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Age- and Sex-Dependent Effects of Footshock Stress on Subsequent Alcohol Drinking and Acoustic Startle Behavior in Mice Selectively Bred for High-Alcohol Preference

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Abstract

Background—Exposure to stress during adolescence is known to be a risk factor for alcohol-use and anxiety disorders. This study examined the effects of footshock stress during adolescence on subsequent alcohol drinking in male and female mice selectively bred for high-alcohol preference (HAP1 lines). Acoustic startle responses and prepulse inhibition (PPI) were also assessed in the absence of, and immediately following, subsequent footshock stress exposures to determine whether a prior history of footshock stress during adolescence would produce enduring effects on anxiety-related behavior and sensorimotor gating.

Methods—Alcohol-naïve, adolescent (male, $n = 27$; female, $n = 23$) and adult (male, $n = 30$; female, $n = 30$) HAP1 mice were randomly assigned to a stress or no stress group. The study consisted of 5 phases: (1) 10 consecutive days of exposure to a 30-minute footshock session, (2) 1 startle test, (3) one 30-minute footshock session immediately followed by 1 startle test, (4) 30 days of free-choice alcohol consumption, and (5) one 30-minute footshock session immediately followed by 1 startle test.

Results—Footshock stress exposure during adolescence, but not adulthood, robustly increased alcohol drinking behavior in both male and female HAP1 mice. Before alcohol drinking, females in both the adolescent and adult stress groups showed greater startle in phases 2 and 3; whereas males in the adolescent stress group showed greater startle only in phase 3. After alcohol drinking, in phase 5, enhanced startle was no longer apparent in any stress group. Males in the adult stress group showed reduced startle in phases 2 and 5. PPI was generally unchanged, except that males in the adolescent stress group showed increased PPI in phase 3 and females in the adolescent stress group showed decreased PPI in phase 5.

Conclusions—Adolescent HAP1 mice appear to be more vulnerable to the effects of footshock stress than adult mice, as manifested by increased alcohol drinking and anxiety-related behavior in adulthood. These results in mice suggest that stress exposure during adolescence may increase the risk for developing an alcohol-use and/or anxiety disorder in individuals with a genetic predisposition toward high alcohol consumption.

Keywords

Acoustic Startle; Adolescence; Alcoholism; Anxiety; Genetics

Adolescence is a period during which exposure to environmental stimuli can result in enduring changes in physiological and psychological functioning (Spear, 2000). Exposure to stressful experiences, such as physical or emotional abuse, during adolescence is known to be a risk factor for various psychiatric disorders, including anxiety and alcohol-use disorders (see reviews by Arnsten and Shansky, 2004; Enoch, 2006; Heim and Nemeroff, 1999; Koehl et al., 2002). Encouraging progress has been made toward understanding how environmental and genetic factors may interact to influence the risk for alcohol-use disorders in humans (see review by Zimmermann et al., 2007), yet this endeavor has proved to be quite challenging. For instance, genetic factors are known to increase risk for developing alcohol-use disorders (Enoch, 2006) but familial alcoholism is also linked with adolescent alcohol use, which in turn appears to be a risk factor for later alcohol-use disorders regardless of family history (Grant and Dawson, 1998; Grant et al., 2006; King and Chassin, 2007). Exposure to stress during adolescence may further complicate these relationships. For instance, a link exists between degree of perceived stress and adolescent alcohol use (Wagner, 1993), and adolescents with a family history of alcoholism may be exposed to more stressful life experiences due to both genetic and environmental causes (see reviews by Johnson and Leff, 1999; Zimmermann et al., 2007).

Potentially confounded factors in human studies, such as history of stress and/or alcohol exposure, can be better controlled in studies using animal models. These models have provided important information on how stress exposure during development may influence risk for psychiatric disorders in humans (see reviews by Heim and Nemeroff, 1999; Koehl et al., 2002). Although there are abundant reports in the animal literature demonstrating effects of early life stress on anxiety- and alcohol-related behavior (e.g., Huot et al., 2001; Ladd et al., 2000; Roman and Nylander, 2005), relatively fewer studies have been conducted in adolescent rodent models. Adolescence in the rodent has been defined as the developmental time period after weaning that encompasses an inexact range of postnatal day (PND) 24-55, with PND 37-48 approximating middle adolescence (Adriani et al., 2002; Hefner and Holmes, 2007; Spear, 2000). Spear et al. have reported that isolate housing, a form of chronic stress that adolescent rodents seem to be particularly vulnerable to (Hall, 1998), did not alter home-cage alcohol consumption in adolescent versus adult rats (Doremus et al., 2005). In another study, adolescent rats exposed to daily footshock stress, during a period of concurrent access to alcohol in the home cage, showed reduced alcohol consumption relative to their adult counterparts exposed to the same stress procedure (Brunell and Spear, 2005). To our knowledge, there are no reports of adolescent stress effects on alcohol drinking behavior in mice.

The primary goal of this study was to compare the effects of a stress manipulation during middle adolescence versus adulthood on subsequent alcohol drinking behavior in male and female mice selectively bred for high-alcohol preference. Selectively bred rodent lines provide a useful animal model to examine how environmental influences, such as stress,

may interact with genetic vulnerability to influence high alcohol drinking behavior. The stress manipulation in this study consisted of 10 consecutive days of exposure to footshock. Another goal of this study was to determine whether a prior history of footshock stress during adolescence would produce enduring effects on anxiety-related behavior and sensorimotor gating. Anxiety-related behavior and sensorimotor gating were quantified using acoustic startle responses and prepulse inhibition (PPI) of the acoustic startle response both in the absence of, and immediately following, re-exposure to foot-shock stress in adulthood. Enhanced acoustic startle responses have been used as a sensitive index of acute and persistent arousal or anxiety produced by stress in rodents (Conti and Printz, 2003; Davis, 1989, 1990; Dubovicky et al., 2007; Faraday, 2002; Garrick et al., 2001; Servatius et al., 1994). PPI is a cross-species phenotype that reflects sensori-motor gating mechanisms. Disrupted PPI is often used as an index of cognitive deficits that occur with several psychiatric disorders, most notably, schizophrenia (Braff et al., 1978). In both rats and mice, exposure to various forms of stress during development and adulthood have been shown to produce deficits in PPI (e.g., Bakshi and Geyer, 1999; Dubovicky et al., 2007; Ellenbroek et al., 1998; Varty et al., 2006; but see Millstein et al., 2006; Pijlman et al., 2003). It was hypothesized that exposure to footshock stress during adolescence would increase subsequent alcohol drinking behavior in HAP1 mice. Further, adolescent mice exposed to footshock stress were expected to show greater behavioral reactivity to subsequent footshock stress exposures in adulthood, as measured by enhanced startle and disrupted PPI. Finally, based on prior studies in rats (Roman et al., 2004) and in HAP1 mice (Chester et al., 2006), we posited that females might be more resistant to the stress manipulation as measured by effects on alcohol drinking, startle responses, and PPI.

