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Effects of Alcohol on the Acquisition and Expression of Fear Potentiated Startle in Mouse Lines
Selectively Bred for High and Low Alcohol Preference

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Running Head: Effects of Alcohol on Fear-Potentiated Startle

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Abstract

Rationale: Anxiety disorders and alcohol-use disorders frequently co-occur in humans perhaps because alcohol relieves anxiety. Studies in humans and rats indicate that alcohol may have greater anxiolytic effects in organisms with increased genetic propensity for high alcohol consumption.

Objectives and Methods: The purpose of this study was to investigate the effects of moderate doses of alcohol (0.5, 1.0, 1.5 g/kg) on the acquisition and expression of anxiety-related behavior using a fear-potentiated startle (FPS) procedure. Experiments were conducted in two replicate pairs of mouse lines selectively bred for high- (HAP1 and HAP2) and low- (LAP1 and LAP2) alcohol preference; these lines have previously shown a genetic correlation between alcohol preference and FPS (HAP>LAP; Barrenha and Chester 2007). In a control experiment, the effect of diazepam (4.0 mg/kg) on the expression of FPS was tested in HAP2 and LAP2 mice.

Results: The 1.5 g/kg alcohol dose moderately decreased the expression of FPS in both HAP lines but not LAP lines. Alcohol had no effect on the acquisition of FPS in any line. Diazepam reduced FPS to a similar extent in both HAP2 and LAP2 mice.

Conclusions: HAP mice may be more sensitive to the anxiolytic effects of alcohol than LAP mice when alcohol is given prior to the expression of FPS. These data collected in two pairs of HAP/LAP mouse lines suggest that the anxiolytic response to alcohol in HAP mice may be genetically correlated with their propensity toward high alcohol preference and robust FPS.

Introduction

Alcohol-use disorders (AUDs) co-occur with anxiety disorders in over 35% of the United States population (Kessler et al. 1996). One primary hypothesis put forward to explain the link between AUDs and anxiety disorders is the “tension-reduction” or “self-medication” hypothesis, which states that AUDs arise because individuals consume alcohol to alleviate anxiety symptoms (Bowen et al. 1984; Cappell and Herman 1972; Conger 1956; Sher 1987; Sinha et al. 1998; Weiss and Rosenberg 1985). A cyclic pattern of excessive alcohol consumption in response to anxiety symptoms may be exacerbated to the point that individuals develop full-blown symptoms of alcohol dependence. Another hypothesis is that there are common inherited genetic factors that increase the risk for developing co-occurring anxiety disorders and AUDs (Maier et al. 1993; Merikangas et al. 1994; 1998; Munjack and Moss 1981). Evidence suggests that these hypotheses are not necessarily mutually exclusive and each hypothesis may appropriately explain the co-morbid relationship between anxiety disorders and AUDs in certain subsets of afflicted people.

The question of whether alcohol actually relieves anxiety symptoms remains open. In humans, some studies indicate that anxiety symptoms are reduced (Abrams et al. 2001; Levenson et al. 1980; Moberg and Curtin 2009; Nesic and Duka 2006; Sayette et al. 1990; Terra et al. 2004; Thomas et al. 2003), increased, (McDougle et al. 1995; Terra et al. 2004) or unaltered (Curtin et al. 1998; Himle et al. 1999; Naftolowitz et al. 1994; Zimmermann et al. 2004) in the presence of alcohol. Discrepant results in the literature may be due to several factors including the nature of the anxiety symptoms/disorder and variations in methods for administering alcohol or for assessing anxiety symptoms. In addition, whether or not alcohol has anxiolytic effects is likely influenced by factors such as personal history of alcohol use, trait or state anxiety levels, and family history of alcohol use and/or anxiety disorders. Indeed, several investigators have found differential sensitivity to alcohol’s anxiolytic effects in special

populations at higher risk for AUDs and/or anxiety disorders. For example, alcohol has been shown to reduce anxiety symptoms in individuals with a positive but not negative family history of alcoholism (Sher and Levenson 1982; Sinha et al. 1998), in individuals with a positive but not negative family history of anxiety disorders (Sinha et al. 1998), and in “high anxiety sensitive” but not in “low anxiety sensitive” individuals (Zack et al. 2007). Further, although not direct indices of anxiety symptoms, alcohol reduces stress-related responses, such as muscle tension (Schuckit et al. 1981), heart rate (Finn and Pihl 1987; Stewart et al. 1992), and cortisol (Croissant and Olbrich 2004) and prolactin (Zimmermann et al. 2009) release, in people with a family history of alcoholism compared to controls. Some studies indicate these effects may be sex-dependent; that is, women with a family history of alcoholism or anxiety disorders have shown greater “stress-response dampening” effects of alcohol than men (Levenson et al. 1987; Sinha et al. 1998).

In rodents, alcohol has been shown to reduce anxiety-related behavior in unconditioned anxiety models, such as the elevated plus maze (Abreu-Villaca et al. 2008; Boehm et al. 2002; Correia et al. 2009; Durcan and Lister 1988; LaBuda and Fuchs 2002; Spanagel et al. 1995), the plus maze discriminative learning avoidance task (PMDAT; Gulick and Gould 2009a; 2009b; Kameda et al. 2007), the social interaction test (File et al. 1976), the holeboard test (Durcan and Lister 1988; File 1976), the light/dark box test (Costall et al. 1988), and defensive behavior tasks (Blanchard et al. 1993). There have been a few reports of alcohol effects on anxiety-related behavior in rats that differ in genetic propensity toward alcohol consumption. Stewart and colleagues (1993) found greater anxiety-related behaviors in rats selectively bred for alcohol preference (P) than in rats selectively bred for nonpreference (NP) and alcohol treatment reduced anxiety in P but not NP rats. P (Pandey et al. 2005) and Sardinian alcohol preferring (sP) (Colombo et al. 1995) have shown show greater anxiety in the elevated plus maze than the alcohol-nonpreferring counterparts (NP and sNP) and voluntary alcohol consumption in P and sP rats reduced their anxiety-related behavior. These findings in rats support studies in humans

suggesting that alcohol may have greater anxiolytic effects in organisms with increased genetic propensity for high alcohol consumption.

