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Effects of stress, acute alcohol treatment, or both on pre-pulse inhibition in high- and low-alcohol preferring mice

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Abstract

Pre-pulse inhibition of the acoustic startle reflex (PPI) is a measure of sensorimotor gating frequently used to assess information processing in both humans and rodents. Both alcohol and stress exposure can modulate PPI, making it possible to assess how stress and alcohol interact to influence information processing. Humans with an increased genetic risk for alcoholism are more reactive to stressful situations compared to those without a family history, and alcohol may have stress-dampening effects for those with high genetic risk. The purpose of the present study was to examine the effects of stress, acute alcohol exposure, or both on PPI in male and female mice selectively bred for high- (HAP2) and low- (LAP2) alcohol preference. Experiment 1 assessed the effects of various doses of acute alcohol on PPI. Experiments 2 and 3 assessed the effect of 10 days of restraint stress on subsequent PPI tested at 30 min (Experiment 2) or 24 h (Experiment 3) following the termination of stress exposure. Experiment 3 also examined the effects of acute alcohol treatment (0.75 g/kg) on PPI in mice previously exposed to stress or no stress. Results indicate that 0.75 and 1.0 g/kg doses of alcohol increased PPI in HAP2 but not LAP2 mice. When PPI was tested 30 min after stress exposure, stressed HAP2 mice showed a trend toward decreased PPI and stressed LAP2 mice showed a trend toward increased PPI. The combination of stress and alcohol treatment did not alter PPI in either line 24 h following the termination of stress exposure, suggesting that alcohol does not ameliorate the effect of stress on PPI. Stressed LAP2 mice had increased basal circulating corticosterone on the final stress exposure day compared to non-stressed LAP2 mice, and no difference was

found between stressed and non-stressed HAP2 mice. The results suggest that high genetic risk for alcoholism may be related to increased sensitivity to alcohol and stress effects on PPI, and this sensitivity could signify an endophenotype for increased genetic risk to develop alcoholism.

Keywords: *acoustic startle, pre-pulse inhibition, stress, alcohol, selectively bred mice, corticosterone*

Introduction

Alcohol abuse and alcoholism represent major burdens to society with costs reaching $185 billion and 100,000 lives lost annually (Li, Hewitt, & Grant, 2004). Exposure to stress is an important variable that interacts with many environmental and biological factors to influence alcohol-drinking behavior in both humans and rodents (Sillaber & Henniger, 2004; Uhart & Wand, 2009). For example, rodents show stress-induced changes in alcohol consumption (e.g. Champagne & Kirouac, 1987; Chester, de Paula Barrenha, DeMaria, & Finegan, 2006; Lynch, Kushner, Rawleigh, Fiszdon, & Carroll, 1999), although the direction of effects are inconsistent and influenced by many factors like genetics, history of alcohol drinking, and type/history of stress exposure (Pohorecky, 1991). As well, interactions between these factors are important and likely contribute to the complex and often inconsistent findings in the literature.

Evidence suggests that humans with a family history of alcoholism are more reactive to stress compared to individuals without a family history of alcoholism. For example, adult sons of alcoholics (SOAs) display increased heart rates and greater vein constriction (classic cardiovascular stress responses) preceding an unavoidable electric shock compared to controls (Finn, Zeutouni, & Pihl, 1990), and adolescent SOAs display increased heart rates compared to non-SOAs while performing a mental arithmetic task (psychological stressor; Harden & Pihl, 1995). On the other hand, one study reported similar acoustic startle in response to threat of electric shock in SOAs compared to controls (Zimmermann, Spring, Wittchen, & Holsboer, 2004) and another study showed reduced skin conductance responses in anticipation of electric shock compared

to controls (Finn, Kessler, & Hussong, 1994). These results may suggest that SOAs are more reactive to stressful stimuli under certain experimental conditions; however, more study is needed to clarify the basis of contradictory results. Animal models provide an opportunity to explore the influence of factors such as genetics and history of stress exposure under controlled experimental conditions.

Rodents selectively bred for high- or low-alcohol drinking/preference have been a rich resource for investigators to assess how genetic influences on alcohol drinking behavior may also influence other behavioral traits (Crabbe, Phillips, & Belknap, 2010). With regard to stress reactivity, reports in selectively bred rat lines suggest that alcohol-preferring (P) rats may be more sensitive to stress-related effects on behavior compared to their non-preferring (NP) counterparts. For example, male P rats showed changes in stress-induced drinking while NP rats did not (Chester, Blose, & Froehlich, 2004). In another study that assessed foot shock effects on reinstatement of alcohol drinking in an alcohol deprivation model, Alcohol-Accepting (AA), High-Alcohol-Drinking (HAD), and P rats showed greater alcohol intake than Wistar rats following the foot shock exposure (Vengeliene et al., 2003). Foot shock stress has also been shown to reinstate alcohol responding to a greater degree in Marchigian Sardinian alcohol-preferring (msP) rats compared to Wistar rats (Hansson et al., 2006). Other reports also indicate that high-alcohol-preferring rodent lines are more susceptible to develop stress-induced, conditioned fear-related behavior

than their low-alcohol-preferring counterparts are (Barrenha & Chester, 2007; Chester, Kirchhoff, & Barrenha, 2013; McKinzie et al., 2000).

