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Differential Dietary Ethanol Intake and Blood Ethanol Levels in Adolescent and Adult Rats: Effects on Anxiety-Like Behavior and Seizure Thresholds

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Abstract

Background—Adult rats exhibit increased anxiety-like behavior after exposure to repeated cycles of chronic ethanol and withdrawal. While adolescent rats have differential responses to both acute and chronic ethanol treatments, the potential differences in the effects of repeated withdrawals in this population have yet to be determined.

Methods—Male adult and adolescent rats received three 5-day cycles of either a 4.5% or 7% ethanol diet (ED) separated by two 2-day withdrawal periods. Five hours into the final withdrawal, rats were tested for social interaction (SI) deficits (an index of anxiety-like behavior) and then assessed for seizure thresholds (audiogenic and bicuculline-induced). Ethanol intake was monitored throughout, and blood ethanol concentrations (BEC) were obtained from a separate group of rats.

Results—Adolescent rats have reduced SI during the final withdrawal from either ED and exhibit a greater reduction in SI compared to adult rats when exposed to a 7%ED. Audiogenic seizures were not increased during withdrawal from either ED in adult rats, but adolescent rats that received 7% ED displayed increased seizures. The bicuculline seizure thresholds were decreased in both ages exposed to a 7%ED, but only adolescent rats showed this decreased threshold after 4.5%ED. Ethanol intakes and BECs were higher in adolescent rats compared to similarly treated adults. However, ethanol intakes and BECs were comparable between 4.5%ED-treated adolescent and 7%ED-treated adult rats.

Conclusions—Behavioral results from the 7%ED-treated groups suggested that adolescent rats may be more vulnerable to repeated withdrawals from ethanol than adults; however, differences in ethanol intake and BECs may be at least in part responsible. When ethanol intakes and BECs were similar between 4.5%ED-treated adolescent and 7%ED-treated adult rats, behavioral effects were not different. Importantly, these data illustrated that adolescent rats can exhibit anxiety and reduced seizure thresholds following this repeated withdrawal paradigm.

Keywords

Repeated Withdrawal; Anxiety; Seizures; Development; Adolescent Rats

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Adolescence is a period of development that is marked with increased experimentation with drugs of abuse, including alcohol. Studies have indicated that 73% of high school students have used alcohol by the time they graduate, and the rate of relapse from alcohol abuse in adolescence is at least as high as adults (Brown, 1993; Johnston et al., 2007). The consequences of drinking during adolescence are important to understand because evidence showed that the strongest predictor for alcohol dependence in adulthood was alcohol use before the age of 14 (Grant, 1998). Additionally, the course to alcohol dependence seemed to be more rapid in adolescents than adults (Clark et al., 1998). These and other clinical data have made it evident that understanding the effects of alcohol use and abuse during this developmental period is critical.

Basic research has demonstrated clear differences between adolescent and adult rats in their responses to acute ethanol administration. For example, adolescent rats had lower sensitivity to the sedative (Little et al., 1996; Silveri and Spear, 1998) and motor impairing (White et al., 2002) effects of acute ethanol injections compared to adult rats. However, it has also been illustrated that although adolescent rats are less sensitive to some of the effects of ethanol, they were more sensitive to ethanol-induced memory impairment and social facilitation relative to adult rats (Markwiese et al., 1998; Varlinskaya and Spear, 2002, 2006). These differences in sensitivity to ethanol displayed by adolescent rats were also maintained into adulthood (Slawecki, 2002; White et al., 2000, 2002). Additionally, Crews et al. (2000) showed that a 4-day binge ethanol exposure during adolescence caused brain damage in select regions which did not occur in adult rats. Further, in P rats it was illustrated that prior ethanol exposure during adolescence but not in adulthood affects later operant responding for ethanol (Rodd-Henricks et al., 2002a,b). This evidence indicated that ethanol treatment during the adolescent period can affect future responses to ethanol as adults.

In evaluating the differential responses of adult versus adolescent rats on alcohol-related measures, it was also important to assess the effects of alcohol withdrawal (a measure of physical dependence). There are a number of symptoms that are associated with ethanol withdrawal including seizures, anxiety, and activity suppression among others. With regard to alcohol withdrawal seizures, Acheson et al. (1999) demonstrated that seizure induction following 5-days of intragrastic ethanol infusions was more pronounced in adult than in adolescent rats. More recent studies, using 2-week vapor chamber exposure, demonstrated no change in anxiety in ethanol-treated groups compared to controls (light/dark box), decreased acoustic startle response, and increased prepulse inhibition during withdrawal, but no differences between ages (Slawecki et al., 2006). However, high frequency power in the parietal cortical electroencephalogram was selectively increased in adolescent rats, while hypo-activity in the light/dark box was produced only in adult rats (Slawecki et al., 2006). Doremus et al. (2003) used the elevated plus maze to measure anxiety 18 hours following acute intraperitoneal (i.p.) ethanol administration and found that adolescent rats displayed no anxiety-like behavior in this test during this acute withdrawal. In contrast, adult rats reliably displayed anxiety at this time. Finally, Varlinskaya and Spear (2004) found that adult, but not adolescent rats, exhibited increased anxiety-like behavior with the social interaction (SI) test 18 hours following acute i.p. ethanol administration. Therefore, it appears that a sensitivity difference to ethanol withdrawal between adolescent and adult rats depends on the behavioral test used and possibly on the type of ethanol exposure.

Together, these data indicate that exposure to ethanol during adolescence can illicit a response that is unique in many cases from that of adults. It has been well characterized that teenagers typically consume alcohol in a ‘‘binge manner’’ where high levels of intake are followed by periods of abstinence (Hiller-Strurmhofel and Swartzwelder, 2004/2005). This pattern suggests that they likely experience repeated withdrawals. In adult humans and rodents, it has been demonstrated that repeated withdrawals (detoxifications) from alcohol increased the

susceptibility for seizures (Ballenger and Post, 1978; Becker and Hale, 1993; Kokka et al., 1993; McCown and Breese, 1990). Further investigations demonstrated that other symptoms of withdrawal (i.e., anxiety) could also undergo a kindling-like process in adult rodents (Breese et al., 2004; Overstreet et al., 2002). This repeated withdrawal model provided a novel way to address the effects of ethanol withdrawal in adolescent rats and offered a potentially valuable comparison with previous studies that showed little indication of acute withdrawal anxiety during this developmental period.

