drinking compared to when drinking followed extinction. Results revealed no differences in drinking levels between experiments (Fig. S4).

3.9. Arc/arg3.1 immunochemistry

In the next experiment, Arc/arg3.1 (Arc) immunohistochemistry was used to identify age-dependent EtOH-induced patterns of synaptic plasticity in the mPFC following remote fear extinction retrieval. Arc is a key regulator of long-term synaptic plasticity associated with memory (Zhang and Bramham, 2020). Prior to analyses, data were transformed (rank ordered) to correct for a violation of equality of variance-covariance matrices (Box's test <0.05). Multivariate ANOVA (MANOVA) revealed a significant Age × Drug interaction (N = 54, n = 5–9/sex/age/drug) on Arc expression in the mPFC (Pillai's Trace, $V = 0.312$, F [4, 43] = 4.87, $p = 0.002$, $d = 0.936$). A significant omnibus MANOVA permitted follow-up Bonferroni corrected ($p < 0.012$) ANOVAs. Bonferroni corrected ANOVAs revealed significant Age × Drug interactions in the PL shallow (F [1, 46] = 7.52, $p = 0.009$, $d = 0.77$) and IL shallow (F [1, 46] = 7.32, $p = 0.01$, $d = 0.76$) layers of the mPFC. In the PL shallow, adolescent EtOH drinkers showed an increased density of Arc^+ cells compared with controls (Welsch, F [1, 24.95] = 8.37, $p = 0.008$, $d = 0.73$). No differences were detected in the adult group. In the IL shallow layer, adult EtOH drinkers showed a decreased density of Arc^+ cells compared with controls (F [1, 24] = 12.54, $p = 0.002$, $d = 0.93$). No differences were detected in the adolescent group. These data indicate a bidirectional influence of EtOH on the density of Arc^+ neurons following remote fear memory extinction retrieval in the mPFC that depended on the age of EtOH exposure. A correlation analysis (Pearson's r) showed the density of Arc^+ neurons in the PL L2/3 correlated ($R^2 = 0.35$, $p = 0.001$) with freezing during the remote time point in the adolescent group (Fig. 4). No other correlations were detected. Next, we ran a correlated activity analysis (Pearsons r) across all mPFC subregion Arc^+ cell density values (Fig. S6). Then, we calculated % change (control to EtOH) in correlation coefficients.

Results revealed a greater change in correlation in the PL L2/3 ←→ IL L2/3 and PL L2/3 ←→ IL 5/6 in the adolescent group. In the adult group, there was a greater change in correlation between PL L2/3 ←→ IL L5/6 only (Fig. 4). These data suggest that voluntary EtOH consumption during adolescence increases the correlated activity of Arc in the mPFC following remote fear extinction retrieval relative to comparable adult age exposure. Because we uncovered large differences in extinction performance between adolescent and adults, we performed a separate analysis of the Arc data in controls only. While MANOVA returned a significant Pillai's Trace test statistic for Age ($V = 0.427$, F [4, 17] = 3.16, $p = 0.041$, $d = 0.69$), none of the mPFC subregions reached statistical significance.