MATERIALS AND METHODS

Subjects, Group Assignments, and Environmental Conditions

Subjects were 27 males and 23 females (experiment 1: adolescent footshock stress) and 30 males and 30 females (experiment 2: adult footshock stress) alcohol-naïve mice selectively bred for high-alcohol preference (HAP1 line) (generation 27). The selectively bred HAP1 mouse line was derived from a foundation stock of outbred Hs/Ibg mice (Institute for Behavioral Genetics, Boulder, CO) at the Indiana Alcohol Research Center (IARC). In every generation of selection, HAP1 was established during a 4-week, 24-hour, free-choice preference test (Grahame et al., 1999). During the preference test, mice were given free-access to food and two 25-ml graduated cylinders, 1 that contained 10% alcohol (v/v) in distilled water and the other that contained distilled water. Mice that drank more than 5.0 g/kg of alcohol per day (averaged over the 4-week test period) were selected to be breeders for the next generation of HAP1 mice (Grahame et al., 1999).

Subjects were generated at Purdue University from HAP1 breeders obtained from the IARC. Pregnant dams were checked for births daily, and the day of birth was considered PND 1. Mice were weaned at 21 to 23 days of age (in order to avoid individually housing weaned mice) and were immediately housed in groups of 2 to 5 in polycarbonate cages (11.5 × 7.5 × 5.0 in.) with aspen wood shavings. Mice were usually housed with same-sex siblings unless mice from another family were ready to be weaned, at which time same-sex mice

from different families were housed together within a home cage. Mice remained group-housed until the free-choice alcohol consumption phase of the experiments (phase 4).

All mice housed within a particular home cage were assigned to either the experimental (stress) or control (no stress) groups. Subjects were derived from 20 different HAP1 families (7 for experiment 1 and 13 for experiment 2). To the best extent possible, balanced numbers of male and female mice from a particular family (litter) were assigned to the different experimental groups; however, it was unavoidable that some families are not represented in every group. The number of mice from each family assigned to each group was as follows (the first number refers to family number followed by the number of mice in parentheses): for experiment 1, stress/male: 1(5), 2(2), 3(3), 4(5); no stress/male: 2(2), 3(1), 4(2), 5(2), 6(2), 7(3); stress/female: 1(1), 2(5), 5(2); no stress/female: 1(1), 3(5), 6(5), 7(4); and for experiment 2, stress/male: 9(4), 10(1), 12(3), 14(1), 15(2), 17(2), 18(1), 19(1); no stress/male: 8(3), 11(4), 13(3), 16(5); stress/female: 8(1), 9(3), 10(1), 11(4), 12(3), 15(2), 20(1); no stress/female: 8(2), 10(4), 11(1), 12(5), 13(3).

Ambient temperature in the colony room was maintained at $21 \pm 1^\circ\text{C}$ and mice had free-access to food (Rodent Lab Diet 5001; Purina Mills Inc., St. Louis, MO) and water in the home cage. The colony room was maintained on a 12:12 light:dark cycle (lights on at 07:00 hours). Experiments were conducted in accordance with the NIH guidelines for animal care and use, and the experimental procedures were approved by the Purdue Animal Care and Use Committee.

Experimental Timeframe

Table 1 illustrates the timeline for experimental procedures and corresponding ages of the mice used in experiments 1 (adolescent footshock stress) and 2 (adult footshock stress). The experiments consisted of 5 phases: (1) 10 consecutive days of exposure to a 30-minute footshock session, (2) one startle test, (3) exposure to one 30-minute footshock session immediately followed by 1 startle test, (4) 30 days of free-choice alcohol consumption, and (5) one 30-minute footshock session immediately followed by 1 startle test.

Acoustic Startle Apparatus and Testing Procedures

Application of footshock stress and acoustic startle tests occurred in a Coulbourn Instruments Animal Acoustic Startle System (Coulbourn Instruments, Allentown, PA). The system consisted of 4 weight-sensitive platforms that were inside 1 sound-attenuated chamber connected to an interfaced computer. The 4 platforms were equidistant from a speaker located in the ceiling of the chamber. Subjects were placed individually into open-air holders ($8 \times 8 \times 16$ cm) with metal rod floors (rod diameter 0.19 in. with each rod separated by 0.39 in.). Scrambled electric footshock was administered through the metal rod floors of each holder using Coulbourn Shocker Units. 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 30-minute footshock stress session consisted of 15 shock stimuli (0.2 mA, 0.5 seconds duration) presented every 2 minutes. Acoustic startle tests began with a 5-minute acclimation period during which time no acoustic stimuli were presented. A ventilating fan provided continuous background noise (~75 dB). Both startle responses and PPI were measured during the acoustic startle tests. Startle stimuli consisted of broadband noise bursts (frequency range: 20 Hz to 20,000 kHz) with a near instantaneous rise time (<1 ms). Twelve different stimulus types were presented in a random order to avoid habituation to the acoustic stimuli during the startle tests. Each trial type was presented 12 times on a random intertrial interval that ranged from 10 to 20 s, for a total of 144 trials throughout a 45-minute test session. The trial types consisted of 1 blank trial (no pulse), 2 pulse alone trials (110 and 125 dB; 40 ms), 3 prepulse alone trials (79, 85, and 91 dB; 20 ms), and 6 prepulse + pulse trials (79, 85, or 91 dB + 110 or + 125 dB). The interstimulus interval between the prepulse and pulse was 100 ms. During phases 3 and 5, the acoustic startle tests began immediately after the 30-minute foot-shock session ended (mice remained in the chamber for ~75 minutes total). The decibel intensity of each startle stimulus was verified prior to the start of the experiments with a Digital Sound Level Meter (Radio Shack, Ft. Worth, TX).

