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Age differences in fear retention and extinction in male Sprague-Dawley rats: Effects of ethanol challenge during conditioning

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Abstract

Pavlovian fear conditioning is an ideal model to investigate how learning and memory are influenced by alcohol use during adolescence because the neural mechanisms involved have been studied extensively. In Exp 1, adolescent and adult male Sprague-Dawley rats were non-injected or injected with saline, 1 or 1.5 g/kg ethanol intraperitoneally 10 minutes prior to tone or context conditioning. Twenty-four hours later, animals were tested for tone or context retention and extinction, with examination of extinction retention conducted 24 hours thereafter. In Exp 2, a context extinction session was inserted between the tone conditioning and the tone fear retention/extinction days to reduce pre-CS baseline freezing levels at test. Basal levels of acquisition, fear retention, extinction, and extinction retention after tone conditioning were similar between adolescent and adult rats. In contrast adolescents showed faster context extinction than adults, while again not differing from adults during context acquisition, retention or extinction retention. In terms of ethanol effects, adolescents were less sensitive to ethanol-induced context retention deficits than adults. No age differences emerged in terms of tone fear retention, with ethanol disrupting tone fear retention at both ages in Exp1, but at neither age in Exp 2, a difference seemingly due to group differences in pre-CS freezing during tone testing in Exp 1, but not Exp 2. These results suggest that age differences in the acute effects of ethanol on cognitive function are task-specific, and provide further evidence for age differences cognitive functioning in a task thought to be hippocampally-related.

Keywords

Adolescent; Adult; Fear Conditioning; Extinction; Alcohol; Sprague-Dawley rat

1. Introduction

Alcohol use is common among adolescents, with 11% of 8th graders, 28% of 10th graders and 42% of 12th graders reporting alcohol use within 30 days, and 14% of adolescents in 8th, 10th and 12th grades combined reporting binge drinking (5+ drinks in a row) [1]. Due to ethical constraints with conducting alcohol research using human adolescents, animal models have proven useful. According to preclinical studies, one potential contributor to adolescents' propensity to consume large quantities of alcohol is their relative insensitivity

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to certain seemingly undesired effects of acute alcohol exposure (e.g., motor impairment, sedation, etc.) that would normally limit further alcohol consumption (see [2] for review). Adolescents' ability to consume large amounts of alcohol is quite concerning given that they may be more sensitive to certain negative consequences of alcohol exposure, such as ethanol-induced disruption of learning [3] and memory [4] in a spatial navigation task, as well as greater brain damage [5]. Further characterization of alcohol's influences on cognitive functioning during adolescence would be helpful to fully understand consequences of adolescent alcohol use.

To further investigate how learning and memory are influenced by alcohol use during adolescence, other models, such as Pavlovian fear conditioning, may prove useful. Fear conditioning involves one or multiple pairing(s) of a neutral stimulus, such as a tone (conditioned stimulus [CS]), and an aversive stimulus, such as a footshock (unconditioned stimulus [US]), that induces the natural defensive behavior of freezing in rodents and is often elicited by the tone alone after conditioning (conditioned response [CR]). Associations are also made to diffuse cues of the context in which conditioning takes place. Thus, re-exposure to the conditioning context can elicit a CR as well. Pavlovian fear conditioning is an ideal model to assess how alcohol affects learning and memory during adolescent development because the neural mechanisms involved have been extensively investigated, at least in adult animals [6, 7, 8]. For instance, there is evidence for amygdala involvement in conditioning to a discrete cue (e.g., tone) [9, 10, 11], whereas the hippocampus may be more involved in conditioning to contextual cues [12, 13, 14], with such context conditioning previously reported to be preferentially disrupted by alcohol [15]. Although while recognizing limitations and caveats to this simplistic view of the neural control of Pavlovian fear conditioning (see [16]), the approach taken here was to assess both context and tone fear conditioning to examine relatively hippocampally-dependent versus -independent learning during ontogeny and determine whether alcohol disrupts these processes differentially during development.

In addition to measuring the degree of conditioned freezing to either the tone or context, rate of extinction and retention of extinction was used to examine age-related differences in a learning circumstance in which *inhibition* of a CR is necessary, which may also be affected by alcohol differentially in adolescent versus adult animals. Extinction occurs after repeated presentation of the CS (e.g., tone or context) without reinforcement from the US. The decrease in responding (extinction) is likely due to a new association (CS - no US), rather than an erasure of the initial excitatory CS-US association. For instance, evidence that the initial CS-US association remains is supported by restoration of responding to the CS due to passage of time (spontaneous recovery), context shift (renewal), or re-exposure to the US (reinstatement) (see [17] for review). There are two ways to examine extinction: within session extinction and retention of extinction. Within session extinction is characterized by a decrease in responding upon successive presentations of the CS in a single session, whereas extinction retention refers to maintenance of decreased conditioned responding to the CS at a later point in time (typically 24 hrs) after the original extinction session. The neural mechanisms underlying extinction to the CS is thought to involve excitation of inhibitory neurons in the amygdala via activation of the mPFC, which in turn decreases the CR, and the hippocampus mediates inhibition of the CR in particular contexts via connections to both mPFC and amygdala (see [9, 18] for review). Extinction retention is thought to critically involve the infralimbic (IL) portion of the mPFC, with manipulations that decrease or increase activity in this brain structure disrupting or facilitating extinction retention, respectively (see [19] for review). Given the ongoing maturational changes in all of these structures during adolescence (e.g., see [20] review), extinction and extinction retention are also important processes to study in adolescent animals.