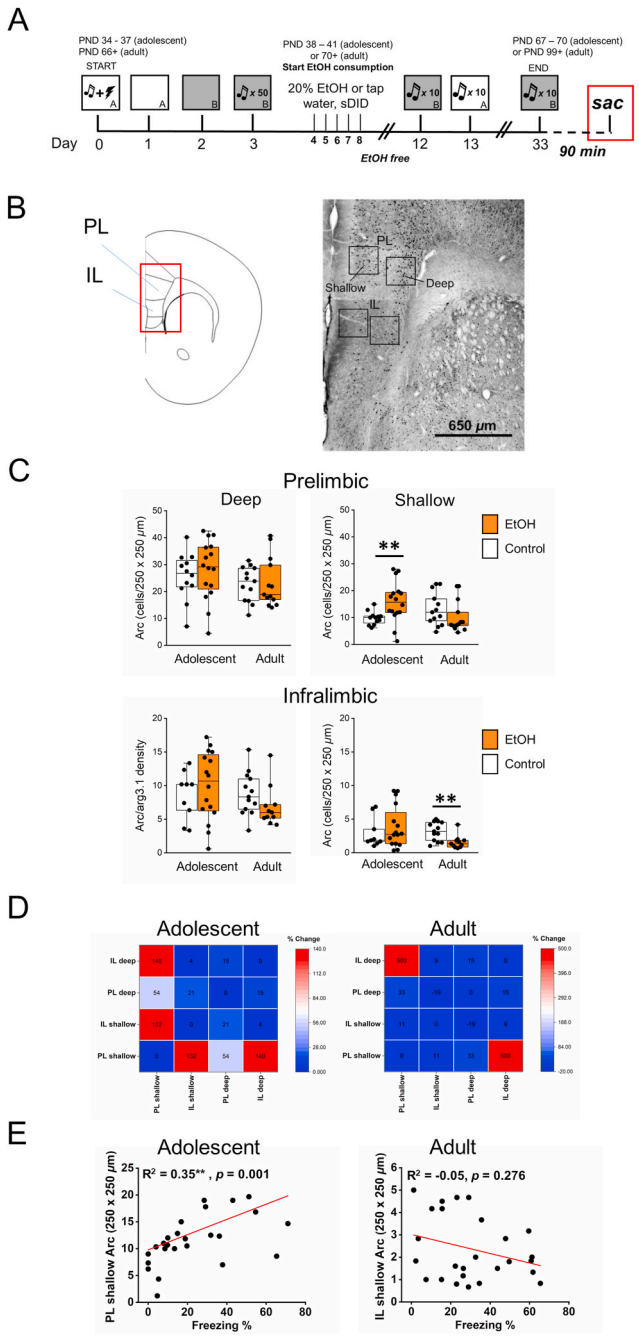

Fig. 4. (A) Schematic depicting the timeframe for sacrifice (sac) for Arc immunohistochemistry. (B) Modified stereotaxic mouse brain atlas image showing the location of the PL and IL (red box; left panel) and representative photomicrograph showing Arc$^+$ immunostaining in the PL and IL, including locations of the counting frames in the shallow (L2/3) and deep (L5/6) layers. (C) In PL shallow layers (L2/3), the number of Arc$^+$ cells were increased in the EtOH-exposed adolescent group compared with controls. In the IL shallow layers (L2/3), the number of Arc$^+$ cells were decreased in the EtOH-exposed adult group compared with controls. (D) Arc$^+$ density correlation matrix showing the % change in correlation between the control and EtOH groups across PL and IL layers. In the adolescent group, there was a greater change in correlation in the IL 2/3 ←→ PL 2/3 and PL 2/3 ←→ IL 5/6. In the adult group, there was a greater change in correlation between PL L 2/3 ←→ IL L 5/6 only. (E) Arc$^+$ neuronal density in PL L2/3 correlated with freezing % in the adolescent group. No correlation was detected in the adult group for Arc$^+$ neuronal density in IL 2/3 and freezing. (N = 54, n = 5–9/sex/age/drug). For box plots, top and bottom of the box represent 75% (upper) and 25% (lower) quartiles, respectively, the center line shows the median, and whiskers represent minimum and maximum values. $^{**}p < 0.01$ versus control.