Therefore, the current studies were designed to evaluate whether repeated withdrawals from ethanol would produce withdrawal symptoms (anxiety-like behavior and seizure susceptibility) in adolescent rats. Additionally, it was investigated whether relative ethanol intake or blood ethanol concentrations (BEC) across ages might affect differences in susceptibility between adolescent and adult rats. These investigations were carried out by testing both ages in SI, audiogenic seizure induction, and bicuculline seizure induction following repeated withdrawals from ethanol diets (ED) known to be effective in adult rats.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Charles-River, Raleigh, NC) were obtained at 7 weeks of age for the adult groups and 3 weeks of age for the adolescent groups. Animals were group housed for 1 day to adapt to the local conditions (light/dark cycle of 12:12, with lights on between 07:00 and 19:00 hour). Rats were then individually housed for the remainder of the experiments with food and fluids monitored as described below. The experiments described here were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Ethanol and Control Diets

Following a day of adaptation, all rats were placed on nutritionally complete liquid diets that have been used previously in this laboratory (e.g., Frye et al., 1983; Knapp et al., 1998; Moy et al., 1997). As it is known that adolescent rats experience rapid growth during this period and liquid diets can modestly retard weight gain (Mason et al., 1992; Sampson et al.,1996), a subgroup of rats was tested to determine if there were differences in SI between adolescent male rats that receive chow or control diet (CD) for the duration of the experiment. The diet is lactalbumin/dextrose-based with vitamins, minerals, and other nutrients from Dyets (Bethlehem, PA). The number of calories from dextrose was equated with calories from the ethanol so that both CD and ED were calorically balanced. Adult rats were then habituated for 3 days on CD and then placed into 1 of 3 treatment groups. Generally, one-third of the rats received 19 days of CD and the other two-thirds received cycled administration (three 5-day cycles of ED separated by two 2-day CD exposures) of either 4.5% or 7% (w /v) ED. Adolescent CD and 4.5%ED groups were treated the same as adult rats, however, a slight modification was made for adolescent 7%ED group. This group was given 4.5%ED for the first cycle of treatment and then exposed to 7%ED for the remaining cycles. This modification was used because of reduced weight gain that occurred when adolescent rats received 7%ED during the first cycle (T. A. Wills, D. J. Knapp, D. H. Overstreet, and G. R. Breese, unpublished observation). The rats were given CD in a volume equivalent to that consumed by the ED group on the previous day. Rats were also weighed weekly and volumes of CD were adjusted to minimize weight gain differences between groups. ED and CD volumes were measured daily at the end of the dark cycle (08:00 hour) to determine g /kg intake /day. All behavioral measures were performed on the 19th day of diet administration during which time all rats maintained on ED were placed on CD. Behavioral tests were then performed 5 hours into withdrawal when blood ethanol levels have fallen to 0 (Breese et al., 2004; Overstreet et al., 2002).

Social Interaction

The SI test was first described by File and Hyde (1978) and has been used regularly in our laboratory to assess anxiety-like behavior 5 to 6 hours following the removal of ED. The results of the SI test have also been confirmed with the use of the elevated plus maze (Overstreet et al., 2002, 2004b). In the SI test, rats were placed into a 60 × 60 cm square open field with a 15 × 15 cm square grid floor under low lighting conditions (30 lx). Two rats, naïve to the testing environment, were paired according to body weight and monitored for 5 minutes. An observer blind to the treatment condition then measured the period of time (in seconds) that each rat was engaged in social behavior (conspecific grooming, sniffing, following, crawling over /under) with its partner. Locomotor activity was also simultaneously measured during the test by the number of times a rat crossed the lines of the grid floor. Ethanol withdrawal has been repeatedly shown to reduce SI and sometimes locomotor activity (Breese et al., 2004; Overstreet et al., 2002, 2003, 2004a,b). However, it is important to note that reductions in SI and locomotor activity seemed to be independent of each other, as they can be independently manipulated with changes in ethanol treatment conditions or drug treatments (Breese et al., 2004, 2005ab Knapp et al., 2005; Overstreet et al., 2002). Additionally, previous work has illustrated that the social behavior of 1 member of a testing pair is independent of the other rat's behavior (Breese et al., 2004; Overstreet et al., 2002, 2003, 2004a). It is therefore possible to use the data from individual animals rather than the average performance of the pair (Overstreet et al., 2003).

Audiogenic Seizure Test

Rats were tested for induction of audiogenic seizures 6 hours into withdrawal (following SI test). Rats were placed individually into a plastic container (30 gallon: 19.5 × 21.75 × 27.5 in), which contained an electric bell (100 db) and view window. Once the rat was placed into the container, the electric bell was turned on for 2 minutes. While the bell was tuned on, an observer blind to the experimental treatment condition scored the degree of seizure (seizure score 1 to 5) and latency to induce seizure. The seizure score was given based on the following criteria: 1 = no change in behavior, 2 = running with no convulsive movement, 3 = running/jumping and masticatory movements with mild facial clonus, 4 = running /jumping with startle followed by complete clonus of the forelimbs, and 5 = running /jumping followed by complete tonic extension of the hindlimbs and then generalized clonus to all limbs. The latency to induce a seizure was measured as the seconds between start of the bell and full seizure episode. Rats that experienced seizures were immediately injected with a lethal dose of pentobarbital. Rats that did not display audiogenic seizures were then tested in response to bicuculline. The results below will illustrate that only adolescent rats given 7%ED demonstrated an induction of audiogenic seizures. As these animals were therefore selected out of the bicuculline test, the *n* size was reduced from 10 to 4. This lowered sample size limited appropriate statistical comparisons involving this group; thus, we tested an additional group of 7%ED treated adolescent rats that received the bicuculline test alone.

Bicuculline Threshold

Immediately following the audiogenic test, rats were infused with 0.05 mg /ml of bicuculline ($GABA_A$ antagonist; MP Biomedicals, Solon, OH) into the lateral tail vein. The drug was injected with a syringe pump at a rate of 1.6 ml /min. The time required for the rat to exhibit a twitch of the head /neck was recorded. From this time, the minimum amount of drug required to produce the first evidence of seizure activity can be calculated.

BEC

A separate group of rats was used for blood ethanol concentration (BEC) analysis and was not included in any of the behavioral tests. This step was taken to prevent potential effects of multiple blood sampling on SI behavior. BECs were taken from groups of adolescent and adult

rats that were cycled on either 4.5%ED or 7%ED in the manner described above. Blood was removed from the tip of the tail during the last hour of the dark cycle (06:00) on the first, fifth, sixth, tenth, and 11th day of ED. Additionally, on the last day of ED (15th day) blood was collected at the time of ethanol removal (hour 0) and then 2, 4, and 6 hours later.

Blood samples were then analyzed with gas chromatographic methods. Tail blood (6 μl) and standards (6 μl; 0 to 200 mg%) were combined with 375 ml of distilled water and 0.5 g NaCl in 12 × 75-mm borosilicate glass culture tubes. These tubes were capped and then heated to 55°C for 10 minutes. After this time 1.5 ml of headspace gas was removed from the tube and injected directly into an SRI 8610C gas chromatograph (SRI Instruments, Inc., Torrance, CA), as previously described (Breese et al., 2004; Navarro et al., 2003; Overstreet et al., 2002).

