2007

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Acknowledgements: Supported by Purdue Research Foundation and AA015424 to JAC. We also acknowledge center grant AA07611 (to Dr. David W. Crabb at the Indiana University School of Medicine) that provides support for the maintenance of the HAP/LAP lines. Special thanks to Dr. Nicholas J. Grahame for providing HAP2 and LAP2 mice and the breeders for the HAP1 and LAP1 mice.

Running Head: Acute Alcohol Withdrawal in Selected Mouse Lines

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Abstract

Background: Previous data in both rat and mouse genetic models suggest that there is a genetic relationship between acute alcohol withdrawal responses and innate alcohol drinking behavior. The purpose of the present study was to examine whether acute alcohol withdrawal responses, as measured by acoustic startle and prepulse inhibition (PPI) of acoustic startle, may be genetically related to innate differences in alcohol preference in two mouse lines selectively bred for high (HAP1 and HAP2) or low (LAP1 and LAP2) alcohol preference. Line differences in startle responses at baseline, prior to alcohol or saline treatment, were also measured.

Methods: Alcohol-naive, male and female HAP1 (n=35) and LAP1 (n=32) and HAP2 (n=43) and LAP2 (n=40) mice were tested under baseline conditions and during withdrawal from a single injection of 4.0 g/kg alcohol or equal volume of saline at 4, 8, and 12 hrs post-injection.

Results: On most trial types, baseline startle responses and PPI were greater in both HAP lines than in both LAP lines and startle responses were greater in males than in females. During acute alcohol withdrawal, both male LAP lines, and LAP1 females, showed reduced startle responses at the 4-hr time point during acute alcohol withdrawal. In contrast, both HAP1 males and females showed a trend toward enhanced startle at 4 hrs in withdrawal. No clear differences in PPI during withdrawal were evident.

Conclusions: These findings indicate good evidence for a genetic relationship between greater baseline acoustic startle responses and PPI and high alcohol preference. Modest support for a genetic correlation between low alcohol preference and reduced startle responses at 4 hrs in withdrawal was found in male mice. The suppression in acoustic startle during acute alcohol withdrawal in male LAP lines but not in male HAP lines suggests that a genetic propensity

toward low alcohol preference may be related to greater sensitivity to alcohol as measured by acoustic startle responses during acute alcohol withdrawal.

Key Words: withdrawal; drinking; acoustic startle; genetics; selected lines

Introduction

Genetic factors contribute a significant portion of the risk for developing alcohol-use disorders, which primarily include alcohol abuse and alcoholism (Crabbe, 2002; Oroszi and Goldman, 2004; Wall et al., 2000). Research indicates that individuals with an increased genetic risk for alcoholism (family history positive) may differ in sensitivity to behavioral, physiological and/or motivational (rewarding or aversive) effects of alcohol compared to individuals without a genetic risk for alcoholism (family history negative) (e.g., Schuckit et al., 2004). Alcohol withdrawal is one of the diagnostic criteria for alcoholism (American Psychiatric Association, 2000) and is thought to contribute to the development and maintenance of alcohol-use disorders (Cappell and LeBlanc, 1979; Koob, 2003). Alcohol withdrawal is typically defined as a constellation of physical signs and subjective symptoms that are evident in alcohol-dependent individuals. Signs and symptoms of alcohol withdrawal include tremor, seizures, anxiety, agitation, hallucinations, and mental confusion (Victor and Adams, 1953). Many of these signs and symptoms of alcohol withdrawal are also seen in milder form following acute alcohol exposure, often termed "hangover," in people that are not necessarily alcohol-dependent (Swift and Davidson, 1998).

The characteristics and severity of the alcohol withdrawal syndrome in humans is influenced by genetic factors (Schmidt and Sander, 2000) and results of several studies suggest that there is a genetic relationship between the frequency or severity of alcohol withdrawal/hangovers and propensity toward alcoholism. Individuals with an increased genetic risk for alcoholism have reported more frequent and more severe withdrawal symptoms than individuals without a genetic risk for alcoholism (McCaul et al., 1991; Newlin and Pretorius, 1990; Piasecki et al., 2005; Slutske et al., 2003; Span and Earleywine, 1999). However, it has

also been reported that a greater severity of alcohol withdrawal signs and symptoms may be protective against the development of alcoholism. For example, Wall and colleagues (2000) have shown that individuals at low risk for developing alcoholism, because they carry a mutated form of an alcohol-metabolizing gene (aldehyde dehydrogenase-2), display greater alcohol withdrawal signs and symptoms.

Interpretation of reports in humans of the relation between alcohol withdrawal frequency and/or severity and genetic risk for alcoholism is potentially complicated by several factors, including genetic heterogeneity of the sample population and differences in history of alcohol exposure. Further, alcohol withdrawal episodes may change in frequency and/or severity with repeated alcohol exposure and the nature of these changes may depend on individuals' genetic risk for alcoholism. For example, Piasecki et al. (2005) conducted a longitudinal study over an 11-year period in subjects from approximately age 18 to 29 and found that a positive association between hangover frequency and family history of alcoholism weakened over time.