3.10. L2/3 PL pyramidal neuron dendritic and spine morphometry

In the next experiment, we analyzed L2/3 PL pyramidal neuron dendrite and spine morphometry in the adolescent group following fear extinction retrieval at the remote time point. We focused our analysis on this age group because EtOH-induced differences in freezing and L2/3 PL Arc expression were found the adolescent group. A total of 60 PL L2/3 pyramidal neurons (n = 3 neurons/subject) were reconstructed in male and female mice from the EtOH and control groups (N = 20, n = 5/sex/drug). For the basal dendrites, results revealed a reduced dendrite complexity index (DCI) ($F[1, 16] = 7.29$, $p = 0.016$, $d = 0.72$) in the EtOH group relative to controls (Fig. 5). No differences were uncovered for the apical tree. To identify the source of DCI variability between drug groups, each component of the dendrite complexity index was analyzed independently. Results revealed significant medium effect sizes throughout, with reduced mean dendrite length ($F[1, 16] = 7.70$, $p = 0.014$, $d = 0.74$), branch tips ($F[1, 15] = 4.74$, $p = 0.046$, $d = 0.53$) (1 outlier removed), and branch tip order ($F[1, 16] = 4.69$, $p = 0.047$, $d = 0.53$), with no significant decrease in overall length ($p = 0.073$), and no differences in the number of primary dendrites. No differences were detected for the apical tree and there were also no sex differences. Next, the distribution of dendrite material (length and intersections) along the extent of the dendrite trees were measured using the Sholl analysis. Results revealed Sholl x Drug (Greenhouse Geisser; $F[2.1, 34.3] = 3.32$; $p = 0.045$) and Sholl x Sex (Greenhouse Geisser; $F[2.1, 34.3] = 3.36$; $p = 0.019$) interactions for basal tree length (Fig. S7). Females exhibited decreased length at 70 μm from the soma and decreased intersections at 50 μm from the soma. There were no differences in apical dendrite length. For intersections, there were no interactions, but there was a main effect for the basal tree ($F[1, 16] = 5.12$; $p = 0.038$) with a significant reduction in intersections in the EtOH group compared with controls.

3.11. Spine density and morphology

For basal dendrites, there were no overall differences in spine density. To determine the distribution of spines at different portions of the dendrite tree, the analysis focused on the first 3 orders of the dendritic tree because all neurons analyzed possessed at least 3 branch orders with spines. Terminal branch order spines were also analyzed across all subjects. Bonferroni corrected ANOVAs revealed increased spine density at the first branch order ($F[1, 15] = 17.17$, $p = 0.004$, $d = 0.95$) (3 outliers removed) (Fig. 5). No other differences were observed. There were also no differences in spine density for the apical tree. Next, the morphology of dendritic spines were also analyzed. Results revealed no differences across mushroom, thin, and stubby spine morphology classifications.

4. Discussion

Does EtOH retroactively interact with the expression of an established fear extinction memory? The present study addressed this question by establishing a cued fear extinction memory prior to a social EtOH "drinking-in-the dark" voluntary consumption paradigm. The extinction memory was then assessed at recent and remote time points following learning. Results showed equivalent fear conditioning between adolescent and adult C57BL/6NCrl mice, but impaired extinction performance in adults. EtOH drinking was greater in adolescents and in females. EtOH-drinking adolescent mice exhibited heightened freezing, an effect that persisted for at least 20 days. Exaggerated remote conditioned freezing correlated with greater L2/3 PL Arc$^+$ neuronal density. This led to a morphometric analysis of L2/3 PL pyramidal neuron dendrites and spines. In the EtOH-exposed group, reduced dendritic complexity was accompanied by increased spine density. To our knowledge, this is the first study demonstrating voluntary EtOH consumption during adolescence impacts fear extinction performance and mPFC neuroplasticity.

