Published in final edited form as:
Alcohol Clin Exp Res. 2002 August ; 26(8): 1259–1268. doi:10.1097/01.ALC.0000023983.10615.D7.

Accentuated Decrease in Social Interaction in Rats Subjected to Repeated Ethanol Withdrawals

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Abstract

Background—Previous work has shown that repeated withdrawals from chronic ethanol exposure can kindle seizures in rodents. In this article, the effects of a three-cycle model of ethanol exposure and withdrawal on the social interaction test of anxiety are summarized.

Methods—Rats were exposed to ethanol (7% or 4.5%) diets over three periods of 5 days, with 2 days of withdrawal between cycles. Between 5 and 6 hr after the ethanol was removed, pairs of rats were placed in open field chambers for the assessment of social interaction behavior and locomotor activity.

Results—After the third cycle of ethanol (7%) presentation, both male and female rats exhibited lower social interaction behavior (more anxiety) and activity than after a single cycle. Rats exposed to a similar amount of ethanol but tested while ethanol was still available did not exhibit a reduction in social interaction. The decrease in social interaction was still present for up to 24 hr but had disappeared by 48 hr after ethanol was withdrawn. When rats were allowed 8 or 16 days to recover from the effects of the three-cycle protocol, a further exposure to 5 days of 7% ethanol diet resulted in a reduction in social interaction on withdrawal similar to that seen from the three-cycle protocol. In contrast, rats exposed continuously to 7% ethanol diet for 15 consecutive days exhibited higher levels of social interaction when maintained on control diet for 8 or 16 days and then reexposed to ethanol. Rats that were exposed to the three-cycle protocol and allowed 32 days to recover before being reexposed to ethanol still had a partial deficit in social interaction. Finally, animals subjected to repeated withdrawals from 4.5% ethanol exhibited a reduction in social interaction without a change in activity after the final withdrawal from ethanol, whereas rats exposed continuously to a 4.5% ethanol diet did not exhibit a reduction in social interaction or activity. Neither blood ethanol concentrations nor changes in body weight could account for these behavioral differences.

Conclusion—Repeated withdrawal from ethanol can lead to accentuated or more persistent anxiety-like behavior in rats, as indicated by a decrease in social interaction. The withdrawal-induced decrease in locomotor activity is not accentuated by repeated withdrawals. This model of repeated withdrawals from ethanol may prove useful in defining the neurochemical basis of this accentuation.

Keywords

Repeated Ethanol Withdrawal; Anxiety; Sensitization; Social Interaction Test

Since ballenger and Post (1978) summarized evidence that increased symptoms after multiple withdrawals from ethanol resemble a kindling process, a number of supportive clinical and animal studies have appeared. McCown and Breese (1990) were the first to report an increase

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in seizure susceptibility of the inferior colliculus after repeated ethanol withdrawals, which supported the kindling hypothesis. This effort demonstrated that seizures generated by electrical stimulation of the inferior colliculus could be elicited more easily in rats withdrawn repeatedly from ethanol, even though this increased sensitivity or sensitization was not observed in the amygdala (McCown and Breese, 1990). Thus, a key observation was that the adaptive changes due to repeated ethanol withdrawal could be different in different brain regions. In further support of this concept, Veatch and Gonzalez (1996, 1999, 2000) reported that several cycles of exposure to an ethanol inhalation chamber followed by an extended withdrawal period resulted in more electrical stimulation being required to kindle seizures from the hippocampus. On the other hand, mice exposed to an ethanol vapor chamber became more dependent with repeated exposures, as revealed by a decrease in seizure threshold or an increase in handling-induced convulsions (Becker and Hale, 1993; Becker et al., 1997, 1998; Jarvis and Becker, 1998). These examples provide convincing evidence for pathologic changes in brain function with repeated exposures to and withdrawals from ethanol. Others, including some studies in humans, have reported a similar sensitization process with repeated ethanol withdrawals (Brown et al., 1988; Holter et al., 1998; Maier and Pohorecky, 1989; Malcolm et al., 2000a,b; Meert and Huysmans, 1994; Ulrichsen et al., 1996, 1998). Thus, evidence supports the presence of a sensitization process in animals subjected to multiple withdrawals from ethanol.

Of particular relevance to this article is the work of Spanagel and Holter (1999). They gave outbred rats access to water and three different concentrations of ethanol over prolonged periods. Periodically, the ethanol solutions were withdrawn. These authors noted that the rats increased their drinking of ethanol during these prolonged exposures and began to prefer the bottle containing the higher concentration of ethanol. Moreover, the animals exhibited anxiety-like behavior as indexed by the elevated plus maze (Holter et al., 1998; Spanagel and Holter, 1999). Although the anxiety-like behavior increased over time, the lengthy duration of these studies precludes detailed mechanistic investigations. The present article demonstrates that anxiety-like behavior can be induced by brief (15 days) exposures to ethanol and that the behavior can be accentuated if the exposure is cycled.