In the few studies that have examined age-related differences in fear conditioning, two important findings have been reported. The first finding is related to alcohol's effects on fear conditioning during development, and the other focused on age differences in extinction in the absence of alcohol. Land & Spear [21] examined alcohol-induced disruption of tone and context fear conditioning in adolescents and adults, and found that 1 g/kg i.p. ethanol 10 minutes prior to conditioning resulted in learning deficits in adults (i.e., less freezing) when tested for retention of conditioned fear to the context or tone 24 hours later, whereas adolescents showed no difference in freezing behavior relative to age-mates given saline. A higher dose of ethanol (1.5 g/kg), however, produced similar retention deficits across age in both tone and context fear. Thus, adolescents may be less sensitive to alcohol-induced disruptions in fear conditioning relative to adults. This finding is surprising for two reasons. First, this contradicts several reports of adolescents being *more* sensitive to alcohol-induced disruptions of learning using the Morris Water Maze (MWM)(see [22, 23] for review). The second unexpected finding in the Land & Spear [21] study was that the alcohol effect was not specific to context conditioning at either age, which contradicts a previous study that found an ethanol-induced deficit in context, but not tone fear retention in adults [15]. This difference across these studies in susceptibility of tone retention to ethanol may be related in part to conditioning parameters, including use of a lower footshock level (0.2 mA vs 0.5) by Land & Spear [21] than in the study by Melia and colleagues [15]. Thus, one goal of the current study was to determine whether adolescents demonstrate a decreased ethanol sensitivity to disruptions of fear retention relative to adults using traditional methods to assess tone conditioning (e.g., using 0.5 mA footshocks during conditioning and testing tone fear retention in a novel context). We also wanted to assess alcohol's effects on context conditioning per se by testing animals that were context conditioned in the absence of the tone CS.

Age differences have not only been reported in terms of sensitivity to ethanol-induced disruptions of fear conditioning, but also extinction. In work focused only on tone conditioning, adolescents were found to learn and extinguish tone conditioning at the same rate as adults, whereas the adolescents failed to *maintain* extinction, exhibiting more freezing than adults to the CS during the extinction retention test that occurred 24 hrs after the extinction session [24, 25]. This effect has been suggested to be related to an inefficient PFC (particularly IL) that is continuing to mature during adolescence (see [25] for discussion). It is not known, however, if a similar extinction retention deficit would occur after context extinction or how alcohol might influence these age differences in extinction retention.

Thus, the purpose of the first experiment of this study was to investigate age differences in fear conditioning to either a tone or context during acquisition, fear retention, extinction, and extinction retention, as well as how acute alcohol exposure during conditioning would influence conditioning and extinction to the tone or context at each age. To this end, adolescent and adult rats were injected with saline, 1 or 1.5 g/kg ethanol intraperitoneally 10 minutes prior to conditioning to either the tone or context, or were non-injected. The following day, animals were tested for retention of the conditioned fear response to either the tone or context, followed by an extinction session. Twenty-four hours after the extinction session, animals were exposed to a second extinction session, with the first portion of this session used to assess retention of extinction. Based on previous studies, we hypothesized ethanol challenge during conditioning would disrupt retention of context conditioning, but not tone conditioning, and predicted that adolescents would be less sensitive to the disrupting effects of 1 g/kg ethanol on context conditioning relative to adults. Furthermore, we hypothesized that adolescents would show a deficit in the retention of tone extinction relative to adults among NM animals.

2. Experiment 1: Materials and methods

2.1. Subjects

A total of 186 adolescent and adult male Sprague-Dawley rats bred and reared in our colony at Binghamton University were used in this experiment. On the day after birth, postnatal day (P) 1, litters were culled to 8–10 pups, with a sex ratio of 6 males and 4 females retained whenever possible. Pups were housed with their mother in a standard clear plastic tub with shavings until pair-housed with a same-sexed littermate at the time of weaning (P21). All animals were maintained in a temperature-controlled vivarium on a 12:12-h light: dark cycle (lights on 0700) with ad libitum access to food (Purina Rat Chow, Lowell, MA) and water. Animals used in this experiment were maintained and treated in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health (8th Ed), using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

2.2. Experimental design

A 2 (age: P34–36 & 70–72) $\times$ 2 (conditioning: tone or context) $\times$ 4 (challenge: non-injected [NM], saline [Sal], 1 g/kg ethanol [E1] or 1.5 g/kg ethanol [E1.5]) factorial design was used, with an $n=10$ –13/group. In order to reduce the impact of litter effects, no more than one animal from a given litter was assigned to any one of the 16 experimental groups [26]. Pair-housed littermates were randomly assigned to 1 of the 4 pre-conditioning exposure groups, and 1 animal of each pair was tone conditioned and the other animal was conditioned to the context.

2.3. Fear Conditioning Apparatus

All behavioral assessments were conducted in 8 identical fear conditioning chambers (32 $\times$ 25 $\times$ 25 cm, Med Associates). Each conditioning chamber was made of clear polycarbonate (top, front walls), white acrylic (back wall), and stainless steel (sides, shock grids, drop pan) material, and equipped with a speaker in the side wall. The grid floors consisted of 19 parallel 4.8-mm diameter rods situated 1 cm apart. At the time of testing for tone retention/extinction sessions (Days 2 & 3), the context was modified by the addition of a smooth floor covering made of white plastic and an A-frame made of black acrylic that fit tightly in the chamber (height: 17.5 cm, side length: 23.5 cm). Chambers and inserts were cleaned with 6% hydrogen peroxide after each trial. Each chamber was encompassed by a sound-attenuated wood box (63.5 cm wide, 35.5 cm high, 76 cm deep) affixed with a ventilation exhaust fan that provided background noise (65 dB). An overhead LED-based light source (Med Associates NIR-100) provided visible broad spectrum White Light (450–650 nm) and near-infrared light (NIR; 940 nm). All behavioral sessions were video recorded by a camera in each conditioning chamber that was connected to a computer in the room. Percent time spent freezing was calculated at 30 frames per second by the Med Associates VideoFreeze system, a validated method for automated assessment of Pavlovian conditioned freezing behavior [27].