Post-traumatic stress disorder (PTSD) is one anxiety disorder that frequently co-occurs with AUDs (Brady et al. 2000; Brown et al. 1998; Kessler et al. 1996; McFarlane 1998), especially among U.S. active military personnel and veterans (Davidson et al. 1990; Hoge et al. 2004; Kang and Hyams 2005; Milliken et al. 2007; Stewart 1996). Fear conditioning models of anxiety, such as fear-potentiated startle (FPS), are commonly used in rodents to study processes that may contribute to fear-related disorders such as PTSD (Kim and Jung 2006). Similar to that seen with models of unconditioned anxiety-related behavior, selectively bred P rats show greater FPS than NP rats (McKinzie et al. 2000). We have also reported that mouse lines selectively bred for high alcohol preference (HAP) show greater FPS than mouse lines selectively bred for low alcohol preference (LAP) (Barrenha and Chester 2007). These data suggest that genes contributing to high or low alcohol preference may also influence propensity to show anxiety- and fear-related behaviors. In this regard, the HAP/LAP mouse lines may be an animal model that represents increased genetic risk to develop AUDs comorbid with PTSD in humans.

To our knowledge, there are only two published studies in which alcohol effects on FPS have been examined in rats; one reported that alcohol decreased FPS (Miller and Barry 1960) while the other reported no effect (Hijzen et al. 1995). The present study examined the effects of various doses of alcohol on the acquisition and expression of FPS in replicate pairs of the HAP/LAP mouse lines. Based on results from human and rat studies previously described, it was hypothesized that HAP lines would be more sensitive than LAP lines to alcohol's anxiolytic effects during the acquisition and expression of FPS. We tested male and female mice from both replicate pairs of mouse lines to adequately address the hypothesis that sensitivity to alcohol's anxiolytic effect on FPS is a genetically correlated response to selection for alcohol

preference (Crabbe et al. 1990) and to determine whether the correlated response depends on sex.

Materials and Methods

Subjects

Subjects were alcohol-naïve adult male and female HAP and LAP mice from replicate lines 1 and 2. The selectively bred HAP1/LAP1 and HAP2/LAP2 mouse lines were derived from a progenitor population of outbred HS/lbg mice (Institute of Behavioral Genetics, Boulder, CO) at the Indiana Alcohol Research Center in Indianapolis, IN (Grahame et al. 1999). Subjects in the present experiments were derived from 25 different HAP1 families, 33 different LAP1 families, 30 different HAP2 families, and 31 different LAP2 families. Replicate 1 HAP mice were from the 27th, 34th, 37th, and 39th generation of selection. Replicate 1 LAP mice were from multiple generations of offspring from generation 27 breeders maintained with relaxed selection. Replicate 2 HAP and LAP mice were from the 27th, 29th, 31st, 34th (HAP2 only) and 35th (LAP2 only) and 37th generation of selection. Multiple replications of the experiments were conducted over a period of 22 months (experiment 1) and 26 months (experiment 2). Experiment 3 was conducted in one replication. Subject representation in each replication was balanced across replicate, line, sex, and litter or origin to the best extent possible. At the start of experimental procedures, mice were between 53 and 100 days old in experiment 1, 67-124 days old in experiment 2, and 72-94 days old in experiment 3. Mice were housed in polycarbonate cages (29.2 x 19.0 x 12.7 cm) with aspen wood shavings in groups of 2-4 per cage. Ambient room temperature was maintained at 21 ± 2 °C. Mice had free access to food (Rodent Lab Diet 5001, Purina Mills Inc., St Louis, MO) and water in the home cage at all times, except when testing procedures took place. Experimental procedures were conducted during the light phase of a 12:12 light:dark cycle (lights off at 19:00).

All experimental procedures were approved by the Purdue Animal Care and Use Committee and were conducted in accordance with the principles of laboratory animal care.

Drugs

Alcohol was diluted from a 95% (v/v) solution to a concentration of 20% (v/v) with physiological saline (0.9%) and was administered as intraperitoneal (IP) injections at doses of 0.5, 1.0, and 1.5 grams per kilogram of body weight (g/kg) and in an injection volume of 3.1, 6.5, and 9.4 milliliters (ml)/kg, respectively. Saline-treated groups received saline in a volume of 6.5 ml/kg. The chosen alcohol doses have been shown to dose-dependently reduce contextual fear-conditioned responses in C57BL/6 mice (Gould 2003) and are below the dose of alcohol shown to produce locomotor stimulation in HAP mice (Grahame et al. 2000). Diazepam (Sigma-Aldrich, St Louis, MO, USA) was dissolved in a 10% 2-Hydroxypropyl- β -cyclodextrin solution (Sigma-Aldrich) and administered IP at a dose of 4.0 mg/kg in an injection volume of 10 ml/kg (Smith et al. 2011). Vehicle-treated groups received 10% 2-Hydroxypropyl- β -cyclodextrin solution in a volume of 10 ml/kg. The 4.0 mg/kg diazepam dose has been shown to significantly reduce FPS in C57BL/6J mice (Smith et al. 2011).

