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Individual differences in conditioned fear are associated with levels of adolescent/early adult alcohol consumption and instrumental extinction

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Abstract

Previous research has shown a relationship between alcohol exposure and conditioned fear, but the nature of this relationship remains unclear. We determined whether chronic intermittent access to alcohol during adolescence and early adulthood would alter or be associated with the level of conditioned fear to an auditory cue in male Long Evans rats. Rats received 6 weeks of chronic intermittent access to 20% alcohol or water from PND 26–66 and began behavioral testing 10 days later. We found no evidence that voluntary alcohol consumption altered fear. However, we found that rats that consumed more alcohol had lower fear, as measured with conditioned suppression of lever-pressing and conditioned freezing to an auditory cue. We have previously shown that higher levels of alcohol consumption are correlated with faster instrumental extinction learning. Therefore, we determined whether instrumental extinction would be directly associated with conditioned fear in rats never given alcohol access. As predicted, we found that rats that exhibited faster instrumental extinction also exhibited lower conditioned fear, as measured with conditioned suppression of lever-pressing and conditioned freezing. Our results suggest that at least part of the relationship between alcohol consumption levels and fear learning differences may be due to pre-existing individual differences. In addition, our finding that conditioned fear and instrumental extinction abilities (both separately associated with alcohol consumption levels) are associated with each other suggests that alcohol consumption levels may be a marker that can distinguish two separate phenotypes that encompass a wide variety of learning traits.

Keywords

Fear conditioning; extinction; alcohol; individual differences

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1. Introduction

There is a high degree of comorbidity between alcoholism and post-traumatic stress disorder (PTSD) [1, 2], but the reason for this relationship is unclear. Individuals may drink to alleviate pre-existing fear/anxiety, alcohol consumption may alter the brain to increase the likelihood of PTSD development, or some pre-existing factor may affect both anxiety and alcohol consumption. Although it is likely that a combination of these factors may be involved in the alcohol-fear/anxiety relationship, most explanations of the relationship have focused on fear and anxiety symptoms leading to increased alcohol use for “self-medication” [3]. However, prospective analyses of symptom development have shown that pre-existing anxiety disorders (including PTSD) increase the probability of later development or worsening of alcohol abuse problems and pre-existing alcohol abuse problems increase the probability of later development of an anxiety disorder [4, 5]. Unfortunately, experiments in humans (including prospective analyses) generally lack the experimental control needed to isolate the causal relationships between alcohol use and fear/anxiety. The control provided by animal models can provide sufficient experimental control to determine the direction of this relationship.

Investigations of relationships between alcohol exposure/consumption and conditioned fear in animal models could help to reveal the nature of alcohol-fear relationships in humans. Pavlovian fear conditioning, in which an initially neutral cue is paired with a foot-shock leading to acquisition of a fear response to the previously neutral cue, is widely used as a model of human fear and anxiety disorders [6, 7]. Notably, individual differences in fear expression after conditioned fear training tend to be relatively stable, making this a good measure to study individual differences in fear reactivity [8]. Previous animal studies have focused more on determining effects of forced exposure to high doses of alcohol [9–15] than examining effects of lower levels of voluntary alcohol consumption on conditioned fear or possible correlations between motivation to consume alcohol and conditioned fear (but see [16]). As a result, the effect of lower levels of alcohol exposure on conditioned fear, and whether pre-existing differences in the motivation to consume alcohol would be associated with pre-existing differences in fear learning abilities, is unknown.

We performed two experiments in the current report. In Experiment 1, we gave rats alcohol or water-alone access during adolescence/early adulthood (post-natal day [PND] 26–66). Our alcohol access method was the chronic intermittent access model (CIA) model, in which animals receive access to alcohol for 24-hour periods that alternate with alcohol-free periods [17]. We then determined whether alcohol consumption would affect conditioned fear (difference between alcohol and water groups) or the level of consumption would be associated with conditioned fear (no difference between alcohol and water groups, but high drinking alcohol rats differing from low drinking rats in their fear behavior). We found a relationship between the level of voluntary alcohol consumption and conditioned fear expression, with no difference between the alcohol and water groups, suggesting that pre-existing differences in the propensity to consume alcohol are associated with individual differences in fear learning abilities.

In Experiment 2, we followed up on this alcohol-fear association and its meaning for our previous finding (in [18]) that alcohol consumption was also associated with the speed of instrumental extinction learning. One possible follow-up experiment would be to determine whether the alcohol consumption-fear conditioning association would be seen if the fear conditioning was assessed first and alcohol consumption was assessed second. Two possible designs for this would be to assess fear conditioning in pre-adolescence so we could keep the age range of alcohol consumption the same, or assess alcohol consumption entirely in adulthood so we could keep the age range for fear conditioning the same. However, as discussed more fully in the Discussion section, there are several reasons to suspect that switching the order of the alcohol consumption and fear conditioning phases could lead to prior stress altering alcohol consumption patterns or that developmental effects could alter the patterns of fear conditioning or alcohol consumption in these designs. Previous research from our lab has also shown that voluntary alcohol consumption is correlated with the rate of instrumental extinction and reversal learning errors in a go/no-go task (with stronger correlations with commission errors [pressing the no-go lever] and maintenance/discontinuation errors [inability to maintain the reversal for multiple days in a row after meeting criterion once]) [18]. For this reason, we performed a different follow-up experiment to Experiment 1. In particular, we determined whether this alcohol consumption-conditioned fear association is part of a larger constellation of behavioral traits that are *all* associated with one another, with a *high alcohol (drinking)-low fear-fast instrumental extinction-low discontinuation errors in reversal* phenotype (which we abbreviate HALF-FIELDER) and a phenotype with none of these traits (which we abbreviate non-HALF-FIELDER). If so, this would broaden our findings beyond an alcoholcentric view of these traits, demonstrate that individual differences in these traits can be found even in rats without alcohol exposure, and show that our alcohol findings have broader significance for individual differences in the general phenotype of behavioral traits. In Experiment 2, we investigated whether instrumental extinction is associated with conditioned fear. We expected that low conditioned fear and fast instrumental extinction (both associated with high drinking in previous studies) would be associated with one another.

2. Methods

2.1 Subjects

Male naïve Long Evans rats (n=36) from Charles River Laboratories (Kingston, NY and Raleigh, NC), PND 21 upon arrival in the facility, were used for Experiment 1. All animals were individually housed and maintained on a 12-hour reverse light-dark cycle with lights off at 07:30 am in a temperature and humidity controlled room. The rats were given 5 days to acclimate to the facility, and then received CIA starting on PND 26. Water and food were available *ad libitum* during this 6-week period. Three days after the final alcohol access period, body weights were recorded and rats were food-restricted and subsequently allowed to grow 1.5 g/day from this initial weight. Water was available *ad libitum* throughout the period of food restriction. In Experiment 2, adult male Long Evans rats (n=12), 175–200 g on arrival in the facility from Charles River Laboratories (Kingston, NY), were used. After 23 days in the facility, they were food restricted and allowed to grow 1.5 g/day from their initial weights. Training took place during the dark cycle and rats were weighed and fed

after the daily sessions. All procedures and animal care were in accordance with the Kansas State University Institutional Animal Care and Use Committee guidelines, the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and United States federal law.

2.2 Apparatus

Experiments were conducted in 12 operant chambers (Med Associates, St Albans, VT). Each chamber had two retractable levers 9 cm above the floor, but only one lever ("active," retractable lever) was extended into the chamber during behavioral sessions. The right lever was the active lever during the fear conditioning phases of the experiments, and the left lever was the active lever during the instrumental extinction phase of Experiment 2. Responding on the active lever activated the pellet dispenser, delivering 45-mg precision pellets (#1811155, 5% fat, 66% carbohydrate, 20.3% protein; TestDiet, Richmond, IN). A red house-light was located in the center at the top of the back wall of the chambers. A tone generator that delivered a 2900 Hz tone (20 dB above background) was located directly to the right of the house-light. The chambers had grid floors connected to electric shock generators that were capable of delivering a 0.5 mA scrambled foot-shock. A camera was mounted above each chamber. These cameras digitally recorded the behavior of the rats on a computer system that allowed later playback so that their behavior could be scored.