A key behavioral pathology associated with ethanol withdrawal is anxiety (e.g., Criswell and Breese, 1992; File et al., 1989; Kampov-Polevoy et al., 2000; Knapp et al., 1998; Moy et al., 1997, 2000). However, except for the studies involving long-term access to alcohol (Holter et al., 1998; Spanagel and Holter, 1999), previous studies have not determined whether repeated withdrawals will sensitize animals to the anxiety associated with withdrawal. The social interaction test has been used widely to study anxiety-related behavior in rodents (e.g., File, 1993; File and Hyde, 1978; File et al., 1999; Gonzalez et al., 1998), including that related to withdrawal from drugs of abuse (Andrews et al., 1997; Costall et al., 1990; File et al., 1989; Irvine et al., 2001; Kampov-Polevoy et al., 2000). Thus, the social interaction test is well suited for determining whether changes in anxiety-related behavior occur during repeated exposures to and withdrawals from ethanol. The present study summarizes the findings of a three-cycle protocol of ethanol exposure and withdrawal on anxiety-like behavior, as measured by the social interaction test.

METHODS

Animals

Male and female Sprague Dawley® rats (Charles-River, Raleigh, NC) were obtained at about 6 weeks of age (140–160 g) and housed in groups of three or four for several days to adapt to the local conditions (light/dark cycle of 12:12, with lights on between 0900 and 2100 hr). Then the rats were housed individually for the remainder of the experiments, with food and fluids

presented as described subsequently. The experiments described here were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Ethanol and Control Diets

After the short period of adaptation, all rats were placed on nutritionally complete liquid diets similar to those used previously in this laboratory (e.g., Frye et al., 1983; Moy et al., 1997, 2000). Briefly, the diet was a lactalbumin/dextrose-based, nutritionally complete diet (with concentrations of vitamins, minerals, and other nutrients derived from ICN Research Diets). Dextrose calories in the control diet (CD) were equated with ethanol calories in the ethanol diet (ED). After 3 days on the CD, approximately 75% of the rats were placed on a diet that contained one of two concentrations of ethanol (4.5 or 7%, w/v) either continuously for 15 or 16 days or for three cycles of 5 days of ED interspersed with 2 days of CD. Preliminary studies suggested that rats subjected to three cycles of ethanol exposure might have lower ethanol intake than rats exposed continuously to ethanol. Therefore, the lower 4.5% ED was used in addition to the standard 7% to produce a lower daily intake of ethanol. Inclusion of this group allowed us to determine whether social interaction or locomotor activity would be differentially affected by ethanol concentration.

A modified pair-feeding design was used in all of the diet studies. The rats maintained on the CD were given a volume of diet equivalent to the average volume consumed the previous day by the rats maintained on the ED. The rats were weighed at weekly intervals and volumes of diet were adjusted to ensure that the groups had similar body weights. In general, behavioral assessments were conducted after 15 or 16 days of exposure to the ED between 5 and 6 hr after the removal of the ethanol. This time point was selected on the basis of previous observations of anxiety-like behavior in our laboratory (e.g., Knapp et al. 1998; Moy et al., 1997, 2000). For each squad of 40 rats, they were tested in subgroups of 20, with balanced numbers of rats in each treatment group. Thus, some rats were tested after 15 days of exposure and some after 16 days. No differences were observed between the subgroups.

Social Interaction Test

The social interaction test was first introduced by File and Hyde (1978). This test involves placing a pair of animals in an arena and measuring the amount of time engaged in such behaviors as grooming, sniffing, and boxing; locomotor activity is recorded simultaneously and provides a measure that is independent of social interaction (File, 1993). Social interaction has been validated repeatedly as an index of anxiety-related behavior because it is decreased after anxiety-provoking stimuli such as bright lights or exposure to cat odor (File, 1993; File and Hyde, 1978), after administration of anxiogenic drugs (e.g., Bhattacharya et al., 1997; File and Lister, 1984; Guy and Gardner, 1985; Sams-Dodd, 1995), or after withdrawal from drugs of abuse, including alcohol (Andrews et al., 1997; Costall et al., 1990; File et al., 1989; Irvine et al., 2001; Kampov-Polevoy et al., 2000). Conversely, social interaction can be increased by prior exposure to the test arena (File, 1980; File and Hyde, 1978) or the administration of anxiolytic drugs at doses that have little effect on locomotor activity (Barnes et al., 1990; File, 1980; Lightowler et al., 1994).

A modification of the standard social interaction test was used to reduce the number of animals needed for experiments. According to File (1993), the most sensitive procedure is to match up pairs of rats that have the same treatment on the basis of their body weights and then treat the total number of interactions by the pair as the unit of measure. However, for other experiments where the index rat may have an implanted cannula (Gonzalez et al., 1998; Irvine et al., 2001), an untreated dummy partner is used and only the interactions of the index rat are recorded. In the present studies, pairs of rats with the same treatment were placed in the arena and the social interactions initiated by each member of the pair were recorded, which thereby

required fewer rats. Statistical analyses of several data sets revealed that this approach provided the same statistical outcome as treating the scores of the pair as a unit. Furthermore, in a study of 25 pairs of rats maintained on CD and 25 on ED, the rats exhibited essentially independent behavior, because there was no significant correlation in either group (0.03 for CD, −0.13 for ED).

Experienced observers who were blind to the experimental condition carried out the social interaction test in a square open field (2 ft by 2 ft, with 16 squares marked out on the floor). The rats were unfamiliar with the open field, and the lighting conditions were low to generate an intermediate level of anxiety-related behavior. Rat pairs were matched on the basis of alcohol intakes, body weights, and treatment conditions and were placed simultaneously in the open field. During the 5-min session, line crosses (by two forepaws) and time spent in social interaction (grooming, sniffing, following) were scored individually for each rat (Kampov-Polevoy et al., 2000).