2.4. Fear Conditioning Procedure

2.4.1. Handling & weighing acclimation—Animals were transported to a room adjacent to the conditioning room to be weighed and handled once daily for 3 days prior to conditioning. All manipulations throughout the fear conditioning procedure took place between the hours of 11 am and 1 pm.

2.4.2. Conditioning (DAY 1)—Eight animals at a time were transported in their homecages to a room adjacent to the conditioning room to be weighed and injected if

appropriate (i.e., all groups but NM). For injections, both animals in each littermate pair received the same intraperitoneal injections-- either 1 or 1.5 g/kg ethanol (20% v/v) or saline (equivalent volume to a 1.5 g/kg ethanol dose)-- and were returned to their home cage. NM animals were weighed and immediately placed back in their homecage. Ten minutes later (an interval sufficient to allow absorption of ethanol [28]), animals were transported to the conditioning room and placed in the conditioning chambers. The conditioning context was the same for both tone and context conditioning, and consisted of a grid floor delivering footshock, and white light illuminating the chambers. After a 2 minute habituation period, 1 animal of the littermate pair received tone conditioning and the other received context conditioning. Animals that were assigned to tone conditioning received 3 CS-US pairings of a 10 second (s) tone (80 dB, 2000 Hz) coterminated with a 1 s footshock (0.5 mA) presented on a 110 s variable ITI. Animals in the context conditioning group received 3 presentations of 1s footshock (0.5mA) at the same time intervals as for tone conditioning, but without any tone presentations. All animals remained in the chamber for 2 minutes following the final footshock. Total conditioning time for each conditioning procedure was about 8 minutes. After conditioning, animals were re-paired with their littermate and immediately returned to the colony room.

2.4.3. Fear Retention Test/Extinction 1 (Day 2, Test 1)—Approximately 24 hrs after conditioning, freezing to the context or tone (without additional US exposure) was measured depending on conditioning group assignment for 12 minutes, with the first portion of the session used to index fear retention. As on conditioning day, animals were transported to a room adjacent to the conditioning chambers where they were weighed and placed back in their homecage. Ten minutes later, animals were transported to the conditioning room. Animals that were tone conditioned were placed in a novel context created by the addition of a smooth floor and an A-frame to the original conditioning chamber. After a 2 min acclimation period, 30 presentations of the 10s tone alone, with an ITI of 10s were delivered (total time= 12 min). Animals that received context conditioning were placed in their original conditioning chamber and context for a test/extinction session that lasted 12 minutes. At the end of the session, animals were reunited with their littermates and returned to the colony room as on Day 1.

2.4.4. Extinction Retention Test/Extinction 2 (Day 3, Test 2)—Animals were tested for retention of extinction to the tone or context depending on their original conditioning group assignment, with extinction retention and extinction assessed during the 12 minute session. Procedures were identical to the previous retention test/extinction day.

2.5. Data Analysis

2.5.1. Exclusion Criteria—For tone conditioning, animals were excluded if they showed consecutive baseline freezing of >50% during the 2 min habituation period (i.e., before CS presentation) on both retention/extinction sessions (Tests 1 & 2). This resulted in the exclusion of 1 adolescent (NM) and 7 adults (4 NM; 3 SAL) in Exp 1. Additional animals were added to these groups to maintain an n=12–13/group. For both types of conditioning, percent freezing during the fear retention test (bin 1 on Day 2) was checked for outliers at each age, with scores > 2 standard deviations from the mean of each experimental condition excluded from analysis. For tone conditioning this resulted in exclusion of 1 adolescent (NM), and for context conditioning, 1 adolescent (SAL) and 3 adult animals (1 animal from NM, SAL and E1 groups).

2.5.2. Statistics—The percentage of time spent freezing on the conditioning and two extinction days were separated into time bins for each type of conditioning. For the tone conditioning day, percent freezing was calculated for the 2 minutes prior to the first tone, the

9 second duration of each of the three tones prior to footshock and the 2 minutes following the final CS-US pairing (total of 5 bins). Pre-CS freezing was included in this analysis to directly compare potential effects of EtOH during baseline versus the first tone. For context conditioning, percent freezing was calculated for the 2 minutes prior to the first footshock, the two time periods between footshock exposures (1st–2nd and 2nd–3rd), as well as the 2 minutes following the final footshock (total of 4 bins). For analysis of data during the extinction sessions, the 30 tone presentations were divided into 5 bins, each consisting of 6 tones and their associated 10s interstimulus intervals [ISI] for tone conditioning and context extinction sessions were separated into 2 minute time bins (total of 6 bins). For each extinction session, the first CS bin for tone conditioning and the first 2 minute bin for context conditioning were used to analyze fear retention (Test 1) and extinction retention (Test 2). Factorial ANOVAs with age and challenge groups included as independent variables were used to analyze pre-CS baseline freezing on tone extinction days, as well as fear retention and extinction retention. Acquisition and extinction over time bin was analyzed separately at each age with repeated measures ANOVAs. Significant effects involving time bin were investigated as planned comparisons of challenge groups across bins via Fisher's LSD to avoid inflating the possibility of type 2 errors [28]. In contrast, when exploring the locus of significant main effects or interactions not involving time bin, Tukey's HSD post hoc were used to reduce the risk of type 1 errors in these assessments. All groups of animals showed evidence of acquisition of conditioned fear and of extinction during extinction sessions; therefore, only interactions involving bin and challenge or atypical bin effects (i.e., an opposite pattern than expected) are discussed in Results.