All mice were weighed before being placed into the open-air holders for startle testing. Testing procedures occurred between 07:20 and 16:10 hours for phase 2 and between 07:10 and 20:15 hours for phases 3 and 5. Startle testing occurred over the course of 2 days due to time constraints associated with the duration of the test sessions and the number of subjects to be tested. Across the 2 startle testing days, mice in each home cage were tested in each session in a counter-balanced fashion based on group (stress/no stress) and sex (male/female). In most cases, 4 mice were tested in each startle test session; however, there were a few instances where fewer than 4 mice were tested during a session due to an odd number of subjects per home cage.

Experimental Procedures

Phase 1: Footshock Stress—During the 10 consecutive days of footshock stress, mice assigned to the stress groups were placed 1 at a time into the open-air holder and the holder was immediately carried into the adjacent room and placed inside the Coulbourn apparatus. Stress sessions lasted 30 minutes and occurred between 07:00 and 13:00 hours. The order of stress sessions was counterbalanced based on sex across this time period. Mice in both the stress and no stress groups were weighed on the first and last day of the 10-day footshock stress phase.

Phase 2: Startle Test—The purpose of phase 2 was to test whether mice previously exposed to footshock stress during adolescence or adulthood showed an enduring change in startle responses and PPI. All mice received the startle test either 6 (experiment 1) or 7 (experiment 2) days after the final footshock stress session in phase 1.

Phase 3: Footshock Stress + Startle Test—The purpose of phase 3 was to test footshock stress reactivity, as measured by startle responses and PPI, in mice previously exposed to footshock stress during adolescence or adulthood. Fourteen days after phase 2, all mice received a 30-minute footshock session immediately followed by a startle test.

Phase 4: Free-Choice Alcohol Consumption—The purpose of phase 4 was to examine the acquisition and maintenance of alcohol drinking behavior in mice previously exposed to footshock stress or no stress during adolescence or adulthood. Mice were individually housed approximately 1 to 2 days after completion of phase 3 testing. Standard water bottles were removed and replaced with two 25-ml plastic graduated cylinders, fitted with steel sipper tubes, and filled with tap water. Mice were introduced to alcohol 2 days later by replacing one of the water-filled cylinders with a cylinder filled with a 10% alcohol solution, alternating in either the left or right position. During the 30 days of free-choice alcohol consumption, alcohol and water intakes were recorded at 16:30 hours using a flashlight to illuminate the cylinders for accurate readings. In experiment 1, cylinders were read every day for 14 days and every 2 days thereafter, and in experiment 2, cylinders were read every day for 8 days and every 2 days thereafter. Fluids were refilled every 2 days and completely replaced every 6 to 8 days when cages were cleaned. Intake measurements represent approximately 23 hours of fluid intake due to time taken to weigh and replace fluids for all subjects. The left–right position of the alcohol and water-filled cylinders was rotated after each reading to control for a potential positional preference.

Phase 5: Footshock Stress + Startle Test—The purpose of phase 5 was to test reactivity to footshock stress after 30 days of alcohol drinking, as measured by startle responses and PPI, in mice previously exposed to footshock stress or no stress during adolescence or adulthood. All mice received the footshock + startle test that occurred over a 2-day period beginning the next day (15 to 27 hours) after the final bottle reading in phase 4. To ensure that all mice were tested within the same time range since alcohol access, alcohol bottles were left on the cages for approximately half the mice for an additional 24 hours but consumption was not recorded.

Data Analyses

Drinking data were analyzed with analysis of variance (ANOVA) and startle data were analyzed with analysis of covariance (ANCOVA) with body weight as the cofactor. Startle data from each phase of the study were analyzed separately because of different environmental conditions surrounding each startle test and the extended experimental timeframe. Startle data on prepulse trial types (79, 85, 91 dB; 20 ms) were analyzed separately from startle data on pulse trial types (110, 125 dB; 40 ms) because these acoustic stimuli, of different intensity and duration, varied along more than 1 continuum. PPI data on the 110- and 125-dB pulse trial types were analyzed using separate ANCOVAs because Pearson correlations indicated a lack of covariance between these repeated measures, which may violate assumptions of multivariate analyses procedures, and because the present design lacked the statistical power to detect subtle group differences in PPI that may depend on interactions with prepulse and/or pulse intensity. Data on no stimulus trials are not reported because analyses of these data did not alter interpretation of the acoustic startle behavior data.

Between-group factors included age of exposure to footshock stress (adolescent, adult), experimental group (stress, no stress), and sex (male, female); and within-group factors included day and startle stimulus type, where applicable. The “age of exposure to footshock

stress” factor is termed “age” in the Results section to simplify terminology but should not be confused with the age at which startle and drinking behaviors were assessed. To simplify the presentation of the results, only significant main effects and highest order interactions are reported. The source of significant higher-order interactions were investigated with lower-order ANOVAs and ANCOVAs (Keppel, 1991). Probability values equal to or less than 0.05 were considered significant.

Alcohol intake was expressed as grams of alcohol per kilogram of body weight (g/kg BW) and as percent alcohol preference (milliliters of 10% alcohol solution/milliliters of total fluid consumed $\times$ 100). Alcohol and water intakes were averaged across 2-day blocks to reduce day-to-day variability in drinking patterns. Prior to averaging data across days, alcohol and water drinking scores on individual days were examined for outliers. The majority of these outliers were on the high end of the normal distribution curve and were most likely due to accidental fluid loss from mouse activity in the cage (e.g., playing with the plastic tubes). A score was considered an outlier if it passed several conservative criteria. The score first had to exceed by 2 standard deviations the mean drinking score for that animal and the mean drinking score for the group. If the score passed these criteria, it was then subjected to the Dixon Extreme Score Test (Dixon, 1950). Scores that passed all criteria were replaced with an average intake score, which was calculated by averaging fluid intake on the day before and the day after the outlier occurred. In one particular case, it was necessary to replace the outlier with the mean of the group because the outlier occurred on the first day of the drinking phase. For experiment 1, valid outliers occurred twice in males (water: 1 stress, 1 no stress) and 4 times in females (alcohol: 3 stress; water: 1 stress). For experiment 2, valid outliers occurred 5 times in males (alcohol: 3 stress, 1 no stress; water: 1 stress) and 5 times in females (alcohol: 3 stress; water: 2 stress). In experiment 1, the number of alcohol and water outliers comprised 0.1% and 0.2% of the total data set for males and females, respectively, and in experiment 2, the outliers comprised 0.2% of the total data set for both males and females.