Rodent models of alcoholism are useful for exploring the relationship between sensitivity to alcohol withdrawal and genetic propensity toward alcohol drinking because many signs of alcohol withdrawal in humans are similar to that seen in rodents (Kalant, 1977) and because alcohol exposure can be controlled. Controlled alcohol exposure in alcohol-naïve animals is particularly useful when trying to study how initial sensitivity to alcohol's effects, such as "hangover" or withdrawal, may be related to genetic risk for developing alcohol-use disorders. Results of previous studies using several genetic mouse models indicate a robust negative genetic correlation between innate propensity toward alcohol drinking and alcohol withdrawal magnitude following acute alcohol treatment (hereafter referred to as "acute alcohol withdrawal"). That is, alcohol-naïve mice that are known to voluntarily drink lower amounts of

alcohol show greater signs of acute alcohol withdrawal than mice that voluntarily drink higher amounts of alcohol, when acute alcohol withdrawal was measured using handling-induced convulsions (HICs) (Crabbe, 1983; Metten and Crabbe, 2005; Metten et al., 1998; Rodgers, 1966). This inverse genetic association between acute alcohol withdrawal and alcohol drinking behavior has also been supported in studies using selectively bred rat lines using several different indices of acute alcohol withdrawal in the rat (Chester et al., 2002, 2003, 2006). Overall, these data suggest that genes that regulate alcohol drinking behavior also influence the magnitude of acute alcohol withdrawal. It appears that, in rodents, increased initial sensitivity to acute alcohol withdrawal may be related to a decreased propensity toward alcohol consumption.

The purpose of the present study was to examine the genetic relationship between acute alcohol withdrawal and innate differences in alcohol drinking behavior in two pairs of mouse lines selectively bred for high alcohol preference (HAP1 and HAP2 lines) or low alcohol preference (LAP1 and LAP2 lines). These replicate mouse lines were selectively bred from a genetically defined progenitor population of outbred HS/Ibg mice (Grahame et al., 1999) that were originally created by an intercross of eight different inbred mouse strains (McClearn et al., 1970). In our work, acute alcohol withdrawal is defined as behavioral changes that occur following acute alcohol exposure at times when blood alcohol levels are falling and after blood alcohol levels have reached zero mg %. Acute alcohol withdrawal in the present study was measured using acoustic startle responses and prepulse inhibition (PPI) of the acoustic startle response. Several investigators have used acoustic startle and PPI as measures of alcohol withdrawal in rodents following both acute (Chester et al., 2003; 2004) and chronic (Chester et al., 2005; Gilliam and Collins, 1986; Macey et al., 1996; Pohorecky and Roberts, 1991; Pohorecky et al., 1976; Rassnick et al., 1992; Slawecki and Ehlers, 2005; Slawecki et al., 2006;

Vandergriff et al., 2000) alcohol treatment. However, there are no studies, to our knowledge, in which acoustic startle responses or PPI during acute alcohol withdrawal have been examined in mice, or, in which the genetic relationship between innate alcohol preference and acute alcohol withdrawal has been tested in mice using acoustic startle responses to measure acute withdrawal. Because both increases and decreases in startle and PPI during withdrawal from both acute and chronic alcohol exposure have been previously reported in rats, it was difficult to predict the outcome of this study in selectively bred mouse lines exposed to acute alcohol treatment. However, it was expected that acoustic startle responses and PPI would provide two different sensitive measures of line differences in acute alcohol withdrawal and that a similar genetic correlation between the acute alcohol withdrawal response and genetic propensity toward high or low alcohol preference would be found in both the HAP1/LAP1 and HAP2/LAP2 replicate lines. We also examined baseline startle and PPI responses in the replicate lines prior to alcohol treatment, as these phenotypes have served as useful measures of cognition and emotion-related brain mechanisms that may be associated with risk for alcoholism (Grillon et al., 2000).

Materials and Methods

***Subjects*:** Subjects were adult male and female, alcohol-naive HAP and LAP mice from replicate 1 (HAP1, n=35; LAP1, n=32) and replicate 2 (HAP2 n=43; LAP2 n=40). Replicate 1 was from the 27th generation and replicate 2 was from the 19th generation of selection for high or low alcohol preference. HAP and LAP lines from both replicates were produced by mass selection from outbred HS/Ibg mice (Boulder, CO) at the Indiana Alcohol Research Center (IARC) in Indianapolis, IN. In every generation of selection, high or low alcohol preference was established during a four-week, 24-hr, free-choice alcohol preference test (Grahame et al., 1999).

Replicate 1 subjects were generated at Purdue University from HAP1/LAP1 breeders obtained from the IARC and replicate 2 subjects were obtained directly from the IARC and were allowed to acclimate to the colony room for a minimum of 2 weeks prior to the start of the experiment.

In the current study, both replicate lines were between 70-81 days old during baseline testing. During alcohol withdrawal testing, replicate 1 lines were between 76-88 days old and replicate 2 lines were between 77-103 days old. Mice were housed in polycarbonate cages (11.5" X 7.5" X 5.0") with aspen wood shavings in groups of 2-4 per cage. Ambient temperature in the colony room was maintained at 21±1ºC and animals had free-access to food (Rodent Lab Diet 5001, Purina Mills Inc.) and water throughout the experiments. Experimental procedures were conducted during the light phase of a 12:12 light/dark cycle (lights on at 0700/off at 1900).

***Drugs*:** Alcohol was diluted from a 95% (v/v) solution to a concentration of 20% (v/v) with physiological saline (0.09%) and was administered intraperitoneally (IP) in a dose of 4.0 g/kg body weight. Each mouse received 25.3 ml of the 20% alcohol solution per kg body weight such that the correct dose was achieved by adjusting the volume of alcohol solution to the weight of each mouse.