Fear-Potentiated Startle Apparatus

FPS was assessed using two dark, sound-attenuated Coulbourn Instruments (Allentown, PA, USA) Animal Acoustic Startle System chambers, as previously described (Barrenha and Chester 2007). Startle stimuli consisted of 100 dB, 40 msec white noise bursts (frequency range: 20 Hz-20 kHz). Subjects' startle responses were measured as the amount of force in grams exerted against a weight-sensitive platform during the 200 msec after the onset of each acoustic stimulus. The force measurement does not include the subject's bodyweight. A ventilating fan provided continuous 70-71 dB background noise.

Fear-Potentiated Startle Procedure

FPS procedures consisted of one conditioning and one test session separated by 24 hrs. During each conditioning trial, fear-conditioned groups received 40 trials of a 30-sec, 7 W light stimulus paired with a 0.5-sec, 0.8 mA footshock [2-min intertrial interval (ITI)]. The footshock occurred during the last 0.5 sec of the light stimulus presentation. Control groups (experiment 1 only) received the same number of light and shock presentations as the fear-conditioned group but these stimuli were explicitly unpaired during each of the 40 2-minute intervals (interstimulus range 13-118 sec). All mice in the control groups received the same sequence of randomized light and shock presentations. The FPS test session occurred 24 hrs after the conditioning session and consisted of a 5-min habituation period followed by 36 total trials (2-min ITI) presented on a random schedule (range: 12-108 sec) to reduce habituation to any single trial type. Twelve of the trials were blank (no stimuli), 12 were noise alone (100 dB, 40 msec), and 12 were light (7 W, 30 sec) + noise (100dB, 40 msec). On light + noise trials, the noise stimulus was presented immediately after the light stimulus ended. FPS parameters were chosen based on our previous work in HAP/LAP replicate lines (Barrenha and Chester, 2007) but the number of conditioning trials was increased from 20 to 40 in this study to avoid a potential floor effect in alcohol-treated LAP groups.

Study Procedures

Experiment 1: Effects of alcohol on FPS expression

Two-hundred and thirteen HAP1 (120 males, 93 females), 234 LAP1 (115 males, 119 females), 272 HAP2 (118 males, 154 females), and 241 LAP2 (101 males, 140 females) were randomly assigned to either a fear-conditioned or control group and further divided into one of four treatment groups: saline (0 g/kg), 0.5, 1.0, or 1.5 g/kg alcohol. The main purpose of including control groups in this experiment was to examine whether alcohol might have

nonspecific effects on behavior which could compromise the interpretation of any effect of alcohol on the expression of FPS.

The conditioning session for experiment 1 began with a 5-min habituation period followed by 10 startle trials of 100 dB [40 msec; 20 sec intertrial interval (ITI)] noise bursts to acclimate mice and reduce their initial startle reactivity. Two min later the first of the 40 conditioning trials began. Analysis of the 10 preconditioning startle trials showed no difference between treatment groups in startle magnitude as a function of replicate, line, or sex. The next day, mice received their respective dose of alcohol or saline 15 min before the start of the FPS test session. A 15-min pretreatment time was chosen based on a study by Gould (2003) in which alcohol disrupted contextual fear-conditioned responses in C57BL/6 mice.

Experiment 2: Effects of alcohol on FPS acquisition

Subjects were 115 HAP1 (57 males, 58 females), 126 LAP1 (65 males, 61 females), 114 HAP2 (52 males, 62 females), and 105 LAP2 (47 males, 58 females). Procedures for this experiment differed from experiment 1 in two ways. First, 20-66 hrs prior to the fear-conditioning session, mice received a baseline startle session to assess initial startle reactivity to 10 trials of 100 dB noise bursts (5 min habituation; 40 ms; 20 sec ITI). Mice within each replicate/line/sex each were then assigned to one of four treatment groups (0.0, 0.5, 1.0, or 1.5 g/kg alcohol), counterbalanced based on average startle response magnitude. This procedure provided an added safeguard against sampling error by attempting to balance any individual differences in overall startle reactivity across drug treatment groups. ANOVA confirmed that there were no treatment group differences as a function of replicate, line, or sex at the start of the conditioning session. Second, mice received their respective dose of alcohol or saline immediately before the start of the conditioning session, which included a 10-min habituation period, to allow for the absorption and distribution of alcohol by the start of the first conditioning trial.

Experiment 3: Effects of diazepam on FPS expression

The purpose of this experiment was to compare the effects of a known anxiolytic drug, diazepam, on the expression of FPS in HAP vs. LAP mice. Forty-three HAP2 (19 males, 24 females), and 41 LAP2 (21 males, 20 females) were randomly assigned to either a vehicle or diazepam (4.0 mg/kg) group. Experimental procedures were the same as that described for Experiment 1. Mice received an injection of diazepam or vehicle 25 min before the start of the FPS test session. This pretreatment time was chosen based on a recent study by Smith et al. (2011) in which diazepam significantly reduced FPS in C57BL/6 mice.