Statistics

Analyses of SI and locomotor activity were conducted with 1-way ANOVAS for each age group because of large differences in baseline SI (seen in CD groups). These baseline differences prevented comparison of data in a 2-way ANOVA for these behavioral tests. Therefore, decrease from baseline scores were used to make comparisons between adolescent and adult rats. When 2 group comparisons were made, *t*-tests were utilized. Two-way ANOVAS were possible for audiogenic and bicuculline tests. Daily ethanol intakes and BECs were analyzed with repeated measures ANOVAS for adolescent and adult rats while ethanol intakes averaged over cycles were analyzed with 1-way ANOVAS. Differences between groups were determined with Fisher's post hoc tests.

RESULTS

SI in Adolescent and Adult Rats

In adult rats, there was a significant difference in SI among groups [$F(2,41) = 4.69$, $p < 0.05$; Fig. 1*A*]. Rats which experienced repeated withdrawals from ED (4.5%ED or 7%ED) spent less time engaged in SI compared to rats that received CD. There were no significant differences in SI between ED groups (4.5%ED and 7%ED). In adolescent rats, there was a significant difference in SI between groups where rats in both the 4.5%ED and 7%ED groups had reduced SI compared to rats in the CD group [$F(2,50) = 45.28$, $p < 0.0001$; Fig. 1*B*]. There was also a significant difference between the ED treated groups, where rats that received 7%ED had significantly reduced SI compared to those receiving 4.5%ED. Regarding adolescent rats that received chow or CD, there was a significant difference between groups [$t(17) = 5.81$, $p < 0.0001$; data not shown] in SI where CD rats had higher SI scores than chow fed rats.

In CD groups, SI in adolescent rats was double that seen in adult rats. Therefore, reductions in SI from ethanol-treated animals were converted to decreases from baseline so the differences between age groups could be better determined. The decreases from baseline were calculated as a percent decrease in SI in the ethanol treatment groups compared to their agematched controls (CD groups). Overall, there was a significant difference among the ethanol treatment groups and ages [$F(3,62) = 3.52$, $p < 0.05$; Fig. 1*C*] in the decrease of SI from baseline. Adolescent rats treated with 7%ED showed a greater decrease from baseline compared to all other groups (4.5%ED adolescent, 4.5% and 7%ED adult). Additionally, no differences were found between adolescent rats given 4.5% ED and adult rats given either 4.5%ED or 7%ED.

Locomotor Activity in Adolescent and Adult Rats

There was a significant difference in locomotor activity in adult groups [$F(2,41) = 10.75$, $p < 0.0005$; Fig. 2*A*), where adult rats treated with 7%ED had reduced line crosses compared to 4.5% ED and CD-treated rats. There was no significant difference in line crosses among the 4.5% ED and CD groups. In adolescent rats, there was a significant difference in line crosses

among groups [$F(2,50) = 114.36$, $p < 0.0001$; Fig. 2*B*] where the ED groups (4.5%ED and 7% ED) had decreased line crosses compared to the CD group and the 7%ED group had reduced line crosses compared to the 4.5%ED group. Regarding adolescent rats that received chow or CD, there was no significant difference between groups [$t(17) = 1.44$, NS; data not shown] in locomotor activity.

In CD groups, adolescent rats were found to have higher baseline activity than adult rats. Therefore, decreases in line crosses from ethanol-treated animals were converted to decreases from baseline, so that the differences between age groups could be better determined. There was a significant difference among ethanol treatment groups and ages [$F(3,62) = 22.41$, $p < 0.0001$; Fig. 2*C*] in the decrease of line crosses from baseline. 7%ED-treated adolescent rats showed the largest decrease of line crosses compared to all other groups. Additionally, adult rats receiving 7%ED had a larger decrease from baseline than adolescent rats given 4.5%ED. No differences were found between adult and adolescent rats given 4.5% ED.

Audiogenic Seizures in Adolescent and Adult Rats

Audiogenic seizures were measured by the amount of time to induce a seizure (latency) and degree of seizure (seizure score). There was a main effect of both diet treatment [$F(2,75) = 17.38$, $p < 0.0001$] and age [$F(1,75) = 5.32$, $p < 0.05$], as well as an interaction between diet treatment and age for latency [$F(2,75) = 8.63$, $p < 0.0005$; data not shown]. Additionally, seizure scores (Fig. 3) also showed a main effect of diet treatment [$F(2,75) = 20.16$, $p < 0.0001$] and age [$F(1,75) = 5.84$, $p < 0.05$] along with an interaction between the two [$F(2,75) = 7.48$, $p < 0.005$]. For both latency and seizure scores, only 7%ED-treated adolescent rats were significantly different from all other groups. This group showed significantly reduced latency to induce audiogenic seizure and higher seizure scores. There were no differences between any other ethanol-treated group and their respective control groups.

Bicuculline Threshold in Adolescent and Adult Rats

Bicuculline thresholds were determined in both ages by calculating the amount of bicuculline (mg /kg) required during the infusion to initiate a seizure. As 7%ED-treated adolescent rats exhibited audiogenic seizures, these animals were excluded from the subsequent bicuculline test. Thus, there was an additional group of 7%ED-treated adolescent rats that received the bicuculline test alone. There were no differences in the amount of bicuculline required for infusion between these groups, so all animals were collapsed into the 7%ED-treated adolescent group for further analysis. For bicuculline thresholds, there was a main effect of diet treatment [$F(2,76) = 7.66$, $p < 0.001$] but no main effect of age [$F(1,76) = 3.18$, NS] or an interaction between the two [$F(2,76) = 1.1$, NS, Fig. 4]. Both 4.5%ED- and 7%ED-treated adolescent rats showed reduced bicuculline thresholds compared to rats given CD. However, only adult rats treated with 7%ED showed this reduction compared to their agematched controls. There were no differences between ethanol-treated groups at either age. Additionally, there were no differences in ethanol-treated groups between adolescent and adults rats.

Daily Ethanol Intake

To determine how ethanol treatment could have influenced behavioral responses in withdrawal, the pattern of daily etha-nol intake was assessed. In adult rats, daily ethanol intake was significantly different between 4.5%ED and 7%ED groups with a repeated measures ANOVA [$F(1,420) = 205.53$, $p < 0.0001$; Fig. 5*A*]. Rats treated with 7%ED had higher g/kg ethanol consumption on these days compared to the 4.5% ED-treated rats.

In adolescent rats, both 4.5%ED and 7%ED groups received 4.5%ED for the first 5 days of ethanol treatment and there were no differences in ethanol intake between groups during these days. There were significant differences in ethanol intake between groups in the second and

third cycles (days 6 to 15) [$F(1,378) = 26.08$, $p < 0.0001$; Fig. 5*B*] measured with a repeated measures ANOVA.

Additionally, to determine differences between adolescent and adult rats ethanol intake was evaluated by cycles (average of daily intake for days 1 to 5 = cycle 1, days 6 to 10 = cycle 2, and days 11 to 15 = cycle 3). During cycle 1, there were significant differences among 4.5% ED- and 7%ED-treated adult and adolescent rats [$F(3,57) = 16.27$, $p < 0.0001$; Fig. 6]. Adolescent rats treated with 4.5%ED and 7%ED drank more than adult rats which received the same ethanol treatments. There were no differences between 4.5%- and 7%ED-treated adolescent rats as all animals received 4.5%ED during this first cycle. However, both these adolescent groups consumed more ethanol than 7%ED-treated adults.