Apparatus and Startle Testing Parameters: Acoustic startle responses were measured in a Coulbourn Instruments Animal Acoustic Startle System (Coulbourn Instruments, Allentown, PA). The system consisted of four weight-sensitive platforms located inside a sound attenuated chamber connected to an interfaced computer. The four platforms were equidistant from speakers located in the chamber's floor and ceiling. Mice were placed individually into open-air

holders (8 x 8 x 16 cm). The holders rested on top of the weight-sensitive platforms that measured subjects' maximum force (in grams) exerted against the platform during the 200 ms after the onset of each acoustic stimulus. All experimental sessions were run in the alternating current coupled mode, which produces output data in absolute grams of force and does not include subjects' body weight in the force measurement.

Each acoustic startle test session began with a 5-min acclimation period during which time no acoustic stimuli were presented. A ventilating fan provided continuous background noise (75 dB). Following the 5-min acclimation period, twelve different stimulus types were presented in a random order to avoid habituation to the acoustic stimuli. Each trial type was presented 12 times on a random intertrial interval that ranged from 10-20 sec, for a total of 144 trials throughout a 50-min test session. The trial types consisted of one blank trial (no pulse), two pulse alone trials (110 and 125 dB; 40 msec), three prepulse alone trials (79, 85, and 91 dB; 20 msec), and six prepulse + pulse trials (79, 85, or 91 dB + 110 or + 125 dB). The dB intensity of each startle stimulus was verified prior to the start of the experiment with a Digital Sound Level Meter (Radio Shack, Ft. Worth, TX).

Startle Testing Procedures: Replicate 1 and replicate 2 lines were tested in separate experiments that were conducted over a period of 9 days (replicate 1) or 13 days (replicate 2) due to time constraints associated with the test session duration, time point in withdrawal, and number of subjects. Mice were tested within and across days in a balanced order with regard to line, sex, and treatment group. Two alcohol-treated and two saline-treated mice were represented in each testing session. Each experiment consisted of two phases: startle testing at baseline and during acute alcohol withdrawal.

For startle testing during alcohol withdrawal, mice received a single IP injection of either alcohol (4.0 g/kg; 20% v/v) or an equal volume of saline (between 0700-0800 hrs) in the colony room and were moved to an adjacent room that contained the acoustic startle apparatus. Startle amplitude and PPI during acute alcohol withdrawal were measured at 4, 8, and 12 hrs post-injection because these are the time points at which alcohol withdrawal signs, as measured by HICs, begin to emerge (4 hrs), peak (8 hrs), and disappear (12 hrs) in mice (Kosobud and Crabbe, 1986), including the HAP1/LAP1 lines (P. Metten, N.J. Grahame, and J.C. Crabbe, unpublished data), following a single i.p. injection of 4.0 g/kg alcohol.

***Blood Alcohol Concentration (BAC) Analyses*:** BAC was assessed at four time points to examine the alcohol pharmacokinetic profile in both replicate lines and to provide a measurement of BAC corresponding to the times of acoustic startle testing during alcohol withdrawal. In replicate 1 mice, BAC was measured in a portion of mice from the saline-treated groups approximately 4-5 weeks after acoustic startle testing. In replicate 2 mice, BAC was measured in a portion of mice from the alcohol-treated groups approximately 6-8 weeks after acoustic startle testing.

HAP1 male (n=7), HAP1 female (n=8), LAP1 male (n=6), LAP1 female (n=9), HAP2 male (n=10), HAP2 female (n=9), LAP2 male (n=8), and LAP2 female (n=12) mice received an IP injection of alcohol (4.0 g/kg; 20% v/v) and blood samples (~20 μl) were obtained from the tip of the tail at 2, 4, 6, and 8 hrs after injection of alcohol. 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).

Data analyses: The amplitude of the acoustic startle response was determined for each mouse by calculating the average amplitude of the twelve startle responses (in grams of force) on each stimulus trial type. PPI was determined for each mouse using the following formula: [average startle response on pulse trials – average startle response on prepulse + pulse trials)/average startle response on pulse trials)] X 100.

All data were analyzed using analysis of variance (ANOVA). Data from replicate 1 lines were analyzed separately from replicate 2 lines because the data were collected in separate experiments. Startle data on prepulse (20 msec) trial types were analyzed separately from startle data on pulse (40 msec) trial types. In addition, PPI data on 110 dB and 125 dB pulse trial types were analyzed separately because pilot work in our laboratory indicated that differences in the intensity of the pulse stimulus may produce qualitative differences in PPI as a function of line and sex. Between-group factors were Line, Sex, and Treatment and within-group factors were Trial Type and Withdrawal Hr. To simplify presentation of the results, only significant main effects and the highest order interactions from within- and between-subject effects are reported from the ANOVA outputs. Significant interactions were followed with lower-order ANOVAs and post-hoc *t*-tests to determine the source of the interactions (Keppel, 1991). Probability values equal to or less than 0.05 were considered significant.

Results

Baseline Startle

HAP1/LAP1 Lines. The prepulse trials ANOVA indicated significant main effects of Line [$F(1,63)=9.4$, $p<0.01$], Sex [$F(1,63)=7.6$, $p<0.01$], Trial Type [$F(2,126)=34.1$, $p<0.01$], and

a Line x Sex x Trial Type interaction [$F(2,126)=3.6$, $p<0.05$]. Further analysis of the three-way interaction (Line x Sex ANOVAs at each trial type) showed that at the 79 dB trial type, LAP1 mice displayed higher startle than HAP1 mice [$F(1,63)=6.6$, $p=0.01$]; however, HAP1 mice displayed greater startle than LAP1 mice at the 85 and 91 dB trial types [$Fs(1,63)\geq 7.2$, $Ps\leq 0.01$]. Further, male mice displayed greater startle than female mice [$Fs(1,63)\geq 4.2$, $Ps<0.05$] at all three prepulse trial types.