In addition to being more reactive to stress, evidence from both humans and rodents indicates that individuals with an increased genetic risk for high alcohol drinking are more sensitive to alcohol's stress-dampening effects. For example, alcohol reduced the physiological stress response (i.e., heart rate and vein constriction) displayed by SOAs in anticipation of shock, but did not change response for those without a family history of alcoholism (Finn et al., 1990). Analogous results were found in high- (HAP) and low- (LAP) alcohol-preferring replicate mouse lines where alcohol reduced the expression of fear-potentiated startle (FPS) in HAP but not in LAP mice (Barrenha, Coon, & Chester, 2011). These results suggest that alcohol's stress-dampening effects on physiological and behavioral responding depend on genetic susceptibility for high- or low-alcohol-drinking behavior.

The mammalian acoustic startle response is an adaptive reflexive behavior in response to a loud acoustic stimulus and has served as a useful phenotype for emotion and cognition-related behavior (Grillon, Sinha, Ameli, & O'Malley, 2000). The startle response can also be decreased by presenting a relatively weaker stimulus directly (e.g., 100 msec) before a startle-inducing stimulus, termed pre-pulse inhibition (PPI). PPI is thought to reflect an inhibitory mechanism (or "gate") that protects the neural processing of the pre-pulse from interruption by the startle pulse (Graham, 1992; Norris & Blumenthal, 1996; Swerdlow, Geyer, Blumenthal, & Hartman, 1999), preventing cognitive overload

(e.g., Braff & Geyer, 1990; Braff, Grillon, & Geyer, 1992). PPI is considered a general measure of information processing and has been utilized to study a range of neuropsychiatric disorders characterized by deficits in sensory and/or cognitive function (Braff, Geyer, & Swerdlow, 2001).

PPI is influenced by both genetic and environmental factors. For example, exposure to stress or stress hormones decreases PPI in rodents (Conti, Murry, Ruiz, & Printz, 2002; Risbrough, Hauger, Roberts, Vale, & Geyer, 2004; Sutherland, Burian, Covault, & Conti, 2010; Sutherland & Conti, 2011), although not all studies find disruptive effects of stress on PPI (Dubovicky, Paton, Morris, Mach, & Lucot, 2007; Faraday, O'Donoghue, & Grunberg, 1999; Pijlman, Herremans, van de Kieft, Kruse, & van Ree, 2003). Evidence of acute alcohol effects on PPI in rodents is sparse, but Jones and colleagues (2000) showed that alcohol disrupts PPI for female P rats but not NP rats, suggesting differential sensitivity to the effects of acute alcohol on PPI that is influenced by genetic susceptibility for high- or low-alcohol preference.

The purpose of the present study was to assess the effects of alcohol, stress, and their combination on PPI in mice that differ in genetic predisposition toward alcohol preference (HAP2/LAP2). We predict that repeated stress exposure will disrupt PPI in both HAP2 and LAP2 mice, based on the majority of evidence that stress exposure reduces PPI (e.g., Conti et al., 2002; Grillon & Davis, 1997; Richter et al., 2011; Risbrough et al., 2004; Sutherland et al., 2010; Sutherland & Conti, 2011). However, we further predict that HAP2 mice will show a greater stress-induced disruption of PPI than LAP2 mice and that alcohol will

ameliorate the stress-induced disruption of PPI in HAP2 but not LAP2 mice. These predictions are based on the previously cited evidence that humans and animals with a genetic susceptibility toward high-alcohol preference may be more sensitive to stress-related effects on behavior (e.g., Chester et al., 2013; Hansson et al., 2006; Vengeliene et al., 2003) and to the stress-dampening effects of alcohol (Barrenha et al., 2011; Finn et al., 1990).

Materials and methods

Subjects

Subjects were alcohol-naïve replicate-line 2 HAP and LAP mice produced by mass selection from outbred HS/Ibg mice (Boulder, CO, USA) at the Indiana Alcohol Research Center (IARC) in Indianapolis, IN, USA (Grahame, Li, & Lumeng, 1999). Subjects in the current studies were generated at Purdue University from HAP2 and LAP2 breeders obtained from the IARC. Mice were housed in groups of 2 to 4 in 11.5 × 7.5 × 5.0 in. polycarbonate cages with aspen wood shavings for bedding. Food and water were available *ad libitum*, except during experimental procedures. Temperature in the colony room was maintained at 21 ± 2 ^{o}C. Experimental procedures were conducted during the light phase of the 12:12 light:dark cycle (lights on at 0700 hrs). Mice were from the 31st and 34th generations of selection for Experiment 1, the 34th and 35th generations of selection for Experiment 2, and the 39th generation of selection for Experiment 3. At the start of experimental procedures, mice were between 57 and 101 days old. 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, 0.75, and 1.0 g/kg of body weight in an injection volume of 3.17, 4.73, and 6.30 mL/kg, respectively.

Testing Apparatus

PPI was assessed using a Coulbourn Instruments Animal Acoustic Startle System (Coulbourn Instruments, Allentown, PA). The startle chamber is sound-attenuated and contains 4 platforms equidistant from center speakers located in the floor and ceiling of the chamber. Each platform is weight-sensitive and records the amount of force in grams produced by the subject in the 200 msec after presentation of the startle stimuli. The force measurement does not include the subjects' body weight. All subjects were placed individually into open-air holders (8 × 8 × 16 cm) with metal rod floors (rod diameter 0.19 in. with each rod separated by 0.39 in.). The holders rest on top of the weight-sensitive platforms during the acoustic startle test sessions. A ventilating fan provided continuous 70–71 dB background noise.

