Adolescent C57BL/6J mice show elevated alcohol intake, but reduced taste aversion, as compared to adult mice: a potential behavioral mechanism for binge drinking

Sarah E. Holstein, Ph.D.[1,*], **Marina Spanos, Ph.D.**[3,*], and **Clyde W. Hodge, Ph.D.**[1,2,3]

[1]Bowles Center for Alcohol Studies, School of Medicine University of North Carolina at Chapel Hill Thurston-Bowles Building, CB 7178 Chapel Hill, NC 27599

[2]Department of Psychiatry, School of Medicine University of North Carolina at Chapel Hill Thurston-Bowles Building, CB 7178 Chapel Hill, NC 27599

[3]Curriculum in Neurobiology School of Medicine University of North Carolina at Chapel Hill Thurston-Bowles Building, CB 7178 Chapel Hill, NC 27599

Abstract

Background—Binge alcohol drinking during adolescence is a serious health problem which may increase future risk of an alcohol use disorder. Although there are several different procedures by which to preclinically model binge-like alcohol intake, limited-access procedures offer the advantage of achieving high voluntary alcohol intake and pharmacologically relevant blood alcohol concentrations (BACs). Therefore, in the current study, developmental differences in binge-like alcohol drinking using a limited-access cycling procedure were examined. In addition, as alcohol drinking has been negatively correlated with sensitivity to the aversive properties of alcohol, we examined developmental differences in sensitivity to an alcohol-induced conditioned taste aversion (CTA).

Methods—Binge-like alcohol consumption was investigated in adolescent (4 wk) and adult (10 wk) male C57BL/6J mice for 2-4 h/day for 16 d. Developmental differences in sensitivity to an alcohol-induced CTA were examined in adolescent and adult mice, with saline or alcohol (3 or 4 g/kg) repeatedly paired with intake of a novel tastant (NaCl).

Results—Adolescent mice showed a significant increase in alcohol intake as compared to adults, with adolescents achieving higher BACs and increasing alcohol consumption over successive cycles of the binge procedure. Conversely, adolescent mice exhibited a dose-dependent reduction in sensitivity to the aversive properties of alcohol, as compared to adult mice, with adolescent mice failing to develop a CTA to 3 g/kg alcohol. Finally, extinction of an alcohol CTA was observed following conditioning with a higher dose of alcohol in adolescent, versus adult, mice.

Conclusions—These results indicate that adolescent mice consume more alcohol, per kg body weight, than adults in a binge-like model of alcohol drinking, and demonstrate a blunted sensitivity to the conditioned aversive effects of alcohol. Overall, this supports a behavioral framework by which heightened binge alcohol intake during adolescence occurs, in part, via a reduced sensitivity to the aversive properties of alcohol.

Corresponding Author: Clyde W. Hodge, Ph.D. Bowles Center for Alcohol Studies University of North Carolina at Chapel Hill Thurston-Bowles Building, CB 7178 Chapel Hill, NC 27599-7178 Tel: (919) 843-4823 Fax: (919) 966-5679 chodge@med.unc.edu.
*These authors contributed equally to this work

Keywords

Development; ethanol; drinking; binge; correlated trait

Introduction

Alcohol use during adolescence is common (Eaton et al., 2010; Hill et al., 2000; Johnston et al., 2007; Miller et al., 2007), with binge drinking being a particularly frequent method of alcohol intake (Miller et al., 2007). Recently, the National Institute on Alcohol Abuse and Alcoholism (NIAAA; 2004) defined binge drinking as a pattern of alcohol consumption that rapidly brings blood alcohol concentrations to 80 mg/dl or above. Approximately one-quarter or more of U.S. high school students report binge alcohol use (Eaton et al., 2010; Miller et al., 2007), with over 60% of current adolescent drinkers engaging in binge drinking (Miller et al., 2007). However, the propensity towards binge-like patterns of alcohol intake drops off precipitously after the transition to adulthood, as adolescents engage in binge-like drinking at over twice the rate of adults (Nelson et al., 2009). These statistics are of concern not only because of the deleterious effects that alcohol may have on the developing brain (McQueeny et al., 2009; Nagel et al., 2005), but also because initiation of alcohol drinking at an early age has been associated with an increased risk of developing an alcohol use disorder later in life (DeWit et al., 2000; Grant and Dawson, 1998; Grant et al., 2001).

Consistent with the epidemiological data, the adolescent developmental period in rodents (which conservatively spans postnatal day [PND] 28-42; Spear, 2000) is associated with an increase in alcohol intake. This age difference, however, may depend on which measure of alcohol consumption is used (i.e. free- vs. limited-, or binge-like, access). For instance, using home-cage models of free-access alcohol consumption, several studies have reported a significant increase in alcohol intake in adolescent mice and rats as compared to adults (Brunell and Spear, 2005; Doremus et al., 2005; Garcia-Burgos et al., 2009; Tambour et al., 2008; Vetter et al., 2007), although no age difference and an opposite pattern of results have also been found (Bell et al., 2006; Fullgrabe et al., 2007; Hefner and Holmes, 2007a; Siegmund et al., 2005). However, using limited-access procedures, a more consistent increase in alcohol intake during adolescence has been reported (Maldonado et al., 2008; Metten et al., in press; Moore et al., 2010; Strong et al., 2010; Vetter-O'Hagen et al., 2009; Walker et al., 2008), which may support a particular vulnerability to binge-like patterns of alcohol intake during this developmental time period.