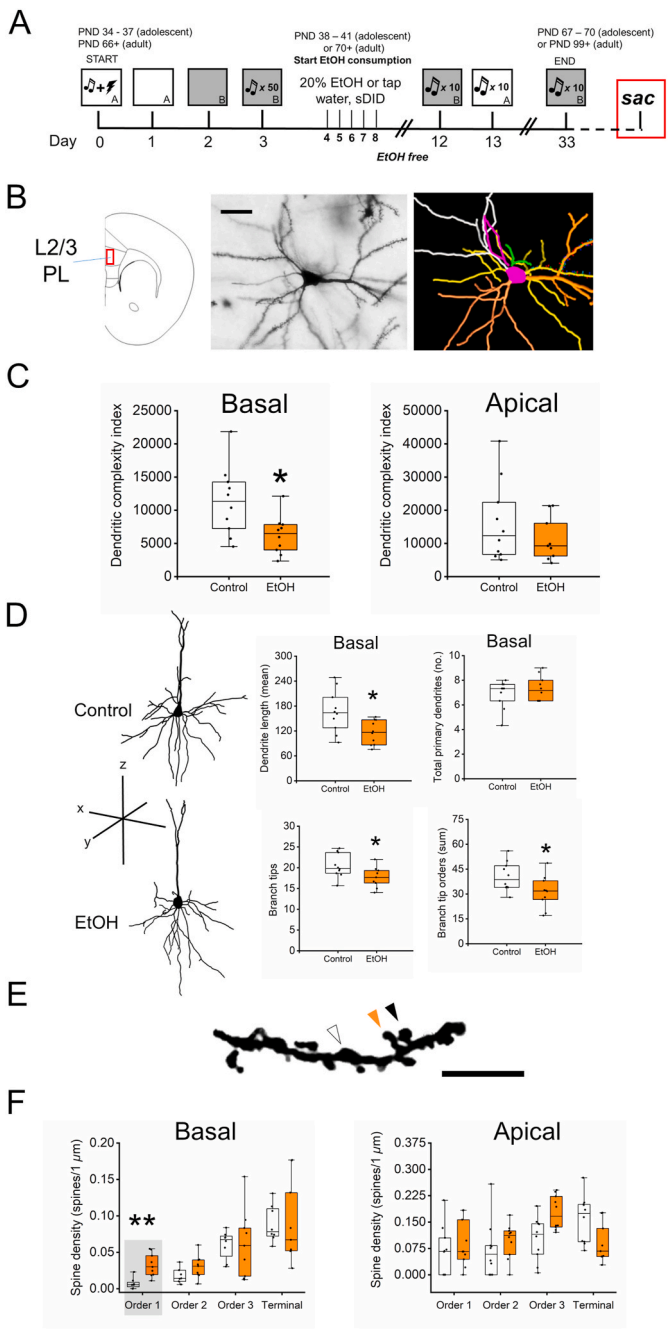

Fig. 5. (A) A modified stereotaxic mouse brain atlas image showing the location of L2/3 PL (red box, left panel), representative photomicrograph showing a Golgi-cox stained L2/3 PL pyramidal neuron (middle panel), and 3D digital reconstruction of the same neuron (right panel). (B) Dendritic complexity index for basal (left panel) and apical (right panel) dendrites. (C) Representative (approximate mean) 3D reconstructions of a neuron from the control and EtOH groups. (D) Box plots showing the mean dendritic length, total primary dendrites, branch tips, and branch tip orders. (E) Representative image of a segment of dendrite containing spines. White arrowhead indicates an example of a stubby spine, orange arrowhead indicates thin spine, and black arrowhead indicates a mushroom spine. (F) Box plots show spine density for the first 3 branch orders and the terminal branch order. For the box plots, the top and bottom of the box represent the 75% (upper) and 25% (lower) quartiles, respectively, the center lines represent the median, and the whiskers represent the minimum and maximum values. Error bars represent ±standard error of the mean. N = 20, n = 5/sex/drug. *$p < 0.05$ and **$p < 0.01$ Scale bar = 20 μm

In adults, there were no EtOH-induced behavioral differences. However, a reduction in L2/3 IL Arc expression was observed after EtOH exposure and remote fear extinction retrieval. Together, with results from adolescent-aged mice, these data support previous findings indicating age-dependent effects of alcohol on mPFC neuroplasticity (Gulley and Juraska, 2013). These findings advance the field of alcohol and memory modulation by showing that EtOH-induced, age-dependent impacts on both auditory cued conditioned fear extinction retrieval and mPFC neuroplasticity.