PPI Parameters

Each PPI session began with a 5-min habituation period (no stimuli) followed by 12 different trial types presented throughout the session for a total of 120 trials (each trial type was presented 10 times). The 12 trial types included 1 blank trial (no stimuli), 2 startle pulse (94,104 dB; 40 msec) trials; 3 pre-pulse (78, 82, 86 dB; 20 msec) trials, and 6 pre-pulse + pulse trials. Multiple pre-

pulse + pulse combinations were used because of evidence that PPI can vary as a function of pre-pulse (Paylor & Crawley, 1997; Plappert, Pilz, & Schnitzler, 2004) and pulse (Chester & Barrenha, 2007) intensities, thus the detection of treatment effects on PPI may be facilitated. Two startle-pulse intensities were also included because HAP2 and LAP2 mice are known to differ in baseline startle responding (HAP2 > LAP2; Chester & Barrenha, 2007). Including multiple startle-pulse intensities allowed comparisons of PPI between HAP2 and LAP2 mice within and across pulse intensities. On pre-pulse + pulse trials, the pre-pulse stimuli preceded the startle pulse by 100 msec. To avoid habituation, trial types were presented randomly with inter-trial intervals (ITI) that ranged from 10–25 sec.

Stress Exposure

During the stress procedure of Experiments 2 and 3, all animals within a cage were assigned to the same stress condition. Mice in the Stress group were moved to an adjacent room during stress administration in order to avoid any disruption to the No Stress group. The stressor consisted of restraining the mice in Plexiglas® tubes (diameter: 25.4 mm; length 83 mm).

On stress days 2–9, the duration (i.e., 20, 40, 80, and 100 min) and start time (i.e., 0900 hrs, 1100 hrs, 1300 hrs) of each stress session were variable and randomized using a random numbers table. However, on stress days 1 and 10, each stress session was the same duration (60 min) and occurred in run order (testing start times ranged from 0900–1600) to match PPI testing times after the termination of stress exposure on day 10 (i.e., 30 min later in Experiment 2 and

24 h later in Experiment 3). Consequently, blood sampling for basal CORT levels in Experiment 3 occurred between 0800 and 1500 on days 1 and 10. Each restraint duration was administered twice across the 10-day stress period for a total duration of 600 min.

Blood Collection

To assess possible changes in hypothalamic–pituitary–adrenal axis (HPA-axis) function caused by the stress procedure, blood samples were taken at the beginning of restraint on days 1 and 10 in Experiment 2 to assess basal CORT levels as a measure of HPA-axis function in response to repeated stress exposure (Katz, Roth, & Carroll, 1981). Blood sampling also afforded the possibility to assess long-term changes in HPA-axis function between HAP2 and LAP2 mice based on evidence that exposure to repeated stress can result in persistently elevated levels of CORT that are evident even when animals are not experiencing acute stress (Ottenweller, Natelson, Pitman, & Drastal, 1989; Pitman, Ottenweller, & Natelson, 1988). Mice were moved into an adjacent room, placed in the restraint tube, and approximately 0.05 mL of blood was collected from the tip of the tail. The blood sampling procedure was completed within 2 min. The No Stress control mice were removed from the restraint tube immediately following blood sampling and returned to the colony room. Mice in the Stress group remained in the restraint tube for the duration of their scheduled stress exposure and then returned to the colony room.

Plasma CORT Analysis

Blood samples were collected in heparinized capillary tubes, placed on ice, and immediately centrifuged at 12,000 rpm for 5 min. The plasma was extracted and frozen at −80° C until analysis using an enzyme immunoassay kit from Assay Designs (Ann Arbor, MI). All samples were run in duplicate, and animals with a coefficient of variance (CV) greater than 30% were excluded from analyses (2 HAP2 male, 2 HAP2 female, and 1 LAP2 male).

PPI Testing

Mice were randomly assigned to experimental groups in a counterbalanced fashion based on line and sex. For all experiments, mice were given two PPI sessions (Baseline and Post-treatment) with body weight measured directly before each session. The Baseline PPI session was conducted to habituate animals to the testing procedure and to provide a baseline measure of acoustic startle and PPI. Mice were given an IP saline injection equal in volume to the 0.75 g/kg dose of alcohol before all baseline PPI sessions. The Post-treatment PPI session for Experiment 2 was conducted 30 min after the termination of stress exposure, which is based on work showing that repeated restraint stress disrupts PPI in rats at this time point (Sutherland et al., 2010; Sutherland & Conti, 2011). For Experiment 3, we tested PPI at a later time point (24 h) to see if the effects of stress on % PPI in HAP2 and LAP2 mice would still be evident. All injections were given 5 min before mice were placed in the apparatus, and each PPI session lasted 42 min.

For Experiment 1, mice were assigned to one of the following treatment groups: Saline, 0.5, 0.75, or 1.0 g/kg alcohol. These doses of alcohol are lower

than the lowest dose (1.25 g/kg) reported to produce acute locomotor activation in HAP and LAP mice (Grahame, Rodd-Henricks, Li, & Lumeng, 2000). Treatment groups within each line had *n*'s ranging from 18 to 29.

For Experiment 2, mice were assigned to either the Stress condition or the No Stress condition. No injections were given before behavioral testing to reduce any acute stress associated with handling and injections. Treatment groups within each line had *n*'s ranging from 13 to 15.

For Experiment 3, the procedure was identical to Experiment 2, except alcohol or saline injections were given prior to behavioral testing, creating the following treatment groups: Stress + Alcohol, Stress + Saline, No Stress + Alcohol, No Stress + Saline. Treatment groups within each line had *n*'s ranging from 22 to 27.