Repeated Versus a Single Withdrawal

This experiment compared the anxiety responses in the social interaction test of four treatment groups: CD throughout the study, 7% ED continuously for 15 days (ED7CON), 7% ED for three cycles of 5 days with two 2-day periods of withdrawal between the 5th and 6th days and the 10th and 11th days (ED7CY3), and 7% ED for one cycle of 5 days (ED7CY1). The ED7CON group was included to provide a group with the same degree of exposure to ethanol as the ED7CY3 group. Between 5 and 6 hr after removal of ethanol, the rats were tested as pairs in the social interaction test, as described previously.

A related experiment addressed whether male and female rats would respond similarly to the repeated withdrawals from ethanol. Investigators seldom study female rats in tests reflecting anxiety (see Fernandes et al., 1999), although considerable data indicate that human females exhibit a higher incidence of anxiety disorders (e.g., Lewinsohn et al., 1998; Pigott, 1999). Even less is known about the relationship between chronic ethanol intake/withdrawal and anxiety in female rats. Male and female Sprague Dawley rats of comparable ages (Charles-River, Raleigh, NC) were obtained and placed on control or ethanol-containing diets as described previously. Rats subjected to a session of one 5-day exposure to 7% ethanol were compared with rats given three 5-day exposures to ED. At the end of the treatment period, the rats were placed in the social interaction test between 5 and 6 hr after the ethanol was withdrawn.

Recovery of Reduced Social Interaction (Anxiety Response)

The purpose of this experiment was to determine when the anxiety-like behavior, as indexed by social interaction, recovered to normal. In one substudy, rats were exposed to CD or 7% ED and tested after 10 days; one ED group had the ethanol continuously and the other had two cycles of 5 days' exposure to ethanol, with 2 days of withdrawal between the 5th and 6th exposures. The rats were exposed to the social interaction arena 5 hr after removal of ethanol. Second, we wanted to know how long it would take for the depressed locomotor activity and reduced social interaction to recover in rats that were exposed to the three-cycle protocol or continuously to 7% ethanol for 15 days. Groups of male rats were withdrawn from their last ethanol session for 5, 24, or 48 hr before being placed in the open field for the social interaction test.

Reinstatement of Reduced Social Interaction On Subsequent Ethanol Exposure

The purpose of this experiment was to carry out reinstatement studies to see how long the adaptive changes that underlie anxiety-like behavior endured. It is proposed that the length of time the rat has been on CD since its 15-day exposure to ethanol will determine whether the

rat will exhibit anxiety-like behavior after being exposed to 5 further days of ethanol and then withdrawn. To investigate reinstatement, rats were placed on the standard 7% ED for three cycles and then were allowed to recover by being placed on a CD for 8, 16, or 32 days (CY8R, CY16R, and CY32R, respectively). Other groups of rats were placed on the standard 7% ED for 15 consecutive days and were allowed to recover by being placed on a CD for 8 or 16 days (CON8R and CON16R). Subsequently, these rats were administered the 7% ED for 5 days and placed in the social interaction arena between 5 and 6 hr after the removal of ethanol. Parallel groups were exposed to CD (CDCY and CDCON) to control for age. Two other groups were maintained on 7% ED for 15 days (EDCON3) or three 5-day cycles (EDCY3) and withdrawn. Testing in the social interaction test was carried out between 5 and 6 hr after withdrawal of ethanol.

Social Interaction in Ethanol-Exposed Rats Before Withdrawal

This experiment was designed to determine whether the changes observed in ethanol-withdrawn rats could have been the consequence of long-term exposure to ethanol and not to the withdrawal process per se. Three groups were established, each containing six rats: CD, 7% ED given continuously for 15 days (ED7CON), and 7% ED given in three cycles of 5 days (ED7CY3). On the 14th day of ethanol exposure, the rats were tested in the social interaction test during the last hour of darkness, when ethanol was still available. On the following day, after being paired with a different partner, they were tested in the social interaction test between 5 and 6 hr after withdrawal of the ED.

Effects of Repeated Withdrawal in Rats Exposed to a Low Ethanol Concentration

The present experiment was designed to determine if repeated withdrawals from exposure to the 4.5% ED would reduce social interaction upon the final withdrawal. Preliminary experiments demonstrated that rats did not exhibit a change in social interaction after exposure to continuous 4.5% ED for 15 days. The results were compared with groups maintained on 7% ethanol with and without repeated withdrawals. Consequently, the following five treatment groups were established: CD, 7% ED for 15 consecutive days (ED7CON), 4.5% ED for 15 consecutive days (ED4.5CON), 7% ED for three cycles of 5 days with 2 days of withdrawal after the first and second cycles (ED7CY3), and 4.5% ED for three cycles of 5 days with 2 days of withdrawal after the first and second cycles (ED4.5CY3). Exposure to ethanol was delayed in the two continuous groups so that their withdrawals coincided with the final withdrawals of the two cycle groups. Rats were tested in the social interaction test between 5 and 6 hr after withdrawal of ethanol.

Blood Ethanol Concentrations

Blood ethanol concentrations (BECs) were not determined in the experiments described previously because of the possibility that taking blood samples might disrupt the social interaction behavior. To obtain evidence of the BECs attained by the rats in the repeated withdrawal protocol, two additional groups of rats ($n = 8$) were established. One group had access to 7% ethanol in the diet for 15 consecutive days, whereas the other was subjected to three separate 5-day cycles of ethanol exposure, as used in the behavioral studies. Blood was taken from the tip of the rat's tail during the last hour of darkness on days 1,6, 11, and 15 of ethanol exposure. In addition, blood was taken from the rats 2 and 4 hr after the ethanol had been removed on the 15th day of exposure.