3. Experiment 1: Results

3.1. Tone Conditioning

The data obtained do not support the hypothesis that tone conditioning and retention are resistant to ethanol, given that the highest ethanol dose disrupted tone acquisition on conditioning day in adolescent animals and produced a deficit in tone fear retention at both ages; this dose also induced evidence of motor impairment at both ages, an effect that was particularly pronounced in adults. The hypothesis that adolescent animals would show an extinction retention deficit was also not supported by the data. A caveat to these findings, however, is that baseline freezing differed significantly across exposure groups regardless of age. Animals that were challenged with ethanol during conditioning showed significantly less baseline freezing prior to the retention test than NM and SAL-challenged animals, which may have influenced the freezing behavior in response to the CS throughout the extinction sessions [30].

3.1.1. Conditioning—A significant bin $\times$ exposure interaction [$F(12, 152) = 2.83, p < .05$] emerged in the analysis of the adolescent data, with adolescents in the E1.5 group showing significantly more freezing than NM adolescents during tone 1 (i.e., prior to footshock), suggestive of ethanol-induced motor impairment. The adolescent E1.5 group also showed attenuated learning, with significantly less freezing observed during tones 2 and 3 (see Fig 1a). In the adult analysis, a significant main effect of challenge [$F(3, 40) = 4.73, p < .05$] and a bin $\times$ challenge interaction [$F(12, 160) = 6.00, p < .05$] emerged, with the E1.5 group showing significantly more freezing relative to NM and SAL adult groups during the pre-CS period and the 1st tone, again indicating ethanol-induced motor impairment; however, this effect was more pronounced in adults than adolescents (see Fig. 1b).

3.1.2. Baseline freezing (Test 1)—A significant main effect of exposure emerged [$F(3,78) = 13.92, p < .05$], with animals that were challenged with ethanol during conditioning

freezing significantly less prior to the tone fear retention test than control groups (see Table 1).

3.1.3. Fear Retention (Test 1)—The 1.5 g/kg ethanol dose disrupted retention of conditioned fear to the tone at both ages, with a significant main effect of exposure condition [$F(3,78) = 7.53, p < .05$] emerging (see bin 1 of Fig. 1c & 1d).

3.1.4. Extinction 1—Significant main effects of challenge [adolescent: $F(3,38) = 6.90, p < .05$; adult: $F(3,40) = 14.90, p < .05$, respectively] and bin $\times$ challenge interactions [$F(12, 152) = 2.92, p < .05$; $F(12, 160) = 2.51, p < .05$] emerged, indicating that the deficit in tone fear retention in the E1.5 group was evident throughout the extinction session at both ages (see Fig. 1c & 1d). Among adolescents, the E1 group also showed significantly less freezing than NM and SAL adolescents observed during bin 3 (see Fig 1c), perhaps reflecting more rapid extinction in this group.

3.1.5. Baseline freezing (Test 2)—Ethanol groups once again tended to freeze less prior to CS exposure than NM and SAL groups, with a trend for a main effect of challenge [$F(3,78) = 1.62, p = .06$] (see Table 1).

3.1.6. Extinction Retention (Test 2)—A significant main effect of challenge condition [$F(3,78) = 18.31, p < .05$] and significant age $\times$ challenge interaction [$F(3,78) = 3.03, p < .05$] emerged in the analysis of extinction retention. Regardless of age, E1.5 animals continued to show reduced freezing during the extinction retention test relative to NM and SAL groups (see bin 1 of Fig. 1e & 1f). There was no evidence of an age-associated extinction retention deficit in adolescent animals, with NM adolescent and adult animals not differing in freezing behavior during the extinction retention test.

3.1.7. Extinction 2—The only effect that emerged in the analysis of the adolescent data was a main effect of bin [$F(5,180) = 2.33, p < .05$], although the adolescent E1.5 group tended to show less freezing throughout the second extinction session (see Fig. 1e). For adults, however, a significant main effect of challenge [$F(3,40) = 5.01, p < .05$] and bin $\times$ challenge interaction [$F(12,160) = 4.84, p < .05$] emerged. The adult E1.5 group showed significantly less freezing than SAL and NM adults through bin 2, an effect that continued relative to the SAL group through bin 4 (see Fig. 1f).

3.2. Context Conditioning

Consistent with our hypotheses, ethanol challenge during context conditioning impaired expression of fear to this context during the retention test, and adolescents were less sensitive to these effects relative to adults. Importantly, these effects were not due to differences in acquisition on conditioning day. Extinction and extinction retention had not previously been examined under these circumstances, but we expected to see more pronounced extinction in animals that showed initially higher levels of conditioned responding (i.e., NM and SAL groups) during the fear retention test (i.e., a greater decline in freezing due to initially high levels of freezing); this expectation was generally supported by the data obtained. Again, as was also the case for tone conditioning, there was no indication of an age-related extinction retention deficit in adolescent animals.

3.2.1. Conditioning—Acquisition did not differ as a function of challenge in the analysis of the adolescent context conditioning data (see Fig. 2a). The adult analysis, however, revealed a significant bin $\times$ challenge interaction [$F(9,105) = 2.40, p < .05$], with both the E1 and E1.5 adult groups freezing significantly more during the 2 minute pre-shock period relative to the NM and SAL adult groups, and the E1.5 adults also freezing significantly

more than SAL adults between shocks 2–3 (see Fig 2b). This enhanced freezing by ethanol-challenged adults during the pre-shock period again likely reflects motor impairing effects of ethanol, an effect not evident in adolescents.