There are a variety of methods used to model high, or 'binge-like', alcohol intake in rodents, including sucrose fading, food and/or water deprivation, and various limited-access procedures (see Finn et al., 2005; Rhodes et al., 2005 for a review and commentary on these various methods). For this experiment, we chose to use an adaptation of the 'drinking in the dark' (DID) procedure (Rhodes et al., 2005). This limited-access protocol results in rapid and high alcohol intake accompanied by behavioral signs of intoxication and physiologically relevant blood alcohol concentrations above 100 mg/dl (Rhodes et al., 2005; 2007). Moreover, this method does not require food or water deprivation to promote high intake levels. Recently, adolescent mice were found to consume significantly more alcohol (per kg body weight) than adult mice using variations of this protocol (Metten et al., in press; Moore et al., 2010). In the current study, we also sought to examine age differences in binge alcohol intake using a limited-access procedure. However, this protocol was modified so as to expose the animals to repeated cycles of binge intake, with 3 days at 2-h/day and 1 day at 4-h, repeated for 4 cycles. One benefit of this cycling procedure is that we can determine whether repeated cycles of binge drinking, with multiple extended-access, 4-h drinking sessions, result in an escalation of alcohol intake. If so, this may correlate with the

observation of increased binge-like alcohol drinking in humans as adolescence progresses (Miller et al., 2007).

In addition to increased alcohol consumption, adolescent mice and rats are also less sensitive to many of the negative effects of alcohol which may serve to curb alcohol intake (Draski et al., 2001; Hefner and Holmes, 2007a; Linsenbardt et al., 2009; Quoilin et al., 2010; Silveri and Spear, 1998; Varlinskaya and Spear, 2002, 2004; for review, see Schramm-Sapyta et al., 2009; Spear and Varlinskaya, 2010). One likely contributor to binge-like alcohol drinking is a relative insensitivity to the aversive properties of alcohol. This can be measured experimentally using conditioned taste aversion (CTA) procedures, in which the potentially aversive motivational effects of alcohol are paired with a novel conditioned stimulus (Chester and Cunningham, 2002). Multiple studies have reported a strong negative genetic correlation between CTA and alcohol intake (Broadbent et al., 2002; Brunetti et al., 2002; Chester et al., 2003; Froehlich et al., 1988; Green and Grahame, 2008; Quintanilla et al., 2001; Rhodes et al., 2007; Risinger and Cunningham, 1995), supporting the hypothesis that high alcohol intake is associated with a decreased sensitivity to the aversive effects of alcohol. Although the negative relationship between alcohol intake and CTA has been well established in adult rodents, only a few studies have investigated this phenomenon during adolescence, and only in rats (Anderson et al., 2010; Schramm-Sapyta et al., 2010; Vetter-O'Hagen et al., 2009). To the best of our knowledge, developmental differences in sensitivity to an alcohol CTA have not been previously reported in mice. Likewise, age differences in the extinction of an alcohol aversion, which may have a significant impact on future drinking, have not been previously examined.

The purpose of the current study was to further examine age-related differences in binge alcohol intake and to determine whether high binge alcohol drinking in adolescents is associated with insensitivity to the conditioned aversive effects of alcohol. Consistent with previous findings (Moore et al., 2010; Strong et al., 2010), we predicted that adolescent C57BL/6J mice would consume significantly more alcohol, relative to body weight, than adult mice in a limited-access procedure. Moreover, we hypothesized that adolescent intake would escalate with successive binge cycles, an effect we did not predict to see in adults. Finally, similar to results in rats (Anderson et al., 2010; Schramm-Sapyta et al., 2010; Vetter-O'Hagen et al., 2009), we predicted that adolescent mice would be less sensitive to the development of an alcohol CTA than adult mice, and would extinguish an alcohol CTA more readily than adult mice (i.e. after a higher alcohol dose).

Materials and Methods

Subjects

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), age 3 weeks (adolescent) and 9 weeks (adult) at the time of arrival, were isolate housed in standard Plexiglas cages with corn-cob bedding and food and water available *ad libitum*, except where noted in individual experiments. For mice in Experiment 2, cages also contained a small PVC tube and a nestlet for the purpose of environmental enrichment. The colony room was maintained on a 12:12 h L:D cycle (lights off at 8 am) at $21 \pm 1°C$. All behavioral testing occurred during the dark cycle. Mice were repeatedly handled for 1 wk prior to the start of experimental testing. All procedures were approved by the UNC-Chapel Hill Institutional Animal Care and Use Committee and were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Drugs

For Experiment 1, the 20% alcohol drinking solution (v/v) was prepared by diluting 95% w/v alcohol (Decon Labs, Inc., King of Prussia, PA, USA) with tap water. For Experiment 2, 0.2M NaCl (Fisher Scientific, Pittsburgh, PA, USA) was prepared in tap water, while injected alcohol solutions were prepared fresh daily and diluted in 0.9% w/v physiological saline (Ricca Chemical Co., Arlington, TX, USA) to a concentration of 20% (v/v). Injections were administered intraperitoneally (IP), with injection volume varied by dose. Saline injections were administered at a volume equivalent to the 4 g/kg dose.

Experiment 1: Binge-like drinking in adolescent and adult mice

Twelve adolescent (4 wk, or PND 28, at start of testing) and 12 adult (10 wk, or PND 71 at the start of testing) C57BL/6J mice were allowed to drink alcohol (20% v/v) in a limited-access procedure, using an adaptation of previously published methods (Rhodes et al., 2005). Briefly, for the first 3 d, the water bottle was removed and replaced with a 50-ml conical tube containing a 20% v/v alcohol solution (bottle on at 11 am). Two hours later (1 pm), the bottle was removed, and the water bottle was immediately returned to the cage. On day 4, the same procedure was used, except the bottle remained on the cage for a total of 4 h (11 am-3 pm). This procedure was repeated four times to bring the total drinking time to 16 d (thereby spanning the entire adolescent period). Alcohol bottles were weighed (to the nearest 0.1 g) before and after presentation on the home cage to determine alcohol consumption, and body weights were taken repeatedly throughout the experiment (prior to DID consumption) to calculate alcohol dose consumed (g/kg).