Blood ethanol samples were analyzed with gas chromatographic methods (Knapp et al., 1993; Ming et al., 2001; Pohorecky, 1974; Pohorecky and Brick, 1982; Wallace and Dahl, 1966). Trunk blood (100 μl) and standards (100 μl; 0–300 mg/dl) were mixed with 375 ml of distilled water and 0.5 g NaCl in 12 × 75 mm borosilicate glass culture tubes. Tubes were capped and heated at 55°C for 10 min in a water bath, whereupon 1.5 ml of headspace gas was

removed with a standard plastic 3 cc syringe and injected directly into an SRI 8610C gas chromatograph (Torrance, CA) equipped with an external syringe adapter and 1 cc external loading loop. The oven temperature was isothermal at 140°C and contained a Hayesep D column and a flame ionization detector. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/min, respectively. Peak retention time was 2 min, and the areas under the curve were analyzed with SRI PeakSimple software for Windows running on a Dell Inspiron 3500 laptop computer.

Data Analysis

Statistical analyses were carried out with the GBStat software package (Dynamic Microsystems, Inc., Silver Spring, MD). The data initially were analyzed by ANOVAs, with one-, two-, or three-way ANOVAs carried out depending on the research design. If the main and/or interaction effects were statistically significant, post hoc analyses were performed by using Tukey's protected *t* tests. The social interaction test has a measure that reflects the general activity level of the rats (line crosses) as well as a measure that reflects the anxiety state (time spent in social interaction). Therefore, these analyses determined whether a selective effect of alcohol withdrawal was observed on social interaction, the measure of anxiety.

RESULTS

Repeated Versus a Single Withdrawal

There were substantial and statistically significant differences in social interaction among the four treatment groups of male Sprague Dawley rats ($F = 6.34$, $p = 0.002$). As indicated in Fig. 1, only the two groups exposed to 7% ethanol for at least 15 days (ED7CON, ED7CY3) exhibited significantly less social interaction than the CD group. The ED7CY1 group was similar to the CD group in their level of social interaction (Fig. 1, upper panel).

The pattern of results for line crosses as an index of activity (Fig. 1, lower panel) also indicated significant differences among the groups ($F = 9.33$, $p < 0.001$). All rats exposed to 7% ethanol were significantly less active than the CD group. The results for time engaged in social interaction (Fig. 1, upper panel) were closely mirrored by the average ethanol intake. The ED7CON (12.8 ± 0.8 g/kg/ day) and ED7CY3 (12.9 ± 0.3 g/kg/day) groups drank significantly more ethanol than the ED7CY1 (10.1 ± 0.3 g/kg/day) group.

Exposure to 7% ethanol produced similar results in male and female rats (data not shown for female rats). A two-way ANOVA indicated a highly significant treatment effect ($F = 112.40$, $p < 0.001$) but no significant gender effect ($F = 0.14$, not significant). Similarly, there was a significant treatment effect for activity ($F = 39.11$, $p < 0.001$) without a significant gender effect ($F = 0.49$, not significant).

Recovery of Reduced Social Interaction (Anxiety Response)

After 10 days of exposure to 7% ethanol, the two-cycle group of male rats had a significantly lower social interaction score (8.9 ±1.2 sec) than the continuous group (20.9 ±1.9 sec). In fact, the score of the two-cycle group was as low as that seen after three cycles (Fig. 1), whereas the value for the continuous group was intermediate between rats exposed to 5 (26.1 ± 3.6 sec) and 15 days (6.8 ± 1.4 sec). Despite this difference in onset of reduced social interaction behavior, there were no differences between the groups in the recovery of this behavior, as illustrated in Fig. 2 (upper panel). The overall analysis with a one-way ANOVA indicated a significant treatment effect for the level of social interaction ($F = 18.71$, $p < 0.001$). As can be seen in Fig. 2, male rats tested 5 or 24 hr after withdrawal from the last ethanol exposure spent significantly less time in social interaction than the rats maintained on CD ($p < 0.01$, Tukey's tests). However, the rats tested 48 hr into withdrawal were not different from the appropriate

CD group, which suggests that anxiety associated with ethanol withdrawal recovers to control levels within 48 hr.

In contrast, the reduced locomotor activity, as reflected by line crossings, was observed at all times after withdrawal from ethanol when exposure was cycled (Fig. 2, lower panel). However, rats exposed continuously to ethanol for 15 days recovered within 24 hr. These differences in locomotor activity were also statistically significant ($F = 13.10$, $p < 0.001$).

Reinstatement of Reduced Social Interaction on Subsequent Ethanol Exposure

There were significant differences in the social interaction behavior of the male rats reexposed to ED at various times after being maintained on CD ($F = 13.95$, $p < 0.001$). The rats exposed to a fourth cycle of ED after either 8 (CY8R) or 16 (CY16R) days on CD were just as anxious as rats exposed to three cycles (EDCY3; Fig. 3, upper panel). The group exposed to the fourth cycle of ED after 32 days (CY32R) on CD had an intermediate social interaction score, which suggested partial recovery (Fig. 3, upper panel). In contrast, rats subjected to a second cycle of ED after a prior period of 15 consecutive days on ED had higher levels of social interaction than their cycled counterparts reexposed to ethanol after 8 (CON8R) and 16 (CON16R) days on CD (Fig. 3, upper panel). Although there were also significant differences in activity across animals ($F = 5.46$, $p < 0.005$), all groups exposed to ED exhibited reduced locomotor activity compared with the CD groups (Fig. 3, lower panel).