The pulse trials ANOVA yielded a significant main effect of Sex [$F(1,63)=7.5$, $p<0.01$], indicating that startle was higher in males than in females, and a Line x Trial Type interaction [$F(1,63)=6.4$, $p=0.01$]. However, follow-up analyses of Line at each trial type were not significant (data shown in Table 1).

HAP2/LAP2 Lines. The prepulse trials ANOVA indicated significant main effects of Line [$F(1,79)=39.5$, $p<0.01$], Sex [$F(1,79)=7.6$, $p<0.01$], Trial Type [$F(2,158)=30.4$, $p<0.01$], and interactions of Line x Trial Type [$(2,158)=5.3$, $p<0.01$] and Sex x Trial Type [$2,158)=5.2$, $p<0.01$]. Follow-up analyses of the two-way interactions indicated that startle was higher in HAP2 mice than in LAP2 mice at all three prepulse trial types [$Fs(1,81)\geq 26.1$, $Ps<0.01$] and that startle was higher in males than in females at the 85 and 91 dB prepulse trial types [$Fs(1,81)\geq 8.0$, $Ps<0.01$].

The pulse trials ANOVA yielded significant main effects of Line [$F(1,79)=17.8$, $p<0.01$], Sex [$F(1,79)=8.2$, $p<0.01$], Trial Type [$F(1,79)=4.6$, $p<0.05$], and a Line x Sex x Trial Type interaction [$F(1,79)=5.3$, $p<0.05$]. Further analysis of the three-way interaction (Line x Sex ANOVAs at each trial type) showed that HAP2 mice displayed greater startle than LAP2 mice [$Fs(1,79)\geq 11.0$, $Ps\leq 0.01$] and that male mice displayed greater startle than female mice [$Fs(1,79)\geq 5.5$, $Ps<0.05$] at both pulse trial types (data shown in Table 1).

Insert Table 1 about here

Baseline PPI

***HAP1/LAP1 lines*.** The prepulse + 110 dB ANOVA showed no significant main effects or interactions. The prepulse + 125 dB ANOVA yielded main effects of Line [$F(1,63)=4.5$, $p<0.05$], indicating that PPI was higher in HAP1 than in LAP1 lines, and PPI Trial Type [$F(2,126)=4.7$, $p=0.01$], indicating prepulse dependent effects on PPI (greater PPI on the 79 + 125 dB trials) (data shown in Table 2).

***HAP2/LAP2 lines*.** The prepulse + 110 dB ANOVA showed a significant main effect of PPI Trial Type [$F(2,158)=4.5$, $p=0.01$] and a Line x PPI Trial Type interaction [$F(2,158)=3.9$, $p<0.05$]. Follow-up analyses of Line at each PPI trial type indicated greater PPI in HAP2 than LAP2 mice on the 79+110 and 85+110 dB trial types ($Fs>4.5$, $Ps<0.05$). The prepulse + 125 dB ANOVA showed a main effect of PPI Trial Type [$F(2,158)=9.0$, $p<0.01$] and a Sex x PPI Trial Type interaction [$F(2,158)=3.7$, $p<0.05$]. Follow-up analyses of Sex at each PPI trial type indicated that males showed greater PPI than females on the 79+125 dB trials [$F(1,81)=5.0$, $p<0.05$] (data shown in Table 2).

Insert Table 2 about here

Body Weight Before Alcohol or Saline Treatment

For replicate 1 mice, analysis of body weights prior to alcohol or saline treatment (Line x Sex x Treatment subgroup ANOVAs) indicated no body weight differences between Treatment subgroups but did show greater body weight in LAP1 than HAP1 mice [$F(1,59)=221.5$, $p<0.01$] and in males than females [$F(1,59)=122.6$, $p<0.01$]. Mean (±sem) body weight was 27.2±0.5 g

and 22.0±0.4 g for male and female HAP1 mice, respectively, and 35.0±0.5 g and 29.1±0.5 g for male and female LAP1 mice, respectively.

For replicate 2 mice, the ANOVA indicated no effects of Line or Treatment subgroup but did show greater body weight in males than females [$F(1,75)=38.8$, $p<0.01$]. Mean (±sem) body weight was 28.0±0.5 g and 25.5±0.4 g for male and female HAP2 mice, respectively, and 29.5±0.8 g and 25.5±0.5 g for male and female LAP2 mice, respectively. Pearson correlations between body weight and startle amplitude in response to the prepulse and pulse acoustic stimuli during both baseline and withdrawal testing were not significant (Pearson coefficients <0.1, n=150).

Startle During Acute Alcohol Withdrawal

***HAP1/LAP1 lines*.** The prepulse trials ANOVA indicated a significant main effect of Trial Type [$F(2,118)=58.5$, $p<0.01$] and Line x Sex x Withdrawal Hr x Trial Type [$F(4,236)=3.0$, $p<0.05$] and Line x Sex x Treatment x Withdrawal Hr [$F(2,118)=4.8$, $p=0.01$] interactions. Because treatment effects were of primary interest, the source of the four-way interaction with the Treatment factor was investigated using lower-order Line x Sex x Treatment ANOVAs at each time point in withdrawal (collapsed across prepulse intensity). These analyses showed a main effect of Line [$F(1,59)=7.94$, $p<0.01$] and a Line x Treatment interaction [$F(1,59)=14.4$, $p<0.001$] at the 4-hr time point in withdrawal. The interaction was due to a strong trend toward enhanced startle in male and female HAP1 mice [$F(1,33)=3.5$, $p=0.07$] and significantly reduced startle in male and female LAP1 mice [$F(1,30)=15.1$, $p=0.001$] at 4 hrs in withdrawal (see Figure 1, top panels). No significant main effects or interactions were found at 8 or 12 hrs in withdrawal.