Blood Alcohol Determination—Blood alcohol measurements from tail blood samples were taken on day 16 (PND 43 and 86), immediately after the last 4 h binge session, a procedure consistent with previous studies in our laboratory (Faccidomo et al., 2009; Stevenson et al., 2009). Mice were briefly placed in a restrainer (Braintree Scientific, Inc., Braintree, MA, USA) and a tail blood sample was collected in a heparinized microcapillary tube, centrifuged, and the resultant plasma (5 μl) was injected into an AM1 Alcohol Analyzer (Anaolx Instruments, Lunenburg, MA, USA) to determine blood alcohol concentration (BAC). As brain and tail blood alcohol concentrations have been found to equilibrate within approximately 30-60 min following a bolus ethanol injection (Gentry et al., 1983; Lumeng et al., 1982; Nurmi et al., 1994), determination of BACs after a 4 h binge session should provide a fairly accurate reflection of brain alcohol levels, and whether these levels meet or exceed the NIAAA definition of binge alcohol intake (National Institute on Alcohol Abuse and Alcoholism, 2004).

Experiment 2: Alcohol-induced conditioned taste aversion (CTA) in adolescent and adult mice

CTA procedures were adapted from previously published methods (Palmer et al., 2004). Adolescent (3 wk; n=10-12/alcohol dose) and adult (9 wk; n=10/alcohol dose) C57BL/6J male mice were isolate housed upon arrival to the animal colony and left undisturbed for 2 d. On day 3, mice were weighed and water bottles were removed (at 2 pm). For the following 4 d, mice were habituated to the fluid restriction schedule, with water provided for 2 h/day (12-2 pm); for these, and all following days, mice were weighed daily prior to fluid access to monitor health and/or calculate alcohol injection volumes. All fluids were provided in a 10 ml calibrated sipper tube (with ball bearing), which was secured to the wire cage top by a medium binder clip. Initial and final fluid volumes (to the nearest 0.1 ml) were recorded to measure fluid intake. Two control tubes on empty cages were also utilized to monitor leakage, and any recorded spillage from these tubes was subtracted from all volume recordings for that day.

Taste Conditioning—Behavioral testing began on day 8 (4 and 10 wks of age, or PND 29 and 71), at which point mice were presented with the novel conditioned stimulus (CS; 0.2M NaCl) in place of water for 1 h (12-1 pm). This initial 1 h exposure (trial 0) was used to habituate the mice to the novel tastant before conditioning trials began. Water was then provided for 30 min, 4 h after the removal of the 0.2M NaCl solution (5 pm) to prevent dehydration. On days 9, 11, 13, 15, and 17, a 2-h water access was provided (12-2 pm). Taste conditioning was performed on the intervening days (10, 12, 14, and 16, or conditioning trials 1-4). Similar to trial 0, mice were provided 1-h access to 0.2M NaCl during the conditioning trials. Immediately after the 1-h CS exposure, the bottle was removed and each mouse was administered an IP injection of 0, 3 or 4 g/kg of alcohol. Each mouse received the same alcohol dose after each of these 4 conditioning trials. Water was provided for 30 min, 4-h after the removal of the CS (5 pm). On day 18 (conditioning trial 5; PND 39 and 81), a 1-h CS access was again provided, but no alcohol injection was administered; instead, a water bottle was returned to the cage immediately after CS removal.

Extinction Testing—In order to examine the extinction of an alcohol-induced CTA in adolescent and adult mice, mice were re-exposed to the CS for 3 additional trials (trials 6-8), in the absence of further vehicle or alcohol injections. Briefly, mice were presented with the CS for 1 h/day (12-1 pm) once every 5 d following the cessation of taste conditioning (days 23, 28, and 33). Water bottles were removed 22 h prior to CS access, and returned immediately after CS removal.

Data Analysis

For Experiment 1, alcohol consumption (g/kg) was first analyzed by repeated measures analysis of variance (RM ANOVA), with age as the between-groups factor and day as the within-groups factor. Significant interactions were followed up by Holm-Sidak post-hoc comparisons. In addition, average alcohol dose consumed and BACs in the two age groups were compared by two-tailed unpaired t-tests. For Experiment 2, the development, and extinction, of a CTA was indexed as the change in CS (0.2M NaCl) consumption from conditioning trial 1, with an aversion defined as a decrease in CS consumption from baseline. Conditioning and extinction data were each analyzed by RM ANOVA (age X alcohol dose X conditioning trial); a significant 3-way interaction was followed up by separate alcohol dose X conditioning trial RM ANOVAs within each age group. In addition, separate age X conditioning trial RM ANOVAs were conducted within each alcohol dose in order to directly compare the two age groups. Significant interactions were followed up by Holm-Sidak post-hoc comparison, where appropriate. Finally, a change in CS consumption over extinction trials (trial 8 – trial 5) was analyzed by a two-way age X alcohol dose ANOVA. Analyses were performed using SigmaPlot (Systat Software Inc., San Jose, CA, USA) and IBM SPSS Statistics 18 software (Chicago, IL, USA). Significance was set *a priori* at $\alpha \leq 0.05$.

Results

Experiment 1: Binge-like drinking in adolescent and adult mice

As seen in Fig. 1, under binge-like alcohol exposure conditions, adolescent mice (PND 28 at the start of testing) consumed significantly more alcohol, relative to body weight, than adult mice (PND 71 at the start of testing). Due to experimenter error, a missing data point from 1 adult mouse (day 1, 2 h consumption) was replaced with the group mean for that day. In an analysis of drinking across the twelve 2-h limited access sessions (days 1-3, 5-7, 9-11, and 13-15), a RM ANOVA revealed main effects of age [$F_{1,22}=15.0$; $p<0.001$] and day [$F_{11,242}=7.1$; $p<0.001$], as well as an age X day interaction [$F_{11,242}=3.9$; $p<0.001$]. As shown in Fig. 1A, adolescent mice consumed significantly higher doses of alcohol than

adults on days 2, 5, 9, and 10 (ps<0.01). When averaged across the twelve 2-h sessions, this age difference was supported, with adolescent mice consuming on average more alcohol, per kg body weight, than adult mice [t_{22}=3.9; p<0.001]. Average alcohol intake ($\pm$ SEM) during the 2-h sessions was 6.3 ($\pm$ 0.2) g/kg for adolescent mice and 5.2 ($\pm$ 0.2) g/kg for adult mice.