2.3 Alcohol access

Rats received 6 weeks of alcohol (using CIA) or water-only access beginning in adolescence and extending into early adulthood (PND 26–66). A two-bottle choice procedure was used in which all rats had access to water in at least one bottle at all times. Twenty-five of the rats in Experiment 1 (the Alcohol group) received 24-h access to 20% (v/v) ethanol mixed with tap water 3X per week in one bottle, while the other bottle contained tap water. Animals in the Alcohol group received alcohol access starting on Sunday, Tuesday and Thursday, with the other days being water-only days in which both bottles contained water. The Water group (n=11) received two water bottles during the six weeks. Bottles were weighed and changed (for alcohol groups) starting at 1 pm and ending by 2 pm every day except Saturday, and placement was counterbalanced to control for any side preference.

2.4 Behavioral training in fear experiments

Behavioral training was largely the same as in previous studies [19, 20]. Rats were given a 40–60-min food-cup training session, with pellet deliveries every 125 sec. The following day, the rats received 2 sessions on a fixed-interval-1 (FI-1) reinforcement schedule for lever-presses on the right lever (lever-presses could earn a pellet each sec) 2–4 h apart. These sessions ended when rats received 50 pellets (with a limit of 1 h). The rats were then given one 90-min session in which pellets were earned on a variable-interval-30 (VI-30) reinforcement schedule (pellet availability for lever-presses ranging from 1 to 59 sec), and 2 daily 90-min sessions on a VI-60 schedule (pellet availability ranging from 1 to 119 sec). Rats were maintained on the VI-60 schedule for the remainder of the behavioral training.

During the fear conditioning session, animals earned pellets on a VI-60 schedule. Sessions began with the extension of the active lever and illumination of a red house-light. On this

day, they received 10 30-second tones (2900 Hz, 20 dB above background) ranging from 3 to 14 min apart, co-terminating with 0.5-mA, 0.5-sec foot-shocks (adjusted for inter-chamber variability) pseudo-randomly throughout a 90-min session.

Rats received a context test followed by a cued fear test (in extinction) on days 1 and 2 after the end of fear conditioning, respectively. The context test consisted of VI-60 lever-press training during which no tones or shocks were presented, and this session lasted 90 min. The extinction test lasted 35 min, during which rats lever-pressed on a VI-60 schedule and 4 presentations of the tone fear cue were presented without any shock deliveries

2.5 Individual Experiments

2.5.1 Experiment 1 - Alcohol-fear associations—Rats (n=36) received CIA during adolescence/early adulthood. The Alcohol group (n=25) received alcohol access as described above and the Water group (n=11) received water-only access. Three days after the last alcohol access period, all rats were food-restricted and behavioral training procedures began 7 days after the start of food restriction. The rats received a 60-min magazine session, followed by lever-press training as described above. Next, the rats received conditioned fear training (1 session of VI-60 with 10 tone-shock pairings per session). One day after the end of fear training, the rats received a context test, followed by a cue test the following day (day 2).

2.5.2 Experiment 2 – Fear-instrumental extinction associations—Rats (n=12) were trained to press the left lever using training procedures nearly identical to those described above to train rats to press the right lever, with one 40-min session of magazine training and four sessions of FI-1 on the left lever across 2 days (2 per day).

Next, rats underwent one daily session of cued instrumental training for two days, designed to mimic the training of an individual lever-food pellet relationship in our previous devaluation-instrumental extinction experiment [18]. Each instrumental training session lasted 40 minutes and consisted of 40 trials. Each trial included the left lever being extended into the chamber and the cuelight above this lever being illuminated for 40 sec. During the cued trials, lever-presses earned 1 pellet at a randomized time in the first 20 seconds, and a second pellet at a randomized time within the second 20 seconds, resulting in the potential to earn a maximum of 2 pellets per trial. There were 40 cue-presentations per session with an average ITI of 20 seconds.

After the cued-trial training, the rats received an instrumental extinction test session. This session was identical to the cued training sessions, except that it lasted 20 min, contained 20 trials rather than 40, and no food pellets were delivered for lever-pressing. The rats then received a single lever retraining session (identical to the first 2 cued training sessions in which lever-presses were reinforced) on the following day, and then a second instrumental extinction test (identical to the first test) 2 days after the initial test.

Next, the rats received training in a limited training fear conditioning procedure nearly identical to that used in Experiment 1, with the main difference being the omission of the magazine training session. Rats received right lever-press training (2 right FI-1 sessions, 1

session of VI-30, 2 sessions of VI-60), fear training (1 session of VI-60 with 10 tone-shock pairings), a context fear test, and a cued fear test.

2.6 Behavioral measures

For alcohol consumption, the alcohol consumed was calculated as the bottle weight difference between the start and end of the access periods, minus 2 g for spillage/evaporation, and then multiplied by 0.162 for the weight of alcohol in 1 g of a 20% v/v alcohol solution. The 2 g subtracted for spillage was determined based on pilot research conducted in our laboratory during which bottles were placed on empty cages and weighed daily to determine general spillage/evaporation.

For Experiments 1 and 2, we used conditioned suppression of lever-pressing and conditioned freezing as our measures of fear [21–27]. Procedures in which conditioned freezing and conditioned suppression are concurrently measured have shown that higher conditioned suppression is associated with higher conditioned freezing [22, 24, 27]. However, it is worthwhile to assess both fear measures, as the two fear measures are neurobiologically dissociable [21, 25, 26] and it is possible to observe reductions in conditioned freezing while conditioned suppression remains at the asymptotic maximum [27]. In addition, conditioned suppression of lever-pressing normalizes the fear measure based on the baseline level of leverpressing, while conditioned freezing to discrete cues is traditionally reported as an absolute number without correction for precue freezing (although the food restriction conditions required to motivate conditioned lever-pressing can lead to lower conditioned freezing compared to lesser or no food restriction) [27]. Thus, assessment of both measures together provides fear measures that both are and are not adjusted for precue activity levels, increasing the likelihood of assessing true fear if both measures show the same pattern.

Lever-presses were recorded during the 30 sec prior to tone presentation (Precue) and during the 30-sec tone presentation (Cue), and were used to calculate a suppression ratio using the formula: $\text{Suppression ratio} = ((\text{Precue} - \text{Cue}) / (\text{Precue} + \text{Cue}))$. The suppression ratio normalizes lever-pressing during the tone based on baseline responding [28, 29]. A value of 1 indicates total conditioned suppression of lever-pressing during tone presentation (high fear), while a value of 0 reflects no lever-press suppression during tone presentation (low fear). When both precue and cue values were 0 on a trial or trial block, the rats' responding in the trials immediately before and after that trial or trial block were averaged to determine a value. Due to a researcher error, video for the cued fear test in Experiment 2 was lost. However, conditioned freezing was scored for fear training in Experiments 1 and 2, and cued fear testing in Experiment 1. Conditioned freezing was defined as immobility other than that required for breathing, and was scored every two seconds during the 30-sec precue and 30-sec cue period [30]. All training phase videos were scored by a trained scorer naïve to the experimental conditions (T.E-W. in the acknowledgements). All testing phase videos were scored by a second trained scorer naïve to the experimental conditions (M.B.). Additional trained scorers who were also blind to the experimental conditions also scored data from Experiment 1 to calculate inter-rater reliability ($r = 0.89$ for the training scoring and $r = 0.87$ for the test scoring).

For Experiment 2, the primary measure of instrumental extinction was the number of lever-presses per trial across the first six trials of the extinction tests. For this measure, we averaged across the two extinction tests to minimize variability. We focused on the first six trials because our previous experiment examining alcohol-instrumental extinction associations demonstrated that the rate of instrumental extinction (decrease in lever-presses divided by the number of trials after the first trial) was the greatest from the first to the sixth trials, with smaller decreases thereafter [18]. The difference in responding from the first to sixth trial was the measure we correlated with alcohol consumption in that report [18], and we used it as our measure to divide the two groups into Fast Extinction and Slow Extinction groups in the current report.