The pulse trials ANOVA indicated a significant main effect of Withdrawal Hr [$F(2,118)=3.2$, $p<0.05$] and a Line x Treatment x Withdrawal Hr interaction [$F(2,118)=5.8$, $p<0.01$]. Follow-up Line x Treatment ANOVAs at each time point in withdrawal (collapsed across pulse intensity) showed only a Line x Treatment interaction at the 4-hr time point in withdrawal [$F(1,63)=9.9$, $p<0.01$]. Similar to that found with the prepulse analyses, the interaction was due to reduced startle in male and female LAP1 mice [$F(1,30)=9.8$, $p<0.01$] and a trend toward enhanced startle in male and female HAP1 mice [$F(1,33)=2.6$, $p=0.1$] at 4 hrs in withdrawal (Figure 1, top panels).

Insert Figure 1 about here

***HAP2/LAP2 lines*.** The prepulse trials ANOVA indicated significant main effects of Line [$F(1,75)=51.1$, $p<0.01$], Treatment [$F(1,75)=9.6$, $p<0.01$], Withdrawal Hr [$F(2,150)=4.9$, $p<0.01$], and Trial Type [$F(2,150)=26.8$, $p<0.01$], and a Line x Sex x Withdrawal Hr x Trial Type interaction [$F(4,300)=2.8$, $p<0.05$]. Interactions with the Treatment factor were not significant; thus, follow-up analyses were not conducted. The significant main effect of Treatment without interactions with other factors suggests that startle was reduced in both the HAP2 and LAP2 lines across all three withdrawal hr time points. However**,** Line x Treatment x Trial Type and Sex x Treatment x Withdrawal Hr x Trial Type interactions were close to statistical significance ($Fs>2.2$, $Ps=0.07$). As can be seen in Figure 2, these interactions seem to be due in part to the robust reduction in startle in alcohol-treated LAP2 males across withdrawal testing time points compared to the other groups.

The pulse trials ANOVA indicated significant main effects of Line [$F(1,75)=31.7$, $p<0.01$], Withdrawal Hr [$F(2,150)=13.6$, $p<0.01$], and a Treatment effect very close to

significance [F(1,75)=3.8, p=0.055]. Line x Withdrawal Hr x Trial Type [F(2,150)=5.9, p<0.01] and Line x Sex x Treatment [F(1,75)=4.3, p<0.05] interactions were also found. Because treatment effects were of primary interest, the Line x Sex x Treatment interaction was investigated two ways. First, Sex x Treatment (collapsed across withdrawal hr and pulse intensity) ANOVAs within each line were conducted which yielded no effects or interactions in the HAP2 line. In the LAP2 line, the ANOVA yielded a main effect of Sex [F(1,36)=4.5, p<0.05] due to higher startle in males than females and a Sex x Treatment interaction [F(1,36)=4.5, p<0.05]. The interaction was due to reduced startle during withdrawal in alcohol-treated LAP2 males but not in LAP2 females (Treatment effect: p=0.09 in LAP2 males, p=0.6 in LAP2 females). Second, Line x Treatment ANOVAs within each sex (collapsed across withdrawal hr and pulse intensity) were conducted which showed Line effects in both females and males (Fs>8.3, Ps<0.01) due to greater startle in HAP2 mice than in LAP2 mice and a Line x Treatment interaction very close to significance in females [F(1,40)=3.8. p=0.058]. The interaction was due to reduced startle throughout withdrawal testing in alcohol-treated HAP2 females but not in LAP2 females (see Figure 2).

Insert Figure 2 about here

PPI During Acute Alcohol Withdrawal

***HAP1/LAP1 lines*.** The prepulse + 110 dB ANOVA showed a significant effect of PPI Trial Type [F(2,118)=16.5, p<0.01] and a Line x PPI Trial Type interaction [F(2,118)=4.7, p=0.01]. The prepulse + 125 dB ANOVA showed significant effects of Sex [F(1,59)=9.3, p<0.01], due to greater PPI in males than females, and PPI Trial Type [F(2,118)=20.9, p<0.01]

and Line x PPI Trial Type [$F(2,118)=6.0$, $p<0.01$] and Line x Withdrawal Hr [$F(2,118)=3.5$, $p<0.05$] interactions (data shown in Figure 3).

***HAP2/LAP2 lines*.** The prepulse + 110 dB ANOVA showed no significant main effects or interactions. The prepulse + 125 dB ANOVA showed significant main effects of Withdrawal Hr [$F(2,150)=5.3$, $p<0.01$] and PPI Trial Type [$F(2,150)=9.9$, $p<0.01$] and a Withdrawal Hr x PPI Trial Type interaction [$F(4,300)=3.5$, $p<0.01$] (data shown in Figure 3).

Insert Figure 3 about here

BAC Analyses

For replicate 1 mice, analysis of body weights taken prior to alcohol injection (Line x Sex ANOVAs) indicated that body weights were similar to that recorded prior to acoustic startle testing [greater in LAP1 than HAP1 mice and greater in males than females ($Ps<0.01$)]. Mean (±sem) body weight was 29.6±0.6 g and 24.0±0.8 g for male and female HAP1 mice, respectively, and 37.2±1.0 g and 32.9±0.6 g for male and female LAP1 mice, respectively.