The amplitude of the acoustic startle response was determined for each mouse by calculating the average amplitude of the 12 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 pre-pulse + pulse trials)/average startle response on pulse trials] $\times$ 100. Pearson correlation coefficients between body weight and startle amplitude in response to the 3 prepulse and 2 pulse acoustic stimuli were generated for data collected in each of the 3 startle testing phases.

RESULTS

Body Weight

Phase 1—The ANOVA for body weights measured at the beginning and the end of the 10 days of footshock stress (age $\times$ group $\times$ sex $\times$ day) indicated main effects of age [$F(1,102) = 146.7, p < 0.01$; adult $>$ adolescent], sex [$F(1,102) = 69.7, p < 0.01$; males $>$ females], day [$F(1,102) = 88.8, p < 0.01$; day 10 $>$ day 1], and an age $\times$ group $\times$ sex $\times$ day interaction [$F(1,102) = 3.8, p = 0.05$]. Follow-up 3-way ANOVAs (group $\times$ sex $\times$ day) were conducted within adolescents and adults to determine the source of the 4-way interaction. In

adolescents, the ANOVA indicated main effects of sex [$F(1,46) = 22.0, p < 0.01$; males > females], day [$F(1,46) = 73.9, p < 0.01$; day 10 > day 1], and a sex $\times$ day interaction [$F(1,46) = 9.5, p < 0.01$]. Follow-up 1-way ANOVAs within each sex yielded significant effects of day for both males and females ($ps < 0.01$). These analyses showed no effect of footshock stress on body weight but males gained more body weight than females from days 1 to 10. In adults, the ANOVA indicated main effects of sex [$F(1,56) = 53.0, p < 0.01$; males > females], day [$F(1,56) = 18.3, p < 0.01$; day 10 > day 1], and a group $\times$ day interaction [$F(1,56) = 15.8, p < 0.01$]. Follow-up 1-way ANOVAs within each group showed a significant effect of day in the no stress groups (collapsed across sex; $p < 0.01$) but not in the stress groups. These analyses showed that the no stress groups gained body weight from days 1 to 10 but the stress groups did not (Table 2).

Phases 2, 3, and 5—The ANOVA for body weights measured prior to each startle testing phase (age $\times$ group $\times$ sex $\times$ phase) indicated main effects of age [$F(1,102) = 32.6, p < 0.01$; adult > adolescent], sex [$F(1,102) = 100.1, p < 0.01$; males > females], and phase [$F(2,204) = 249.2, p < 0.01$; phase 5 > phase 3 > phase 2] and an age $\times$ sex $\times$ phase interaction [$F(2,204) = 4.3, p = 0.01$]. Follow-up 2-way ANOVAs (age $\times$ sex) were conducted within each phase to determine the source of the interaction. These analyses indicated only main effects of age [$F_s(1,106) > 6.3, ps \leq 0.01$, adult > adolescent] and sex [$F_s(1,106) > 94.3, ps < 0.01$; males > females] during each phase (Table 2).

We have previously reported no correlation between body weight and amplitude of the acoustic startle response in mice using the Coulbourn Instruments Animal Acoustic Startle System (Barrenha and Chester, 2007; Chester and Barrenha, 2007). This finding is not necessarily surprising given that (i) the acoustic startle reflex is a short-latency, jerk-like muscular extension response to acoustic stimuli rather than whole-body weight displacement, and (ii) we run acoustic startle sessions in the alternating current-coupled mode, which yields peak startle amplitude responses that reflect the grams of muscular force exerted against the platform and does not include subjects' body weight in the force measurement. However, because it is possible that muscular force may be correlated with body weight (for example, in males vs. females; Adamec et al., 2006; Blaszczyk and Tajchert, 1996), we conducted Pearson correlations between body weight taken prior to each acoustic startle session and average response amplitude to prepulse and pulse stimuli in each of the 3 startle sessions in phase 2, 3, and 5 (15 correlations; $n = 110$). Approximately half (8/15) of the correlations yielded small but significant positive r values that ranged between 0.2 and 0.3 ($ps < 0.05$). Because of these findings, we included body weight as a cofactor in all analyses of startle data in order to remove any variance due to body weight differences that may influence the startle response.

Phase 2: Startle Test

Startle Response—The prepulse ANCOVA (age $\times$ group $\times$ sex $\times$ trial type) indicated a significant group $\times$ sex $\times$ trial-type interaction [$F(2,202) = 8.7, p < 0.01$]. Follow-up group $\times$ sex ANCOVAs at each trial type yielded group $\times$ sex interactions at the 91- and 85-dB trial types [$F_s(1,105) > 10.3, ps < 0.01$]. The interactions were due to significantly higher startle in female stress groups than their no stress counterparts [$F_s(1,50) > 6.1, ps < 0.05$] and

significantly lower startle in male stress groups than their no stress counterparts [$F(1,54) = 4.2, p < 0.05$] on the 91- and 85-dB trial types. Even though these follow-up analyses were collapsed across the age factor due to lack of interactions, the lower startle in the male stress groups was clearly more evident in the mice exposed to footshock stress in adulthood (see top panels, Fig. 1).

The pulse ANCOVA indicated a significant group $\times$ sex interaction [$F(1,101) = 6.5, p = 0.01$]. Follow-up 1-way ANCOVAs of group within each sex (collapsed across pulse stimuli) showed significantly higher startle in female stress groups than their no stress counterparts [$F(1,50) = 4.9, p < 0.05$] but no group difference in males (see top panels, Fig. 1).

Prepulse Inhibition—The prepulse + 110 ANCOVA (age $\times$ group $\times$ sex $\times$ trial type) showed a main effect of trial type [$F(2,202) = 3.6, p < 0.05$], due to greater PPI when the pulses were preceded by the 79- and 85-dB prepulses, and an age $\times$ trial-type interaction [$F(2,202) = 6.2, p < 0.05$]. The interaction was due to significantly greater PPI in experiment 1 mice than experiment 2 mice on the 85 + 110 dB trial type [$F(1,107) = 11.7, p < 0.01$] (see top panels, Fig. 2).