Averages of ethanol intake during the second cycle also illustrated significant differences among 4.5%ED- and 7%ED-treated adult and adolescent rats [$F(3,57) = 30.49$, $p < 0.0001$; Fig. 6]. Again, adolescent rats treated with 4.5%ED and 7%ED drank more than adult rats which received the same ethanol treatments. Additionally, rats of both ages treated with 7% ED showed higher consumption than rats of the same age that were given 4.5%ED. Interestingly, when 4.5%ED-treated adolescent rats were compared to 7%ED-treated adult rats, they did not differ in their amount of consumption.

In the third cycle, group differences were also demonstrated among 4.5%ED- and 7%ED-treated adult and adolescent rats [$F(3,57) = 61.79$, $p < 0.0001$; Fig. 6]. Adolescent rats treated with 4.5%ED and 7%ED drank more than adult rats which received the same ethanol treatments. Rats of both ages treated with 7%ED showed higher consumption than rats of the same age that were given 4.5%ED. However, during this last cycle, 7%ED adult rats and 4.5% ED adolescent rats were shown to be different with adult 7%ED-treated rats consuming more ethanol than 4.5%ED adolescent rats. This difference was caused by a slight increase in intake between cycle 2 and 3 for 7%ED adult rats and a corresponding decrease in ethanol intake for 4.5%ED adolescent rats.

BEC

In adolescent rats, there were significant differences between ED groups (4.5% and 7%ED) in BEC across the days examined [$F(1,352) = 9.36$, $p < 0.005$; Table 1]. BECs in adult rats were also significantly different between ED groups (4.5% and 7%ED) [$F(1,126) = 15.75$, $p < 0.001$; Table 1]. In both adolescent and adult rats, it was determined that BECs had returned to 0 by 6 hours into withdrawal on the final test day (day 15, Table 1). As illustrated in Table 1, 7% ED-treated adult and adolescent rats had higher BECs than their age-matched counterparts receiving 4.5% ED. Additionally, BECs in adolescent rats treated with 4.5%ED were similar to adult rats treated with 7%ED. These data, therefore, compliment ethanol intake data displaying comparable ethanol intakes between these 2 groups.

Body Weights

In adult rats, there was a significant difference between groups in body weight [$F(2,41) = 9.33$, $p < 0.001$, Table 2] where the 7%ED group have reduced body weight compared to CD and 4.5%ED groups. There was no difference between body weights of 4.5%ED and CD groups.

In adolescent rats, there was a significant difference between groups in body weight [$F(2,50) = 24.81$, $p < 0.0001$; Table 2] where the 7%ED group had reduced body weight compared to CD and 4.5%ED groups. There was no difference between body weights of 4.5%ED and CD groups. In a separate study, chow-fed adolescent rats had higher body weights (194 ± 3 g) than CD-exposed rats [$t(17) = 18.0$, $p < 0.0001$; data not shown].

DISCUSSION

Adolescent rats demonstrated reduced SI following repeated withdrawals from both 4.5% and 7% ED. Adult rats also demonstrated this behavioral phenotype which is consistent with previous studies (Overstreet et al., 2002). As prior research showed that the SI test is a validated means to measure anxiety-like behavior (File and Seth, 2003), it would appear that repeated withdrawals from these EDs produced an anxiety-like phenotype in adolescent rats, as it is known to do in adult rats. Although the retardation of weight gain sometimes seen with liquid diets could arguably be stressful and impact negatively on SI, the fact that the CD-treated adolescent rat's SI scores were not lower than those of the chow-fed adolescent rats provides evidence against this hypothesis. Furthermore, the fact that the chow-fed rats and the CD-treated rats had no difference in locomotor activity suggests that neither group was unduly stressed. The presence of this anxiety-like phenotype after repeated ethanol withdrawals in adolescent rats was a novel finding, particularly in light of evidence from acute withdrawal tests that showed anxiety-like behavior in adult but not in adolescent rats (Doremus et al., 2003; Varlinskaya and Spear, 2004). Differences between these studies and the current investigation could be due to the duration and cycling of ethanol exposure. In adult animals it has been shown that repeated withdrawals from 4.5%ED is critical to the expression of anxiety-like behavior (Breese et al., 2004; Overstreet et al., 2002). One 5-day cycle or continuous 15 days of 4.5%ED did not elicit an anxiety-like phenotype in these adult rats. Therefore, the difference between expression of anxiety in this study compared to acute administrations (Doremus et al., 2003) could have been due to the cyclical nature of the ethanol administration. Another potentially relevant difference between these studies was the age of behavioral assessment for anxiety following ethanol withdrawal and the test used. The chronic ethanol administration that was used in the present procedure delays measurement of SI related anxiety until P43, whereas in the study by Doremus et al. (2003), adolescent rats were tested at P33–35 in the elevated plus maze. Therefore, it might be the case that younger adolescent rats are able to undergo adaptations in the brain that contribute to anxiety but may not be able to express this phenotype until later stages of adolescence.

Adolescent rats were also shown to display higher baseline levels of activity and SI than adult rats. This result was consistent with previous reports of elevated exploration and social behavior in adolescent rats (Adriani et al., 1998; Primus and Kellogg, 1989; Vanderschuren et al., 1997). Comparisons between adolescent and adult rats in SI (after correction for baseline differences) demonstrated that adolescent rats treated with the higher concentration of ethanol (7%ED) exhibited a greater reduction in SI than in adult rats. One interpretation of these data is that adolescent rats may have greater sensitivity to the effects of this ethanol administration than adult rats.

The greater sensitivity of adolescent rats is also indicated in the other behavioral measurements (activity, audiogenic seizures, and bicuculline threshold). These data showed that lower doses of ED (4.5%) decreased activity in adolescent but not in adult rats. When corrections for baseline differences were made, it was found that adolescent rats treated with the higher concentration of ethanol (7%ED) exhibited a greater reduction in locomotor activity than adults receiving the same treatment. Further analysis of audiogenic seizures illustrated that the adolescent 7%ED group was the only group in which seizures were induced. For bicuculline thresholds, both EDs were able to decrease thresholds in adolescent rats where only the high concentration was able to do so in adult rats. These data were in contrast with results found by Acheson et al. (1999), who demonstrated that seizure induction following 5-days of intragastric ethanol infusions in mice was more pronounced in adults than adolescents. However, this study differed in a number of ways to those presented here: mice versus rats, single cycle versus repeated cycles, high ethanol doses versus moderate ethanol dose, pentylenetetrazolinduced seizures versus bicuculline and audiogenic induced seizures, and testing 15 versus 5 hours into

withdrawal. Therefore, different results in these studies could have been caused by any one or more of these variables. Together, these data presented here suggested an overall greater sensitivity in adolescent rats compared to adult rats following repeated withdrawals in the behavioral measures that were tested.