3.2.2. Fear Retention (Test 1)—A significant age $\times$ challenge interaction [$F(3,70)=4.50, p<.05$] emerged, with adolescents and adults in the E1.5 group showing significantly less freezing than the NM and SAL groups, indicating disruption of context fear retention. Disruption of context fear retention was also evident in the adult (but not adolescent) E1 group, with significantly less freezing observed relative to NM and SAL groups; freezing in the adult E1 group was also less than in E1 adolescents (see bin 1 of Fig 2c & 2d). Thus, whereas ethanol challenge during conditioning disrupted expression of conditioned fear to the context in both adolescents and adults, adolescents required a higher dose of ethanol than adults for this effect to be apparent.

3.2.3. Extinction 1—The analysis of the adolescent data revealed a significant main effect of challenge [$F(3,36)=5.90, p<.05$] and a significant bin $\times$ challenge interaction [$F(15,180)=2.51, p<.05$], with adolescents in the E1.5 group showing significantly less freezing during extinction, indicating a continuation of the ethanol-induced disruption of context fear. No differences across group were evident by the end of the extinction session on this day (see Fig. 2c). The adult analysis revealed a significant main effect of challenge [$F(3,35)=19.07, p<.05$], with adults in both the E1 and E1.5 groups continuing to show significantly less freezing throughout the extinction session (see Fig. 2d). An atypical bin effect [$F(5,175)=8.49, p<.05$] also emerged in the adult analysis, with freezing significantly increasing from bin 1 to bin 2 when collapsed across exposure group.

3.2.4. Extinction Retention (Test 1)—Significant main effects of age [$F(1,70)=9.10, p<.05$] and challenge [$F(3,70)=7.79, p<.05$], as well as their interaction [$F(3,70)=3.67, p<.05$] were evident, with significantly less freezing again observed in both adult E1 and E1.5 groups than their control groups. Also, NM adolescents showed significantly less freezing than NM adults, with a similar age difference tending to emerge in the SAL groups as well (see bin 1 of Fig. 2e & 2f). These data likely reflect a continuation of the higher levels of freezing (i.e., slower extinction rates), seen at the end of the first extinction session in NM and SAL-exposed adults relative to their adolescent counterparts (compare bin 6 in Figs. 2c and d with Fig. 2e).

3.2.5. Extinction 2—The only effect that emerged in the analysis of the adolescent data was an atypical main effect of bin [$F(5,180)=2.33, p<.05$], with a significant increase from bin 1 to bin 2 (see Fig. 2e). The adult analysis, however, revealed significant main effect of challenge [$F(3,35)=4.10, p<.05$] and a bin $\times$ challenge interaction [$F(15,175)=3.34, p<.05$], with a continuation of extinction observed on this second day of extinction in the adult NM and SAL groups (see Fig 2f), whereas the adult ethanol groups and all the adolescent groups already approached baseline freezing levels at the start of the second extinction session.

4. Experiment 2: Introduction

The tone conditioning results from Experiment 1 were not consistent with previous reports of ethanol disrupting retention of context but not tone fear conditioning [15], nor with prior findings of a fear extinction retention deficit to the tone among adolescents [24, 25]. One potential contributor to these differing results could be the elevated levels of baseline freezing observed on test days in tone conditioned animals that did not receive ethanol during conditioning. Any elevations in baseline freezing could have exacerbated freezing during CS exposure in these NM and SAL groups, potentially making differences more

pronounced between challenge groups and perhaps masking age differences in extinction retention. Thus, a second experiment focusing only on tone fear conditioning was conducted that attempted to decrease high baseline freezing prior to the tone fear retention test.

Despite our efforts to produce a novel context for the tone retention test, it is possible that baseline freezing prior to tone testing may have been elevated in some animals in Exp 1 because aspects of the conditioning context were transferred to the tone testing context. Thus, lower baseline freezing in ethanol challenged animals may be indicative of disruptions in memory for contextual cues, reminiscent of context retention deficits after ethanol challenge in animals that were context conditioned *per se*. In other studies, baseline differences between groups may have been minimized by testing both tone and context fear retention in the same animals after tone conditioning, with the test for contextual fear, which may also serve as a context extinction session, occurring prior to the tone fear test (see [31] for protocol). This approach was used in the following experiment, with a context fear retention and extinction session interpolated between the tone fear conditioning and the tone fear retention test.

5. Experiment 2: Materials and methods

5.1. Subjects

A total of 80 adolescent and adult male Sprague-Dawley rats bred and reared in our colony at Binghamton University were used in this experiment and were treated in accordance with subjects in Exp 1.

5.2. Experimental design

A 2 (age) $\times$ 4 (challenge: NM, Sal, 1 g/kg or 1.5 g/kg EtOH) factorial design was used for this experiment, with an $n=10$ /group. As in Exp 1, no more than one animal from a given litter was assigned to any one of the 8 experimental groups. However, in this experiment pair-housed littermates were randomly assigned to non-ethanol or ethanol challenge groups. In the non-ethanol group, 1 littermate was injected with saline and the other was non-injected (NM), whereas in the ethanol group, 1 littermate was given 1 g/kg *i.p.* and the other was given 1.5 g/kg. All animals were tone conditioned in this experiment.

5.3. Fear Conditioning Procedure

Most of the conditioning procedures were identical to the tone conditioning procedures in Exp 1. However, in this study all animals were placed in the original conditioning context for 12 minutes on Day 2 for a context retention test and extinction session. Tone fear retention and extinction was then conducted on Day 3, with testing for tone extinction retention and tone extinction 2 occurring on Day 4. Testing for tone retention, extinction and extinction retention on Days 3 and 4 was conducted using the same chamber adjustments (*i.e.*, smooth floor and A-frame inserts), CS exposure and session length as in Exp 1.