2.7 Statistical analyses

Data were analyzed by Statistica 5.1 software (Tulsa, OK). The factors used in the statistical analyses are described in the Results section for each ANOVA and significant effects ($p < 0.05$) in the different ANOVAs were followed by post-hoc Tukey's HSD tests.

We also ran power analyses to allay concerns that our non-significant comparisons of the alcohol and water group in Experiment 1 were found because the experiments were underpowered, rather than that the effects of alcohol access were so small that it would require hundreds or thousands of rats per group to find an effect. We used the effect sizes from our ANOVAs and did power analyses to determine the number of subjects required to achieve a power of 0.8 with $\alpha = 0.05$. Power analyses used to estimate required power used G*Power 3.1.9.2. As all power analyses were for comparisons with two groups, all estimates of required subjects with odd numbers had one subject added to them to make them even numbers, so that the proposed experiments would have equal numbers of subjects in both groups.

3. Results

3.1 Experiment 1

3.1.1 Alcohol consumption—When all rats in the Alcohol group were analyzed together, there was relatively steady alcohol consumption across the 6-week access period. While chronic intermittent access to alcohol typically produces an escalation in consumption over time in adult rats [17], adolescent access follows the pattern seen in the present experiment [18, 31, 32]. This may occur because adolescent rats increase their body weights faster than their alcohol consumption (in absolute terms) increases. However, our goal was to examine individual differences in alcohol consumption as well as overall patterns. For all analyses of individual differences in alcohol consumption, we categorized the rats given alcohol access based on their consumption in the last 2 weeks of alcohol access, to be consistent with our previous work on go/no-go reversal learning, devaluation, and instrumental extinction [18]. In our previous experiment, there was an extremely high number of maintenance/discontinuation errors in go/no-go reversal learning in one rat that consumed less than 1.5 g/kg/24-h compared to rats that consumed more than 1.5 g/kg/24-h, but we were unable to pursue this further as there was only one rat that met this criterion. Notably, voluntary alcohol consumption below 1.5 g/kg/day was the original criterion used

to select rats used for breeding to create the NP (non-preferring) and LAD (low alcohol drinking) lines of inbred rats [33]. Likewise, a related study that examined the relationship between alcohol consumption levels and conditioned fear discrimination used 10 g/kg of a 20% (v/v) ethanol solution (or ~1.6 g/kg/24-h of pure ethanol) as their cut-off for heavy drinking [16]. As such, 1.5 g/kg/24-h appeared to be a reasonable cut-off for selecting rats to compare behavioral traits in low drinking vs. high drinking phenotypes in our experiments, and we divided the Alcohol group into two sub-groups based on this cut-off.

In the current experiment, we found that there were 7 rats with consumption below the 1.5 g/kg/24-h level, so we compared conditioned fear behavior in this group (Sub-group Low in the Alcohol group) with conditioned fear behavior in 18 rats with consumption above this level (Sub-group High in the Alcohol group) (Figure 1A). We analyzed the drinking data in these 2 groups with a mixed-factor ANOVA with the between-subjects factor of Alcohol Consumption Group (High vs. Low) and the within-subjects factor of Week (the 6 weeks of consumption). We found a significant effect of Alcohol Consumption Group ($F(1, 23)=25.7$, $p<0.01$), but no significant effect of Week and no significant Alcohol Consumption Group X Week interaction ($p>0.05$). Thus, the two Alcohol sub-groups differed in their level of alcohol consumption.

3.1.2 Conditioned fear training—Prior alcohol consumption had no effect on any behavioral measure in the conditioned fear training. However, the sub-groups of the Alcohol group that were divided based on their alcohol consumption differed in their conditioned fear behaviors during training, suggesting individual differences in alcohol consumption are associated with individual differences in conditioned fear. Notably, conditioned fear in our study was relatively low, which is in accord with prior findings that food restriction leads to lower levels of conditioned freezing- a pattern observed even in rats that were not given a concurrent lever-press task [27].

We first compared the rats that were given access to alcohol vs. those given access to water only. We analyzed all data with a mixed-factor ANOVA with the between-subjects factor of Alcohol Consumption Group (High vs. Low) and the within-subjects factor of Training Block (the five 2-trial blocks). We found no differences (no main effect or interaction) between the two groups on precue freezing (Figure 1B, left), freezing to the tone cue (Figure 1C, left), precue lever-pressing (data not shown), or conditioned suppression of lever-pressing (Figure 1E, left). Results of all ANOVAs are in Table 1. For the three fear measures (precue freezing, cue freezing, conditioned suppression of lever-pressing), the effect sizes of the main effect of Access Group and the Access Group X Training Block interaction were very low, and our analyses suggested that it would require 80–560 rats to achieve a power of 0.80 with our effect sizes (Table 2).

Despite the lack of a difference between the Alcohol and Water groups, we did find differences between the high (>1.5 g/kg/24-h) and low (<1.5 g/kg/24-h) alcohol drinkers, with higher fear responses in the Low Alcohol group. We analyzed all data with a mixed-factor ANOVA with the between-subjects factor of Alcohol Consumption Group (High vs. Low) and the within-subjects factor of Training Block (the five 2-trial blocks). An analysis of precue freezing (Figure 1B, right) found a significant Alcohol Consumption Group X

Training Block interaction. The Alcohol Consumption Group X Training Block interaction was driven by significantly higher freezing in the Low Alcohol Group on the fourth and fifth trial blocks (Trials 7–10) with no differences on other blocks. An analysis of cue freezing (Figure 1C, right) also found a significant effect of Alcohol Consumption Group, but no significant Alcohol Consumption Group X Test Trial interaction. The Alcohol Consumption Group effect reflected higher freezing in the Low Alcohol Group. Because there was a significant effect of Alcohol Consumption Group and no interaction, we analyzed the first trial to determine whether the difference between groups existed on the first trial (before any shocks were delivered). We found no difference between conditioned freezing in the high consumption group ($10.0 \pm 5.6\%$) and the low consumption group ($4.8 \pm 1.9\%$) ($F < 1$), suggesting that there were no pre-existing differences in freezing. An analysis of precue lever-pressing found no effect or interaction of Alcohol Consumption Group (data not shown). Finally, an analysis of conditioned suppression of lever-pressing (Figure 1E, right) found a significant Alcohol Consumption Group X Training Block interaction. The Alcohol Consumption Group X Training Block interaction was driven by significantly higher conditioned suppression in the Low Alcohol Group on the second trial block (Trials 3–4) with no differences on other trials. Full results of all ANOVAs are in Table 1.

We also examined whether high vs. low water consumption would be associated with differences in conditioned fear in the Water group. We had no theoretical reason to choose any particular cut-off, so we performed a median split on the 11 rats in the water group (5 in the high group, 5 in the low group and the rat with the median value excluded). We analyzed precue lever-pressing, conditioned suppression of lever-pressing, precue freezing and cue freezing using separate mixed-factor ANOVAs with the between-subjects factor of Water Consumption Group (High vs. Low) and the within-subjects factor of Training Block (the five 2-trial blocks). None of these ANOVAs found a significant main effect of Water Consumption Group or significant Water Consumption Group X Training Block interaction (all $p > 0.05$).

3.1.3 Conditioned fear testing—Prior alcohol consumption had no effect on any behavioral measure in the conditioned fear test. However, the sub-groups of the Alcohol group that were divided based on their alcohol consumption behavior differed in their conditioned fear behaviors during testing, suggesting that individual differences in alcohol consumption are associated with individual differences in conditioned fear.

We first compared the rats that were given access to alcohol vs. those given access to water only. We analyzed all data with a mixed-factor ANOVA with the between-subjects factor of Access Group (Water vs. Alcohol) and the within-subjects factor of Test Trial (the four test trials). We found no difference between the two groups on precue freezing (data not shown), freezing to the tone cue (Figure 1D, left), precue lever-pressing (data not shown), or conditioned suppression of lever-pressing (Figure 1F, left). Results of all ANOVAs are in Table 1. For the three fear measures (precue freezing, cue freezing, conditioned suppression of lever-pressing, the effect sizes of the main effect of Access Group and the Access Group X Trial interaction were very low, and our analyses suggested that it would require 214–26158 rats to achieve a power of 0.80 with our effect sizes (Table 2).