Statistical Analyses

PPI was calculated as a percent score (% PPI) using the following formula: 1 − (average startle response on pre-pulse + pulse trials/average startle response on pulse-alone trials) × 100.

Prior to analyses, mice with average % PPI responses (i.e., % PPI collapsed across all dB levels) greater than 2 standard deviations from the mean on either the Baseline or Post-treatment PPI test session were considered outliers and removed from the dataset. A total of 6 mice [2 HAP2 (2 male and 0 female) and 4 LAP2 (1 male and 3 female)] were removed from Experiment 1, 0 mice from Experiment 2, and 7 mice [3 HAP2 (2 male and 1 female) and 4

LAP2 (1 male and 3 female)] from Experiment 3. Mice that were removed from the dataset showed pre-pulse facilitation rather than PPI.

Data were analyzed using analysis of variance (ANOVA) with Line (HAP2 and LAP2), Sex (male and female), Dose (0, 0.5, 0.75, 1.0 g/kg alcohol), and Stress Exposure (Stress and No Stress) as between-subjects factors. Within-subject factors were startle trial type [2 pulse (94,104 dB) trials], % PPI (at each of the 6 pre-pulse + pulse trials), and stress day (1 and 10).

Analyses were conducted separately within the Baseline and Post-treatment PPI sessions. *Post hoc* analyses included lower order ANOVAs, Dunnett's test (Experiment 1), and Tukey's HSD tests, where appropriate. For the Post-treatment PPI session analyses, only interactions involving the treatment variables of interest (i.e., stress and alcohol exposure) are reported. The alpha level was set at $p \leq 0.05$.

Pearson product-moment correlations between body weight and startle magnitude on the 94 dB and 104 dB pulse trials were conducted separately even though the force measurement does not include body weight. In the few instances where body weight and startle magnitude were found to be significantly correlated, analyses were conducted with and without body weight as a co-factor which did not affect the results; thus, reported analyses do not include body weight as a co-factor.

Results

Experiment 1

Experiment 1 tested the effects of acute low-dose alcohol (0.5, 0.75, and 1.0 g/kg) on % PPI in male and female HAP2 and LAP2 mice.

Baseline Session

% PPI: Baseline Session: Baseline % PPI data is shown in Table 1 for all experiments. Four-way ANOVA (Pulse × Pre-pulse × Line × Sex) indicated that HAP2 mice showed greater % PPI than LAP2 mice [$F(1,185) = 21.3$, $p < 0.01$]. There was also a Pulse × Pre-pulse interaction [$F(2,370) = 7.2$, $p < 0.01$], due to reduced % PPI when the 86 dB pre-pulse preceded the 94 dB pulse.

Startle on Pulse-Alone Trials: Three-way ANOVA (Pulse × Line × Sex) indicated greater startle to the 104 dB than the 94 dB pulse [$F(1,185) = 91.3$, $p < 0.01$], greater startle in HAP2 mice than LAP2 mice [$F(1,185) = 53.3$, $p < 0.01$], and greater startle in males than females [$F(1,185) = 8.9$, $p < 0.01$]. Analysis uncovered a Pulse × Line interaction [$F(1,185) = 11.3$, $p < 0.01$] due to a greater line difference in startle to the 104 dB pulse (Table 1). A Pulse × Sex interaction was also significant [$F(1,185) = 5.3$, $p < 0.05$], due to a greater sex difference (Male > Female) in startle to the 104 dB pulse (Table 1).

There was a weak positive correlation between body weight and startle magnitude on the 104 dB (n = 189, r = 0.16; $p < 0.05$) but not the 94 dB pulse trials.

Post-treatment Session

Body Weight: Average (± SEM) body weights on the Post-treatment PPI session were 25.2 (± 0.3) g for HAP2 mice (males: 26.7 ± 0.4 g, females: 24.3 ± 0.4 g) and 25.1 (± 0.3) g for LAP2 mice (males: 27.3 ± 0.4 g, females: 23.7 ± 0.2 g). Three-way ANOVA (Line × Sex × Dose) indicated males weighed significantly more than females [$F(1,185) = 83.2$, $p < 0.01$].

% PPI: Five-way repeated measures ANOVA (Pulse × Pre-pulse × Line × Sex × Dose) indicated a Line × Dose interaction [$F(3,173) = 2.6$, $p = 0.05$]. Dunnett's tests run within each line indicated no significant dose effect in LAP2 mice, but HAP2 mice displayed increased % PPI following 0.75 and 1.0 g/kg alcohol compared to saline (P's < 0.05) (Fig. 1).

Startle on Pulse-Alone Trials: Four-way repeated-measures ANOVA (Pulse × Line × Sex × Dose) indicated Pulse × Sex × Dose [$F(3,173) = 3.4$, $p < 0.05$] and Line × Dose [$F(3,173) = 5.2$, $p < 0.01$] interactions. Follow-up Sex × Dose ANOVAs at each pulse showed no significant effects. Dose comparisons within each line indicated increased startle in HAP2 mice pretreated with 0.75 or 1.0 g/kg alcohol compared to saline (Dunnett's: p's < 0.05); no effects were seen in LAP2 mice (Fig. 1).

There was no correlation between body weight and startle magnitude on either the 94 dB (n = 189, r = −0.01, p = 0.9) or the 104 dB pulse trials (n = 189, r = 0.12, $p = 0.09$).