5.4. Data Analysis

5.4.1. Exclusion Criteria—Animals were excluded if they showed consecutive baseline freezing of $>50\%$ during the 2 min habituation period (*i.e.*, before CS presentation) of both tone retention/extinction sessions (Tests 1 & 2), as in Exp 1. This resulted in the exclusion of 2 adolescents (1 SAL; 1 E1) and 1 adult (NM) prior to further analysis. Percent freezing during the tone fear retention test (CS bin 1) was then checked for outliers at each age, with scores > 2 standard deviations from the mean of each experimental condition excluded from analysis. This resulted in exclusion of 2 adolescents (1 E1, 1 E1.5). Final n 's were 8–10/group.

5.4.2. Statistics—Statistical analyses were conducted using an identical strategy as in Exp 1.

6. Experiment 2: Results

6.1. Tone Conditioning

Context extinction prior to tone testing was sufficient to eliminate challenge group differences in baseline freezing, and ethanol challenge prior to tone conditioning did not affect tone retention at either age. Under these test circumstances, retention of tone fear (bin 1) was not influenced by ethanol, findings consistent with previous data [15]. Tone extinction, however, was influenced by ethanol challenge during conditioning in adults during the first extinction session and in both adolescents and adults during the extinction retention session the following day. Thus, ethanol does not appear to leave tone fear memory completely spared. Consistent with Exp 1, no age differences emerged in terms of an extinction retention deficit.

6.1.1. Conditioning Day—Acquisition of tone conditioning was not influenced by challenge among adolescents (See Fig 3a). Analysis of the adult data, however, revealed a significant bin $\times$ challenge interaction [$F(12,144) = 2.16, p < .05$], with adults in the E1.5 group freezing significantly more than NM and SAL groups during the pre-CS period and the first tone (see Fig. 3b). These results suggest that adults showed significant ethanol-induced motor impairment, while adolescents did not.

6.1.2. Context Fear Retention Test—Analysis of the context retention data revealed a significant main effect of challenge [$F(3,67) = 8.96, p < .05$], with context fear retention disrupted in both ethanol groups regardless of age (see bin 1 of Fig. 3c & 3d).

6.1.3. Context Extinction—A significant main effect of challenge [$F(3,30) = 3.04, p < .05$] emerged in the analysis of the adolescent context extinction data, with adolescents in the E1.5 group continuing to show significantly less freezing than NM adolescents throughout the extinction session (see Fig. 3c). Analysis of the adult data revealed a significant main effect of challenge [$F(3,37) = 3.13, p < .05$] and bin $\times$ challenge interaction [$F(15,185) = 2.36, p < .05$], with SAL, E1 and E1.5 adults freezing significantly less than NM animals during bins 2 and 3, and the significant difference in freezing between E1.5 and NM adults continuing through bin 4 (see Fig. 3d). Although no significant effects of age were evident in the context retention analysis (above), the across bin analysis of extinction indicated that adolescents again showed decreased sensitivity to ethanol-induced disruptions of context memory, given that the adult (but not the adolescent) E1 group showed significantly less freezing during the context extinction session.

6.1.4. Baseline 1 (Tone Test 1) and Baseline 2 (Tone Test 2)—No significant effects emerged in the analysis of baseline freezing on either test day, suggesting that the context extinction session on Day 2 was sufficient to eliminate challenge group differences in baseline freezing (see Table 1).

6.1.5. Tone Fear Retention (Test 1)—No effects of age or exposure emerged in the analysis of tone fear retention (see bin 1 of Fig. 3e & 3f), suggesting that ethanol challenge during conditioning did not affect tone fear retention.

6.1.6. Tone Extinction 1—No challenge effects emerged in the analysis of the adolescent data (see Fig. 3e). For the adult data, a significant bin $\times$ challenge interaction [$F(12,148) =$

2.16, $p < .05$] emerged, with adults in the E1 and SAL groups extinguishing slightly faster than NM animals (see Fig. 3f).

6.1.7. Tone Extinction Retention (Test 2)—A significant main effect of challenge emerged [$F(3,67) = 4.37$, $p < .05$], with both ethanol groups showing significantly less freezing than NM and SAL groups regardless of age, although the E1 effect was more pronounced in adults (see bin 1 of Fig. 3g & 3h). As in Exp1, no evidence of age differences in extinction retention emerged in the NM exposure group.

6.1.8. Tone Extinction 2—A significant bin $\times$ challenge interaction [$F(12, 120) = 2.54$, $p < .05$; $F(12, 148) = 3.11$, $p < .05$] emerged in the analysis of the adolescent data, with the E1.5 showing significantly less freezing early in the second tone extinction session (see Fig. 3g). In adults, aside from the above mentioned challenge group differences during bin 1, no significant differences emerged between challenge groups throughout the extinction session in adults (see Fig. 3h).

7. Discussion

These experiments examined potential age differences during acquisition, fear retention, extinction and extinction retention for tone and context conditioning, and determined how these learning and memory processes may be affected by ethanol challenge during conditioning at each age. In terms of tone conditioning, adolescent and adult males were found to be similar with respect to basal levels of acquisition, fear retention, and extinction. Surprisingly, age differences were also not observed during extinction retention, findings which contrast with previous reports of an adolescent-associated deficit in retention of the extinguished response [24, 25]. When assessing context conditioning, acquisition and context fear retention was found to be similar between adolescents and adults. However, adolescent animals showed faster context extinction than adults, while again not differing from adults in retention of the extinguished response.