Social Interaction in Ethanol-Exposed Rats Before Withdrawal

The data for the male rats while on ethanol and during ethanol withdrawal are presented in Table 1. Note that the group exposed to CD had a lower social interaction level than previous groups; this might be related to the fact that this group was tested during the dark period. The two-way ANOVAs for these data were most notable for the highly significant interaction effect ($F = 9.42$, $p < 0.01$), which was a consequence of the social interaction scores of the CD and ED groups going in opposite directions. As expected, the CD group exhibited an increase in social interaction on the second exposure because it is well known that prior exposure to the open field arena increases subsequent social interaction behavior (File, 1980,1993). In contrast, the two ED groups exhibited decreases, a reflection of withdrawal-induced anxiety (Table 1). Thus, there were no differences among the groups while ethanol was present, but there were substantial differences during ethanol withdrawal.

Effects of Repeated Withdrawal in Rats Exposed to a Low Ethanol Concentration

The findings for the social interaction test in the rats repeatedly exposed to 4.5% vs. 7% ethanol and then withdrawn are illustrated in Fig. 4. For social interaction (upper panel), there were highly significant group differences ($F = 14.14$, $p < 0.001$). The CD group exhibited a level of social interaction that was similar to other groups maintained on CDs in previous experiments (Figs. 1–3). The group maintained on the 4.5% ED continuously had a similar level of social interaction to the group maintained on CD. The two groups maintained on 7% ED interacted significantly less than the CD group and did not differ from each other, which confirmed previous findings (Fig. 1). The group maintained on the 4.5% ED but given repeated withdrawals exhibited a reduction in social interaction as great as that shown by the two groups that were exposed to 7% ethanol, with its social interaction score significantly different from that of the CD group, but not those of the two 7% ED groups (Fig. 4, upper panel).

The results for locomotor activity (Fig. 4, lower panel) suggested that withdrawal-induced suppression of locomotor activity depended on the concentration of ethanol in the diet. After we confirmed overall significant differences with a one-way ANOVA ($F = 4.10$, $p < 0.01$), Tukey's tests were carried out. The rats maintained on 7% ethanol had reduced activity, whereas the rats maintained on 4.5% ethanol did not.

To gain a fuller understanding of how the different exposures to ethanol might influence withdrawal behavior, we calculated ethanol intakes for each group for days 2 through 14 of exposure. The findings are presented in Fig. 5. The results of the three-way ANOVA of these data indicated that the pattern of findings was complex. It is clear that the two groups maintained on 7% ethanol received more ethanol than the two groups maintained on 4.5% ($F = 160.78$, $p < 0.001$). There was also a tendency for the 7% groups to maintain a stable level of alcohol intake over the exposure period, whereas the 4.5% groups tended to reduce their alcohol intake over time (Fig. 5). This finding was confirmed by the significant day effect ($F = 3.88$, $p < 0.001$) and the significant day × concentration interaction ($F = 9.34$, $p < 0.001$). Overall, there was no significant effect of cycle ($F = 0.41$, $p = 0.52$). However, the day × cycle interaction was significant ($F = 4.79$, $p < 0.001$), probably because the 7% group that was cycled drank less than the continuous 7% group early in the second cycle (Fig. 5). Therefore, the alcohol intakes of the groups appear to be more closely related to the locomotor activity results (Fig. 4, lower panel) than the social interaction results (Fig. 4, upper panel).

Blood Ethanol Concentrations

The BECs for rats exposed to 7% ethanol are illustrated in Fig. 6. Both groups had higher BECs on the final day of exposure, but there were no differences between the groups for any treatment condition. In particular, the groups did not differ in BEC on days 6 and 11 of ethanol exposure, when the ED7CY3 group had been reexposed to ethanol. In addition, the BEC declined in both groups at a similar rate when the ethanol was removed. There were also no differences between the ethanol intakes of the two groups at any time (data not shown).

Testing the rats in the social interaction test 5 hr after withdrawal from the 15th exposure to ethanol confirmed that the CD group (25.5 ± 1.3 sec) had higher social interaction than the ED7CY (4.6 ± 7.1 sec) or ED7CON (7.1 ± 1.5 sec) groups ($F = 63.72$, $p < 0.001$), but the ethanol-exposed groups did not differ from each other. Rats were killed immediately after these tasks and blood samples were taken for BECs. No detectable ethanol was found.

Body Weights of Rats Did Not Differ Before or After Dietary Exposure

The body weights of the treatment groups involved in the various experiments were analyzed by ANOVAs, and in virtually every case there were no significant differences among the groups (data not shown). Therefore, the modified pair-feeding method used in this study was effective in producing groups with similar body weights.