Although the SI, locomotor, and seizure data appear to support the hypothesis that adolescent rats were more sensitive to the repeated ethanol withdrawal experiences, alternative interpretations should be considered based on other data collected from these animals. These behavioral differences could also be explained by differences in BEC and intake between ages. Analysis of ethanol intake between ages consistently showed that adolescent rats consumed higher g /kg of ethanol than their respective ED groups in adult rats. Other investigators have reported greater ethanol intake in adolescent rats compared to adult rats using ethanol administration paradigms (Bell et al., 2006; Doremus et al., 2005; Rodd-Henricks et al., 2002a,b; Vetter et al., 2007).

The results presented here emphasize the importance of monitoring ethanol intake between adolescent and adult rats to fully address potential age differences in behavioral responding to chronic ethanol exposure and withdrawal. While significant efforts were made in the current study to control blood ethanol levels and intake across age, the multiple variables involved (differential intake, blood levels, physiological /metabolic differences) make this task formidable. When comparing the behavioral effects of treatments herein across 4.5%ED-treated adolescent rats and 7%ED-treated adult rats, it could be argued that relatively comparable behavioral effects were present. There were no differences between these groups in SI (after corrections were made for differences in baseline), audiogenic seizure measures (latency and seizure score), or bicuculline threshold. Furthermore, these groups were the 2 most closely matched in ethanol intake and BECs. Therefore, it could be concluded that when corrections were made for differences in ethanol intake and BECs between adolescent and adult rats, that behavioral responses following repeated withdrawals from ethanol were similar. Additionally, it should be noted that high BECs obtained in adolescent rats given 7%ED, which appear to peak near hour 0 on day 15, might function as an acute stress. This additional stress exposure in the adolescent group should also be considered when evaluating the present results. Interpretations of behavioral differences between adolescent and adult rats with the use of liquid diet might be bolstered by further reducing dietary ethanol concentration in adolescent rats so that intakes and/or BECs are more tightly comparable across age. Regardless of the outcomes of such studies, one interpretation that seemed less challenging was that, like adult rats, adolescent rats showed relevant withdrawal responses on all of these measures in our model, and that these responses deserve further study.

With additional refinement of the liquid diet regimen, further study of other relevant variables known to impact this anxiety-like behavior in adult rats could be examined. For example, anxiety-like behavior from repeated withdrawal is known to be sensitized in adult rats in that only repeated cycles of ethanol, but not a single or continuous exposure, produced anxiety-like behavior. This behavioral/physiological process, however, has not been evaluated in adolescent rats. Additionally, the anxiety-like behavior that is produced from repeated withdrawals is known to be persistent in adult rats. This effect was determined by re-exposing rats to a nonanxiogenic (subthreshold) chronic ethanol treatment up to 32 days following a repeated withdrawal regimen (Overstreet et al., 2002). Additionally, a corticotropin-releasing factor (CRF) type 1 receptor antagonist, benzodiazepine receptor antagonist, or a 5-HT_{1A} receptor agonist given during the early withdrawals blocked the induction of anxiety-like behavior (Knapp et al., 2004; Overstreet et al., 2003) in adult rats. Findings from these latter studies indicated the importance of GABA, 5-HT, and CRF in the adaptations that occur. It has also been illustrated that stress substitutes for early withdrawals in the production of anxiety-like behavior (Breese et al., 2004) in adult rats. This knowledge on the effects of repeated

withdrawals in adult rats will be used in appropriately refined models to guide future research analyzing the effects of repeated withdrawals in adolescent rats.

In summary, the results of these experiments suggested initially that adolescents might be more sensitive to the consequences of repeated withdrawal from chronic ethanol exposure. This impression was based on greater decreases in SI behavior, locomotor deficits, and increased seizure sensitivity. However, further examination of the different blood ethanol levels and ethanol intake across age suggest that caution should be employed in interpreting such age-dependent effects. Adolescent rats, like adult rats, showed relevant withdrawal responses on all of these measures in our model. Given the relatively limited data of this type available for this age group, these responses should be further explored and studies should be expanded to assess additional relevant variables such as persistence, drug effects, and interactions with stress.

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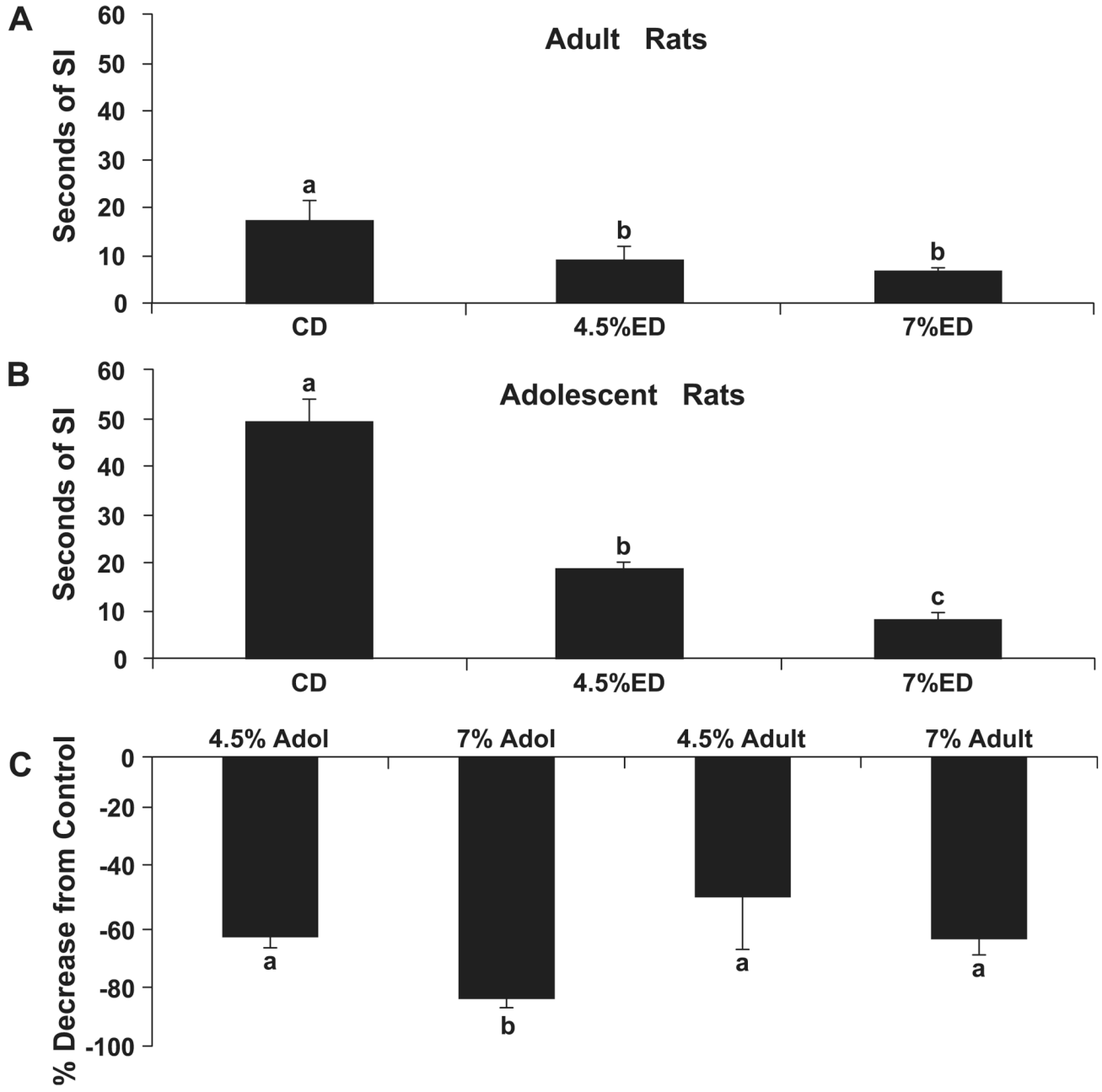