Despite the lack of a difference between the Alcohol and Water groups, we did find differences between the high (>1.5 g/kg/24-h) and low (<1.5 g/kg/24-h) alcohol drinkers. We analyzed all data with a mixed-factor ANOVA with the between-subjects factor of Alcohol Consumption Group (High vs. Low) and the within-subjects factor of Test Trial (the four test trials). An analysis of precue freezing found a significant interaction of Alcohol Consumption Group X Test Trial. Post-hoc analyses found that the Alcohol Consumption Group X Test Trial was due to a significant difference in freezing that was limited to the third conditioning trial. The trial by trial freezing was: High group: Trial 1- 2.6 ± 1.9 , Trial 2- 0.4 ± 0.4 , Trial 3- 1.1 ± 0.6 , Trial 4- 3.0 ± 1.3 ; Low group: Trial 1- 0.0 ± 0.0 , Trial 2- 4.8 ± 3.8 , Trial 3- 13.3 ± 9.1 , Trial 4- 6.7 ± 4.6 . An analysis of cue freezing (Figure 1D, right) found a significant Alcohol Consumption Group X Test Trial interaction. The Alcohol Consumption Group X Test Trial interaction reflected higher conditioned freezing in the Low Alcohol group on the first test trial, which was not significant on subsequent trials. An analysis of precue lever-pressing found no significant main effects or interactions (all $p > 0.05$) (data not shown). An analysis of conditioned suppression of lever-pressing (Figure 1F, right) found a significant main effect of Alcohol Consumption Group, but no interaction of Alcohol Consumption Group X Test Trial interaction ($F < 1$). The effect of Alcohol Consumption Group reflected higher conditioned suppression in the Low Alcohol Group. Results of all ANOVAs are in Table 1.

We also examined whether high vs. low water consumption would be associated with differences in conditioned fear in the water-only group, with the same median split used for the training data. We analyzed precue lever-pressing, conditioned suppression of lever-pressing, precue freezing and cue freezing using separate mixed-factor ANOVAs with the between-subjects factor of Water Consumption Group (High vs. Low) and the within-subjects factor of Test Trial (the four test trials). None of these ANOVAs found a significant main effect of Water Consumption Group or significant Water Consumption Group X Test Trial interactions (all $p > 0.05$).

3.1.4 Correlations between alcohol consumption and fear measures—We also calculated Pearson's correlation coefficients between alcohol consumption and the three fear measures (precue freezing, cue freezing and conditioned suppression of lever-pressing) in training and testing. Our measure of alcohol consumption was the average consumption in the last 2 weeks of access (g/kg/24-h). Our measure of conditioned fear during training was the value of each measure from trials 2–10, as trial 1 behavior reflected behavior before the rats had received any shocks. Our measure of conditioned fear during testing was the average value of each measure from test trials 1–4.

These correlations revealed that the low alcohol consumption group contained a disproportionate number of rats with high fear intermixed with a few rats with low fear. For the four measures in which the high alcohol consumption group differed from the low consumption group (with a significant main effect), the Low Alcohol consumption group had a disproportionate number of rats with high fear scores. With only 7 rats in the low alcohol consumption group and 18 rats in the high alcohol consumption group, the low alcohol consumption group had the 3 rats with the highest suppression ratio during the test, 5 out of 6 rats with the highest precue freezing in training, 4 out of 5 rats with the highest

cue freezing in training, and 4 out of 5 rats with the highest suppression in training. For the two measures in which the high alcohol consumption group did not differ from the low consumption group with a main effect but there were significant interactions of Alcohol Consumption Group (with Training Block or Test Trial), the low alcohol group had 5 out of 8 rats with the highest precue freezing in the test and 4 out of 7 rats with the highest cue freezing in the test. These patterns led to insignificant correlations between alcohol consumption and all of the fear measures (correlations for precue freezing for training and testing were $r = -0.30$ and -0.21 , respectively; all other correlation coefficients are in Figures 1G and 1H).

3.2 Experiment 2

3.2.1 Cued lever training—We first divided our rats into those who decreased responding faster (Fast Extinction) or slower (Slow Extinction) during the extinction test, for use in all analyses within this experiment. We averaged the response rate in the two instrumental responding extinction tests, and then performed a median split ($n=6/\text{group}$) (Figure 2A) based on the same measure that was correlated with voluntary alcohol consumption in our previous paper [18]: responses on trial 1 minus responses on trial 6.

In the fast extinction group compared to the slow extinction group, there was a trend for higher response rates for the cued lever training before all extinction testing began, but this did not reach significance. The response rates for the two days of cued lever training prior to all extinction testing were (in responses/trial): Fast Extinction-Day 1 = 14.7 ± 1.5 , Fast Extinction-Day 2 = 15.8 ± 2.5 , Slow Extinction-Day 1 = 11.2 ± 1.1 , Slow Extinction-Day 2 = 10.7 ± 1.3 . A mixed-factor ANOVA of the 2 days of cued lever training with the between-subjects factor of Instrumental Extinction Group (Fast vs. Slow) and the within-subjects factor of Training Day (the two training days) found a non-significant trend towards higher responding in the fast extinction group [main effect of Instrumental Extinction Group ($F(1, 10)=4.1$, $p=0.07$)]. No other effects or interactions were significant (all $F < 1$). There was also no significant difference in responding between the two groups in the cued lever retraining session between the two extinction tests [Fast Extinction = 18.07 ± 2.4 , Slow Extinction = 13.1 ± 1.5 ; ($F(1, 10)=2.3$, $p=0.12$)].

3.2.2 Instrumental extinction—A mixed-factor ANOVA of the first 6 extinction trials using with the between-subjects factor of Instrumental Extinction Group (Fast vs. Slow) and the within-subjects factor of Test Trial (the first 6 test trials) found a significant effect of Test Trial ($F(5, 50)=15.0$, $p < 0.01$) and a significant interaction of Instrumental Extinction Group X Test Trial ($F(5, 50)=5.4$, $p < 0.01$). The main effect of Instrumental Extinction Group was marginally significant ($F(5, 50)=4.0$, $p=0.07$). The Instrumental Extinction Group X Test Trial interaction had an odd form, in which the two groups differed on the first 2 trials and converged on later trials, rather than starting with equal responding and diverging as extinction progressed. Although we cannot explain the higher initial responding in the fast extinction group, this group clearly exhibited significant extinction within the first 6 trials (responding on trials 3–6 significantly differed from responding in trial 1, $p < 0.05$), while the slow extinction group did not (responding on trial 1 did not differ from trials 2–6, all $p > 0.05$). However, the lack of an extinction effect in the slow extinction group was not due

to a floor effect, as both groups were able to decrease their responding further. When we examined the change in fear from trial 6 (the end of our range to determine fast vs. slow extinction) to trial 20 (the end of the test session), we found that both the slow extinction group (responses/trial: trial 6=10.0±0.8, trial 20=2.8±1.2) and the fast extinction group (responses/trial: trial 6=9.9±2.5, trial 20=3.8±0.9) exhibited decreases in responding. This conclusion was confirmed by a mixed-factor ANOVA with the between-subjects factor of Instrumental Extinction Group (Fast vs. Slow) and the within-subjects factor of Test Trial (trial 6 vs. trial 20). This analysis found a marginally significant effect of Test Trial ($p=0.07$), but no effect of Instrumental Extinction Group and no Instrumental Extinction Group X Test Trial interaction (all $F<1$). This suggests that, while the fast extinction group exhibited an extinction effect earlier in the session, both groups were capable of further extinction and decreased their responding within the extinction test.