DISCUSSION

The present results demonstrate the effects of ethanol concentration, chronic cycling, and gender on anxiety-related behavior and locomotor activity in rats withdrawn from chronic ethanol exposure. First, our findings confirmed that rats withdrawn from continuous exposure to modest concentrations of ethanol exhibit anxiety-related behavior (Criswell and Breese, 1992; File et al., 1989; Kampov-Polevoy et al., 2000; Knapp et al., 1998; Moy et al., 1997, 2000) that can last up to 24 hr (Pandey et al., 1999). Importantly, the decrease in social interaction, the measure of anxiety used in the present study, was clearly greater in rats subjected to three cycles of ethanol exposure and withdrawal than in rats subjected to just one withdrawal. The rats subjected to a single 5-day exposure to ethanol (7%, w/v) diet exhibited either no change or only a small decrease in social interaction. In contrast, the rats subjected to three cycles of ED of 5 days exhibited large reductions in social interaction, as large as those exhibited by rats that had been on ethanol (7%, w/v) diet for 15 continuous days. Both genders showed this increased effect on anxiety-related behavior after cycled exposure to ethanol.

The most important observation of this study was that the group subjected to repeated withdrawals from 4.5% ethanol exhibited reduced social interaction behavior even though they drank equivalent amounts of ethanol as the rats that were exposed to 4.5% ethanol continuously and exhibited no change in social interaction. Moreover, the reduced social interaction in the repeatedly withdrawn 4.5% group was similar to that in the two groups exposed to 7% ethanol, but the ethanol intake of this 4.5% group was significantly lower. This finding provides strong evidence for the conclusion that repeated withdrawals from ethanol can lead to a "sensitization" of anxiety as measured by social interaction. Therefore, the sensitization of the social interaction response during multiple ethanol withdrawals would be consistent with other responses altered by repeated withdrawals (Becker and Hale, 1993; Becker et al., 1997, 1998; McCown and Breese, 1990; Veatch and Gonzalez, 1996, 1999, 2000).

Despite the similarity in social interaction behavior after exposure to the same amount of 7% ethanol, other data suggest that continuous and cycled administrations of 7% ethanol have different consequences. After just 10 days of exposure, the continuous group exhibits only a mild anxiety reaction, whereas the cycled group exhibits a very strong anxiety reaction. Recovery from exposure follows a similar time course in the two groups. However, the fact that the anxiety response can be reinstated almost completely in the cycled group even after 16 days on CD (Fig. 3) suggests that cycled exposure to 7% ethanol has a more persistent effect than continuous exposure. Thus, cycled exposure of 7% ethanol led to both a more rapid onset and a greater persistence of anxiety-like behavior (reduced social interaction on reinstatement of ethanol and withdrawal) than continuous exposure.

The present findings have demonstrated that the exaggerated social interaction response can be elicited in a much shorter time frame than demonstrated previously. For example, Holter et al. (1998) and Spanagel and Holter (1999) showed that rats allowed to drink alcohol voluntarily for a very long time (up to 1 year) also become more anxious in the elevated plus maze test with repeated withdrawals. The most striking aspect of our findings is that anxiety could be induced in animals exposed to 4.5% ethanol in just 15 days by withdrawing them from ethanol on just two occasions for 48 hr each. The much shorter time frame of our findings allows for a whole range of studies to elucidate the underlying mechanisms responsible for sensitization of social interaction after repeated ethanol withdrawals (e.g., Knapp et al., 2001).

The BECs illustrated in Fig. 6 demonstrate that the rats exposed continuously or discontinuously to 7% ED exhibited similar BECs at the end of their drinking days and similar declines in BECs on being withdrawn from ethanol. It is unlikely, therefore, that differences in BECs between the two groups could explain the greater persistence in the cycled group. A similar study of rats exposed continuously or discontinuously to 4.5% ethanol failed to reveal any consistent pattern that could explain the differential social interaction responses (data not shown). Also, there was no evidence that the cycled group exhibited an increase in ethanol intake when returned to ethanol on the 6th and 11th days of exposure (Figs. 5 and 6). In contrast, alcohol-preferring rats exhibit a substantial increase (20–40%) in ethanol intake when ethanol is returned after a period of deprivation (e.g., Rodd-Henricks et al., 2000b). This phenomenon of increased ethanol intake has been referred to as the "alcohol deprivation effect." Although commonly observed in alcohol-preferring rats (see Rodd-Henricks et al., 2000a,b;Sinclair and Li, 1989), it is rarely seen in rats that do not voluntarily self-administer ethanol. Thus, the outbred rats used in these studies behave more like other outbred rats given sweetened alcohol to drink (e.g., Gardell et al., 1997) and not like alcohol-preferring P rats that significantly increase their alcohol intake when deprived. The differential social interaction responses in the rats exposed continuously or discontinuously to EDs cannot be accounted for by differences in alcohol intake or BECs.

Both an increase in anxiety symptoms (decrease in social interaction) and a decrease in general activity were observed in ethanol-withdrawn rats exposed to the 7% ED. These findings reinforce the notion that the withdrawal syndrome associated with ethanol exposure is complex (e.g., Moy et al., 1997). It cannot be argued that these two behaviors were merely components of the same process, because the data presented here clearly indicated that they responded differently to various manipulations. It appears that general activity was more sensitive because the rats exposed to just 5 days of the 7% ED had fewer line crosses than rats maintained on CD but showed little change in social interaction. In addition, the depressed locomotor activity was still observed at 48 hr after removal of the ethanol in rats exposed to three cycles of ED, so this behavioral consequence of ethanol withdrawal was even more protracted than the social interaction response that had returned to control levels. However, the reduction in locomotor activity was not accentuated in rats subjected to three cycles of ethanol exposure. Moreover, in rats exposed to three cycles of the lower 4.5% ethanol, there was no change in locomotor activity despite a reduction in social interaction (Fig. 4). Thus, the social interaction response can be enhanced without the locomotor response being affected by repeated ethanol withdrawals. Regardless, these findings confirm previous studies which suggest that social interaction and line crosses are independent behavioral measures.