Analysis of BAC showed no line differences. A main effect of Sex [$F(1,26)=8.5$, $p<0.01$] and Hr [$F(3,78)=448.8$, $p<0.01$] and a Sex x Hr interaction [$F(3,78)=4.6$, $p<0.01$] were found. The interaction was due to significantly greater BAC in males than in females at hr 4 ($p<0.05$) and hr 6 ($p<0.001$) (Table 3).

For replicate 2 mice, the ANOVA indicated that body weight was greater in LAP2 than HAP2 mice ($p<0.05$) and greater in males than females ($p<0.01$). Mean (±sem) body weight was 30.4±0.8 g and 26.6±0.6 g for male and female HAP2 mice, respectively, and 34.3±1.9 g and 28.8±1.2 g for male and female LAP2 mice, respectively. This line difference in body weight was not previously seen in these mice (from the alcohol-treated groups in the acoustic startle

portion of the experiment) and can be attributed to a greater increase in body weight in LAP2 mice since the time of acoustic startle testing. Analysis of BAC indicated a main effect of Line [$F(1,35)=4.4$, $p<0.05$], Hour [$F(3,105)=718.4$, $p<0.01$] and a Line x Hour interaction [$F(3,105)=2.8$, $p<0.05$]. The interaction was due to significantly greater BAC in LAP2 than HAP2 mice at the 6 hr sampling time point ($p=0.01$) (Table 3).

Insert Table 3 about here

Discussion

Alcohol withdrawal has long been thought to influence alcohol drinking behavior and the risk for alcoholism (Cappell and LeBlanc, 1979). In humans, there is some evidence that common genetic mechanisms influence both alcohol withdrawal severity and propensity to consume alcohol (McCaul et al., 1991; Newlin and Pretorius, 1990; Piasecki et al., 2005; Slutske et al., 2003; Span and Earleywine, 1999; Wall et al., 2000), but the nature of this association is not well-understood. Further, little is known about how individual differences in initial response to alcohol withdrawal in alcohol-naïve individuals may influence subsequent alcohol drinking behavior and risk for alcoholism. One way to assess the relationship between alcohol withdrawal and alcohol drinking is to examine whether a genetic correlation between the two traits exists using a genetic animal model. In the present study, replicate mouse lines selectively bred for high (HAP lines) or low (LAP lines) alcohol preference were tested for differences in acute alcohol withdrawal following a single alcohol treatment using acoustic startle responses and PPI. We found modest support for a genetic correlation between alcohol preference and the acoustic response to alcohol at 4 hrs in acute alcohol withdrawal in male mice. That is, both male LAP lines showed reduced startle responses at the 4-hr time point when BAC was falling

during acute alcohol withdrawal. In contrast, HAP1 males showed a trend toward enhanced startle, whereas HAP2 males showed no change in startle, at 4 hrs in withdrawal despite showing comparable BAC. LAP2 but not LAP1 males showed a trend toward reduced startle at 8 and 12 hrs in withdrawal. Similar to HAP1 males, HAP1 females showed a trend toward enhanced startle at 4 hrs in withdrawal, whereas HAP2 females evidenced a trend toward reduced startle throughout withdrawal testing. Overall, the suppression in acoustic startle during acute alcohol withdrawal in both male LAP lines but not in male HAP lines suggests that a genetic propensity toward low alcohol preference may be related to greater sensitivity to alcohol's effects during acute alcohol withdrawal, as measured by acoustic startle responses.

Under baseline conditions, good evidence was found for a positive genetic correlation between startle responses and innate alcohol preference. Both male and female HAP lines showed greater startle responses than both male and female LAP lines. Overall, male mice displayed greater startle responses than female mice, a finding consistent with published reports in both rats (Blaszczyk and Tajchert, 1996; Lehmann et al., 1999) and mice (Plappert et al., 2005). The greater startle responses in HAP than in LAP mice is consistent with prior results in the selectively bred alcohol-preferring (P) and alcohol-nonpreferring (NP) rat lines in which male (Chester et al., 2003; 2004) and female (Jones et al., 2000) P rats showed greater startle reactivity than NP rats. Given that the acoustic startle response has been suggested to measure anxiety-related behavior, these data suggest that there may be overlapping genetic mechanisms that contribute to a greater level of baseline anxiety and high innate preference for alcohol in certain selectively bred rodent models, including the HAP/LAP replicate lines tested in the present study. Thus, the HAP/LAP replicate lines may be an excellent model to study emotion-related brain mechanisms that may be associated with genetic risk for alcoholism.

PPI, which refers to a reduced startle response when the startling stimulus is preceded by a weak pre-stimulus, is an adaptive response displayed by most mammalian species that reflects sensorimotor gating mechanisms regulating attention and sensory information (Swerdlow et al., 2000). This measure is often used to assess cognitive processes associated with psychiatric disease states in normal, at-risk, and affected populations (e.g., Braff et al., 2001). Deficits in PPI have been reported in children (Grillon et al., 1997) and adults (Grillon et al., 2000) with increased genetic risk for alcoholism, suggesting that disrupted PPI might be a marker for alcoholism vulnerability. Comparable levels of PPI have previously been reported in the selectively bred P and NP rat lines (Jones et al., 2000). In contrast, results of the present study indicated that PPI was higher in mice with a genetic propensity toward high alcohol preference (HAP lines) than low alcohol preference (LAP lines). Specifically, HAP1 lines showed greater PPI than LAP1 lines on the prepulse + 125 dB trial types and HAP2 lines showing greater PPI than LAP2 lines on the 79+110 and 85+110 dB trial types. These results suggest that there may be common genetic mechanisms that regulate PPI and alcohol preference in these selected mouse lines.