The prepulse + 125 dB ANCOVA showed no main effects or interactions (data not shown).

Phase 3: Footshock + Startle Test

Startle Response—The prepulse ANCOVA (age $\times$ group $\times$ sex $\times$ trial type) indicated a main effect of trial type [$F(2,202) = 5.2, p < 0.01$; 79 dB < 85 and 91 dB] and a group $\times$ sex $\times$ trial-type interaction [$F(2,202) = 3.5, p < 0.05$]. Follow-up group $\times$ sex ANCOVAs at each trial type yielded group $\times$ sex interactions at the 91- and 85-dB trial types [$F(1,105) > 4.8, p < 0.05$]. The interactions were due to significantly higher startle in female stress groups than their no stress counterparts [$F(1,50) > 5.2, p < 0.05$] but no group difference was seen in males.

The pulse ANCOVA showed an age $\times$ group interaction [$F(1,101) = 5.1, p < 0.05$]. The interaction was due to significantly higher startle on the 110- and 125-dB trial types in both male and female mice that had been exposed to foot-shock stress in adolescence compared to their no stress counterparts [$F(1,47) = 10.8, p < 0.01$]. No group difference in startle was seen in mice exposed to footshock stress in adulthood (see middle panels, Fig. 1).

Prepulse Inhibition—The prepulse + 110 dB ANCOVA (age $\times$ group $\times$ sex $\times$ trial type) indicated a group $\times$ sex $\times$ trial-type interaction [$F(2,202) = 3.9, p < 0.05$]. Follow-up group $\times$ sex ANCOVAs at each trial type yielded a group $\times$ sex interaction on the 91 + 110 dB trial type [$F(1,105) = 3.9, p = 0.05$]. The interaction was due to significantly greater PPI on the 91 + 110 dB trial type in male mice exposed to footshock stress compared to their no stress counterparts [$F(1,54) = 4.0, p < 0.05$]. Even though this follow-up analysis was collapsed across the age factor due to lack of interactions, the greater PPI was clearly more prominent in the males exposed to footshock stress during adolescence than adulthood (see middle panels, Fig. 2). No group difference in PPI was seen in female mice.

The prepulse + 125 dB ANCOVA showed no main effects or interactions (data not shown).

Phase 4: Free-Choice Alcohol Consumption

Alcohol Intake and Preference—The ANOVA for g/kg alcohol intake (age $\times$ group $\times$ sex $\times$ blocks) yielded main effects of age [$F(1,102) = 5.1, p < 0.05$; adolescent $>$ adult], group [$F(1,102) = 10.9, p < 0.01$; stress $>$ no stress], sex [$F(1,102) = 10.0, p < 0.01$; females $>$ males], blocks [$F(14,1428) = 3.4, p < 0.01$], and an age $\times$ group $\times$ sex $\times$ blocks interaction [$F(14,1428) = 2.4, p < 0.01$]. Follow-up 3-way ANOVAs were conducted separately for mice exposed to footshock stress during adolescence versus in adulthood. In the mice exposed to footshock stress in adolescence, the ANOVA indicated main effects of group [$F(1,46) = 14.1, p < 0.01$; stress $>$ no stress], sex [$F(1,46) = 4.8, p < 0.05$; females $>$ males], and blocks [$F(14,644) = 2.9, p < 0.01$]. In the mice exposed to footshock stress in adulthood, the ANOVA indicated main effects of sex [$F(1,56) = 5.1, p < 0.05$; females $>$ males] and blocks [$F(14,784) = 2.2, p < 0.01$].

The ANOVA for alcohol preference (age $\times$ group $\times$ sex $\times$ blocks) yielded main effects of group [$F(1,102) = 4.3, p < 0.05$; stress $>$ no stress] and blocks [$F(14,1428) = 4.0, p < 0.01$] and an age $\times$ group $\times$ sex $\times$ blocks interaction [$F(14,1428) = 2.3, p < 0.01$]. Follow-up 3-way ANOVAs were conducted separately for mice exposed to footshock stress during adolescence versus in adulthood. For mice exposed to footshock stress during adolescence, the ANOVA indicated main effects of group [$F(1,46) = 8.3, p < 0.01$; stress $>$ no stress] and blocks [$F(14,644) = 4.4, p < 0.01$]. For mice exposed to footshock stress during adulthood, the ANOVA indicated only a main effect of blocks [$F(14,784) = 2.1, p = 0.01$] and a sex $\times$ blocks interaction [$F(14,784) = 1.9, p < 0.05$].

These analyses indicate that both male and female adult mice exposed to footshock stress during adolescence showed significantly greater g/kg alcohol intake and alcohol preference compared to the no stress control groups throughout the 30-day period (Fig. 3). However, footshock stress exposure during adulthood did not significantly alter g/kg alcohol intake or alcohol preference (Fig. 4).

Total Fluid Intake—Total fluid intake collapsed across the 30-day period was as follows for each group: adolescent stress/male, 247.6 ± 8.1 ; adolescent stress/female, 324.1 ± 19.1 ; adolescent no stress/male, 260.0 ± 7.8 ; adolescent no stress/female, 295.1 ± 9.7 ; adult stress/male, 217.6 ± 9.9 ; adult stress/female, 236.1 ± 6.1 ; adult no stress/male, 202.0 ± 6.0 ; adult no stress/female, 238.0 ± 5.4 . The ANOVA for total fluid intake yielded main effects of age [$F(1,102) = 86.9, p < 0.01$; adolescent $>$ adult], sex [$F(1,102) = 44.9, p < 0.01$; females $>$ males], blocks [$F(14,1428) = 5.4, p < 0.01$], and age $\times$ sex $\times$ blocks [$F(14,1428) = 2.8, p < 0.01$] and age $\times$ group $\times$ sex [$F(1,102) = 5.4, p < 0.05$] interactions. The age $\times$ group $\times$ sex interaction was investigated by conducting group $\times$ sex ANOVAs separately for mice exposed to footshock stress during adolescence versus in adulthood, which yielded only main effects of sex for both groups of mice ($F_s > 15.3, p_s < 0.01$). Overall, these analyses indicate that female mice consumed more total fluid than male mice during the 30-day period but exposure to footshock stress did not alter total fluid intake.