Two important ethanol effects emerged in these studies. First, adolescents were less sensitive than adults to ethanol – both in terms of motor impairing effects of ethanol during context and tone conditioning, as well as when examining deficits in context retention following ethanol challenge during conditioning. The second ethanol effect of interest was that context fear retention was disrupted by an ethanol challenge during conditioning, but tone fear retention was not influenced by ethanol when the baseline (pre-CS) freezing differences across challenge groups seen on test day in Exp 1 were corrected in Exp 2. These studies provide more evidence that ethanol challenge during conditioning disrupts context, but not tone retention, and highlight the importance of reducing group differences in pre-CS freezing to appropriately assess tone fear retention in future studies.

When examining extinction retention after tone conditioning, both adolescents and adults showed substantial freezing during the tone fear extinction test. These results were unexpected given that previous studies found that adults showed low levels of freezing to the tone after extinction and that only adolescents showed the substantial levels of freezing consistent with limited retention of extinction memory [24, 25]. Procedural differences may account for the discrepant findings between the prior work and the present study. While the same procedures for tone conditioning and extinction were used in prior work as in this study, the previous studies tested for extinction retention using a continuous 3 minute tone. Given the change in tone duration from that which was used during conditioning and extinction, it is possible that the extinction retention may have also tested stimulus (and hence fear) generalization [16]. This may be an important consideration, given that age differences have been reported in degree of stimulus generalization between adolescents and

adults, with adolescent mice showing more fear generalization in terms of tone pattern (i.e., duration) and frequency than adults during a fear retention test [32]. Perhaps a lack of stimulus generalization in adult animals in prior work using an extended CS presentation contributed to the attenuated freezing seen during the extinction retention test, whereas adolescents in these experiments may have showed more freezing under these test conditions due to their propensity to generalize stimuli. It would be useful for a study to be conducted in rats that varied CS duration during tone fear testing to determine if previously reported age differences in extinction retention deficits would emerge as the CS duration increased beyond that used during conditioning.

Another procedural difference between this study and the previous studies is that in prior work animals were exposed to the conditioning chamber daily for 3 days prior to conditioning in order to decrease associations made to the context during conditioning [24, 25]. This procedure was not used for these experiments because littermates were housed in pairs, with one littermate receiving tone conditioning and the other context conditioning, necessitating similar preconditioning procedures for both animals. It is possible that context pre-exposure in tone conditioned animals may have somehow facilitated retention of tone extinction memory among adults, but not their adolescent counterparts.

Our finding of an age difference in expression of ethanol-induced context retention deficit, with adults but not adolescents showing a deficit at the 1 g/kg ethanol dose, is reminiscent of that of Land & Spear [21], although that study also reported a similar effect after tone conditioning. The differences across studies in terms of ethanol-induced tone fear deficits may be related to differences in the assessment of tone fear retention, with the Land & Spear [21] study using the original conditioning context to test tone fear, whereas the present study used a novel context for tone testing. These age differences in sensitivity to ethanol-induced deficits in context retention are unlikely to be due to pharmacokinetic factors, given prior reports of equivalent blood ethanol concentrations across age after i.p. administration of a 1 g/kg ethanol dose in adolescent and adult rats [21, 33] -- the dose that affected context retention in adults, but not adolescents in the present study. These results are consistent with numerous reports that adolescents are less sensitive than adults to a variety of acute ethanol effects, including ethanol-induced sedation [34], motor impairment [35, 36], and social inhibition [37], but contradict other reports of a greater adolescent sensitivity to ethanol-induced disruptions of memory using the MWM [4]. This contradiction is surprising since both performance of the MWM (see [22, 23] for review) and contextual fear conditioning [12, 13, 14] are thought to rely on the hippocampus. Such differential ethanol sensitivities across tasks between adolescents and adults could be related to differences in task demands, with the MWM requiring spatial navigation and fear conditioning typically expressed via freezing behavior. With this in mind, it would not be surprising for the two tasks to also differ in underlying neural pathways, with one pathway more potently disrupted by ethanol than the other at particular points in ontogeny. Indeed, it has been suggested that the dorsal region of the hippocampus is more involved in spatial learning and memory, whereas context conditioning may rely more on circuitry involving the ventral hippocampus (see [38] for review). These results suggest that adolescents are not more sensitive than adults to ethanol-induced disruptions of all learning and memory tasks thought to rely at least in part on the hippocampus. Further studies are needed to determine why age differences in ethanol sensitivity are task-specific, perhaps starting with direct examination of potential age differences in ethanol sensitivity across different hippocampal regions.

In the present study, ethanol challenge during conditioning influenced tone retention only when differences emerged across groups in freezing at baseline (pre-CS) on test day. An interesting study by Jacobs et al. [30] found that pre-CS freezing interacts with freezing during test, with more baseline freezing resulting in greater freezing to the CS, and

concluded that to accurately assess freezing to the tone CS, group differences in pre-CS freezing need to be eliminated. In line with the findings of Jacobs et al. [30], when tone fear retention was examined in Exp 1, the ethanol groups showed significantly less baseline freezing as well as less freezing to the CS. In Exp 2, however, where procedures were modified to eliminate baseline differences across challenge groups, no effect of ethanol was detected during the tone fear retention test, suggesting that the ethanol effect on CS freezing observed in Exp 1 had likely been influenced by pre-CS freezing.

Differences in baseline freezing among challenge groups were also evident during acquisition, with elevated freezing observed at the highest ethanol dose, particularly among adults. This complicates interpretation of the degree to which adult E1.5 animals in Exp 1 acquired conditioned fear, thereby possibly masking any potential disruption in learning that could have contributed to the reduced context and tone fear retention seen among this group. Results from Exp 2, however, suggest that this may not be the case since similar differences in baseline freezing were observed during acquisition as in Exp 1, yet freezing during the tone fear retention test did not differ among the challenge groups.