Disruptions in PPI during withdrawal from chronic alcohol exposure have been reported in rats (Rassnick et al., 1992) and in human alcoholics (Keedwell et al., 2001), suggesting that cognitive processes may be disordered during alcohol withdrawal. On the other hand, a recent report in rats indicated that PPI was enhanced during withdrawal from chronic alcohol exposure (Slawecki et al., 2006). In the present study, no clear differences in PPI during acute alcohol withdrawal were found in any line. Future studies are planned to examine PPI in the HAP/LAP mouse lines during withdrawal from chronic alcohol treatment.

To our knowledge, this study is the first to report the effects of a single alcohol treatment on acoustic startle responses during acute alcohol withdrawal in mice. Although alcohol intoxication following acute (Brunell and Spear, 2006; Owens et al., 2003; Pohorecky et al., 1976) and chronic (Rassnick et al., 1992) alcohol exposure has been shown to reduce startle reactivity in rodents, the suppression in startle at the 4-hr time point during acute alcohol withdrawal observed in the LAP lines does not seem related to BAC. This interpretation is supported by the fact that all groups showed comparable BAC at the 4-hr time point; however, the HAP1 male and female lines showed a trend toward enhanced startle whereas the male LAP lines showed suppressed startle at 4 hrs in withdrawal. Further, LAP2 males and HAP2 females showed a trend toward suppressed startle at 8 and 12 hrs in withdrawal after BAC was very low or completely metabolized. These reductions in acoustic startle during acute alcohol withdrawal are consistent with prior reports in mice (Gilliam and Collins, 1986) and rats (Chester et al., 2005; Slawecki and Ehlers, 2005; Slawecki et al., 2006) in which startle was reduced during withdrawal from chronic alcohol exposure at time points well beyond that which alcohol was still present in the blood. In fact, in the Gilliam and Collins (1986) study, startle was suppressed to a similar extent throughout withdrawal testing, that is, at early time points when alcohol was presumably still present in the blood (e.g., 1 hr in withdrawal after measured BACs were approximately 200 mg %) and at later withdrawal time points after alcohol was metabolized (e.g., 27 hrs in withdrawal). Taken together, these findings suggest that reduced acoustic startle during acute alcohol withdrawal may not be due to effects of alcohol intoxication on startle. Still, we cannot rule out the possibility that the present results reflect line differences in sensitivity to alcohol intoxication on startle, rather than sensitivity to alcohol withdrawal per se.

Additional studies are planned to examine the timecourse of effects on startle reactivity in the HAP/LAP selected lines following acute administration of various alcohol doses.

It is generally accepted that the acoustic startle reflex is a good measure of both inhibitory and excitatory changes in central nervous system (CNS) excitability (Davis, 1984) and has been used to index CNS hyperexcitability that is known to occur during alcohol withdrawal (Finn and Crabbe, 1997). Indeed, a number of studies in rats have shown enhanced startle responding during withdrawal from chronic alcohol exposure (Macey et al., 1996; Pohorecky and Roberts, 1991; Pohorecky et al., 1976; Rassnick et al., 1992; Vandergriff et al., 2000). However, the reduced startle during acute alcohol withdrawal observed in the present study, and in prior reports during withdrawal from chronic alcohol exposure (Chester et al., 2005; Gilliam and Collins, 1986; Slawecki and Ehlers, 2005; Slawecki et al., 2006), suggests that CNS excitability may be suppressed during withdrawal under certain conditions. Gilliam and Collins (1986) provided good evidence to support this concept by showing that both increased and decreased behavioral responses could be detected in mice during alcohol withdrawal, and that the direction of these responses depended on genetic background and the endpoint used to index alcohol withdrawal. For example, mice selectively bred for greater resistance to alcohol's narcotic effect [Short-Sleep (SS) line] showed reduced acoustic startle responses and elevated heart rate during withdrawal from 7 days of alcohol exposure whereas their Long-Sleep (LS) counterparts showed little change in startle responses but did display elevated heart rate. Moreover, although SS mice showed reduced startle throughout withdrawal testing, SS mice have previously demonstrated greater HICs, a common measure of CNS excitability in the mouse (Goldstein, 1973), during alcohol withdrawal compared to the LS line (Goldstein and Kakihana, 1975). It is interesting to note that LAP1 mice have previously been shown to exhibit

greater HICs than HAP1 mice during the same acute alcohol withdrawal timecourse as that used in the present study (P. Metten, N.J. Grahame, and J.C. Crabbe, unpublished data). Together, these findings suggest that reduced acoustic startle and increased HICs during alcohol withdrawal reflect different components of the alcohol withdrawal syndrome, a conclusion offered by Gilliam and Collins (1986) over 20 years ago. Certainly, an alternative interpretation for reduced startle during alcohol withdrawal that must be considered is that alcohol intoxication or withdrawal interferes with motor function regulated by the startle reflex pathway and is not reflective of CNS excitability per se. We plan to further examine the genetic relationship between responses during acute alcohol withdrawal and propensity toward alcohol drinking in the HAP/LAP mouse lines using a variety of alcohol withdrawal measures. It will also be very important to test how innate differences in alcohol preference may be related to alcohol withdrawal responses following chronic alcohol exposure, especially in light of evidence suggesting that different genetic mechanisms regulate withdrawal from acute versus chronic alcohol exposure (Buck et al., 2002; Chester et al., 2005).