Phase 5: Footshock + Startle Test

Startle Response—The prepulse ANCOVA (age $\times$ group $\times$ sex $\times$ trial type) indicated a group $\times$ sex $\times$ trial-type interaction [$F(2,202) = 4.3, p < 0.05$]. Follow-up group $\times$ sex ANCOVAs at each trial type yielded group $\times$ sex interactions at the 91- and 85-dB trial types [$F_s(1,105) > 5.9, p_s < 0.05$]. The interactions were due to lower startle in male mice exposed to footshock stress compared to their no stress counterparts, an effect that was statistically significant on the 85-dB trial type [$F(1,54) = 6.7, p = 0.01$]. No group difference was seen in females (see bottom panels, Fig. 1).

The pulse ANCOVA showed no main effects or interactions.

Prepulse Inhibition—The prepulse + 110 ANCOVA (age $\times$ group $\times$ sex $\times$ trial type) indicated an age $\times$ group $\times$ sex interaction close to statistical significance [$F(1,101) = 3.1, p = 0.08$]. Because of this trend and the fact that observed power to detect this interaction was low (0.4), lower-order ANCOVAs (age $\times$ group) were separately conducted within each sex to determine the source of the interaction. These analyses showed a significant age $\times$ group interaction on the 91 + 110 dB trial type in female mice [$F(1,48) = 6.0, p < 0.05$]. The interaction was due to significantly lower PPI in female mice exposed to footshock stress during adolescence compared to their no stress counterparts [$F(1,20) = 5.9, p < 0.05$]. No group differences were seen in female mice exposed to stress during adulthood (see bottom panels, Fig. 2).

The prepulse + 125 ANCOVA showed a main effect of age [$F(1,101) = 4.5, p < 0.05$; adult $>$ adolescent; data not shown].

DISCUSSION

The present results showed that footshock stress exposure during adolescence but not adulthood robustly increased alcohol drinking behavior in mice with a genetic propensity toward high-alcohol preference. This effect did not depend on sex, as both males and females showed a significant and stable increase in both g/kg alcohol intake and alcohol preference throughout the 30-day drinking period. To our knowledge, this is the first study to report the effects of a stress manipulation during adolescence on subsequent alcohol drinking behavior in a mouse model. These data in mice are not consistent with previous findings where isolate housing did not affect (Doremus et al., 2005), or footshock decreased (Brunell and Spear, 2005), alcohol consumption in adolescent rats. The many experimental differences between the present study and prior reports, including species and stress/drinking procedures, make it very difficult to compare results across studies. However, one major experimental variable worth noting is that, in the previous rat studies, effects of stress were assessed in adolescent rats with concurrent access to alcohol. Effects of stress on alcohol consumption in these prior rat studies could have been influenced by the fact that adolescents consumed more alcohol than adults (Brunell and Spear, 2005; Doremus et al., 2005). It may also be that it takes time for the adolescent brain to develop stress-induced neurobiological changes that result in increased alcohol consumption. This view is supported by studies in rats where chronic stress has been shown to produce delayed

morphological changes in brain regions such as the hippocampus (see review by Romeo and McEwen, 2006).

One of the goals of the present study was to determine whether mice exposed to footshock stress during adolescence would show enduring effects on anxiety-related behavior and whether these mice would be more reactive to subsequent footshock stress in adulthood as evidenced by increased acoustic startle (Conti and Printz, 2003; Davis, 1989, 1990; Dubovicky et al., 2007; Faraday, 2002; Garrick et al., 2001; Servatius et al., 1994). In phase 2 when startle was tested without footshock stress, both adolescent and adult female stress groups showed greater startle compared to controls. In contrast, the adult male stress group showed reduced startle. In phase 3, following re-exposure to the footshock stress, both adolescent and adult female stress groups showed greater startle, as was seen in phase 2. However, in males, the adolescent but not adult stress group showed greater startle (on the pulse trial types) than controls, suggesting that these males were showing greater anxiety-related behavior following the re-exposure to footshock stress. After alcohol consumption and re-exposure to the footshock stress in phase 5, enhanced startle was no longer apparent in any stress group but both adolescent and adult male stress groups showed reduced startle (on the 85-dB prepulse trial type). Overall, these data suggest that females in the stress groups seem to show greater anxiety-related behavior regardless of the age at which they were exposed to the footshock stress. On the other hand, there was some evidence for age-dependent changes in anxiety-related behavior in males, with the adolescent stress group showing greater startle responses with footshock re-exposure and the adult stress group showing reduced startle both with and without footshock re-exposure. It should be noted that we did not expose the no stress groups to the footshock/startle chambers during phase 1; thus, it is possible that differential familiarity with the startle testing environment may have contributed in some way to the observed group differences. The enhanced acoustic startle seen in the stress groups may actually reflect a conditioned anxiety response elicited by contextual cues of the startle chamber because, due to equipment limitations, footshock and startle testing occurred in the same chamber. This alternative interpretation of the data does not diminish the relevance of the group differences seen here; however, there may be different underlying neural mechanisms that contribute to nonspecific versus cue-elicited anxiety-related behavior (e.g., Davis, 1998). Future studies may be designed to specifically disentangle how enduring effects of prior stressor exposure on alcohol drinking and anxiety-related behavior may be influenced by environmental conditions and contextual cues associated with the stressor.

The startle data do not support our hypothesis, based on prior examinations of stress/alcohol drinking interactions (Chester et al., 2006; Roman et al., 2004), that females would be more resistant to stress than males. In fact, these data suggest that females may be more prone to exhibit stress-related anxiety than males. This suggestion is consistent with a few other reports showing that female rodents seem to be more susceptible to the behavior-impairing effects of stress than males under certain conditions that depend on age, type of stressor, housing conditions, and adrenal and gonadal hormone profiles (Baker and Bielajew, 2007; Mathews et al., 2008; Pohl et al., 2007; for reviews see Laviola et al., 2002; Shors and Leuner, 2003). Although reduced acoustic startle in the male adult stress group was an unexpected result, this finding is generally consistent with at least 2 other reports where a

prior history of repeated footshock stress in developing mice (Millstein et al., 2006) and in adult rats (Pijlman et al., 2003) suppressed acoustic startle compared to controls.