Assessment of Blood Alcohol Content

In a separate experiment, blood alcohol content (BAC) was assessed in saline-treated mice from fear-conditioned (experiments 1 and 2) and control (experiment 1) groups. HAP1 male (n=31), HAP1 female (n=25), LAP1 male (n=31), LAP1 female (n=35), HAP2 male (n=31), HAP2 female (n=39), LAP2 male (n=20), and LAP2 female (n=38) mice received an IP injection of alcohol (0.5, 1.0, or 1.5 g/kg; 20% v/v) and blood samples (~30 μ l) were obtained from the tip of the tail at 15 and 75 min after injection of alcohol; these two time points corresponded with the beginning and the end of the FPS test session. Tail blood was collected into heparin-coated capillary tubes, immediately centrifuged, and plasma was extracted and frozen at -80°C until analyzed for BAC using an AM1 Analyzer (Analox Instruments, MA, USA).

Statistical Analyses

All 12 startle responses on each trial type (noise-alone, light+noise) were averaged for each mouse. Mice that did not meet the minimum startle response criterion of 11 g of force and whose data were affected by equipment/experimenter errors were removed from all analyses. The % FPS measure was obtained using proportional change scores calculated using the following formula: $[(\text{startle amplitude on light + noise trials} - \text{startle amplitude on noise-alone$

trials)/startle amplitude on noise-alone trials) x 100]. The % FPS measure adjusts for individual and group differences in startle reactivity. It also adjusts for potential non-specific drug treatment effects on startle reactivity and thus is indicated to be an accurate and sensitive way to detect selective effects of pharmacological compounds on FPS (Walker and Davis 2002).

Data were analyzed using analysis of variance (ANOVA) with Replicate (1, 2), Line (HAP, LAP), Sex (male, female), Conditioning Group (fear-conditioned, control) and Treatment Group (saline, 0.5, 1.0, 1.5 g/kg alcohol or vehicle, 4.0 mg/kg diazepam) as between-group factors and time (15 min, 75 min post-injection) as a within-group factor, where applicable. Lower-order ANOVAs and Tukey's t-test were used to explore interactions and main effects. Pearson product moment correlation coefficients were generated to assess the relationship between body weight and BAC. Probability values less than 0.05 were considered to be significant.

Results

Experiment 1: Effects of alcohol on the expression of FPS

A 5-way ANOVA (Replicate x Line x Sex x Conditioning Group x Treatment Group) conducted on % FPS scores revealed significant main effects of Replicate [$F(1,896)=4.8$, $p<0.05$; $1>2$], Line [$F(1,896)=24.3$, $p<0.01$; HAP>LAP], Sex [$F(1,896)=8.0$, $p<0.01$; male>female], Conditioning Group [$F(1,896)=142.4$, $p<0.01$; fear-conditioned>control] and Replicate x Sex [$F(1,896)=4.6$, $p<0.05$], Replicate x Conditioning Group [$F(1,896)=3.9$, $p=0.05$], and Line x Conditioning Group [$F(1,896)=15.6$, $p<0.01$] interactions, and a three-way Line x Conditioning Group x Treatment Group interaction close to significance [$F(3,896)=2.4$, $p<0.07$]. To further explore the three-way interaction, Conditioning Group x Treatment Group ANOVAs were conducted separately for HAP and LAP mice. A main effect of Conditioning Group was found for both HAP and LAP mice [$F_s>38.9$, $P_s<0.01$; fear-conditioned>control] and a significant Conditioning Group x Treatment Group interaction was found in HAP but not LAP

mice [$F(3,477)=3.2$, $p<0.05$]. The source of the interaction was a main effect of Treatment Group close to significance in the HAP fear-conditioned but not control groups [$F(3,241)=2.3$, $p<0.08$], due to reduced % FPS in the 1.5 g/kg alcohol group compared to the saline group ($p<0.05$ with Tukey's t-test). Data are shown in Figure 1, collapsed across replicate and sex.