Experiment 2

The purpose of Experiment 2 was to assess the effect of 10 days of repeated stress exposure on basal circulating CORT levels and PPI measured 30 min after the termination of stress exposure.

Baseline Session

% PPI: A four-way ANOVA (Pulse × Pre-pulse × Line × Sex) indicated main effects of Pulse [$F(1, 52) = 6.6$, $p = 0.01$; 104 dB > 94 dB] and Pre-pulse [$F(2,104) = 4.1$, $p < 0.05$; % PPI decreased as pre-pulse dB increased] (Table 1).

Startle on Pulse-Alone Trials: Three-way ANOVA (Pulse × Line × Sex) indicated a main effect of Pulse [$F(1,52) = 18.5$, $p < 0.01$; 104 dB > 94 dB], Line [$F(1,52) = 15.1$, $p < 0.01$; HAP2 > LAP2], and Sex [$F(1,52) = 8.4$, $p < 0.01$; Male > Female] (Table 1).

There was no correlation between body weight and startle magnitude on either the 94 dB (n = 56, r = 0.25, $p = 0.07$) or the 104 dB pulse trials (n = 56, r = 0.24, $p = 0.08$).

Post-treatment Session

Body Weight: Average (± SEM) body weights on the Post-treatment PPI session were 25.3 (± 0.3) g for HAP2 mice (males: 27.7 ± 0.5 g, females: 22.9 ± 0.5 g) and 25.0 (± 0.4) g for LAP2 mice (males: 2.6 ± 0.5 g, females: 23.3 ± 0.5 g). ANOVA (Line × Sex × Stress Exposure) indicated males weighed more than females [$F(1,48) = 70.3$, $p < 0.01$], and No Stress mice weighed more than Stress mice [$F(1, 48) = 22.2$, $p \leq 0.01$].

% PPI: A five-way ANOVA (Pulse × Pre-pulse × Line × Sex × Stress Exposure) indicated a Pulse × Pre-pulse × Line × Stress Exposure interaction [$F(2,96) = 4.9$, $p = 0.01$]. The four-way interaction was explored with three-way ANOVAs (Pre-pulse × Line × Stress Exposure) at each Pulse. For the 94 dB pulse, there was a Pre-pulse × Line × Stress Exposure interaction [$F(2,104) = 5.7$, $p < 0.01$], and follow-up Line × Stress Exposure ANOVAs at each pre-pulse dB indicated greater % PPI in Stress vs. No Stress groups on 78 and 82 dB pre-pulse trials [F's(1,52) > 5.9, p's < 0.05].

For the 104 dB pulse, there was a Line × Stress Exposure interaction [$F(1,52) = 6.6$, $p = 0.01$]. Analyses of Line within each Stress Exposure indicated greater % PPI in HAP2 than LAP2 mice in the No Stress groups [$F(1,26) = 11.1$, $p < 0.01$] only. Analyses of Stress Exposure within each line uncovered trends for opposing line-dependent effects of stress on % PPI (HAP2: $p = 0.11$; LAP2: $p = 0.06$), such that HAP2 Stress mice showed disrupted % PPI and LAP2 Stress mice showed enhanced % PPI (Fig. 2).

Startle on Pulse-Alone Trials: Four-way repeated-measures ANOVA (Pulse × Line × Sex × Stress Exposure) indicated a significant Pulse × Line × Sex × Stress Exposure interaction [$F(1,48) = 4.7$, $p < 0.05$]. Three-way follow-up ANOVAs were conducted within each between- and within-subject variable, and there was only a significant interaction when data were split by sex; in males, a significant Pulse × Line × Stress Exposure interaction [$F(1,23) = 6.0$, $p < 0.05$] was uncovered, but lower-order ANOVAs did not indicate significant effects.

There was no correlation between body weight and startle magnitude on either the 94 dB (n = 56, r = 0.26, $p = 0.06$) or the 104 dB pulse trials (n = 56, r = 0.15, $p = 0.26$).

Basal CORT Levels: Repeated-measures ANOVA of basal CORT levels on days 1 and 10 of the stress exposure (Day × Line × Sex × Stress Exposure) indicated a Day × Stress Exposure interaction [$F(1,40) = 9.6$, $p < 0.01$] that was due to significantly higher basal CORT levels in the Stress groups compared to the No Stress groups on stress exposure day 10 only [$F(1,51) = 6.9$, $p = 0.01$].

The ANOVA also indicated a Line × Stress Exposure interaction [$F(1,40) = 4.3$, $p < 0.05$]. Comparisons of Line within each Stress Exposure group and Stress Exposure within each Line indicated the interaction was due to significantly higher basal CORT (collapsed across sampling days) in Stress LAP2 than Stress HAP2 mice [$F(1,24) = 5.3$, $p < 0.05$] and basal CORT was higher in Stress than in No Stress LAP2 [$F(1, 23) = 4.9$, $p < 0.05$] but not HAP2 groups. Although these effects are based on data collapsed across sampling days, it appears the main effect of Stress in LAP2 mice is due to an effect on day 10 (Fig. 3).

Experiment 3

Experiment 3 assessed the effects of acute alcohol, repeated stress, and their combination on % PPI and startle in male and female HAP2 and LAP2 mice. A dose of 0.75 g/kg alcohol was used based on the results of Experiment 1, in which this dose significantly enhanced % PPI in HAP2 mice.