3.2.3 Conditioned fear acquisition—The two instrumental extinction groups also exhibited differences in conditioned fear behaviors to an auditory stimulus during acquisition of conditioned fear. The fast extinction group exhibited lower conditioned fear, as seen in lower conditioned freezing and lower conditioned suppression of lever-pressing. All analyses were performed with a mixed-factor ANOVA with the between-subjects factor of Instrumental Extinction Group (Fast vs. Slow) and the within-subjects factor of Training Block (the five 2-trial blocks). An analysis of precue freezing found no significant effects or interactions (all $p>0.05$) (data not shown). An analysis of conditioned freezing during the tone (Figure 2B) found significant effects of Instrumental Extinction Group, but the Instrumental Extinction Group X Training Block interaction was only marginally significant ($p=0.09$). The effect of Instrumental Extinction Group reflected that the slow extinction group exhibited higher freezing than the fast extinction group. Because there was a significant effect of Instrumental Extinction Group and no interaction, we analyzed the first trial to determine whether the difference between groups existed before any shocks were delivered. We found that conditioned freezing in the fast extinction group ($4.4\pm2.2\%$) and the slow extinction group ($7.8\pm2.0\%$) were not different from one another ($F(1, 10)=1.2$, $p>0.05$), suggesting that there were no pre-existing differences in conditioned freezing. An analysis of precue lever-pressing (Figure 2C) found a significant Instrumental Extinction Group X Training Block interaction. The interaction reflects the fact that the two groups did not differ in their response rates in blocks 1 or 2, but the slow extinction group developed lower responding as the session progressed, likely reflecting decreased lever-pressing due to contextual fear in the slow extinction group. An analysis of conditioned suppression of lever-pressing (Figure 2D) found a significant effect of Instrumental Extinction, but no significant Instrumental Extinction Group X Training Block interaction. The effect of Instrumental Extinction Group reflected that the slow extinction group exhibited higher conditioned suppression than the fast extinction group. Because there was a significant effect of Instrumental Extinction Group and no interaction, we analyzed the first trial to determine whether the difference between groups existed before any shocks were delivered. We found that conditioned suppression in the fast extinction group (-0.05 ± 0.06) was significantly lower than in the slow extinction group (0.39 ± 0.10) ($F(1, 10)=13.9$, $p<0.01$) suggesting that there were differences in reactivity to the tone before fear conditioning had occurred.

(although conditioned freezing did not exhibit this pattern). Results of all ANOVAs are in Table 3.

3.2.4 Conditioned fear testing—The groups that were divided based on their instrumental extinction behavior also exhibited differences in conditioned fear behaviors to an auditory stimulus during testing of conditioned fear. A researcher error prevented the video recording of conditioned behavior to score conditioned freezing. However, the fast extinction group exhibited lower conditioned fear, as seen in lower conditioned suppression of lever-pressing. All analyses were performed with a mixed-factor ANOVA with the between-subjects factor of Instrumental Extinction Group (Fast vs. Slow) and the within-subjects factor of Test Trial (the four test trials). Analysis of precue lever-pressing (data not shown) found no significant effects or interactions of Instrumental Extinction Group. An analysis of conditioned suppression of lever-pressing (Figure 2E) found a significant Instrumental Extinction Group X Training Block interaction. The interaction reflected that the slow extinction group exhibited higher conditioned suppression than the fast extinction group for the first 3 test trials, but this difference disappeared on the fourth trial. Results of all ANOVAs are in Table 3.

3.2.5 Correlations between instrumental extinction and fear measures—We also calculated Pearson's correlation coefficients between instrumental extinction scores and the three fear measures (precue freezing, cue freezing and conditioned suppression of lever-pressing) in training and conditioned suppression in testing. Because we found a significant decrease in lever-pressing that developed across the course of the fear conditioning training session (which suggests that it was a response to fear conditioning), we also calculated correlations between instrumental extinction and precue lever-pressing in fear training and testing. We found significant correlations of instrumental extinction with cue freezing during training ($r = -0.62$; Figure 2F) and precue lever-pressing during training ($r = 0.66$; Figure 2G) and a nearly significant negative correlation between instrumental extinction and conditioned suppression of lever-pressing during training ($r = -0.55$; $p = 0.07$; Figure 2H). These patterns show that rats with faster instrumental extinction exhibited lower fear to the tone cue and less of a depression of baseline lever-pressing in the fear conditioning session. There was no significant correlation of instrumental extinction with precue freezing in the training session ($r = -0.32$) or precue lever-pressing or conditioned suppression of lever-pressing (Figure 2I) in the fear test ($r = 0.43$ and -0.49 , respectively).

4 Discussion

We found that relatively low levels of voluntary alcohol consumption in adolescence and early adulthood did not alter conditioned fear during fear training or a cued fear test, but there were individual differences in alcohol consumption that were associated with the level of conditioned fear. Very low alcohol consumption in early adulthood (<1.5 g/kg/24-h) was associated with high conditioned fear during training and testing. We also found that rats that exhibited low conditioned fear also exhibited faster instrumental extinction. Below, we discuss all these effects (or lack of effects).

4.1 Methodological considerations in the lack of an effect of alcohol access on conditioned fear learning or expression

We found no evidence that low levels of voluntary alcohol access affects fear learning or expression, as we found no group differences between the Water and Alcohol groups in Experiment 1. The lack of an effect seems convincing for 2 reasons. First, the lack of an effect was not due merely to insufficient power to detect an effect, as we used the effect sizes from our ANOVAs to determine the number of subjects that would be required to detect differences between the alcohol and water groups, and found that the minimum number for any of the fear measures in any phase of Experiment 1 was 80 (40/group) for a main effect of Access Group for the data from cued freezing during training. We examined the estimated number of subjects required for our power analyses for the three direct measures of fear (precue freezing, cue freezing, and conditioned suppression of lever-pressing) in training and testing for a total of 6 comparisons. For each comparison, we determined whether a lower number of subjects would be required to achieve the desired power for the main effect of Access Group or the interaction (ex: fewer subjects would be required for the main effect [80] than the interaction [256] for training cue freezing, but fewer subjects would be required for the interaction [368] than the main effect [560] for testing conditioned suppression of lever-pressing). When we examined the minimum number of subjects estimated to achieve adequate power in the 6 comparisons (using just the lower value for each comparison), we found that the median number of subjects required would be 352 (or 176 per group). These results of the power analysis suggest that any effect of alcohol on fear behavior is negligible. Second, as can be seen in Table 4, in 5/6 cases, the level of fear responding in the Water group was lower than in the Low Alcohol consumption group and higher than in the High Alcohol consumption group. In the one case where average fear responding for the Water group was not between the average for the Low and High alcohol consumption group (cued freezing during training), the average for the Water group only exceeded the average for the Low alcohol consumption group by 0.8%. This pattern (average fear responding in the Water group higher than in the Low Alcohol group) was not seen in concurrently measured conditioned suppression of lever-pressing during training or in the cued freezing in the test session two days later, so it does not represent an overall pattern in the data. We are unaware of any prior findings that extraordinarily low alcohol consumption would have long-term effects that are the opposite of higher alcohol consumption. This suggests that alcohol access does not affect conditioned fear under the parameters used in the current experiment. This contrasts with the results of several experiments in which higher levels of alcohol exposure led to alterations in auditory fear conditioning ([9–12], but see [13–15]). Below, we discuss several possibilities for why our results are different from these previous experiments.

First, it is possible that our adolescent/early adult alcohol access regimen was sufficient to lead to alterations in auditory fear conditioning, but these effects faded over the interval between alcohol access and the test. Quinones and colleagues [12] found that an identical alcohol injection and testing procedure produced increased fear expression if there was a 3-day interval between the final alcohol injection and the test, but not if there was an 11-day interval between the final alcohol injection and the test. Thus, it is possible that alcohol access (even at our relatively low levels in adolescence/early adulthood) is sufficient to cause

alterations in fear conditioning, but the interval between alcohol access and the beginning of training and testing (10 days in Experiment 1) was too long for these effects to remain. Other studies in which alcohol exposure (via intragastric injections) did not alter conditioned fear to tones also had intervals of ~ 3 weeks between the end of alcohol exposure and fear conditioning [13–15], which could also support a role of the alcohol-conditioning interval on alcohol's effects on conditioned fear. However, a comparison between our result and those of Bergstrom and colleagues [9] does not support the time interval as the only factor responsible for our lack of an effect of alcohol access on conditioned fear. In that experiment, rats were trained and tested 30 days after the end of alcohol access and the rats exhibited lower conditioned fear. This suggests that alcohol access *can* lead to alterations in conditioned fear that last at least 30 days, and our day 2 conditioned fear test was 18 days after the final alcohol access period. Thus, the time interval alone seems like an unlikely explanation for our results.