Fig. 1.

Effects of repeated ethanol exposure on social interaction (SI) in adult and adolescent rats (Panels **A** & **B**). Male adult and adolescent rats were given control diet (CD), 4.5% ethanol diet (ED), or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. Rats were tested 5 hours after removal of ethanol during the final withdrawal. Data represent means ± SEM for 8 to 10 rats / group. Panel **C** represents these data as a decrease from baseline to correct for differences in baseline (CD group) social interaction between ages. Groups with different letters are significantly different from each other ($p < 10.05$).

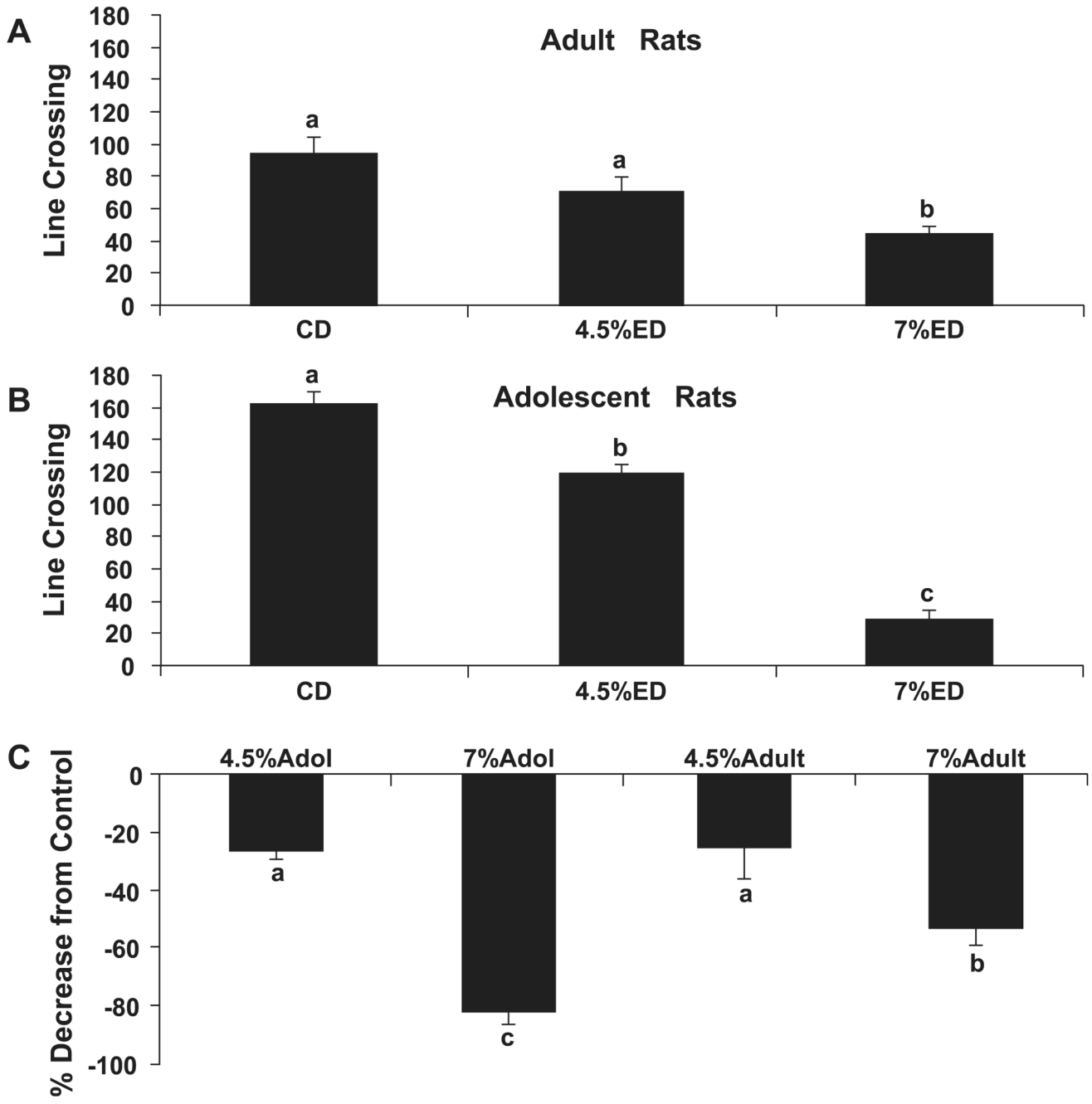

Fig. 2.

Effects of repeated ethanol exposure on locomotor activity in adult and adolescent rats (Panels **A** & **B**). Male adult and adolescent rats were given control diet (CD), 4.5% ethanol diet (ED), or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. Rats were tested 5 hours after removal of ethanol during the final withdrawal. Locomotor activity was measured concurrently with social interaction. Data represent means ± SEM for 8 to 10 rats/group. Panel **C** represents these data as a decrease from baseline to correct for differences in baseline (CD group) activity between ages. Groups with different letters are significantly different from each other ($p < 0.05$).