Baseline Session

% PPI: A four-way ANOVA (Pulse × Pre-pulse × Line × Sex) indicated that HAP2 mice showed greater % PPI than LAP2 mice [$F(1,197) = 29.6$, $p < 0.01$] similar to Experiment 1. A Pulse × Pre-pulse interaction was also uncovered [$F(1,197) = 29.6$, $p < 0.01$], and similar to Experiment 1, was due to reduced % PPI when the 86 dB pre-pulse preceded the 94 dB pulse (Table 1).

Startle on Pulse-Alone Trials: A three-way ANOVA (Pulse × Line × Sex) indicated a Pulse × Line interaction [$F(1,197) = 15.9$, $p < 0.01$] that was due to a

greater line difference (HAP2 > LAP2) at the 104 dB pulse than the 94 dB pulse (F's > 40.0, p's < 0.001) (Table 1).

There was a weak positive correlation between body weight and startle magnitude on both the 94 dB (n = 251, r = 0.24) and 104 dB (n = 251, r = 0.21) pulse trials.

Post-treatment Session

Body Weight: Average (± SEM) body weights on the Post-treatment PPI session were 25.1 (± 0.2) g for HAP2 mice (males: 26.2 ± 0.3 g, females: 24.0 ± 0.3 g) and 23.5 (± 0.2) g for LAP2 mice (males: 25.0 ± 0.3 g, females: 22.1 ± 0.3 g). ANOVA (Line × Sex × Stress Exposure) indicated a significant three-way interaction [$F(1,193) = 6.7$; $p = 0.01$]. Follow-up Sex × Stress Exposure ANOVAs were run within each line. For HAP2 mice, males weighed more than females [$F(1,95) = 29.7$; $p < 0.01$] and No Stress mice weighed more than Stress mice [$F(1,95) = 14.8$; $p < 0.01$]. For LAP2 mice, a Sex × Stress Exposure interaction was uncovered [$F(1,98) = 4.3$, $p < 0.05$]. One-way ANOVAs (Stress Exposure) within each Sex indicated a significant difference between Stress and No Stress LAP2 male mice [$F(1,40) = 7.3$, $p = 0.01$] but not in LAP2 female mice.

% PPI: A six-way ANOVA [Pulse × Pre-pulse × Line × Sex × Stress Exposure × Dose (0.75 g/kg alcohol or saline)] indicated a Pulse × Pre-pulse × Stress Exposure interaction [$F(2,370) = 6.2$, $p = 0.01$]. Two-way follow-up ANOVAs within each Pulse indicated a significant Pre-pulse × Stress interaction at the 94 dB Pulse [$F(2,398) = 5.9$, $p < 0.01$] only, resulting from lower in Stress

mice compared to No Stress mice on 78 + 94 pre-pulse + pulse trials [$F(1,199) = 7.3$, $p < 0.01$].

A Pre-pulse × Line × Sex × Stress Exposure × Dose interaction was also uncovered, and follow-up ANOVAs (Line × Sex × Stress Exposure × Dose) within each pre-pulse (collapsed by pulse) indicated lower % PPI in Stress mice compared to No Stress mice at the 78 dB pre-pulse [$F(1,185) = 4.7$, $p < 0.05$] and a trend for the same effect was also found at the 82 dB pre-pulse ($p = 0.09$).

Based on the results of Experiments 1 and 2, we did focused analyses to compare the effects of Dose (0.75 g/kg alcohol and saline) and Stress Exposure (Stress and No Stress) on % PPI within each line. For the Dose comparison, the ANOVA (Sex × Dose; collapsed across all Pulse and Pre-pulse dBs) on % PPI indicated no significant effects (Fs < 1.0, $p > 0.37$; Fig. 4A). For the Stress Exposure comparison, the ANOVA (Sex × Stress Exposure; collapsed across all Pulse and Pre-pulse dBs) on % PPI indicated no significant effects (Fs < 0.5, $p > 0.37$; Fig. 4B).

Startle on Pulse-Alone Trials: Five-way repeated-measures ANOVA (Pulse × Line × Sex × Stress Exposure × Dose) indicated a significant Pulse × Dose interaction [$F(1,185) = 8.7$, $p < 0.01$]. Although follow-ups did not reach significance, the interaction was due to a trend for increase startle in alcohol- compared to saline-pretreated mice on the 104 dB pulse trials.

There was a weak positive correlation between body weight and startle magnitude on the 94 dB (n = 251, r = 0.20; $p <0.05$) and 104 dB (n = 251, r = 0.22; $p < 0.05$) pulse trials.

Discussion

The purpose of this study was to assess the effects of alcohol, stress, and their combination on PPI in mice that differ in genetic predisposition toward alcohol preference (HAP2/LAP2). A 0.75-g/kg dose and a 1.0-g/kg dose of alcohol increased PPI in HAP2 but not LAP2 mice, suggesting that HAP2 and LAP2 mice are differentially sensitive to the effects of low-dose alcohol (Experiment 1). Ten days of repeated stress exposure decreased PPI in HAP2 mice and increased PPI in LAP2 mice, but these effects did not reach statistical significance (Experiment 2). In Experiment 3, the combination of stress and alcohol treatment did not alter PPI, which did not support the hypothesis that alcohol would ameliorate the effect of stress on PPI (Experiment 3).

Line differences in baseline PPI were observed between HAP2 and LAP2 mice (HAP2 > LAP2), which is consistent with our prior findings in these mice (Chester & Barrenha, 2007). Reports using selectively bred rats, however, indicate no baseline differences in PPI between high- and low-alcohol preferring animals (Acewicz et al., 2012; Jones et al., 2000). The reason for this inconsistency is unclear, but evidence suggests that strain and species differences can influence PPI under baseline conditions (Palmer et al., 2000; van den Buuse, 2003; see Pian, Criado, & Elhers, 2008 for a discussion of this issue).