4.1. Alcohol effects on fear extinction versus acquisition

EtOH elevated conditioned freezing following extinction, supporting data indicating that EtOH impairs PFC-dependent tasks (Cannady et al., 2020). There are several possible mechanisms: 1) alcohol may have degraded the extinction memory or interacted with the retrieval of the extinction memory, allowing for greater expression of the original fear memory, 2) EtOH may have sensitized or strengthening the original fear memory, or a retrieval mechanism, to enhance fear expression, 3) or both. To test the question of whether EtOH may have interacted with the original fear memory, an additional experiment was conducted in which EtOH drinking was timed after fear conditioning and the memory tested for the cued response (without extinction). Results revealed no differences between alcohol and control groups (Fig. 3). Together, with the effects observed following extinction, we can speculate option number 1 is more likely: alcohol preferentially interacted with the extinction memory itself (i.e., degradation), an extinction retrieval mechanism, or both. These data are inconsistent with other reports indicating that alcohol has the potential to strengthen the cued response (Pajser et al., 2018; Quiñones-Laracuente et al., 2015). Nevertheless, the present data fall in line with several studies indicating that alcohol impairs fear extinction processes (Cincotta et al., 2021; Holmes et al., 2012; Scarlata et al., 2019).

It is commonly thought that extinction represents a new form of learning that establishes an inhibitory memory that competes with the original memory for behavioral control. This is supported by the discovery of separable fear acquisition and extinction neuronal ensembles in the dentate gyrus (Lacagnina et al., 2019). This model suggests that Pavlovian fear conditioning and extinction memories are dissociable and potentially separately modifiable by alcohol. An outstanding question is whether EtOH has the potential to preferentially target fear extinction versus fear acquisition neuronal ensembles. According to a neuronal ensemble hypothesis of memory organization, EtOH may have degraded or inhibited the extinction neuronal ensemble, leading to more freezing, because the acquisition ensembles are no longer inhibited by the extinction ensemble.

4.2. Alcohol consumption in a social DID paradigm varied by age and sex

The total amount of EtOH (g/kg/2hrs) consumed in the sDID protocol 2 h after light off (Day 4: 2.72 ± 0.13 g/kg) was comparable to voluntary intake reported in previous study using single housed DID protocols (Day 4: 2.79 ± 0.29 g/kg) (Rhodes et al., 2005), suggesting the sDID procedure does not significantly alter voluntary EtOH drinking. In the sDID paradigm, adolescents consumed more alcohol than adults, a finding supported in the literature (Bergstrom et al., 2006; Doremus et al., 2005; Maldonado et al., 2008; Spear, 2018; Strong et al., 2010; Vetter et al., 2007). However, water drinking was also higher in the adolescent group, suggesting that perhaps adolescent mice drink more volume of liquids, and not EtOH per se. Elevated EtOH drinking levels in adolescents may have conferred greater, and more long-lasting, extinction impairments relative to adults, as evidenced in the present study (Fig. 3). Females also drank more EtOH than males, which supports previous work (Bauer et al., 2020; Sneddon et al., 2019; Strong et al., 2010). In addition, adolescent females escalated EtOH consumption over drinking days (Day 1 drinking = 2.94 ± 0.30 g/kg/2 h to Day 5

drinking = 3.57 ± 0.36 g/kg/2 h). This finding is supported in the preclinical literature as B6J females exhibited increased drinking by day 10 of a two-bottle drinking-in-the-dark paradigm (Sneddon et al., 2019). Across nearly all behavioral assays, higher drinking in females did not confer differences in conditioned freezing relative to males, which ran counter to our initial hypothesis. Together, these data indicate the adolescent period, but not sex, heightens vulnerability to EtOH effects on fear extinction. While estrus cycle does not generally drive EtOH consumption patterns (Finn, 2020; Radke et al., 2021; Satta et al., 2018), one unresolved question is how controlling for estrus stage may have revealed sex differences in fear extinction (Milad et al., 2009a).