Figure 1

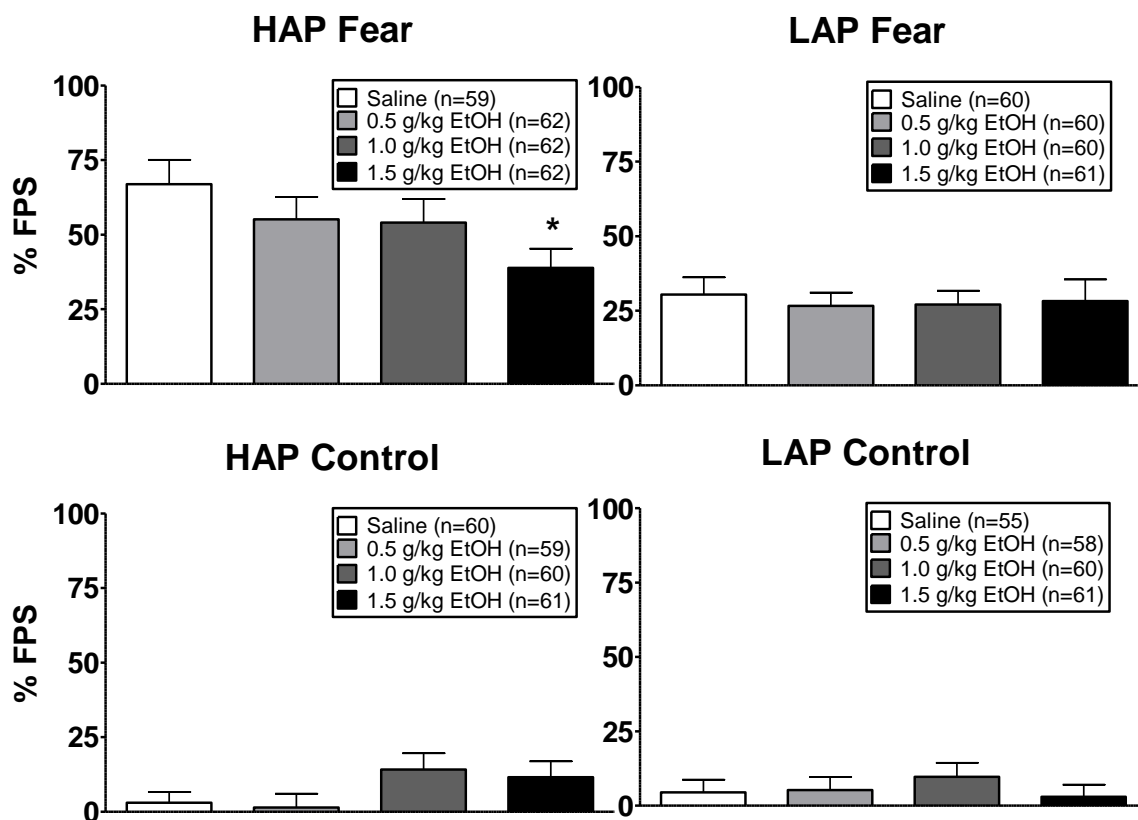


Table 1. Effects of alcohol on the expression of FPS

| Treatment Group | Saline | 0.5 g/kg EtOH | 1.0 g/kg EtOH | 1.5 g/kg EtOH |
|---------------------|-----------|---------------|---------------|------------------------|
| HAP1 Male Fear | 81.5±22.2 | 56.0±15.5 | 69.7±19.4 | 33.3±13.8 ^a |
| HAP1 Male Control | 12.1±6.4 | 7.4±11.2 | 23.7±12.6 | 13.4±15.9 |
| LAP1 Male Fear | 40.4±15.0 | 12.1±8.4 | 27.9±9.7 | 18.8±11.6 |
| LAP1 Male Control | 19.0±9.3 | 9.5±9.2 | 12.7±7.5 | 23.8±9.9 |
| HAP1 Female Fear | 75.7±18.8 | 62.4±25.5 | 60.8±28.4 | 23.9±12.7 ^a |
| HAP1 Female Control | 10.9±11.2 | 18.4±11.1 | 39.7±16.8 | 11.5±10.9 |
| LAP1 Female Fear | 22.1±9.2 | 34.2±8.0 | 29.9±9.0 | 27.1±10.5 |

| | | | | |
|---------------------|-----------|-----------|-----------|------------------------|
| LAP1 Female Control | 5.9±9.7 | -1.2±6.8 | 10.6±11.7 | -1.2±9.8 |
| HAP2 Male Fear | 82.6±15.2 | 70.4±11.9 | 32.4±8.6 | 52.7±13.2 ^a |
| HAP2 Male Control | -6.1±5.8 | 2.5±8.9 | 3.4±7.3 | 16.7±7.5 |
| LAP2 Male Fear | 44.9±13.9 | 33.9±12.1 | 33.3±10.9 | 68.1±24.8 |
| LAP2 Male Control | 0.2±8.4 | 29.0±8.9 | 1.7±7.4 | -6.1±5.3 |
| HAP2 Female Fear | 39.0±10.2 | 38.3±8.6 | 54.5±8.1 | 42.0±12.1 ^a |
| HAP2 Female Control | -2.5±6.1 | -13.3±5.2 | -0.1±5.7 | 6.2±6.8 |
| LAP2 Female Fear | 19.1±8.4 | 27.2±7.2 | 19.6±8.7 | 7.1±7.3 |
| LAP2 Female Control | -6.5±6.2 | -9.4±8.3 | 12.6±9.6 | -2.8±5.6 |

Mean (±SEM) % FPS data from in Figure 1 shown separately for replicate lines 1 and 2 and male and female mice in fear-conditioned and control groups despite the lack of significant statistical interactions with these factors. ^a indicates statistical comparisons collapsed across replicate and sex (p<0.05 saline vs. 1.5 g/kg group).

Experiment 2: Effects of alcohol on the acquisition of % FPS

A 4-way ANOVA (Replicate x Line x Sex x Treatment Group) on % FPS scores revealed significant main effects of Replicate [$F(1,428)=5.2$, $p<0.05$; Replicate 2>Replicate 1] and Line [$F(1,428)=16.9$, $p<0.01$; HAP>LAP] and a Replicate x Treatment Group interaction [$F(3,428)=2.8$, $p<0.05$] but follow up analyses of treatment group within each replicate yielded no significant effects. The initial ANOVA also yielded a Replicate x Line x Sex [$F(1,428)=6.3$, $p<0.05$] interaction that was followed with Line x Sex ANOVAs within each replicate. For both replicates, HAP mice showed greater % FPS than LAP mice [$F_s>6.2$, $P_s\leq 0.01$]. A Line x Sex [$F(1,215)=9.6$, $p<0.01$] interaction was also found in Replicate 2 mice due to a sex effect in LAP2 [$F(1,103)=6.3$, $p=0.01$; female>male] but not HAP2 mice. Table 1 shows these data separately for replicate lines 1 and 2 and male and female mice in fear-conditioned and control groups despite the lack of significant statistical interactions with these factors.