HAP2 mice also exhibited greater baseline startle magnitude than LAP2 mice, replicating earlier reports in these lines (Barrenha & Chester, 2007; Chester & Barrenha, 2007). The line difference is also consistent with reports in selectively bred rats in which P, High-Alcohol Drinking (HAD1), HAD2 (replicate

line of HAD1), and Warsaw Alcohol High-Preferring rats all showed greater startle than their low-alcohol preferring counterparts (Acewicz et al., 2012; Chester, Blose, & Froehlich, 2003; Chester et al., 2004; Jones et al., 2000). The consistent line differences in baseline startle seen between these selectively bred rodents suggest there is overlap in the genes contributing to both alcohol preference and startle reactivity in these animals. This interpretation is based on the concept of pleiotropy, in which multiple traits may be influenced by the same genetic mechanisms, often described as a genetic correlation (Crabbe, Phillips, Kosobud, & Belknap, 1990). Evidence for a genetic correlation comes from observed differences in non-selected traits occurring between lines that differ in a particular trait of selection (Crabbe et al., 1990), in this case, startle reactivity and alcohol preference, respectively. Enhanced startle reactivity under baseline conditions is suggested to reflect greater emotional reactivity (i.e., higher anxiety state) (Davis, 1992; Davis, Walker, & Lee, 1997), and therefore the current findings support other data in which rodents with a genetic predisposition for high-alcohol preference show increased anxiety-like behaviors (e.g., Barrenha & Chester, 2007; McKinzie et al., 2000; Stewart, Gatto, Lumeng, Li, & Murphy, 1993).

Alcohol increased PPI for HAP2 mice and had no effect in LAP2 mice in Experiment 1, but this effect did not replicate in Experiment 3, likely due to differences in experimental procedures and a small effect size. A prior study by Jones et al. (2000) indicated a line difference in the opposite direction, that is, adult female P rats showed decreased as opposed to increased PPI following

low-dose alcohol. The current finding that alcohol increases PPI in HAP2 mice is also in contrast to reports in adult outbred rats (Wistar and Sprague-Dawley), in which alcohol had no effect on PPI (Acewicz et al., 2012; Brunell & Spear, 2006). The current findings in HAP2 mice and prior report in P rats suggest that a genetic predisposition for high-alcohol preference may be generally related to increased sensitivity to the effects of alcohol on PPI, independent of the direction of the effect. Sensitivity to the effect of alcohol on PPI may therefore be a phenotypic marker for increased genetic risk to develop alcoholism. We plan to further explore this possibility in another independently selected set of HAP/LAP lines (i.e., HAP3/LAP3).

It should be noted that, in Experiment 1, alcohol also increased startle on pulse-alone trials. However, evidence suggests that this effect is not related to the alcohol-induced increase in % PPI. The % PPI measure, calculated using the following formula: 1 − (average startle response on pre-pulse + pulse trials/average startle response on pulse-alone trials) × 100, adjusts for treatment group differences in startle responses on noise-alone trials and thus is an accurate method for detecting selective effects of drugs on PPI (Swerdlow, Braff, & Geyer, 2000). Further, numerous studies have shown no consistent relationship between startle response and PPI with drugs that increase startle magnitude (see Swerdlow, Geyer, & Braff, 2001).

The alcohol-induced increase in PPI in HAP2 mice could also be related to the line difference in baseline PPI (HAP2 > LAP2). Similar to our finding that alcohol increased PPI for HAP2 mice only, Hutchison, Rohsenow, Monti, Palfai,

& Swift (1997) found that low-dose alcohol increased PPI for humans with high baseline PPI, and decreased it in those with low baseline PPI. Hutchison speculated that baseline-dependent effects of alcohol on PPI might be related to differences in basal dopamine function that manifests as differential sensitivity to drugs that activate the dopamine system, like alcohol. Indeed, multiple studies comparing the dopamine system between rats selectively bred for high- and low-alcohol preference suggest basal line differences (reviewed by Murphy et al., 2002), lending support to Hutchison's speculation.

Experiments 2 and 3 assessed the effects of repeated restraint stress on PPI 30 min and 24 h after the termination of stress exposure, respectively. We previously showed that a history of repeated restraint stress had no effect on PPI in HAP2 mice (Chester et al., 2006), and other studies showed that stress (Sutherland et al., 2010; Sutherland & Conti, 2011) or stress hormones (Conti et al., 2002; Risbrough et al., 2004) decreased PPI in rodents, although one study did report an increase in PPI following foot shock stress (Pijlman et al., 2003). Stress exposure had no effect on PPI when assessed 24 h after stress termination; however, when PPI was assessed at 30 min after stress, stressed HAP2 mice showed a trend toward decreased PPI while stressed LAP2 mice showed a trend toward increased PPI compared to controls (Experiment 2). These results suggest that the direction of stress effects on PPI may depend on genetic predisposition toward alcohol preference, and that HAP2 mice are more sensitive to the disrupting effects of stress exposure (as measured by PPI) than LAP2 mice, who displayed an enhancement of PPI following stress exposure.