In a separate analysis of the extended 4-h binge sessions (days 4, 8, 12, and 16), an increase in alcohol intake in adolescence was again found, with a RM ANOVA revealing a significant effect of age [$F_{1,22}$=22.8; p<0.001], as well as a significant age X day interaction [$F_{3,66}$=3.9; p=0.01] (Fig. 1B). Post-hoc comparisons revealed a significant increase in alcohol consumption on day 12 in adolescents relative to day 4 (p=0.01), suggesting an escalation in alcohol intake with repeated binge cycles in adolescent mice. In adults, however, alcohol consumption decreased with successive cycles, with alcohol intake significantly reduced on day 16 as compared to day 4 (p=0.02). Moreover, alcohol intake was significantly higher in adolescents, as compared to adults, on days 8, 12, and 16 (ps<0.05). This age difference was confirmed when the data over the 4-h binge sessions were averaged, with adolescent mice consuming significantly more alcohol (per kg body weight) than adult mice [t_{22}=4.8; p<0.001]. Average alcohol intake ($\pm$ SEM) was 9.2 ($\pm$ 0.3) g/kg for adolescent mice and 7.5 ($\pm$ 0.1) g/kg for adult mice. BAC's taken on day 16, immediately after the 4-h binge, also supported an age difference in alcohol dose consumed, with adolescent mice (204.7 $\pm$ 9.3 mg/dl) having a significantly higher BAC than adult mice (119.6 $\pm$ 9.5 mg/dl) after a 4-h binge session [t_{20}=6.4; p<0.001] (Fig. 1C).

Experiment 2: Alcohol-induced conditioned taste aversion (CTA) in adolescent and adult mice

Taste Conditioning—When examined across conditioning trials (trials 1-5), repeated alcohol administration decreased CS (NaCl) consumption; however, as shown in Fig. 2A, adolescent mice did not develop a significant aversion (compared to baseline) to the 3 g/kg dose of alcohol, whereas adult mice did. These results suggest that adolescent C57BL/6J mice are less sensitive than their adult counterparts to these aversive effects of alcohol, which may explain, in part, their heightened binge-like alcohol intake.

Briefly, during the course of the experiment, 1 adolescent mouse (4 g/kg) died, 1 adolescent mouse (4 g/kg) did not respond to the first alcohol injection, and 1 adult mouse (0 g/kg) did not consume any of the CS on conditioning trial 1; therefore, these data were removed from the analysis. In addition, due to a recording error, data from 1 adolescent mouse (4 g/kg) from conditioning trial 3 was replaced with the group mean. The resultant n was 9-10 per age and alcohol dose. Initial body weights for the two age groups prior to implementation of the water restriction schedule (day 3) were 10.7 $\pm$ 0.2 g for adolescents and 24.2 $\pm$ 0.3 g for adults. Early into the water deprivation schedule, some minor weight loss was observed in both age groups (-1.1 $\pm$ 0.1 g in adolescents, -3.0 $\pm$ 0.2 g in adults after 2 days of limited water access), but body weights stabilized rapidly, with adolescents and adults weighing 12.4 $\pm$ 0.2 g and 21.8 $\pm$ 0.3 g, respectively, at the start of testing (T0).

In order to examine the development of an alcohol-induced CTA in adolescents and adults, data were expressed as change in CS intake from Trial 1 (CS intake immediately prior to the first alcohol injection). Development of an aversion within each age and or dose group was operationally defined as a decrease in CS intake from Trial 1. Mean ($\pm$ SEM) NaCl intake was 2.1 $\pm$ 0.3 mls, 2.1 $\pm$0.3 mls, and 2.4 $\pm$ 0.3 mls for the adolescent 0, 3, and 4 g/kg groups, respectively, and 2.4 $\pm$ 0.4 mls, 2.8 $\pm$ 0.5 mls, and 2.8 $\pm$ 0.2 mls for the adult 0, 3, and 4 g/kg groups, respectively. A RM ANOVA of change in CS intake from Trial 1 revealed a significant age X alcohol dose X conditioning trial interaction [$F_{8,212}$=2.0; p=0.049]. In order to examine the effect of alcohol on CS intake within each age group, separate alcohol dose X conditioning trial ANOVAs were first conducted. Within adolescent mice, there

were main effects of alcohol dose [$F_{2,27}$=28.1; p<0.001] and conditioning trial [$F_{4,108}$=3.9; p=0.005], as well as a significant alcohol dose X trial interaction [$F_{8,108}$=8.7; p<0.001]. Follow-up post-hoc analyses revealed a significant increase in CS consumption in the vehicle-treated group at conditioning trial 5, as compared to conditioning trial 1 (p<0.001). Although a moderate dose of alcohol (3 g/kg) did not affect CS intake in adolescent mice, the 4 g/kg alcohol dose did significantly decrease CS consumption in conditioning trials 2-5 (as compared to trial 1; ps<0.001), indicative of a CTA. In adult mice, there were main effects of alcohol dose [$F_{2,26}$=33.9; p<0.001] and conditioning trial [$F_{4,104}$=18.8; p<0.001], as well as a significant alcohol dose X conditioning trial interaction [$F_{8,104}$=11.7; p<0.001]. CS intake in adults increased over trials in the vehicle group (conditioning trials 2, 3, and 5; ps<0.01), and decreased over trials in the adult 4 g/kg treatment group (conditioning trials 2-5; ps<0.001). Unlike the adolescent group, however, adult C57BL/6J mice treated with 3 g/kg alcohol did significantly reduce CS intake in trials 2-5 (relative to trial 1; ps<0.001), indicating the development of a CTA to this moderate dose of alcohol in adults.