4.3. Alcohol-induced neuroadaptations in the mPFC

Following remote fear retrieval, L2/3 PL Arc$^+$ neuron density was greater in mice exposed to EtOH during adolescence. This finding supports a robust collection of studies in adults indicating the PL as a target for EtOH-induced neuroadaptions (Avchalumov et al., 2021; Crowley et al., 2019; Dao et al., 2021; Galaj et al., 2020; Holmes et al., 2012; Hughes et al., 2020, 2021; Joffe et al., 2020; Jury et al., 2017; McGinnis et al., 2020; Pleil et al., 2015; Salling et al., 2018; Smiley et al., 2021; Trantham-Davidson et al., 2017; Varodayan et al., 2018). There is evidence for protracted developmental regulation of the PL, which may render the adolescent PL differentially susceptible to EtOH-induced neuroadaptations as compared with the adult PL (Galaj et al., 2020; Jury et al., 2017). In line with this, we showed that comparable adult-age EtOH exposure impacted the mPFC differently: Arc $^+$ neuron density was *decreased* in L2/3 IL. This finding is consistent with a previous study from our lab, showing decreased Arc$^+$ neuron density in L2/3 IL was associated with increased fear generalization (enhanced freezing) following systemic EtOH injections in adult B6N mice (Scarlata et al., 2019). Behaviorally, while differences in freezing between drug groups in adult mice at the remote time point were statistically insignificant, the directionality of the small effect ($d = 0.26$) would point to a slight impairment in extinction retention. An associated reduction in IL L2/3 Arc$^+$ cell density in the EtOH-exposed group may line up with this result, considering the role of the IL in suppressing freezing during extinction (Bloodgood et al., 2018). Overall, these data indicate a bi-directional interaction of EtOH on mPFC subregion plasticity after remote fear memory retrieval that depends on the age of exposure.

In both adolescents and adults, EtOH-induced changes in Arc$^+$ neuron density were restricted to superficial PL and IL layers. mPFC L2/3 cells reciprocally connect with the basolateral amygdala (Anastasiades and Carter, 2021), a key association hub for fear processing. In a recent study, chronic EtOH exposure was found to increase glutamate release from the PL-BLA (strengthen synaptic connectivity) and decrease glutamate release (weaken synaptic connectivity) from the IL-BLA (McGinnis et al., 2020). Considering a role for the PL in promoting conditioned fear responses (Burgos-Robles et al., 2009; Corcoran and Quirk, 2007), and the IL in fear extinction (Bukalo et al., 2015), these data support a model whereby alcohol-induced neuroadaptations in a PL/IL ←→ BLA circuit augmented conditioned freezing (McCool, 2021; Scarlata et al., 2019). Through a correlated activity analysis, we showed greater plasticity in the adolescent group relative to adults, as evidenced by a greater change in correlated activity between control and EtOH groups (Fig. 4). A common shift in correlation values between age groups was observed for PL 2/3 ←→ IL 5/6, but a unique shift (PL L2/3 ←→ IL L2/3) was observed in the adolescent group. This finding supports the idea that EtOH exposure during early ontogenetic stages uniquely alters the trajectory of neurodevelopment (Smith et al., 2015).

4.4. Age and mouse substrain differences in fear extinction, context renewal, and spontaneous recovery

Several age-related differences in extinction performance were uncovered in the B6N mouse substrain, including extinction performance, context renewal, and spontaneous recovery. Extinction performance was superior in adolescents compared with adults. This finding is in apparent contrast to several previous reports showing impaired extinction (Hefner and Holmes, 2007) and extinction retention (Pattwell et al., 2012) in B6J early adolescence (PND 32 and 29, respectively) as compared with adult mice. In the present study, extinction training was conducted during mid-adolescence PND 37–41 (Brust et al., 2015), which may have fallen outside of the extinction impairment neurodevelopmental window. We conducted an additional experiment to assess baseline differences in locomotor activity between adolescent and adult B6N mice. Adolescent mice exhibited greater locomotor activity in the first 300 s of the novel open field test, suggesting baseline differences in locomotor behavior may contribute for age-related differences in conditioned freezing.