We had also posited that adolescent mice exposed to foot-shock stress would show disruptions in sensorimotor gating, particularly after footshock stress application, as measured by lower PPI (Bakshi and Geyer, 1999; Dubovicky et al., 2007; Ellenbroek et al., 1998; Varty et al., 2006). The data as a whole do not support this hypothesis, although there were some interesting group differences in PPI that warrant further investigation. In phase 3 following re-exposure to footshock stress, male mice in the stress groups showed significantly higher PPI compared to controls on the 91 + 110 dB trial type. Although this effect is based on data collapsed across age group due to the lack of statistical interaction, the effect was clearly more prominent in the male mice exposed to foot-shock stress during adolescence (Fig. 2, middle panels). In phase 5 after alcohol consumption and re-exposure to the footshock stress, female mice in the adolescent stress group showed lower PPI compared to controls on the 91 + 110 dB trial type (Fig. 2, bottom left panel). These effects were not seen in mice exposed to the same footshock stress during adulthood. Although these PPI findings are subtle, they suggest that in male HAP1 mice, exposure to stress during adolescence may be adaptive and facilitate cognitive processing in response to stressors. This interpretation is generally consistent with previous findings where enhanced PPI was observed in rats that had a history of footshock stress (Pijlman et al., 2003) or that were anticipating imminent footshock (Grillon and Davis, 1997). On the other hand, the data in female HAP1 mice suggest that females may be particularly sensitive to stress-induced cognitive disruption after alcohol exposure, as measured by PPI. More data are needed to address possible sex- and age-specific effects of stress, alcohol, and their combination on PPI.

Alcohol-use disorders in the human population frequently co-occur with anxiety disorders (Kessler et al., 1997). One popular hypothesis proposed to explain this relationship is the “self-medication hypothesis,” which suggests that alcohol may be especially reinforcing in individuals with anxiety, which in turn promotes a cycle of excessive alcohol consumption (Cappell and Herman, 1972; Conger, 1956; Sher, 1987). An interesting aspect of the present data that agrees with the self-medication hypothesis is that the significantly greater startle responses in the stress groups were no longer evident in phase 5 after alcohol consumption. Although alcohol was not present during startle testing in phase 5, this result suggests that a prior history of alcohol consumption may have produced anxiolytic effects sufficient to reduce the enhanced startle to the level of controls. In this context, it is interesting to note that rats selectively bred for alcohol preference (Sardinian alcohol preferring) have shown reduced anxiety-related behavior when tested after a period of voluntary alcohol consumption (Colombo et al., 1995). However, a possible alternative interpretation for this effect could simply be that experiential factors, for example, repeated exposure to the footshock session and/or startle testing, may have produced extinction of a conditioned anxiety state. These observations may be viewed with caution in the absence of statistical comparisons between phases 3 and 5. Additional work is planned to examine the conditions under which alcohol may have anxiolytic effects on stress-related anxiety behavior and determine whether HAP mice may be more sensitive to alcohol's anxiolytic effects than their low-alcohol-preferring counterparts (LAP lines).

In rodents and nonhuman primates, stress exposure in early life produces profound changes in hypothalamic–pituitary–adrenal (HPA) axis function and in brain areas known to regulate response to stress and to drugs of abuse (Anisman et al., 1998; Coplan et al., 1996; Koehl et al., 2002; Romeo and McEwen, 2006). These biological changes have been suggested to be primary mechanisms that facilitate the acquisition of drug self-administration in both humans and animals (see reviews by Piazza and Le Moal, 1998; Sinha, 2001; Spear, 2000). For example, several lines of evidence indicate that repeated stress-induced activation of reward-related brain areas, such as the mesolimbic dopamine system, may result in increased sensitivity to the rewarding effects of drugs (see review by Koob and Le Moal, 2001). HPA axis hormones and their interaction with the dopamine system has been suggested to be a mechanism by which increased sensitivity to drug effects may occur following stressor exposure and vice versa, termed “cross-sensitization” (see review by Phillips et al., 1997). Thus, footshock stress in adolescence may have subsequently produced greater alcohol consumption in HAP1 mice because they were more sensitive to the neurobiological and rewarding effects of alcohol consumption [e.g., release of corticosterone (Tabakoff et al., 1978) and/or dopamine (Weiss et al., 1993)]. This view is further supported by a recent study indicating that adolescent mice exposed to chronic stress were more sensitive to the rewarding effects of alcohol, as assessed using a conditioned place preference procedure, compared with controls not exposed to stress (Song et al., 2007). It is also possible that the greater alcohol drinking in the adolescent stress groups was influenced by the change from group to isolate housing conditions prior to the alcohol drinking phase. For example, isolation stress may have produced a different neuroendocrine response in the adolescent stress groups because of their prior history of footshock stress (i.e., they were more vulnerable to cross-sensitization).

A limitation of this study was that we did not measure physiological correlates of footshock stress. It is quite possible that age- and/or sex-related differences in the HPA axis response to footshock stress produced enduring changes in neuroendocrine response profiles that contributed to the observed behavioral differences in alcohol drinking and startle behavior. Some studies have shown that adolescent rodents display extended corticosterone release in response to an acute stressor (e.g., Goldman et al., 1973; Romeo et al., 2006) and show alterations in HPA axis recovery depending on the stage of puberty during the adolescent period (Romeo et al., 2006). Some of the age-dependent differences in HPA axis function differ between males and females, and there is conflicting evidence regarding the role of gonadal hormones in these effects (see review by McCormick and Mathews, 2007). It must also be considered that exposure to footshock stress and alcohol drinking may have altered estrous cyclicity in females (Baker et al., 2006), which could have influenced startle behavior. Although published reports in mice indicate no effect of stage of estrous cycle on startle and PPI (Farr et al., 1995; Meziane et al., 2007; Plappert et al., 2005), there are at least 2 conflicting reports in rats in which PPI was altered during proestrous (Kinkead et al., 2008; Koch, 1998). It has also been shown that many anxiety-related behaviors are greater when females are in certain stages of the estrous cycle (e.g., Marcondes et al., 2001; Schneider and Popik, 2007). Future work is needed to study the role of HPA and/or hypothalamic–pituitary–gonadal axes functions and their effects on stress-sensitive brain regions in regulating behavioral differences between adolescent and adult mice.