CONCLUSION

The present findings have clearly established that the social interaction (anxiety) response to withdrawal from ethanol is accentuated (sensitized) with repeated withdrawals. The reduction in general activity associated with withdrawal from ethanol is affected by the various manipulations in a manner different from the anxiety response (File et al., 1993).

Acknowledgments

Supported by Grants AA 00243, AA 11605, and AA 00214 from NIAAA.

We wish to thank Ying Qian, Lee Gause, Matthew Crist, Ryan Hill, and Dhritiman Mukherjee for technical assistance.

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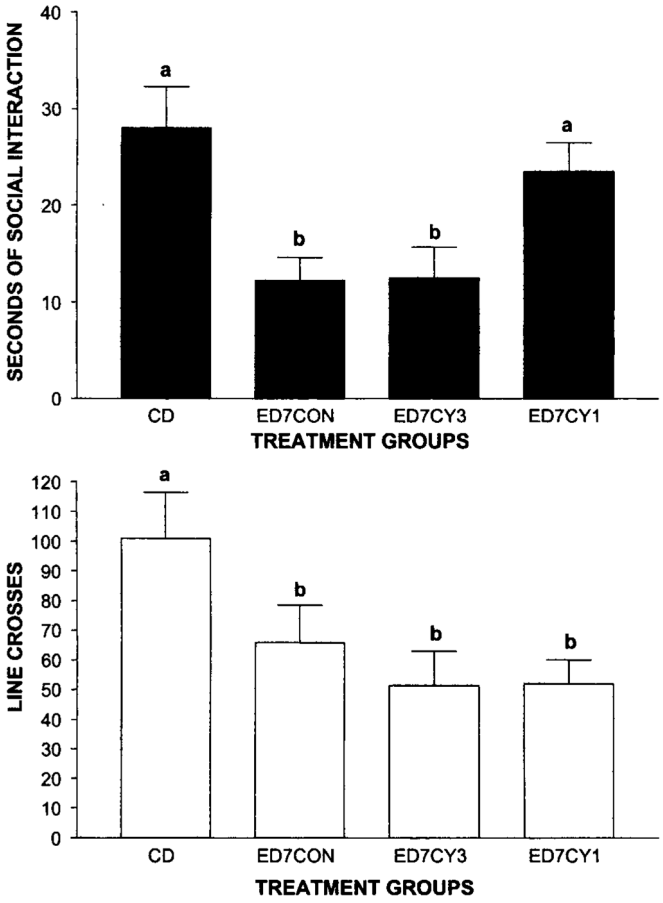

Fig. 1.

Effects of one versus three cycles of ethanol exposure on behavior in the social interaction test. Male rats were exposed to a control diet (CD), 15 consecutive days of a 7% ethanol diet (ED7CON), or one (ED7CY1) or three (ED7CY3) cycles of a 7% ethanol diet. The three cycles were each 5 days of exposure, and 2 days of control diet were interspersed between the first and second and the second and third cycles. The group given only one cycle was maintained on a control diet until the three-cycle group started their third cycle. The test was conducted between 5 and 6 hr after the ethanol was removed. Data represent mean ± SEM for 8 to 10 rats. Groups with different letters are significantly different, according to Tukey's tests.