Although a taste aversion developed to 3 g/kg alcohol only in adult C57BL/6J mice, this does not provide a direct statistical comparison between the two age groups, and therefore separate age X conditioning trial RM ANOVAs were also conducted at each alcohol dose. In the 0 g/kg alcohol group, there was a main effect of trial [$F_{4,68}$=3.3, p=0.02], but no significant effect of age or an age X trial interaction. Similar results were observed in the 4 g/kg alcohol group, with a main effect of trial [$F_{4,72}$=57.5, p<0.001], but no effect of age or an age X trial interaction, reflecting no differences between adolescents and adults repeatedly treated with vehicle or a high dose of alcohol (4 g/kg). However, in mice repeatedly administered 3 g/kg alcohol, there were significant effects of age [$F_{1,18}$=16.3, p=0.001] and conditioning trial [$F_{4,72}$=15.8, p<0.001]. In addition, there was a significant age X conditioning trial interaction [$F_{4,72}$=13.1, p≤0.001], with the change in CS intake being significantly larger in adults than adolescents on conditioning trials 3-5 (ps<0.02). For ease of viewing, the age difference in overall sensitivity to an alcohol CTA is highlighted in Fig. 2B, which visually depicts the overall change in CS intake after the 5 conditioning trials (trial 5-trial 1). These results support the hypothesis that adolescent mice are less sensitive to the aversive properties of alcohol than adults.

Extinction—In order to examine the extinction of an alcohol-induced CTA, mice were re-exposed to the CS (in the absence of a vehicle or alcohol injection) once every 5 d for 3 trials (trials 6-8). As shown in Fig. 2A, extinction of the CTA was observed in adult mice at 3 g/kg and in adolescent mice at 4 g/kg, a dose at which no change in CS intake was observed in adults.

Due to a transcription error, data from 1 adolescent mouse on trial 7 (3 g/kg) was replaced by the group mean for that trial. However, in trial 6, data from 4 adolescent mice (2 in the 0 g/kg group, and 1 each from the 3 and 4 g/kg groups) were improperly recorded; due to this larger loss of data, these data were dropped from the RM analysis of extinction (resultant n was 8-10 per age and alcohol dose). In an analysis of the 3 extinction trials (trials 6-8), a RM ANOVA revealed a significant age X alcohol dose X extinction trial interaction [$F_{4,98}$=4.3; p=0.003], which prompted separate analyses within each age group. In adolescent mice, there were main effects of alcohol dose [$F_{2,23}$=10.5; p<0.001] and extinction trial [$F_{2,46}$=8.6; p<0.001], but no alcohol dose X extinction trial interaction (p=0.16). This lack of an interaction was likely mediated by a generalized increase in CS intake over extinction trials; however, as seen in Fig. 2A, there was a clear extinction of the CTA in the adolescent 4 g/kg treatment group. Bonferroni-corrected planned comparisons between trials 6 and 8 in adolescent mice were therefore conducted in order to examine whether an extinction of an alcohol CTA was observed in the 4 g/kg group (see Keppel and Wickens, 2004 for a discussion of planned comparisons when the interaction term is not significant). As visually

observed in Fig 2A, there was a significant increase in CS consumption between trials 6 and 8 [t_{16}= -3.2; p=0.006] in adolescents previously conditioned with 4 g/kg alcohol, indicative of extinction. However, there was no significant change in CS intake in adolescents conditioned with 0 or 3 g/kg alcohol. In adult mice, an alcohol dose X extinction trial RM ANOVA revealed a main effect of alcohol dose [$F_{2,26}$=31.5; p<0.001] and extinction trial [$F_{2,52}$=8.2; p<0.001], as well as an alcohol dose X extinction trial ANOVA [$F_{4,52}$=6.0; p<0.001]. Post-hoc comparisons showed a significant increase in CS intake in the adult 3 g/kg treatment group over extinction trials (ps<0.001), but unlike the adolescent mice, there was no change in CS intake in the 4 g/kg group.

In order to directly compare the two age groups, and due to the different baselines from which extinction started, the data were also indexed as change across extinction sessions (see extinction summary, Fig 2B). Briefly, CS intake on the final conditioning trial (trial 5, the first trial in which alcohol did not immediately follow the CS) was subtracted from CS consumption on the final extinction trial (trial 8), thereby summarizing the change in CS intake over 15 d after taste conditioning. Positive values represent an increase in CS intake during the extinction period, indicative of a decrease in aversion. An age X alcohol dose ANOVA revealed a main effect of alcohol dose [$F_{2,53}$=9.8; p<0.001] and a significant age X alcohol dose interaction [$F_{2,53}$=7.0; p=0.002]. Post-hoc analyses revealed a significant difference between the two age groups at both the 3 and 4 g/kg alcohol doses (ps<0.05), with adults showing an increase in CS intake after 3 g/kg alcohol (significantly different from vehicle; p=0.001). In adolescents, an increase in CS intake, indicative of extinction, was observed in the 4 g/kg alcohol group (p<0.001), supporting the results from the planned comparisons performed in the previous analysis. In summary, although adolescent mice conditioned with 4 g/kg alcohol showed an extinction of an alcohol CTA over the 15 d extinction period, adult mice did not. Rather, extinction of an alcohol-induced CTA was only observed at a lower dose (3 g/kg) in adults.

Discussion

Risk taking behavior, including heavy episodic, or binge, drinking, is common during adolescence and occurs at rates higher than that seen in adults (Eaton et al., 2010; Miller et al., 2007; Nelson et al., 2009). The results of the current study support this characterization. Briefly, we observed increased binge-like alcohol intake and higher BACs in adolescent C57BL/6J mice as compared to adults. In addition, the current results indicate that adolescent C57BL/6J mice show a blunted sensitivity to the aversive effects of alcohol as compared to adults, and extinction of a CTA is observed following a higher dose of alcohol in adolescents as compared to adults. These results add to the growing literature highlighting adolescence as a time of increased sensitivity to the positive (hedonic) effects of alcohol and decreased sensitivity to the negative (aversive) effects of alcohol, which may promote increased alcohol intake during this developmental stage (Schramm-Sapyta et al., 2009; Spear and Varlinskaya, 2010).