A second possibility is that the rats in our experiment did not consume enough alcohol to cause effects on fear conditioning. The previous experiments that have shown that preconditioning alcohol exposure alters conditioned fear have achieved estimated BECs of ~80 mg/dl [10, 11], ~188 mg/dl [9] or 200 mg/dl [12]. It is difficult for us to currently estimate BECs in our high drinking group, but in previous work from our laboratory, food-restricted adult rats given CIA consumed an average of 10.5–11 g/kg/24-h and reached blood alcohol levels of 85–90 mg/dl [34]. As the high drinking rats in Experiment 1 of the current report only consumed an average of 8.1 and 6.9 g/kg/24-h in weeks 5 and 6 of alcohol access, the BECs of our high drinking group should be well below this 85–90 mg/dl. Likewise, we can estimate the BECs for the low drinking rats based on some pilot data from our lab. We ran a pilot experiment (n=7) in which rats were given CIA from PND 26–66 and blood alcohol analyses were performed from blood taken 30 min after the start of alcohol access on the last alcohol access period in weeks 2, 4 and 6. We found that the average consumption in weeks 2, 4 and 6 was 0.7 ± 0.2 , 1.2 ± 0.8 and 1.8 ± 0.7 g/kg/24-h, respectively, and the BECs were 6.2 ± 1.3 , 11.4 ± 0.7 and 14.3 ± 1.0 , respectively. Thus, it is possible that our lower BECs achieved in adolescence and early adulthood did not reach the levels required to alter conditioned fear in either the high drinking and low drinking rats. Although this explanation works well to explain why our experimental manipulations did not alter conditioned fear, this explanation cannot explain all of the findings in the literature, as intragastric injections that lead to peak BECs of 175–225 mg/dl [35] do not lead to long-term effects on fear conditioning to tones [13–15]. As such, there may be some interaction between BECs, the alcohol-conditioning interval, and/or the particular fear conditioning procedure that determines whether alcohol exposure will alter conditioned fear.

Our results also differed from a pattern in which voluntary drinking led to impaired conditioned fear discrimination, using the same strain of rats, methods of alcohol access, and an overlapping age of exposure (PND 25–60 in the other study, PND 26–66 in our study) [16]. Notably, this study reported their consumption in terms of g/kg of a 20% ethanol solution, so their cut-off of 10 g/kg/24-h of the solution (~1.6 g/kg/24-h of pure ethanol) was close to our cutoff of 1.5 g/kg/24-h. They found that rats with alcohol consumption above the ~1.6 g/kg/24-h cut-off were impaired in a conditioned fear discrimination task, while rats with consumption below this cut-off were unimpaired. This pattern differs from our pattern,

in which the alcohol access group as a whole did not differ from the water group, but consumption levels within the alcohol group were associated with the level of conditioned fear. One possibility is that the differing tasks are responsible for the different pattern of results. DiLeo and colleagues [16] used a discrimination task in which rats had to differentiate between 3 cues that predicted different probabilities of shock (0, 25, 100%) and trained for 16 days. We used a simpler task in which the rats were only exposed to a single discrete cue that predicted shock 100% of trials and were only fear conditioned trained for a single day. As such, either the difference in training length or the relative complexity of the task may be responsible for our differing results. Notably, an examination of the first 2 days of DiLeo and colleagues' [16] data (the first 12 trials in which cues were paired with shock) found no difference between alcohol-access and water-only rats, although randomized order of CS-US and CS-noUS trials made relationships between individual differences in alcohol consumption and fear acquisition difficult to assess (personal communication, M. McDannald). Future research will be needed to determine the experimental parameters that allow voluntary alcohol access to alter fear learning.

4.2 Methodological considerations for the instrumental extinction-conditioned fear association

One potential alternate explanation for the individual differences patterns in fear conditioning and instrumental extinction was that the low alcohol consumption group had lower motivation for appetitive reinforcers overall, including the food reinforcers used in our behavioral tasks. If so, then this lower motivation could have led to higher fear expression in the fear test (as motivation to lever-press for reinforcers was less able to counteract fear) and led to a floor effect in instrumental responding rate in the instrumental extinction tests that was unable to be decreased by extinction. Notably, several rat strains that are selectively bred for higher alcohol consumption (P and/or AA) exhibit higher consumption of certain concentrations of saccharin and sucrose solutions than rat strains bred for low alcohol consumption (NP and/or ANA) [36–38]. Likewise, outbred albino rats selected for consuming a greater amount of alcohol in their first week of access were found to have higher voluntary consumption of a saccharin solution compared with rats that drank less alcohol in their first week of access [39]. Similarly, outbred Wistar rats selected for high saccharin intake consumed greater amounts of higher concentration alcohol solutions than rats selected for low saccharin consumption [40]. Thus, it is possible that differing motivation for food reinforcers are driving our effects.

However, we believe that this explanation for our data is unlikely for several reasons. First, while the lever-press rate for the fast extinction group trended higher during cued leverpress training and was significantly higher during the beginning of extinction training and in the instrumental extinction phase of Experiment 2, there was no significant difference between the fast and slow extinction groups in precue lever-pressing in the first block of fear conditioning training in Experiment 2 (block 1 in Figure 2C). Likewise, in Experiment 1, there was no significant difference in precue lever-pressing between the high and low alcohol groups either across the 5-blocks of cued fear training or in precue lever-pressing before the first training trial (before any shocks were delivered: in lever-presses/min: high consumption group = 13.6 ± 1.4 , low consumption group = 11.1 ± 3.2). This suggests that the

difference in response rate between the two groups was not seen in behavioral testing beyond the cued lever training and extinction phase of Experiment 2. Second, the suppression ratio formula for suppression of lever-pressing takes into account the precue rate of responding and is designed to correct for differences in individual response rate [28]. This is supported by previous findings from an experiment that examined the effects of differing food restriction conditions on fear conditioning using the same parameters as the current experiment for their daily conditioning sessions (90-min session containing 10 30-sec tones paired with shocks) [27]. In that experiment, differences in food restriction conditions led to precue lever-press rates that were more than doubled in one group compared to another during fear conditioning, but led to no significant difference between the two groups in suppression ratios across 10 days of fear conditioning acquisition. Third, although lever-pressing was higher in the fast extinction group compared to the slow extinction group across the first two trials of instrumental extinction in Experiment 2, the slow extinction group was not experiencing a floor effect that prevented extinction. The slow extinction group did not decrease their responding between the first and sixth trial (which was the comparison used to assess the speed of extinction, as related to our previous findings [18]), but did decrease their responding from the 6th trial to the 20th trial (with no interaction to indicate a different magnitude of decrease compared to that of the fast extinction group). Thus, it seems unlikely that increased motivation for food is responsible for our results.

4.3 Individual differences in conditioned fear are associated with voluntary alcohol consumption levels

Despite the lack of an effect of alcohol consumption on fear learning or expression, we found that individual differences in conditioned fear were associated with voluntary alcohol consumption. The associations we found with the last two weeks of drinking are in accord with research that shows that human alcohol drinking is not stable over the course of development [41–43], with later drinking as a better representation of drinking due to trait-like characteristics. In particular, Nealis and colleagues [43] found that trait-like characteristics accounted for a greater amount of variance in drinking in older samples (people followed from 9th–11th grade vs. people followed from 7th–9th grade). If rodents exhibit a similar pattern, then drinking in the last 2 weeks of alcohol consumption (which corresponds to early adulthood) may be a better measure of trait-like drinking levels that could be correlated with other trait-like behavioral abilities than drinking at an earlier age. Notably, another set of experiments from our laboratory has found similar associations between alcohol drinking in the last 2 week of our access period and instrumental extinction and errors in a go/no-go reversal learning task, and these associations are not seen for drinking across the entire 6-week alcohol access period [18]. This suggests that consumption in the early adulthood phase of our alcohol access period may be a good indicator of traits that can be correlated with other behavioral measures.