Table 2. Effects of alcohol on the acquisition of FPS

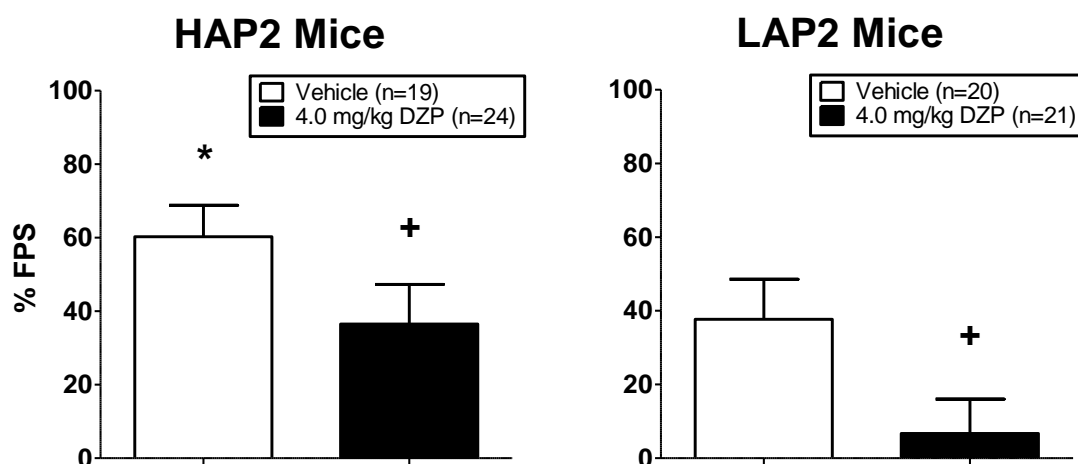
| Treatment Group | Saline | 0.5 g/kg EtOH | 1.0 g/kg EtOH | 1.5 g/kg EtOH |
|-----------------|------------|---------------|---------------|---------------|
| HAP1 Male | 87.7±35.2 | 37.8±15.9 | 145.9±61.2 | 28.6±18.9 |
| LAP1 Male | 43.9±18.5 | 40.7±16.5 | 54.7±11.9 | 22.5±11.3 |
| HAP1 Female | 79.1±32.7 | 109.1±45.7 | 86.3±23.7 | 53.6±16.4 |
| LAP1 Female | 21.3±11.7 | 28.2±12.1 | 43.3±14.2 | 35.8±7.8 |
| HAP2 Male | 92.8±19.7 | 78.2±14.1 | 97.5±17.3 | 149.1±35.3 |
| LAP2 Male | 45.5±19.9 | 22.4±10.6 | 43.6±12.3 | 55.1±14.7 |
| HAP2 Female | 68.4±11.9 | 95.9±16.9 | 64.7±20.4 | 78.6±17.3 |
| LAP2 Female | 109.4±31.6 | 92.8±23.3 | 61.2±20.9 | 73.3±37.0 |

Mean (\pm SEM) % FPS in male and female HAP1/LAP1 and HAP2/LAP2 lines treated with 0 (saline), 0.5, 1.0, and 1.5 g/kg alcohol prior to the fear conditioning session.

Experiment 3: Effects of diazepam on the expression of FPS

A 2-way ANOVA (Line x Sex x Treatment Group) conducted on % FPS scores revealed significant main effects of Line [$F(1,76)=7.3$, $p<0.01$; HAP2>LAP2] and Treatment Group [$F(1,76)=7.3$, $p<0.01$; vehicle>diazepam] but no interaction. Data are shown in Figure 2, collapsed across sex.

Figure 2



Blood Alcohol Content

Pearson product moment correlations between body weight and BAC indicated a significant correlation at the 15-min time point ($r=0.2$, $p<0.01$). Thus, statistical analyses of BAC included body weight as a co-factor. A 5-way repeated measures analysis of co-variance (Replicate x Line x Sex x Treatment Group x Time with body weight as a co-factor) revealed a significant main effect of Treatment Group [$F(2,225)=299.6$, $p<0.01$] and a Replicate x Treatment Group x Time interaction [$F(2,225)=4.0$, $p<0.05$] but no line or sex differences in BAC. Data are shown in Table 3.

Table 3 Blood Alcohol Content in HAP1/LAP1 and HAP2/LAP2 lines

| Treatment Group | 0.5 g/kg | | 1.0 g/kg | | 1.5 g/kg | |
|-----------------|----------|---------|-----------|-----------|------------|-----------|
| | 15 | 75 | 15 | 75 | 15 | 75 |
| HAP1 Male | 48.0±6.2 | 6.1±1.2 | 102.2±9.3 | 32.7±10.3 | 147.6±18.5 | 84.6±11.4 |
| LAP1 Male | 40.6±5.8 | 5.8±1.1 | 96.3±9.6 | 31.9±5.1 | 160.5±13.6 | 102.5±7.2 |

| | | | | | | |
|-------------|----------|---------|-----------|----------|------------|-----------|
| HAP1 Female | 39.0±5.6 | 5.3±1.0 | 90.6±12.4 | 18.3±3.3 | 165.0±13.4 | 93.4±4.9 |
| LAP1 Female | 44.7±5.0 | 5.9±2.2 | 94.8±15.3 | 22.0±5.6 | 156.1±13.1 | 103.3±5.9 |
| HAP2 Male | 41.7±4.0 | 3.4±0.8 | 80.3±11.8 | 21.8±4.5 | 114.1±14.6 | 89.8±10.8 |
| LAP2 Male | 36.8±5.2 | 6.0±0.9 | 84.0±16.3 | 27.9±7.4 | 134.1±19.9 | 90.6±20.0 |
| HAP2 Female | 37.2±3.9 | 4.0±0.9 | 78.9±7.7 | 23.5±5.4 | 115.8±15.2 | 98.2±14.5 |
| LAP2 Female | 34.2±2.4 | 4.5±0.8 | 83.9±8.4 | 26.0±4.3 | 108.7±8.3 | 91.1±9.6 |

Mean (\pm SEM) BAC (mg of alcohol/dl of plasma) at 15 and 75 min in HAP and LAP mouse lines treated with IP injections of 0.5, 1.0, and 1.5 g/kg alcohol.