Elevated alcohol intake during adolescence has repeatedly been observed in the preclinical literature; however, this result may be dependent on the method of alcohol consumption used. Although contradictory results have been observed for free-access alcohol drinking studies (Bell et al., 2006; Brunell and Spear, 2005; Doremus et al., 2005; Fullgrabe et al., 2007; Garcia-Burgos et al., 2009; Hefner and Holmes, 2007a; Siegmund et al., 2005; Tambour et al., 2008; Vetter et al., 2007), in limited-access drinking studies, adolescent mice and rats have consistently shown an increase in alcohol intake relative to adults (Maldonado et al., 2008; Metten et al., in press; Moore et al., 2010; Strong et al., 2010; Vetter-O'Hagen et al., 2009; Walker et al., 2008). The results of Experiment 1 further corroborate this increase in binge-like alcohol consumption during adolescence, and may

support an escalation of binge drinking as adolescence progresses. The current data also corroborate the utility of this type of limited-access procedure for eliciting high or binge-like patterns of alcohol intake that result in physiologically significant BACs (Moore et al., 2010; Rhodes et al., 2005; Strong et al., 2010). In adolescent mice (as shown in Fig. 1), the increase in alcohol intake was supported by a significantly higher BAC after a 4-h binge session (205 mg/dl), a level that was almost twice that of adult mice (120 mg/dl).

Increased alcohol intake in adolescence may suggest heightened reward sensitivity during this developmental stage (but see Dickinson et al., 2009; Song et al., 2007). Combined with other studies that have reported increased sensitivity to the social facilitating (Varlinskaya and Spear, 2002), behavioral stimulant (Hefner and Holmes, 2007a; Quoilin et al., 2010; Stevenson et al., 2008), and anxiolytic effects of alcohol (Hefner and Holmes, 2007a), these results suggest increased sensitivity to the positive motivational effects of alcohol during adolescence (Schramm-Sapyta et al., 2009; Spear and Varlinskaya, 2010). In contrast, adolescents appear to be less sensitive than their adult counterparts to the sedative-hypnotic (Draski et al., 2001; Hefner and Holmes, 2007a; Linsenbardt et al., 2009; Quoilin et al., 2010; Silveri and Spear, 1998), social inhibitory (Varlinskaya and Spear, 2002, 2004), and aversive effects of alcohol (Anderson et al., 2010; Schramm-Sapyta et al., 2010; Vetter-O'Hagen et al., 2009). The results of Experiment 2 further support this hypothesis. Specifically, adolescent mice did not develop a CTA to alcohol (3 g/kg), indicating dose-dependent reduced sensitivity to the aversive properties of alcohol in adolescents as compared to adults. These results are in striking similarity to recent reports demonstrating decreased sensitivity to the development of an alcohol-induced CTA in adolescent rats (Anderson et al., 2010; Schramm-Sapyta et al., 2010; Vetter-O'Hagen et al., 2009), and add to the growing literature suggesting an overall decreased sensitivity of adolescents to the negative effects of alcohol (Schramm-Sapyta et al., 2009; Spear and Varlinskaya, 2010).

A survey of the published literature supports a negative correlation between alcohol consumption and expression of an alcohol CTA (Green and Grahame, 2008), a finding supported by the current results. For instance, mice and rats selectively bred for high alcohol consumption and preference show reduced sensitivity to an alcohol-induced CTA than rodents selectively bred for low alcohol consumption and preference (Brunetti et al., 2002; Chester et al., 2003; Froehlich et al., 1988; Quintanilla et al., 2001). A similar pattern of results has been observed in several inbred strain panels, with an inverse genetic correlation between alcohol consumption (including binge consumption in the DID procedure) and magnitude of an alcohol CTA (Broadbent et al., 2002; Rhodes et al., 2007; Risinger and Cunningham, 1995). The current results further support this hypothesis, and suggest that heightened sensitivity to an alcohol-induced CTA in adults may act as an internal stimulus cue to curb or curtail alcohol intake, whereas the blunted sensitivity to an alcohol CTA in adolescents may promote heightened binge intake (Anderson et al., 2010; Broadbent et al., 2002; Elkins, 1991; Garcia et al., 1974; Green and Grahame, 2008; Schramm-Sapyta et al., 2009).

In adolescents, there was also a modest escalation in alcohol intake with successive binge cycles. Conversely, in adults, alcohol intake gradually decreased with successive binge cycles. This modest decrease in binge-like drinking in adults could suggest the development of an alcohol CTA as drinking progressed. Additional studies are needed to determine if a CTA is responsible for reducing alcohol intake in adults in a cycling binge procedure, and whether alcohol consumption during adolescence precludes this effect when mice reach adulthood. In general, pre-exposure to the unconditioned stimulus (US), such as alcohol, prior to taste conditioning reduces the aversive properties of that US (Lessov et al., 2001; Rabin et al., 1988; Randich and LoLordo, 1979). A history of alcohol use during adolescence may then promote heightened alcohol intake during adulthood (Blizard et al.,

2004; Ho et al., 1989; Moore et al., 2010) and increase the risk for later alcohol dependence (DeWit et al., 2000; Grant and Dawson, 1998; Grant et al., 2001) due to a dampening of sensitivity to the aversive effects of alcohol that would normally have developed during adulthood.