Our data fit well with the previous literature on behavioral differences between rodent strains, as differences in conditioned fear across strains are well-established [44–47]. These differences also extend to inbred strains that are selected for alcohol consumption. Although alcohol-preferring (P) and non-preferring (NP) rats do not differ in baseline startle, P rats exhibit greater contextual fear-induced increases in a fear-potentiated startle task and also

exhibit light cue-induced fear-potentiated startle with more limited training than is required in NP rats [48]. Although the direction of this relationship (higher drinking rats with higher conditioned fear) is the opposite of what would be expected based on our findings, these data do support a potential relationship between alcohol consumption levels and conditioned fear.

Notably, the developmental period of alcohol access may play some role in our findings, as the age of initiation to alcohol exposure can alter alcohol consumption and seeking patterns. For example, even a difference between initiation on PND30 vs. PND45 leads to higher drinking with escalation in consumption (PND30) or lower drinking without escalation (PND45) in rats [49] (see also [50] for a similar effect in mice). These increases are seen whether consumption is measured based on age or weeks since initiation of alcohol access. Likewise, instrumental responding for an alcohol solution appeared to become insensitive to contingency degradation in rats pressing for alcohol during adulthood (PND72–96), while remaining sensitive to contingency degradation in rats pressing for alcohol in adolescence (PND30–54) [51]. The differential sensitivity to contingency degradation suggests that there may be different neural substrates of alcohol seeking in adolescent vs. adult rats. As such, giving fear conditioning during adulthood and then associating fear behavior with alcohol consumption exclusively during adulthood might miss neurobiological/genetic traits that are revealed by adolescent/early adult drinking.

4.4 Individual differences in conditioned fear are associated with instrumental extinction-suggesting a generalized phenotype

Because we had previously shown that individual differences in alcohol consumption are associated with instrumental extinction [18], we investigated whether the relationship would exist between conditioned fear and instrumental extinction. We believed that examining whether conditioned fear would be associated with instrumental extinction (in rats never exposed to alcohol) would be the most effective method to determine whether the alcohol consumption-fear conditioning association represented a real effect for several reasons.

First, any design in which alcohol consumption and fear conditioning were both assessed had the potential to have whichever assessment occurred first lead to alterations in the subsequent test for the other behavior. As described above, there are several reports of prior alcohol exposure altering later fear conditioning [9–12], so it would be impossible to completely exclude the possibility that alcohol consumption altered later fear conditioning. Likewise, there are many reports of prior stress affecting the level of voluntary alcohol consumption (as reviewed in [52]). Although these prior studies generally found effects after chronic stress, it would be impossible to completely exclude the possibility that prior shock exposure affected subsequent alcohol consumption if fear conditioning preceded alcohol consumption. It seemed likely that performing an instrumental extinction procedure as a first behavioral assessment had a lower probability of leading to interference with subsequent behavioral assessments than having either alcohol consumption or fear conditioning as an initial assessment.

Second, neurobiological changes that occur during development could complicate attempts to follow up Experiment 1 by conducting fear conditioning before alcohol consumption.

Comparing fear conditioning conducted in adulthood with fear conditioning conducted before PND26 would be complicated by the brain development/maturation that occurs after PND30 in basolateral amygdala [53] and ventral prefrontal cortex [54], which are both important components of the conditioned fear circuitry (see [55–57] for reviews). Likewise, conducting conditioned fear training in adulthood and subsequently assessing alcohol consumption would lead to all alcohol consumption occurring after adolescence. Several experiments have found that adolescent alcohol exposure leads to different patterns of voluntary drinking/seeking in rodents than those seen if alcohol exposure begins at a later age [49–51]. As such, associating fear behavior with alcohol consumption assessed exclusively during adulthood might miss neurobiological/genetic traits that are revealed by adolescent/early adult drinking.

Third, the many phenomena that have behavioral levels associated with the level of alcohol consumption in our assessments (instrumental extinction, go/no-go reversal learning, conditioned fear) suggests that there might be a broad constellation of behavioral traits that are associated with one another, even in rats that were not exposed to alcohol. If so, our individual differences findings would have broader significance for the overall study of individual differences in behavioral traits (beyond alcohol consumption exclusively).

We found that lower conditioned fear and faster instrumental extinction (both associated with higher drinking) were associated with each other. This finding is in accord with previous findings of individual differences in fear within outbred strains, with some of these differences correlating with other behavioral traits. For example, Morrow and colleagues [58] tested Sprague-Dawley rats in a fear incubation procedure in which the rats underwent extended 10-day fear conditioning training designed to induce low fear initially that grows over time (termed “fear incubation”) [19, 20, 27, 59]. In this experiment, sign-trackers and intermediate responders showed the fear incubation pattern with low fear on day 3 that increased on day 33, while goaltrackers exhibited high fear 3 days after fear training that did not grow [58]. Cued fear discrimination between a CS+ and CS– was also found to correlate with anxiety in the elevated plus maze in Lewis rats, with worse discrimination in rats that spent more time in the closed arms of the maze [60]. Lower contextual fear and discrete auditory cued fear in tests 1–2 days after training was also associated with better place discrimination in a place recognition task [61]. Likewise, there is a growing literature on correlations between fibroblast growth factor 2 and conditioned fear, with higher fibroblast growth factor 2 associated with lower fear. Higher hippocampus (but not amygdala and prefrontal cortex) fibroblast growth factor 2 (measured 2 hours or ~3 months after fear testing) was associated with lower contextual fear and discrete auditory cued fear recall in Sprague-Dawley rats [61, 62]. Likewise, serum fibroblast growth factor 2 was negatively correlated with contextual fear recall in Sprague-Dawley rats, and salivary fibroblast growth factor 2 was negatively correlated with fear to a CS– during discriminative fear conditioning and fear to the CS+ and CS– during the early phase of a recall test in humans [63]. Thus, our findings add to a growing literature on individual differences in conditioned fear that correlate with other behavioral or neurobiological traits. Future research will be needed to determine the neurobiological correlates of our individual differences, and whether these would resemble the neurobiological correlates present in the previously described patterns of individual differences.

Our findings that high conditioned fear in our task (associated with low alcohol consumption in Experiment 1) was also associated with faster instrumental extinction (also associated with low alcohol consumption [18]), suggests that there may be 2 separate phenotypes of Long-Evans male rats, with each phenotype encompassing multiple behavioral traits. One potential phenotype may exhibit high alcohol consumption, low conditioned fear, fast instrumental extinction and few maintenance/discontinuation and commission errors in go/no-go reversal learning (the HALF-FIELDER phenotype), and the other potential phenotype may exhibit low alcohol consumption, high conditioned fear, slow instrumental extinction and many maintenance/ discontinuation and commission errors in go/no-go reversal learning (the non-HALF-FIELDER phenotype). We interpreted our earlier findings [18] as suggesting a relationship between alcohol consumption and behavioral measures that assess the ability to inhibit previously reinforced responses. Commission errors in our go/no-go reversal learning task and within-session extinction both measure the ability to inhibit responses that do not lead to reinforcement, without requiring learning of a new response or representing response-outcome information. It is possible that this relationship with inhibiting responses also generalizes to the inhibition of conditioned fear responses, suggesting a general relationship between alcohol consumption and response inhibition across both appetitive and aversive outcomes. Future research should investigate the boundary conditions and neurobiological substrates of these correlations.

4.5 Conclusion

In conclusion, we found that low levels of voluntary alcohol consumption did not alter conditioned fear in a subsequent test several weeks later. However, the level of alcohol consumption was associated with the level of conditioned fear, suggesting that some of the relationship between alcohol consumption and conditioned fear may be caused by individual differences, rather than a neurotoxic effect of alcohol on the neural substrates of fear. We also found that the level of conditioned fear was associated with the rate of instrumental extinction, which is a behavioral trait we have previously shown to be associated with the level of alcohol consumption. This suggests that there may be separate phenotypes of rats that are associated with multiple behavioral traits that can be differentiated by their voluntary alcohol consumption, the HALF-FIELDER and non-HALF-FIELDER phenotypes. Although the current data allow for the possibility that the developmental period of initiation of alcohol access could play a role in the associations between alcohol consumption and other behavioral traits, our results in Experiment 2 show that alcohol access is not necessary for these associations between other behavioral traits to be revealed. Future research will examine other behavioral traits and neurobiological substrates associated with these putative phenotypes.