The typical HPA-axis response to stress is increased circulating levels of corticosteroids, which progressively return to basal levels following removal of the stressor (Björntorp, 2001). The gradual return to basal levels is mediated through a negative feedback system, where high levels of circulating corticosteroids inhibit the release of corticotropin-releasing hormone (CRH) from the hypothalamus, ultimately inhibiting subsequent release of CORT. Exposure to repeated stress can result in persistently elevated levels of basal circulating CORT (basal CORT) (Ottenweller et al., 1989; Pitman et al., 1988), possibly through stress-induced changes in brain glucocorticoid receptor numbers (McEwen, De Kloet, & Rostone, 1986; Sapolsky, Krey, & McEwen, 1984).

A previous report in HAP2/LAP2 mice (Chester et al., 2013) reported basal levels of CORT between about 10–20 ng/mL for HAP2 mice and between about 12–15 ng/mL for LAP2 mice, which are similar to another report in C57BL/6 (Harpaz et al., 2013). In the current study, basal CORT levels were slightly higher than what was reported in Chester et al. (2013): HAP mice ranged from about 15–50 ng/mL and LAP mice ranged from about 30–50 ng/mL. With regard to basal CORT levels after repeated stress exposure, the main variable of interest in the current study, we have no data in HAP/LAP mice to compare to because this experiment is the first study designed to examine this variable. But, in the literature, a study that examined basal CORT levels in C57BL/6 mice following repeated neck restraint stress (Spyrka & Hess, 2010) reported basal CORT levels comparable to what we report for LAP2 mice (75–130 ng/mL), but higher levels than what we found in HAP2 mice (40–50 ng/mL). Recall that the

basal CORT levels increased following stress only in LAP2 but not HAP2 mice. In the Spyrka & Hess study, C57BL/6 mice showed average basal CORT levels of 176 ng/mL and 126 ng/mL following 3 and 14 days of neck restraint, respectively.

It is interesting that stressed and non-stressed HAP2 mice did not show a difference in basal CORT levels following 10 days of stress exposure (Fig. 3). This may suggest that HAP2 mice are resistant to HPA-axis adaptations resulting from repeated stress exposure. This differential adaptability of HPA-axis function indicates a response that may be genetically correlated with alcohol preference (see Crabbe et al., 1990), an idea supported by evidence that dysregulation of the HPA-axis is a consequence of, and risk factor for, alcoholism (see review by Haddad, 2004).

Lower levels of basal circulating CORT following repeated stress in HAP2 compared to LAP2 mice is also interesting based on emerging evidence that HAP2 mice may be a useful model of human PTSD (Chester et al., 2013), which has been associated with altered HPA-axis function. Some studies in humans indicate reduced basal circulating CORT in PTSD patients compared to controls (see review by Golier & Yehuda, 1998). Although more work is needed assessing the utility of HAP2 mice as a model of PTSD, the current results encourage further characterization of HAP/LAP mice as a model for PTSD and/or alcoholism.

Our hypothesis that stress would reduce PPI for both lines was partially supported with HAP2 mice showing a trend toward reduced PPI and LAP2 mice showing a trend toward increased PPI (Experiment 2). Our further hypothesis

that alcohol would ameliorate stress-induced reduction of PPI in a line-dependent manner was not supported (Experiment 3). While alcohol did not ameliorate the stress-induced disruption of PPI observed in HAP2 mice, alcohol did increase PPI for HAP2 mice (Experiment 1). The major finding of the current study is that mice genetically predisposed for high- and low-alcohol preference are differentially sensitive to the effects of alcohol and stress on PPI. Therefore, these behaviors may represent phenotypic markers for increased genetic risk to develop alcoholism.

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FIGURE LEGENDS

Figure 1. Mean (± SEM) startle (top panels) and % PPI (bottom panels; collapsed across dB) on the Post-treatment PPI session of Experiment 1 in HAP2 (left panels) and LAP2 (right panels) mice. Mice received IP injections of either saline or alcohol (0.5, 0.75, or 1.0 g/kg) 5 min before PPI testing. *indicates main effect of Pulse ($p < 0.05$); #indicates significant difference from Saline ($p < 0.05$) using Dunnett's test.

Figure 2. Mean (± SEM) % PPI on the Post-treatment PPI session of Experiment 2 in HAP2 (left panel) and LAP2 (right panel) mice. Mice received either 10 days of repeated unpredictable stress exposure or were left undisturbed, depending on treatment group. Mice were tested for PPI 30 min following the termination of stress exposure. No IP injections were given prior to the PPI session. Data are presented collapsed by dB level. PP indicates that data are collapsed across pre-pulse dB level.

Figure 3. Mean (± SEM) levels of basal circulating CORT for HAP2 and LAP2 mice. Blood samples were taken at the start of the 1st and 10th stress exposure day for Experiment 2. *indicates LAP2 > HAP2, $p < 0.05$; #indicates Stress day 10 > Stress Day 1, $p < 0.05$.

Figure 4. Mean (± SEM) % PPI on the Post-treatment PPI session of Experiment 3 in HAP2 and LAP2 mice. Mice received either 10 days of repeated unpredictable stress exposure or were left undisturbed, depending on treatment group. Mice were tested for PPI 24 h following the termination of stress exposure and were given IP injections of saline or 0.75 g/kg alcohol 5 min before the start of the PPI session. Data are shown collapsed by pre-pulse and each pulse dB level. Top panels (A) show % PPI in Stress and No Stress mice separated by Line and collapsed across alcohol treatment. Bottom panels (B) show % PPI in mice pretreated with either saline or alcohol (0.75 g/kg), separated by Line, and collapsed across stress exposure.