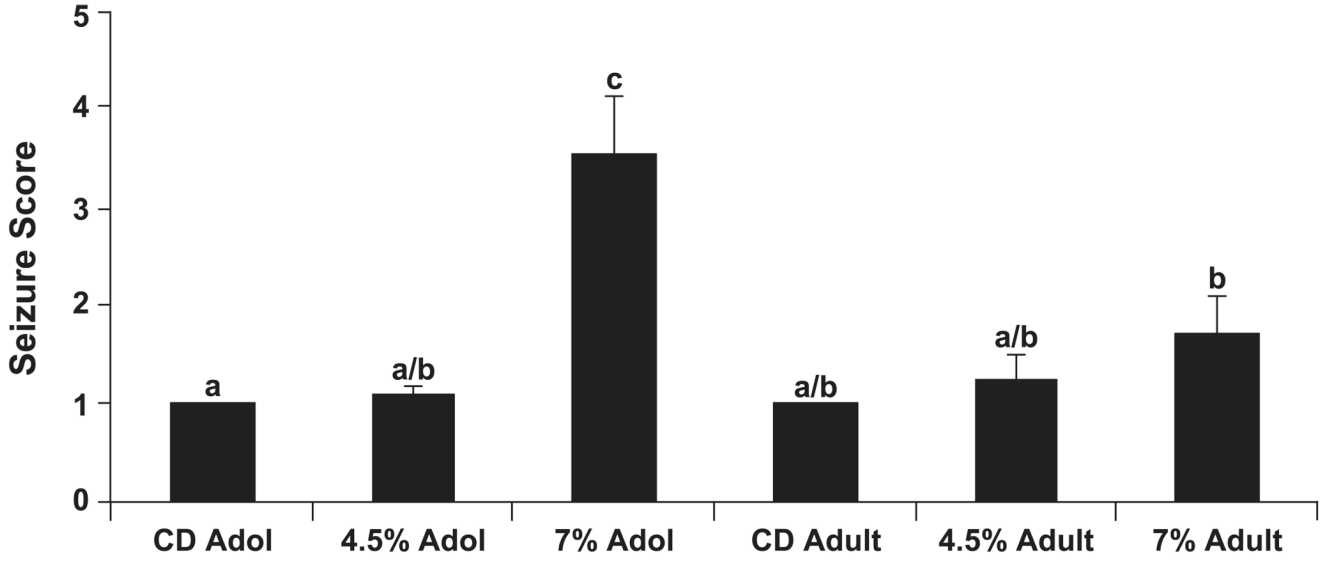

Fig. 3.

Effects of repeated ethanol exposure on audiogenic-induced seizures in adult and adolescent rats. Male adult and adolescent rats were given control diet (CD), 4.5% ethanol diet (ED), or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. Rats were tested 6 hours into final withdrawal following social interaction. See Materials and Methods for seizure scoring. Data represent means ± SEM for 8 to 10 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

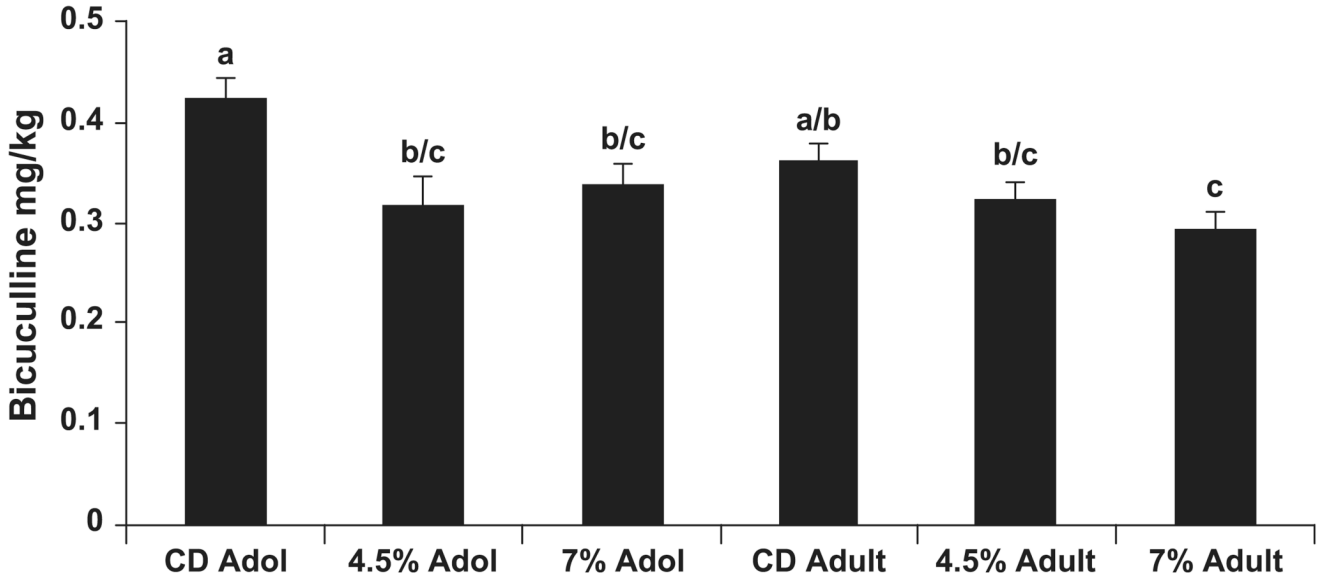

Fig. 4.

Effects of repeated ethanol exposure on bicuculline-induced seizures in adult and adolescent rats. Male adult and adolescent rats were given control diet (CD), 4.5% ethanol diet (ED), or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. Rats were tested 6 hours into final withdrawal in those rats in which audiogenic seizures were not detected. The amount of bicuculline infused until the first sign of head/neck twitch was recorded. Data represent means ± SEM for 8 to 10 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