Overall, the present findings suggest that adolescent HAP1 mice are more vulnerable to footshock stress effects than adult mice, as manifested by increased alcohol drinking and enhanced anxiety-related behavior following re-exposure to footshock stress in adulthood. This interpretation is consistent with a number of studies indicating that adolescent rodents show greater stress-induced alterations in behavior and physiology compared to adults (e.g., Brunell and Spear, 2005; Hascoet et al., 1999; Spear, 2000; Stone and Quartermain, 1997). The present findings in HAP1 mice suggest that individuals with a family history of alcoholism may be more vulnerable to stress during adolescence, which may increase the risk for developing an alcohol-use and/or anxiety disorder. However, this suggestion should be viewed with caution until additional studies are conducted to examine stress effects in adolescent mice that have a genetic propensity for low alcohol preference to see if the present findings are specific to high-alcohol-preferring mice. Future research utilizing genetic animal models of alcohol drinking will continue to serve as useful tools to study how environmental factors interact with genetic propensity toward alcohol drinking to influence the risk for psychiatric disorders in humans. This work may uncover mechanisms of resilience and perhaps lead to specific and effective risk-reduction therapies aimed at reducing exposure to stress in vulnerable individuals.

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Fig. 1.

Mean ($\pm$ SEM) startle amplitude on prepulse (79, 85, 91 dB; 20 ms) and pulse (110, 125 dB; 40 ms) trials for male and female HAP1 mice exposed to footshock stress during adolescence (experiment 1—left panels) or adulthood (experiment 2—right panels) for phases 2 (top panels), 3 (middle panels), and 5 (bottom panels). *No stress > stress (collapsed across age; $p < 0.05$); +stress > no stress (collapsed across age; $p < 0.05$); #stress > no stress (collapsed across age and pulse stimuli; $p < 0.05$); &stress > no stress (collapsed across sex and pulse stimuli; $p < 0.01$).

Fig. 2.

Mean ($\pm$ SEM) % prepulse inhibition (PPI) on prepulse (79, 85, 91 dB; 20 ms) + 110 dB (40 ms) pulse stimuli for male and female HAP1 mice exposed to footshock stress during adolescence (experiment 1—left panels) or adulthood (experiment 2—right panels) for phases 2 (top panels), 3 (middle panels), and 5 (bottom panels). *Adolescent > adult (collapsed across group and sex; $p < 0.01$); +stress > no stress (collapsed across age; $p < 0.05$); #no stress > stress ($p < 0.05$).

Experiment 1 (Adolescent Footshock Stress)

Fig. 3.

Mean ($\pm$ SEM) alcohol intake in g/kg BW and percent alcohol preference (insets) in male (left panel) and female (right panel) HAP1 mice exposed to footshock stress during adolescence (experiment 1) averaged across 2-day blocks during the 30-day period (phase 4).

Experiment 2 (Adult Footshock Stress)

Fig. 4. Mean ($\pm$ SEM) alcohol intake in g/kg BW and percent alcohol preference (insets) in male (left panel) and female (right panel) HAP1 mice exposed to footshock stress in adulthood (experiment 2) averaged across 2-day blocks during the 30-day period (phase 4).

Table 1

Experimental Timeline for Experiments 1 and 2

<u>Phase of experiment</u>	<u>(1) Footshock stress</u>	<u>(2) Startle test</u>	<u>(3) Footshock stress + startle test</u>	<u>(4) Alcohol drinking</u>	<u>(5) Footshock stress + startle test</u>
Number of days of experiment	10; interim (6-7)	2; interim (13)	2; interim (3-4)	30; interim (1)	2
<i>Age of mice (postnatal day) at start of phase</i>					
Experiment 1 (adolescent footshock stress)	36-37	51-52	65-66	69-70	100-101
Experiment 2 (adult footshock stress)	72-77	88-93	102-107	106-111	137-142

Table 2

Mean ($\pm$ SEM) Body Weight in Male and Female HAP1 Mice Exposed to Footshock Stress or No Stress as Adolescents in Experiment 1 or as Adults in Experiment 2

	1				
Phase	Day 1	Day 10	2	3	5
Adolescent footshock stress (experiment 1)					
Male					
Stress	20.0 ± 0.5	21.0 ± 0.7 ^a	22.5 ± 0.7 ^b	24.6 ± 0.7	25.8 ± 0.7
No stress	19.9 ± 0.5	21.7 ± 0.4 ^a	22.9 ± 0.4 ^b	24.8 ± 0.5	26.0 ± 0.4
Female					
Stress	17.5 ± 0.3	18.1 ± 0.3 ^a	19.1 ± 0.3	20.3 ± 0.2	22.8 ± 0.4
No stress	18.0 ± 0.6	18.7 ± 0.4 ^a	19.4 ± 0.4	20.7 ± 0.5	22.7 ± 0.5
Adult footshock stress (experiment 2)					
Male					
Stress	25.3 ± 0.8	25.5 ± 0.8	26.4 ± 0.9 ^{b, c}	27.1 ± 0.9	27.5 ± 0.8
No stress	26.0 ± 0.3	26.7 ± 0.4 ^a	26.9 ± 0.4 ^{b, c}	27.4 ± 0.4	27.5 ± 0.3
Female					
Stress	22.6 ± 0.5	22.5 ± 0.4	22.5 ± 0.4 ^c	23.2 ± 0.5	23.5 ± 0.5
No stress	21.2 ± 0.4	22.0 ± 0.4 ^a	21.9 ± 0.5 ^c	22.3 ± 0.5	22.6 ± 0.5

Body weights were measured on days 1 and 10 of footshock stress exposure in phase 1 and prior to startle testing in phases 2, 3, and 5.

^a Day 10 > day 1 in phase 1 (experiment 1 collapsed across group, experiment 2 collapsed across sex; $ps < 0.01$).

^b Males > females (collapsed across experiment and group; $ps < 0.01$).

^c Experiment 2 mice > experiment 1 mice (collapsed across group and sex; $ps < 0.01$).