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Highlights

Alcohol consumption in adolescence/early adulthood did not affect conditioned fear

Alcohol consumption in early adulthood was associated with conditioned fear levels

The rate of operant extinction was associated with conditioned fear levels

With previous findings, phenotypes affecting multiple behaviors are revealed

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Figure 1.

A. Weekly alcohol consumption in g/kg/24-h (mean $\pm$ SEM) across the 6 weeks of access in Experiment 1. White circles indicate high alcohol drinkers while black circles indicate low drinkers. **B.** Conditioned freezing in the 30-sec precue period across 10 training trials subdivided into two-trial blocks. **C.** Conditioned freezing in the 30-sec cue period across 10 training trials subdivided into two-trial blocks. **D.** Conditioned freezing in the 30-sec cue period across 4 test trials. **E.** Suppression ratios for the 30-sec cue period across 10 training trials subdivided into two-trial blocks. High values indicate high fear and low values indicate low fear. **F.** Suppression ratios for the 30-sec cue period across 4 test trials. For **1B–F**,

alcohol (gray) and water (white) groups are shown on the left and the high (white) and low (black) subgroups of the alcohol group are shown on the right. * = $p < 0.05$ compared to the corresponding measure in the high vs. low alcohol drinking group. **G.** Correlation between alcohol consumption in the last 2 weeks of access and percent time freezing during the tone cue during conditioned fear training (left) and conditioned fear testing (right). **H.** Correlation between alcohol consumption in the last 2 weeks of access and conditioned suppression of lever-pressing during the tone cue during conditioned fear training (left) and conditioned fear testing (right).

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Figure 2.

A. Lever-presses/trial across first 6 test trials of the instrumental extinction tests in Experiment 2. **B.** Conditioned freezing in the 30-sec cue period across 10 training trials subdivided into two-trial blocks. **C.** Lever-presses during the precue period across 10 conditioned fear training trials subdivided into two-trial blocks. **D.** Suppression ratio during the cue across 10 conditioned fear training trials subdivided into two-trial blocks. **E.** Suppression ratio during the cue on the 4 trials of the conditioned fear test. For **2A–E** the fast extinction group is depicted by black circles and the slow extinction group is depicted by white circles. * = $p < 0.05$ compared to the corresponding measure in the fast vs. slow alcohol drinking group. **F.** Correlation between instrumental extinction scores and percent time freezing during the tone cue during conditioned fear training. **G.** Correlation between instrumental extinction scores and precue lever-pressing during conditioned fear training. **H.** Correlation between instrumental extinction scores and conditioned suppression of lever-pressing during conditioned fear training. **I.** Correlation between instrumental extinction scores and conditioned suppression of lever-pressing during the conditioned fear test. For

2F-Ithe extinction score represents the difference in lever-press rates per trial on trials 1 and 6 of instrumental extinction. For **2F-I** values in bold with an asterisk represent significant effects.

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Table 1

Results of ANOVAs for main effects and interactions in Experiment 1.

	Effect of Access Group: Alcohol vs. Water			Effect of Alcohol Consumption Group: High vs Low		
	Main effect: Group	Main effect: Training Block	Interaction:	Main effect: Group	Main effect: Training Block	Interaction:
Training-Precue freezing	F(1, 34) = 0.8	F(4, 136) = 2.8 *	F(4, 136) = 0.9	F(1, 23) = 7.0 *	F(4, 92) = 3.8 *	F(4, 92) = 5.9 *
Training-Cue freezing	F(1, 34) = 3.5	F(4, 136) = 6.6 *	F(4, 136) = 1.6	F(1, 23) = 6.1 *	F(4, 92) = 3.3 *	F(4, 92) = 2.1
Training-Precue lever-press	F(1, 34) = 1.7	F(4, 136) = 4.5 *	F(4, 136) = 0.5	F(1, 23) = 0.4	F(4, 92) = 4.5 *	F(4, 92) = 0.5
Training-Suppression ratio	F(1, 34) = 0.5	F(4, 136) = 4.9 *	F(4, 136) = 1.1	F(1, 23) = 4.3 *	F(4, 92) = 3.2 *	F(4, 92) = 2.8 *
	Main effect: Group	Main effect: Test Trial	Interaction:	Main effect: Group	Main effect: Test Trial	Interaction:
Test-Precue freezing	F(1, 34) = 0.0	F(3, 102) = 1.7	F(3, 102) = 0.1	F(1, 23) = 2.6	F(3, 69) = 3.0 *	F(3, 69) = 4.0 *
Test-Cue freezing	F(1, 34) = 0.0	F(3, 102) = 2.1	F(3, 102) = 0.1	F(1, 23) = 3.3	F(3, 69) = 3.0 *	F(3, 69) = 3.2 *
Test-Precue lever-press	F(1, 34) = 1.0	F(3, 102) = 2.3	F(3, 102) = 0.3	F(1, 23) = 0.3	F(3, 69) = 1.3	F(3, 69) = 0.9
Test-Suppression ratio	F(1, 34) = 0.0	F(3, 102) = 4.0 *	F(3, 102) = 1.8	F(1, 23) = 4.7 *	F(3, 69) = 3.3 *	F(3, 69) = 0.2

Values in bold with an asterisk represent significant effects.

Table 2

Results of power analyses on the Access Group main effect or Access Group interaction in Experiment 1

Comparison	Main effect- Power (partial η^2)	Main effect- N required for significance	Interaction- Power (partial η^2)	Interaction- N required for significance
Fear training-Precue freezing	0.0232	334	0.0256	460
Fear training-Cue freezing	0.0928	80	0.0456	256
Fear training-Conditioned suppression	0.0139	560	0.0319	368
Fear testing-Precue freezing	0.0013	6032	0.0022	4950
Fear testing-Cue freezing	0.0004	26,158	0.0041	2654
Fear testing-Conditioned suppression	0.0004	26,158	214	214

The “Main effect” columns represents the effect size and number of subjects to achieve a power of 0.8 based on the effect size for the main effect of Access Group. The “Interaction” columns represents the effect size and number of subjects to achieve a power of 0.8 based on the effect size for the Access Group X Training Block interaction (for fear training) or Access Group X Trial interaction (for fear testing).

Table 3

Results of ANOVAs for main effects and interactions in Experiment 2.

	Main effect: Group	Main effect: Test Trial	Interaction:
Instrumental Extinction	$F(1, 10) = 4.0$	$F(5, 50) = 15.0^*$	$F(5, 50) = 5.4^*$
	Main effect: Group	Main effect: Training Block	Interaction:
Training-Precue freezing	$F(1, 10) = 2.5$	$F(4, 40) = 1.2$	$F(4, 40) = 2.2$
Training-Cue freezing	$F(1, 10) = 9.6^*$	$F(4, 40) = 7.9^*$	$F(4, 40) = 2.2$
Training-Precue lever-press	$F(1, 10) = 13.5^*$	$F(4, 40) = 4.8^*$	$F(4, 40) = 3.9^*$
Training-Suppression ratio	$F(1, 10) = 27.8^*$	$F(4, 40) = 6.1^*$	$F(4, 40) = 1.5$
	Main effect: Group	Main effect: Test Trial	Interaction:
Test-Precue lever-press	$F(1, 10) = 27.8^*$	$F(3, 30) = 6.1^*$	$F(3, 30) = 1.5$
Test-Suppression ratio	$F(1, 10) = 23.5^*$	$F(3, 30) = 14.0^*$	$F(3, 30) = 4.4^*$

Values in bold with an asterisk represent significant effects.

Table 4

Average fear measures for training and testing in Experiment 1.

	Training Suppression Ratio	Training Precue Freezing	Training Cue Freezing	Test Suppression Ratio	Test Precue Freezing	Test Cue Freezing
Low Drinking	0.39±0.13	12.2±5.1	17.2±5.5	0.49±0.15	6.2±0.6	26.2±8.4
Water	0.29±0.08	8.6±2.7	18.0±4.8	0.29±0.12	3.5±1.7	15.6±5.0
High Drinking	0.16±0.05	3.4±0.8	6.6±1.7	0.20±0.06	1.8±0.6	12.3±3.5

All freezing values represent % of total behavior.