Discussion

The “tension-reduction” and “stress-response dampening” hypotheses of alcohol use (Cappell and Herman 1972; Conger 1956; Sher 1987) set forth the idea that alcohol may reduce anxiety symptoms, which has been suggested as a primary reason for the high rate of co-morbidity between AUDs and anxiety disorders. It has also been hypothesized that common inherited genes could be mediating risk factors in the development of these co-morbid disorders because some studies indicate people with a positive family history of AUDs and/or anxiety disorders are more sensitive to the effects of alcohol on anxiety symptoms (Sher and Levenson 1982; Sinha et al. 1998; but see Zimmermann et al. 2004) and other stress-related indices (Croissant and Olbrich 2004; Finn and Pihl 1987; Levenson et al. 1987; Schuckit et al. 1981; Stewart et al. 1992; Zimmermann et al. 2009). In the present study, we used the selectively bred HAP/LAP mouse lines to explore the idea that organisms with a genetic propensity toward high alcohol preference may be more sensitive to the anxiolytic effects of alcohol than organisms that do not have this genetic propensity. We have previously suggested the HAP/LAP lines to be an animal model that may represent increased genetic risk to develop AUDs and a co-morbid anxiety disorder, specifically, PTSD (Barrenha and Chester 2007), in

humans because these mouse lines show a positive association between alcohol preference and FPS (HAP>LAP).

One of the primary goals of this study was to examine whether alcohol has anxiolytic effects in the FPS procedure in mice because to our knowledge this has not yet been demonstrated. Although many studies have consistently shown anxiolytic effects of alcohol on unconditioned anxiety-related behaviors in both mice (Abreu-Villaca et al. 2008; Boehm et al. 2002; Correia et al. 2009; Costall et al. 1988; Durcan and Lister 1988; Gulick and Gould 2009a; 2009b; Kameda et al. 2007) and rats (Blanchard et al. 1993; Colombo et al. 1995; File 1976; File et al. 1976; LaBuda and Fuchs 2002; Spanagel et al. 1995; Stewart et al. 1993), there are only two published studies in rats that examined alcohol effects on the expression of FPS. Miller and Barry (1960) reported that alcohol decreased FPS whereas Hijzen et al. (1995) reported no effect of alcohol on FPS. In the present study, alcohol had no effect on the acquisition of FPS but the highest dose of alcohol (1.5 g/kg) moderately reduced FPS in HAP but not LAP mice when given during the expression test. This effect was similar in both male and female mice. It should be noted that the magnitude of alcohol's effect on FPS was small and that some statistical terms did not quite reach the $p < 0.05$ significance criterion [the effect size of the line x conditioning group x treatment group interaction term was small ($\eta^2 = 0.03$; power = 0.6; $p < 0.07$)].

The other primary goal of this study was to determine whether alcohol's effect on FPS is a genetically correlated response to selection for alcohol consumption. The question of whether two traits of interest are genetically correlated can be rigorously tested using rodent lines selected in replicate. If both pair of lines show the correlated trait, this outcome provides stronger evidence that the correlated trait is influenced by common genes that also mediate the selection phenotype in the replicate lines (Crabbe et al. 1990). In the current study we replicated our previous findings of a genetic correlation between alcohol preference and FPS in both pairs of the HAP/LAP mouse lines (Barrenha and Chester 2007). In addition, we found

support for the hypothesis that HAP lines would be more sensitive than LAP lines to the anxiolytic effects of alcohol with the finding that the highest dose of alcohol reduced the expression of FPS in HAP but not LAP mice. The similar reduction in % FPS in both HAP1 and HAP2 lines suggests that the anxiolytic response to a moderate dose of alcohol during the expression of FPS may be genetically correlated with predisposition toward high alcohol preference. Analyses of BAC indicate that the effect of alcohol on FPS in HAP mice is not due to line differences in alcohol pharmacokinetics. Anxiolytic effects of alcohol in HAP but not LAP mice is a result consistent with prior reports in selected rat lines where alcohol reduced unconditioned anxiety-related behavior in alcohol-preferring but not in alcohol-non-preferring rat lines (Colombo et al. 1995; Pandey et al. 2005; Stewart et al. 1993). Interestingly, Rorick et al. (2003) showed that, under certain conditions, alcohol reversed avoidance learning deficits in rats selectively bred for high alcohol drinking (HAD) but not in their low alcohol drinking (LAD) counterparts, an effect the authors suggested might be due to greater anxiolytic effects of alcohol in HAD than LAD rats. However, HAP and LAP mice from both replicates 1 and 2 have shown no differences in anxiety-related behavior or in the anxiolytic response to 1.25 and 2.0 g/kg alcohol in the elevated plus maze (Dr. Nicholas J. Grahame, personal communication). Taken together, these data suggest that perhaps line differences in anxiety-related behavior are necessary to observe line-specific anxiolytic effects of alcohol. In addition, whether alcohol produces anxiolytic effects may depend on the type of anxiety-related behavior being measured (conditioned vs. unconditioned) and the method of alcohol exposure (experimenter-administered vs. voluntary self-administration). Further research should address these issues. A primary question we are currently exploring is whether fear-conditioning alters subsequent voluntary alcohol consumption and, conversely, whether alcohol consumption reduces the expression of FPS.