Interestingly, extinction of an alcohol-induced CTA was also enhanced in adolescent mice, with CS intake during extinction (when no alcohol was administered) increasing in adolescents conditioned with 4 g/kg alcohol. There was no recovery of CS intake in adult mice conditioned with this same dose of alcohol (though there was a recovery of CS intake in adult mice conditioned with 3 g/kg alcohol), suggesting that this aversion memory is more powerful and/or prolonged in adults as compared to adolescents. Interestingly, results from other Pavlovian learning procedures, such as fear conditioning, have shown no age difference in the rate of extinction of a conditioned fear response (Hefner and Holmes, 2007b; McCallum et al., 2010), which may suggest that adolescents do not simply show a generalized enhanced rate of extinction. However, age differences in severity of an alcohol CTA may preclude interpretation of these results as simply adolescents extinguishing this response faster or more readily. For instance, although both adolescents and adults repeatedly administered 4 g/kg alcohol similarly avoided the CS, if this aversion is simply not as strong in adolescents, then they may be more likely to extinguish this response than adults. Therefore, the age difference in extinction of an alcohol CTA may not only reflect a weaker aversion memory in adolescents, but may also simply reflect a weaker alcohol aversion in this age group. Further studies are needed to distinguish these possibilities. Despite this caveat, this abbreviated persistence of a CTA in adolescents is yet another factor that likely promotes binge alcohol consumption in adolescents and facilitates adolescents returning to reckless or high levels of alcohol intake sooner than that observed in adults.

Although considerable work has been done on the association between alcohol intake and CTA, it must be noted that the relative insensitivity of adolescents to the development of a CTA may not be unique to alcohol, as adolescents are also less sensitive to the aversive properties of other drugs of abuse, including amphetamine (Infurna and Spear, 1979), cocaine (Schramm-Sapyta et al., 2006), and nicotine (Shram et al., 2006). This suggests a general decrease in sensitivity to drug aversions, which likely contributes to the increase in drug experimentation and use during adolescence (Eaton et al., 2010). However, decreased sensitivity to the conditioned aversive properties of alcohol and other drugs of abuse may also suggest a deficit in associative learning during adolescence (Schramm-Sapyta et al., 2006, 2009). For instance, adolescent mice are not only less sensitive to the aversive properties of drugs of abuse, but are also less sensitive to the aversive properties of the emetic agent lithium chloride (Schramm-Sapyta et al., 2006). This generalized reduction in aversive responding during development may indicate that adolescents are less able to associate the aversive properties of a drug, such as alcohol, with the properties (taste) of the CS (Schramm-Sapyta et al., 2006, 2009). For instance, in the current study, adolescent C57BL/6J mice may experience similar aversive reactions to a moderate or high dose of alcohol as adults, but may simply not associate it with the CS (NaCl), despite the temporal contiguity between these two stimuli. Further studies are required to definitively conclude whether adolescents are truly less sensitive than adults to the aversive properties of alcohol, as shown through CTA, or whether they are simply less effective at associating the aversive stimulus properties of alcohol with the consumed tastant.

In summary, results of the current study demonstrate that adolescent C57BL/6J mice consume significantly more alcohol (g/kg) than adult mice in a model of binge-like drinking, and achieve higher BACs than their adult counterparts. Moreover, preliminary evidence suggests that adolescent mice escalate their binge-like alcohol intake, whereas adult mice

decrease alcohol intake, with repeated binge cycles. Additionally, the current study supports the hypothesis that adolescent mice are less sensitive to an alcohol-induced CTA than adult mice. This relative insensitivity to the aversive effects of alcohol in adolescence likely contributes to the high alcohol intake (binging) observed during adolescence, and may contribute to increased risk of alcohol abuse in adulthood. These results provide additional data to support the hypothesis that adolescence is a sensitive developmental time period during which the motivational response to alcohol is unique. Additional studies are required to further examine how this may affect risk of an alcohol use disorder later in life, and what behavioral and pharmacological interventions may be best suited to treat binge alcohol use during adolescence.

Acknowledgments

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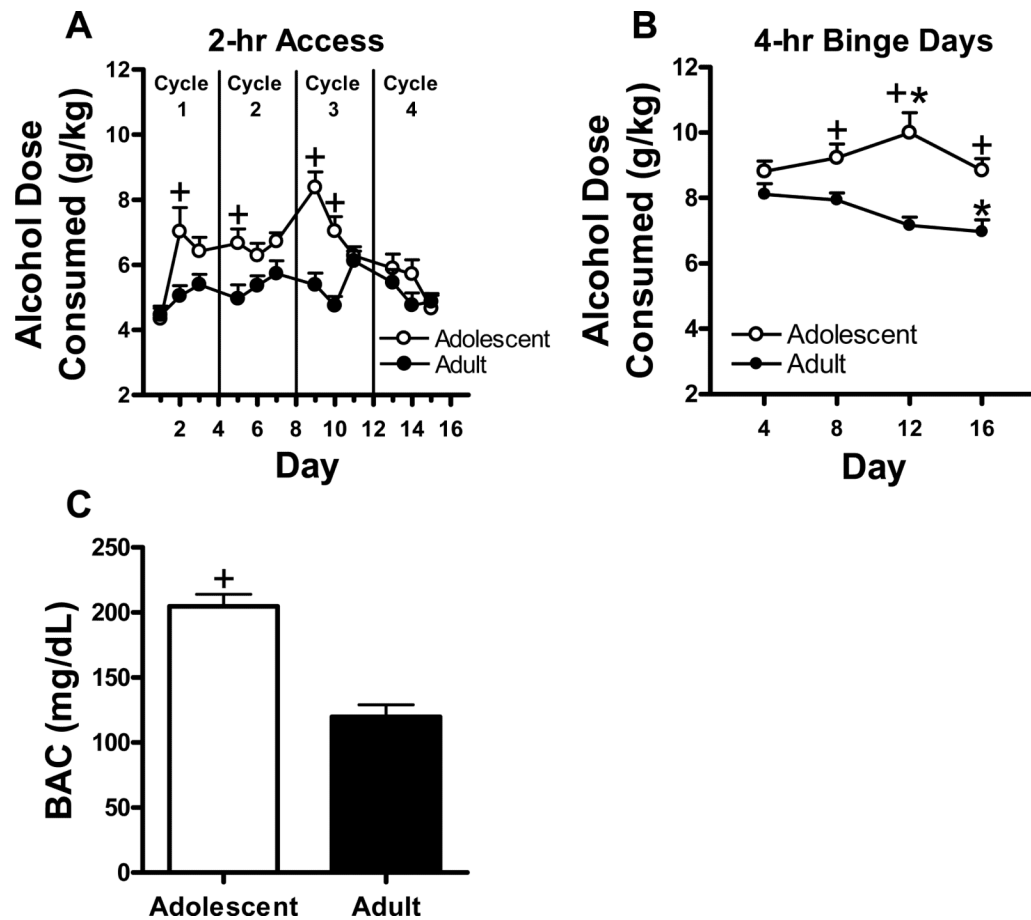
Figure 1. Adolescent C57BL/6J mice consume significantly more alcohol, per kg body weight, than adult mice in a limited-access paradigm