These two experiments collectively suggest that preconditioning ethanol challenge does not affect tone retention when baseline freezing differences at the time of the retention test were eliminated. These results are consistent with previous reports that delay tone conditioning, where the CS and US overlap, is insensitive to ethanol [15, 39, 40]. Given that the amygdala is essential for delay tone conditioning [9, 10, 11], these results suggest that the amygdala is relatively resistant to acute disruption by ethanol. However, there was evidence that ethanol during conditioning enhanced extinction rate relative to saline or NM controls in Exp 2, suggesting that tone fear conditioning is not completely unaffected by ethanol. Extinction rate has been shown to be related to degree of initial learning and differ as a function of US intensity [41]. Hence, it is possible that when animals received tone conditioning under the influence of ethanol in the present study, their learning may have been slightly impaired, although these differences may not have been detectable in terms of freezing during conditioning or retention, but instead emerged at the time of extinction. It is also possible that ethanol challenge during conditioning may have enhanced tone extinction by way of state-dependent effects on learning. That is, because the ethanol cue was not present during extinction, these animals may not have demonstrated as strong of a CS-US association as animals that were trained under non-ethanol conditions. Few studies have examined state-dependent effects of ethanol during Pavlovian conditioning (e.g., [42]), and to our knowledge, no studies have examined state-dependent effects in terms of extinction processes. Further studies are needed to address whether ethanol's enhancement of tone extinction may be due to state-dependent learning deficits.

Another important point is that this study and many of the other studies assessing age differences in fear conditioning and extinction only included males. Therefore, it is unknown whether similar effects would be observed with female rats. Indeed, there is some evidence to suggest that adult males and females differ in terms of fear conditioning, with males generally showing greater acquisition and resistance to extinction than females (see [43] for review). Future studies should be conducted to examine the effects of age and ethanol on fear conditioning in females.

Examination of learning and memory after context or tone conditioning provides a unique opportunity to detect potential age differences in cognitive processes thought to be relatively dependent on the hippocampus and amygdala, respectively [44]. Given that age differences in ethanol sensitivity in this study emerged during context retention and extinction, these results indicate that ethanol affects cognitive processes thought to involve the hippocampus differently between adolescent and adult males, as has previously been suggested (see [21,

22] for review), with these differences possibly due to the relative immaturity of the hippocampus during adolescence (see [45] for review). Since adolescents commonly engage in binge level consumption of ethanol at a time when the hippocampus is continuing to mature, repeated ethanol exposure during this stage of development could reflect a regional vulnerability for lasting ethanol effects. Future studies should examine how repeated ethanol exposure during adolescence influences context and tone conditioning to further understanding of the consequences of adolescent alcohol consumption on cognition.

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Research Highlights

- Ethanol disrupted context fear retention more than tone fear.
- Adolescents were less sensitive to these ethanol-induced disruptions.
- These results suggest hippocampal processing is particularly vulnerable to ethanol.
- Adolescents respond differently to these hippocampal disruptions.

Fig 1.

Adolescent and adult tone fear conditioning (a, b), fear retention (bin 1) and extinction day 1 (c, d), extinction retention (bin 1) and extinction day 2 (e, f) in Exp 1. Note: Retention data was analyzed across age. One asterisk (*) indicates a significant difference from NM (except for Fig 1f, which indicates a significant difference from SAL) and two asterisks (**) indicates a significant difference from SAL and NM groups.

Fig 2.

Adolescent and adult context fear conditioning (a, b), fear retention (bin 1) and extinction day 1 (c, d), extinction retention (bin 1) and extinction day 2 (e, f) in Exp 1. Note: Retention data was analyzed across age. One asterisk (*) indicates a significant difference from NM (except for Fig. 2b, which indicates a significant difference from SAL) and two asterisks (**) indicates a significant difference from SAL and NM groups. Number signs (#) indicate a significant age difference.

Fig 3.

Adolescent and adult tone fear conditioning (a, b), context fear retention (bin 1) and extinction after tone conditioning (c, d), tone fear retention (bin 1) and extinction day 1 (e, f), tone extinction retention (bin 1) and extinction day 2 (g, h) in Exp 2. Note: Retention data was analyzed across age. One asterisk (*) indicates a significant difference from NM and two asterisks (**) indicates a significant difference from SAL and NM groups.

Table 1Baseline freezing (mean $\pm$ SEM) after tone conditioning in Exp 1 and 2

	Baseline 1				Baseline 2			
	Adolescents		Adults		Adolescents		Adults	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
NM	49.1 $\pm$ 8.8	25.9 $\pm$ 8.0	40.1 $\pm$ 7.6	27.1 $\pm$ 6.1	25.8 $\pm$ 7.7	21.9 $\pm$ 6.7	27.3 $\pm$ 5.8	35.0 $\pm$ 7.3
SAL	31.1 $\pm$ 6.0	25.6 $\pm$ 10.7	28.8 $\pm$ 4.4	13.2 $\pm$ 5.9	17.5 $\pm$ 6.4	34.8 $\pm$ 7.2	35.9 $\pm$ 8.5	28.2 $\pm$ 9.7
E1	19.8 $\pm$ 7.7 ⁺	21.8 $\pm$ 7.4	15.2 $\pm$ 8.4 ⁺	7.5 $\pm$ 2.5	13.5 $\pm$ 3.7	24.6 $\pm$ 7.0	13.6 $\pm$ 4.3	28.6 $\pm$ 8.3
E1.5	7.1 $\pm$ 2.3 [#]	15.2 $\pm$ 7.6	3.8 $\pm$ 1.3 [#]	8.8 $\pm$ 3.6	18.1 $\pm$ 9.1	16.0 $\pm$ 7.5	5.4 $\pm$ 3.5	29.8 $\pm$ 7.8

Significance relative to SAL & NM (#) or NM only (+)