Extinction is thought to represent a less stable and malleable form of memory compared with acquisition (Rescorla, 2004). This idea stems from behavioral evidence showing that conditioned responding can return with time (spontaneous recovery) or context change (renewal) after extinction. In the present study, even though adolescents exhibited superior within- and between-session extinction performance, they showed a high degree of context (ABA) renewal and spontaneous recovery (Fig. S4), achieving similar levels of freezing as adults. These data indicate potential age-related differences in the strength of fear extinction. EtOH preferentially targeted the extinction memory, indicating that relatively less robust aspects of emotional memories (i.e., extinction processes) are more sensitive to disruption by EtOH. One outstanding question, and limitation of the current study, is how generally high levels of freezing in adult B6N mice may have produced a ceiling effect, obscuring the effect of EtOH on conditioned freezing.

4.5. L2/3 PL pyramidal neuron dendrite and spine morphometry following adolescent EtOH

To our knowledge, this is the first study showing prolonged (at least 25 days) restructuring of L2/3 PL pyramidal dendrites following a drinking-in-the-dark EtOH consumption procedure in adolescent mice. The few number of studies that have addressed how adult alcohol exposure impacts dendrite morphology found *increased* PL dendrite complexity (CADETE-LEITE et al., 1990; Frost et al., 2019; Kim et al., 2015; Klenowski et al., 2016; Navarro and Mandyam, 2015) (although see (Holmes et al., 2012). Here, *decreased* L2/3 PL basal dendrite morphology complexity was found, potentially indicating another age-dependent effect of alcohol on neuroplasticity. There is some evidence in rat showing PL pyramidal neurons increase in dendritic complexity from adolescence to adulthood (Koss et al., 2014; Markham et al., 2013). While the evidence is not direct (potential species differences), they lead to the hypothesis that EtOH exposure during adolescence may inhibit the normal developmental trajectory of PL basal dendrite complexity. Considering that dendrite structure shapes neuronal firing properties (van der Velden et al., 2012), and PL neurons play an integrative role in fear extinction (Marek et al., 2018), EtOH-induced adaptations in L2/3 PL basal dendrite complexity may have contributed to fear extinction memory performance.

Reduced L2/3 PL basal pyramidal neuron dendrite complexity in the EtOH-exposed group was accompanied by increased basal dendritic spine density at proximal locations of the basal dendritic tree. Previous results showing the effects of adolescent EtOH exposure on PL neuron spine density and morphology are equivocal, with increased “long/thin” spines (Trantham-Davidson et al., 2017), decreased overall density and “non-thin” spines (Galaj et al., 2020), and no effects of alcohol on PL spine density (Jury et al., 2017). Interestingly, we found increased spine density at proximal regions of the dendritic tree. Considering principles of synaptic integration (i.e., spatial summation), greater synaptic input at proximal portions of the dendritic tree may produce different action potential output patterns (Magee, 2000). In combination with the Arc/arg3.1 data, both L2/3 PL Arc expression and spine density were

increased. This finding is significant as there is a well-established link between spine plasticity and Arc/arg3.1 expression (Peebles et al., 2010).

5. Conclusions

We conclude that EtOH drinking, especially during adolescence, has the potential to modify the expression of established fear extinction memory and mPFC neuroplasticity. These data advance our understanding of the relationship between alcohol exposure and fear learning by showing age-dependent and persisting effects of voluntary alcohol drinking on the expression of fear extinction. These data support clinical data indicating that alcohol drinking, following traumatic stress, may exacerbate the symptoms of traumatic fear. Further, these data identify the PL and IL (ventromedial prefrontal cortex) as loci for alcohol-induced neuroadaptations that may be associated with the expression of traumatic fear.

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CRediT authorship contribution statement

K. Lawson: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Visualization. **M.J. Scarlata:** Conceptualization, Methodology, Investigation, Writing – review & editing. **W.C. Cho:** Investigation, Formal analysis, Visualization. **C. Mangan:** Investigation, Formal analysis, Visualization. **D. Petersen:** Methodology, Investigation, Formal analysis, Visualization. **H.M. Thompson:** Investigation. **S. Ehnstrom:** Investigation. **A.L. Mousley:** Investigation. **J.L. Bezek:** Investigation. **H.C. Bergstrom:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition.

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Appendix A. Supplementary data

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