Adolescent and adult mice were presented with a 20% v/v alcohol solution in place of water for 2-h (days 1-3, 5-7, 9-11, and 13-15) or 4-h (days 4, 8, 12, and 16) per day, and intake was recorded. Shown are mean ± SEM values. **(A)** Analysis of alcohol intake over the twelve 2-h binge sessions revealed an increase in dose consumed in adolescents, as compared to adults, on days 2, 5, 9 and 10. **(B)** Analysis of alcohol intake during the 4-h binge sessions revealed a significant increase in alcohol consumption on day 12 (relative to day 4) in adolescents, but a significant decrease in alcohol consumption on day 16 (relative to day 4) in adults. The two age groups differed in alcohol intake on days 8, 12, and 16. **(C)** BACs, taken immediately following the final binge session on day 16, were significantly higher in adolescents as compared to adults. + significant difference from adults, ps<0.01. * significant difference from day 4 in the respective age group, ps<0.05.
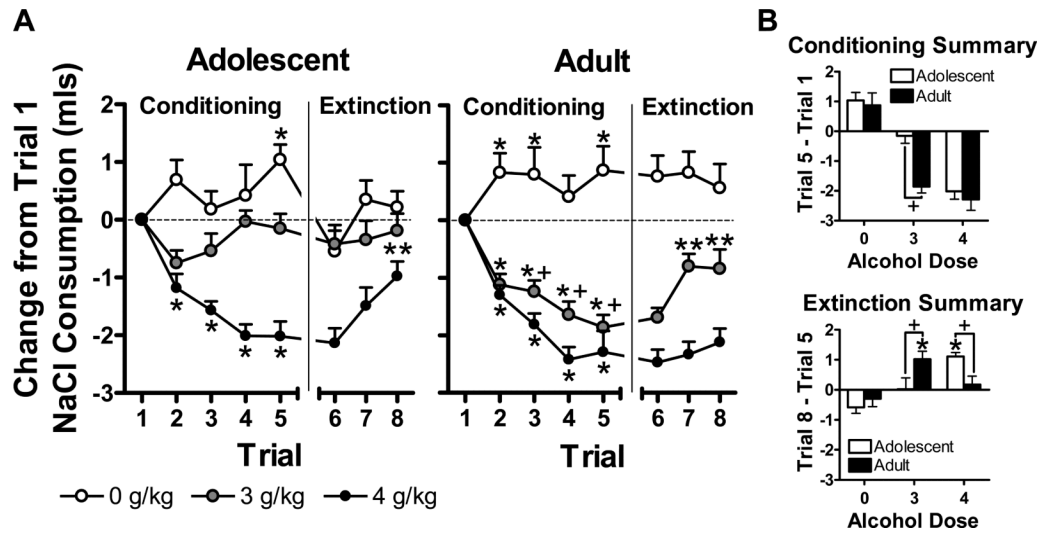
Figure 2. Adolescent mice are less sensitive to the aversive properties of alcohol as compared to adult mice

During the conditioning phase of the experiment (Trials 1-5), mice were presented with the CS (0.2M NaCl) for 1-h/day; on conditioning trials 1-4, this 1-h access was immediately followed by an IP injection of saline or alcohol (3 or 4 g/kg). No alcohol treatment followed trial 5. Extinction of the conditioned response was assessed once every 5 d for 3 trials (6-8). Shown are mean ± SEM values. **(A)** In adolescent mice, only the 4 g/kg alcohol dose induced a conditioned taste aversion (CTA), and this aversion diminished after three extinction trials. In adults, however, both doses of alcohol (3 and 4 g/kg) produced a significant taste aversion, and a reduction of this aversion was only observed in adult mice treated with 3 g/kg alcohol prior to extinction. * significant difference from conditioning trial 1 (ps<0.05). ** significant difference from trial 6 (extinction) (ps<0.05). + significant difference from adolescents at the same alcohol dose and conditioning trial (ps<0.05). The dashed line represents no change from trial 1. **(B)** Shown in this figure is a summary of the change in CS consumption after conditioning ('Conditioning Summary', Trial 5 – Trial 1, the same data as plotted for Trial 5 in Fig. 2A) and the overall change in CS intake following 3 extinction trials ('Extinction Summary', Trial 8 – Trial 5). These data are re-graphed to emphasize the age difference in sensitivity to a CTA and in extinction of a CTA. Adult mice were more sensitive to the aversive effects of 3 g/kg alcohol as compared to adolescent mice (+ p<0.05), but did not differ from adolescents after 4 g/kg alcohol. Following 15 d of extinction, adolescent mice in the 4 g/kg alcohol group increased CS intake (indicative of extinction), but adult mice did not. Rather, an extinction of aversion was only observed in adult mice at a 3 g/kg alcohol dose (* p<0.05 as compared to the 0 g/kg group in the respective age group). Adolescent and adult mice differed significantly in the change in CS intake over extinction trials at both the 3 and 4 g/kg alcohol doses (+ p<0.05 at respective alcohol dose).