Different neural pathways and chemical substrates regulate the acquisition vs. the expression of fear-conditioned behaviors (Davis, 2006; Fendt and Fanselow, 1999). For

example, Davis and colleagues have shown that activation of the basolateral nucleus of the amygdala is particularly important for the acquisition of FPS whereas the central nucleus of the amygdala (CeA) is critical for the expression of FPS (Davis, 2006). It is known that alcohol affects neuronal function in the CeA (see review by McBride 2002) and, specifically, increases inhibitory neurotransmission in the CeA via interaction with gamma-aminobutyric acid (GABA_A) receptors (Roberto et al. 2003). Our finding that alcohol affected the expression but not the acquisition of FPS suggests that the CeA in HAP mice may be particularly sensitive to alcohol and that differences in GABA_A receptor function could partly account for the observed line difference in alcohol's anxiolytic effect on FPS expression. In order to explore this idea, we examined the effect of diazepam, the prototypical anxiolytic drug and agonist at the GABA_A/benzodiazepine receptor binding site (Löw et al. 2000) on the expression of FPS in HAP2 and LAP2 mice. Similar to previous reports in rats (Davis 1979) and mice (Risbrough et al. 2003; Smith et al. 2011), diazepam (4.0 mg/kg) significantly reduced the expression of FPS but no line difference in the response to diazepam was seen. This small piece of evidence suggests that the line difference in anxiolysis may be specific to alcohol and is perhaps not mediated by the GABA_A receptor and/or that the lines do not differ in GABA_A receptor function. But, certainly more research is needed to explore the neurochemical basis for the effects reported here. Future studies are planned to investigate line-based differences in amygdala function that may be related to alcohol- and anxiety-related behaviors in HAP and LAP mice.

Data from both experiments are generally inconsistent with a number of reports in which effects of alcohol were examined on contextual fear-conditioned freezing behavior in rodents. Most of these studies indicate that alcohol disrupts the acquisition (Gould, 2003; Gulick and Gould 2007; Land and Spear 2004; Melia et al. 1996; Wehner et al. 2004; Weitemeier and Ryabinin 2003; but see Stromberg and Hammond 1997) but not the expression of fear-conditioned behavior (Gould 2003; Land and Spear 2004). Lattal (2007) further explored this issue and reported that alcohol (1.5 g/kg) appears to interfere with the acquisition of extinction

learning in mice. Findings from these studies were interpreted primarily in the context of alcohol's effect on learning and memory mechanisms, rather than anxiety-related mechanisms per se, and were suggested to indicate that alcohol disrupts neural processes involved in memory encoding but not memory retrieval. It is possible that alcohol's effect on FPS in this study was due to its amnesic effect, but this interpretation seems unlikely given the inconsistencies between the present and prior published data. However, it is important to keep in mind that comparison between present vs. prior data may be complicated by the fact that FPS is primarily mediated by the amygdala whereas contextual fear-conditioned behavior is primarily mediated by the hippocampus (Logue et al. 1997; Phillips and LeDoux 1992) and these two brain areas are differentially sensitive to alcohol (Ryabinin et al. 1997). It would be necessary to conduct additional experiments specifically designed to disentangle alcohol's effects on fear-vs. learning/memory-related mechanisms to adequately address whether alcohol has possible amnesic effects in the FPS procedure. On this point, it is notable that alcohol's effects on anxiety-related behavior seem to be dissociable from its effects on learning/memory (Gulick and Gould, 2007; Kameda et al. 2007).

In summary, this is the first study to report anxiolytic effects of alcohol in mice using an FPS procedure, but, the observed effect depended on genetic background of the mice. Although the overall effect size in this study was small, these data collected in two pairs of male and female HAP/LAP mouse lines suggest that the anxiolytic response to alcohol in HAP mice may be genetically correlated with their propensity toward high alcohol preference and robust FPS. Because FPS is thought to be a particularly relevant model for certain features of PTSD in humans (Grillon 2002; Kolb 1984), the presence of this genetic correlation in selected mouse lines could suggest that the high rate of co-morbidity between PTSD and AUDs in humans may be partly due to the influence of common genes on mechanisms related to the development of both psychiatric disorders. Results of this study provide additional evidence that one of the relevant mechanisms of co-morbidity could be that organisms with a genetic propensity toward

AUDs/high alcohol consumption are more sensitive to the anxiolytic, and therefore, reinforcing effects of alcohol (Colombo et al. 1995; Pandey et al. 2005; Sher and Levenson 1982; Sinha et al. 1998; Stewart et al. 1993). More studies are encouraged to explore the effects of alcohol on other types of stress- and anxiety-related behaviors in these unique selectively bred mouse lines.

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Figure Legends

Fig 1. Mean (\pm SEM) % FPS in fear-conditioned (top panels) and control (bottom panels) HAP (left panels) and LAP (right panels) mouse lines treated with IP injections of alcohol (0.5, 1.0, and 1.5 g/kg) or saline before the FPS test session. * $p < 0.05$ saline vs. 1.5 g/kg group.

Fig 2. Mean (\pm SEM) % FPS in HAP (left panel) and LAP (right panel) mouse lines treated with IP injections of 4.0 mg/kg diazepam or vehicle before the FPS test session. * $p < 0.01$ HAP2 vs. LAP2; † $p < 0.01$, vehicle vs. diazepam groups.