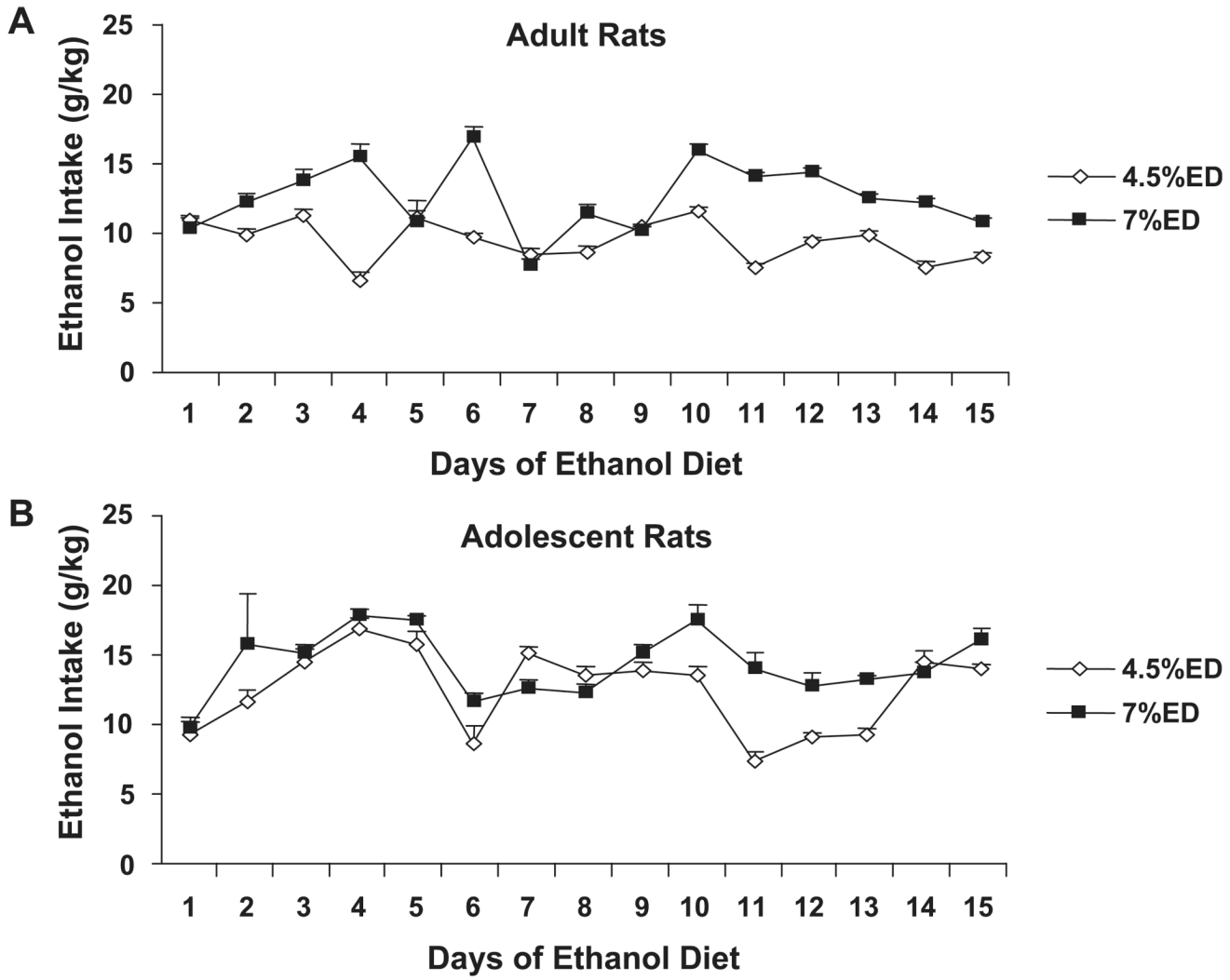

Fig. 5.

Daily ethanol intake in adult and adolescent rats exposed to 4.5% ethanol diet (ED) and 7% ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. Data represent means ± SEM for 8 to 10 rats/group.

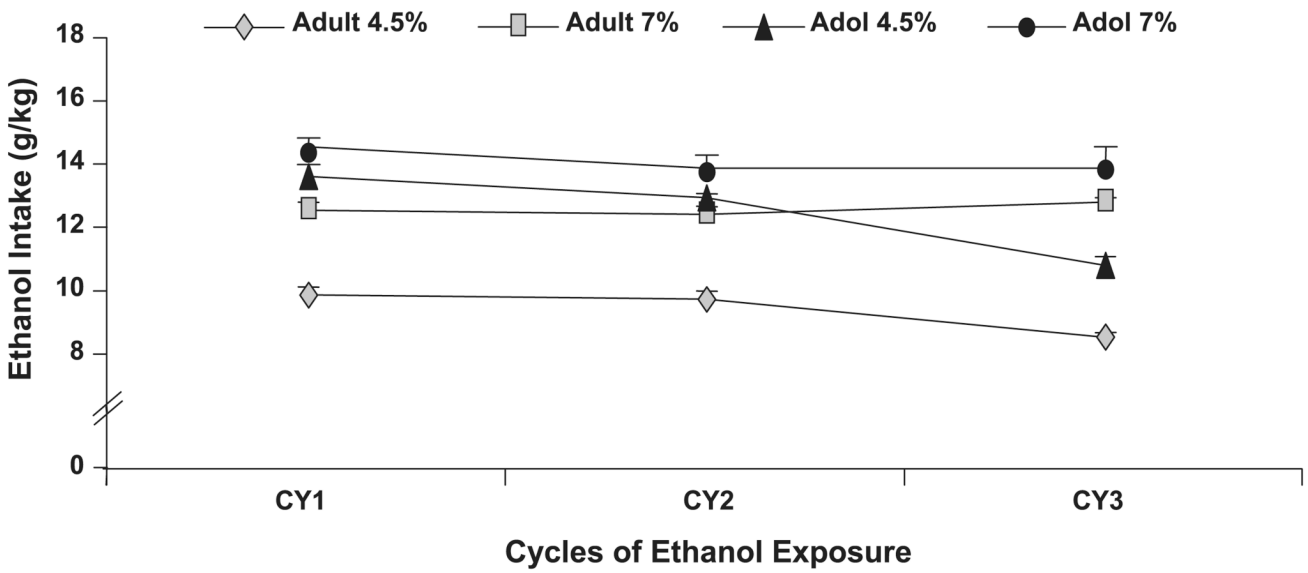

Fig. 6.

Ethanol intake averaged by cycles for adult and adolescent male rats. Ethanol diet (ED) groups were exposed to three 5-day cycles (CY) of ED interspersed with two 2-day withdrawal periods, during which rats received control diet (CD). These data are an average of daily intake for each 5-day cycle. Data represent means ± SEM for 8 to 10 rats /group.

Table 1

Blood Ethanol Concentrations in Adult and Adolescent Rats

	Day 1	Day 5	Day 6	Day l0	Day 11	Day 15 (H0)	Day 15 (H2)	Day 15 (H4)	Day 15 (H6)
Adult 4.5%ED	63 ± 11	88 ± 10	94 ± 14	107 ± 12	95 ± 12	40 ± 17	8 ± 8	0 ± 2	0 ± 0
Adult 7% ED	70 ± 11	149 ± 18	123 ± 18	187 ± 24	121 ± 17	187 ± 19	76 ± 15	3 ± 4	0 ± 0
Adolescent 4.5%ED	121 ± 9	160 ± 15	139 ± 15	183 ± 19	130 ± 11	160 ± 30	75 ± 18	21 ± 7	0 ± 1
Adolescent 7% ED	154 ± 12	158 ± 13	129 ± 16	246 ±16	171 ± 10	284 ±17	154 ± 15	30 ± 10	0 ± 1

H, hour; ED, ethanol diet.

Adolescent and adult rats given 4.5%ED and 7%ED, which were exposed to three 5-day cycles of ethanol diet interspersed with two 2-day withdrawals. Blood was collected from the tip of the tail during the last hour of darkness on day 1, 5, 6, 10, and 11 of ethanol diet. In addition, blood was collected when ethanol was removed on day 15 (H0) and during withdrawal (2, 4, and 6 hours). Data represent mean mg % ± SEM for 23 to 25 rats per group.

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Table 2

Body Weights in Adult and Adolescent Rats

	CD	4.5%ED	7% ED
Adult	311 ± 5	310 ± 7	283 ± 5[a]
Adolescent	153 ± 4	165 ± 6	116 ± 6[a]

CD, control diet; ED, ethanol diet.

Body weights for the 3 treatment groups (CD, 4.5%ED, and 7%ED) were collected the day before behavioral tests. Data represent means in g ± SEM for 8 to 10 rats/group.

[a]Significantly different from CD and 4.5% groups for both adolescents and adults ($p < 0.05$).