That different behavioral indices of alcohol withdrawal may be tapping into diverse manifestations of the alcohol withdrawal syndrome suggests that any interpretations regarding a genetic relationship between alcohol withdrawal responses and propensity toward alcohol drinking should be made with caution. We have previously found this to be true when using the acoustic startle paradigm to assess acute alcohol withdrawal in alcohol naïve rat lines selectively bred for high or low alcohol drinking behavior. In these studies, the direction of the startle response during acute alcohol withdrawal was influenced by the acoustic characteristics of the startle stimulus. That is, male rats selectively bred for high alcohol drinking (P, HAD1, and HAD2 lines) showed reduced startle whereas those selectively bred for low alcohol drinking

(NP, LAD1, LAD2 lines) showed enhanced startle during withdrawal when tone stimuli were used (Chester et al., 2003). However, in a separate series of studies, the P, HAD1, and HAD2 lines showed enhanced startle and the NP, LAD1, and LAD2 lines showed no change in startle during withdrawal when white noise stimuli were used (Chester et al., 2004). In the current experiments, white noise stimuli similar to that employed in the Chester et al. (2004) study were used to assess acute alcohol withdrawal in the HAP/LAP selected mouse lines. However, we found little overlap in the pattern of acoustic startle responses during acute alcohol withdrawal in the HAP/LAP mouse lines compared to that previously observed in selected rat lines, except for the observed trends toward enhanced startle during withdrawal in the HAP1 line.

In the present study, we found good evidence for a genetic correlation between baseline startle and PPI responses and innate alcohol preference in two replicate HAP/LAP mouse lines. This is the first study in which the genetic relationship between responses during acute alcohol withdrawal and innate alcohol preference has been examined using a measure of acute alcohol withdrawal other than HICs. After acute alcohol treatment, we found modest support for a genetic correlation between startle responses during acute alcohol withdrawal and alcohol preference in male but not female mice at the 4-hr time point in withdrawal. Because all groups had comparable BAC at this 4-hr time point, we cautiously interpret the present findings as evidence that male mice with a genetic propensity toward low alcohol preference (LAP lines) may be more sensitive to the startle suppressing effects of alcohol during acute alcohol withdrawal than mice with a genetic propensity toward high alcohol preference (HAP lines). A similar genetic relationship was not seen in female HAP and LAP lines, suggesting that the genetic relationship between acoustic startle during acute alcohol withdrawal and innate alcohol preference may depend on sex. However, future studies are needed to delineate whether

suppressed startle during acute alcohol withdrawal represents a measure of sensitivity to alcohol, to alcohol withdrawal, or to both. Interestingly, Grillon and colleagues (2000) reported a greater alcohol-induced suppression in startle reactivity in male subjects without a family history of alcoholism compared to those with a family history of alcoholism. These findings are similar to the present results in selected mouse lines when interpreted in the context of overall sensitivity to alcohol's effects on startle. Our suggestion that low alcohol preferring mice may be more sensitive to alcohol's effects during acute alcohol withdrawal, as measured by acoustic startle responses, may agree with a large body of data, collected in several genetic mouse models, indicating that mice with an innate tendency toward lower alcohol consumption appear to be more sensitive to acute alcohol withdrawal as measured by HICs than mice that tend to drink higher amounts of alcohol (Crabbe, 1983; Metten and Crabbe, 2005; Metten et al., 1998; Rodgers, 1966). This relationship has been further supported by findings in rat genetic models where alcohol-naïve rats selectively bred for low alcohol drinking showed greater sensitivity to acute alcohol withdrawal than their high alcohol drinking counterparts when withdrawal was assessed using acoustic startle response to tone stimuli (Chester et al., 2003), a behavioral rating scale (Chester et al., 2002), and a brain stimulation reward procedure (Chester et al., 2006). Although genetic correlations between alcohol-related traits suggest that the traits may be functionally related, it remains to be determined how different facets of the alcohol withdrawal syndrome may be predictive of an increased or decreased risk for alcohol consumption and how these findings may relate to humans. This will be a challenging endeavor given that signs and symptoms of the alcohol withdrawal syndrome reflect the disruption of many interacting neurobiological systems which, in turn, are influenced by both genetic and environmental variables.

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Figure Legends

Figure 1. Mean (±sem) startle amplitudes in response to prepulse (averaged across 79, 85, and 91 dB) and pulse (averaged across 110 and 125 dB) acoustic stimuli in male and female HAP1 lines (left panels) and LAP1 lines (right panels) at 4 (top panels), 8 (middle panels), and 12 (bottom panels) hrs after a single injection of 4.0 g/kg i.p. alcohol or equal volume of saline.

Figure 2. Mean (±sem) startle amplitudes in response to prepulse (averaged across 79, 85, and 91 dB) and pulse (averaged across 110 and 125 dB) acoustic stimuli in male and female HAP2 lines (left panels) and LAP2 lines (right panels) at 4 (top panels), 8 (middle panels), and 12 (bottom panels) hrs after a single injection of 4.0 g/kg i.p. alcohol or equal volume of saline.

Figure 3. Mean (±sem) PPI on the 110 and 125 pulse trial types (collapsed across prepulse intensity and withdrawal hr) after a single injection of 4.0 g/kg i.p. alcohol or equal volume of saline in male and female HAP1/LAP1 lines (top panels) and HAP2/LAP2 lines (bottom panels).