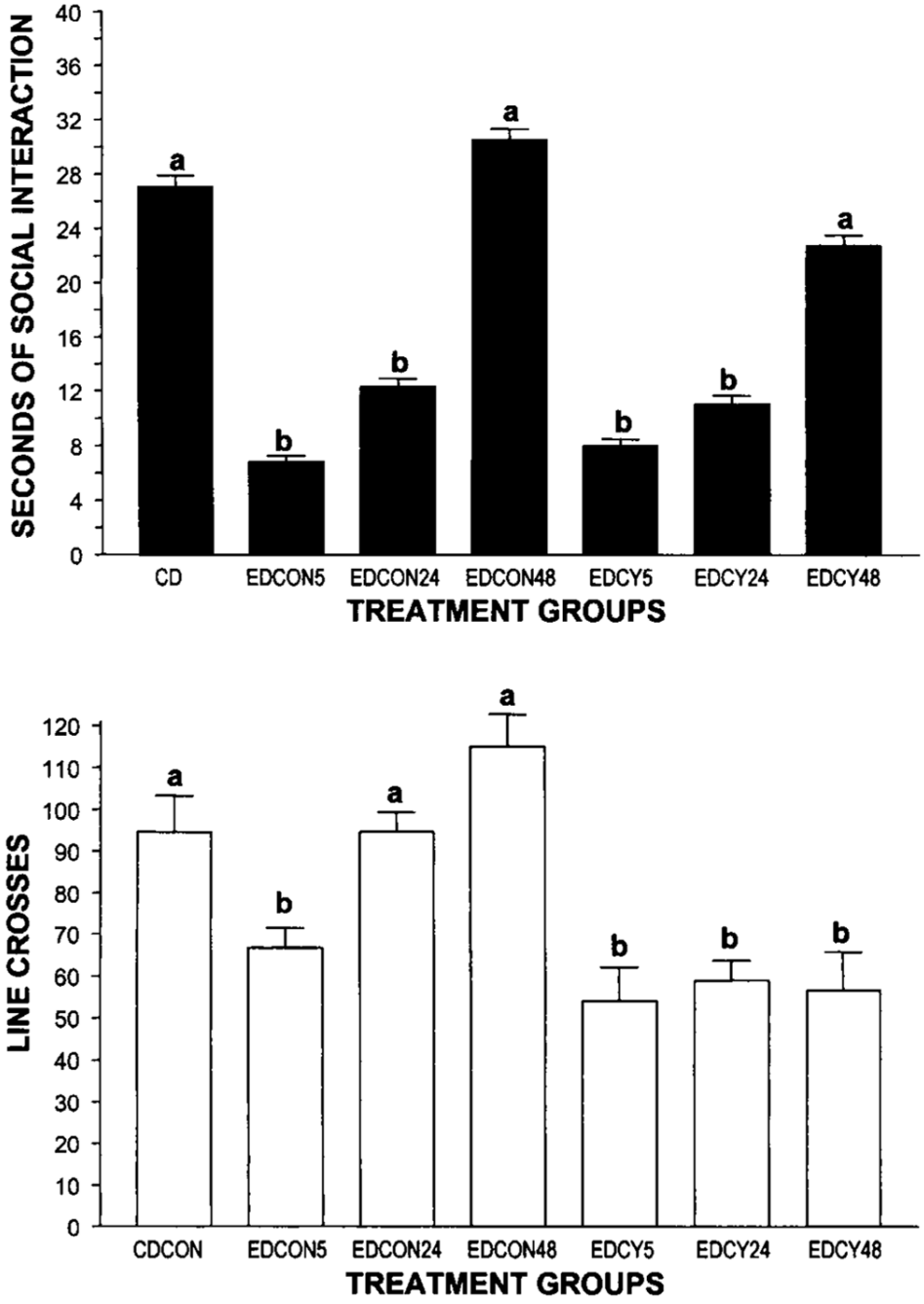

Fig. 2.

Recovery of anxiety responses in the social interaction test after withdrawal from three cycles of alcohol exposure or a continuous exposure of 15 days. Male rats were exposed to a control diet (CD), three cycles of a 7% ethanol diet, or 15 consecutive days of a 7% ethanol diet and tested at either 5 hr (EDCON5, EDCY5), 24 hr (EDCON24, EDCY24), or 48 hr (EDCON48, EDCY48) after the ethanol was withdrawn. Data represent mean ± SEM for 8 to 10 rats. Groups with different letters are significantly different, according to Tukey's tests.

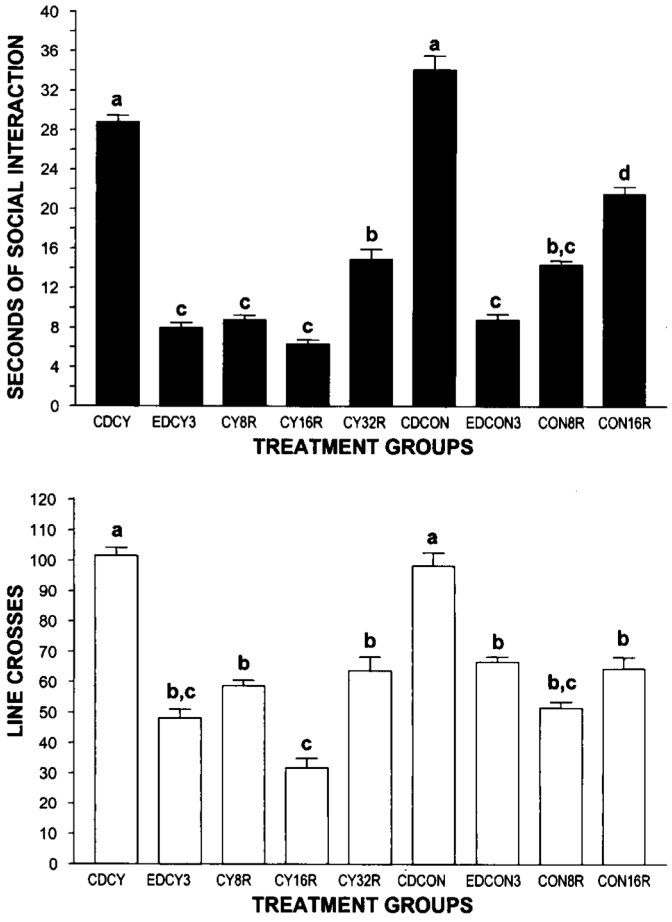

Fig. 3.

Social interaction behavior after reinstatement of ethanol exposure and withdrawal in rats previously exposed to ethanol. Male rats were given three cycles of 7% ethanol diet (EDCY3), 15 consecutive days of 7% ethanol diet (EDCON3), or control diet (CDCY, CDCON). Then they were maintained on control diet for 8 (CON8R, CY8R), 16 (CON16R, CY16R), or 32 (CY32R) days. Reinstatement involved exposing the rats to 5 days of 7% ethanol diet and withdrawing them. The social interaction test was conducted between 5 and 6 hr after the last exposure to ethanol. Data represent mean ± SEM for 8 to 10 rats. Groups with different letters are significantly different, according to Tukey's tests.

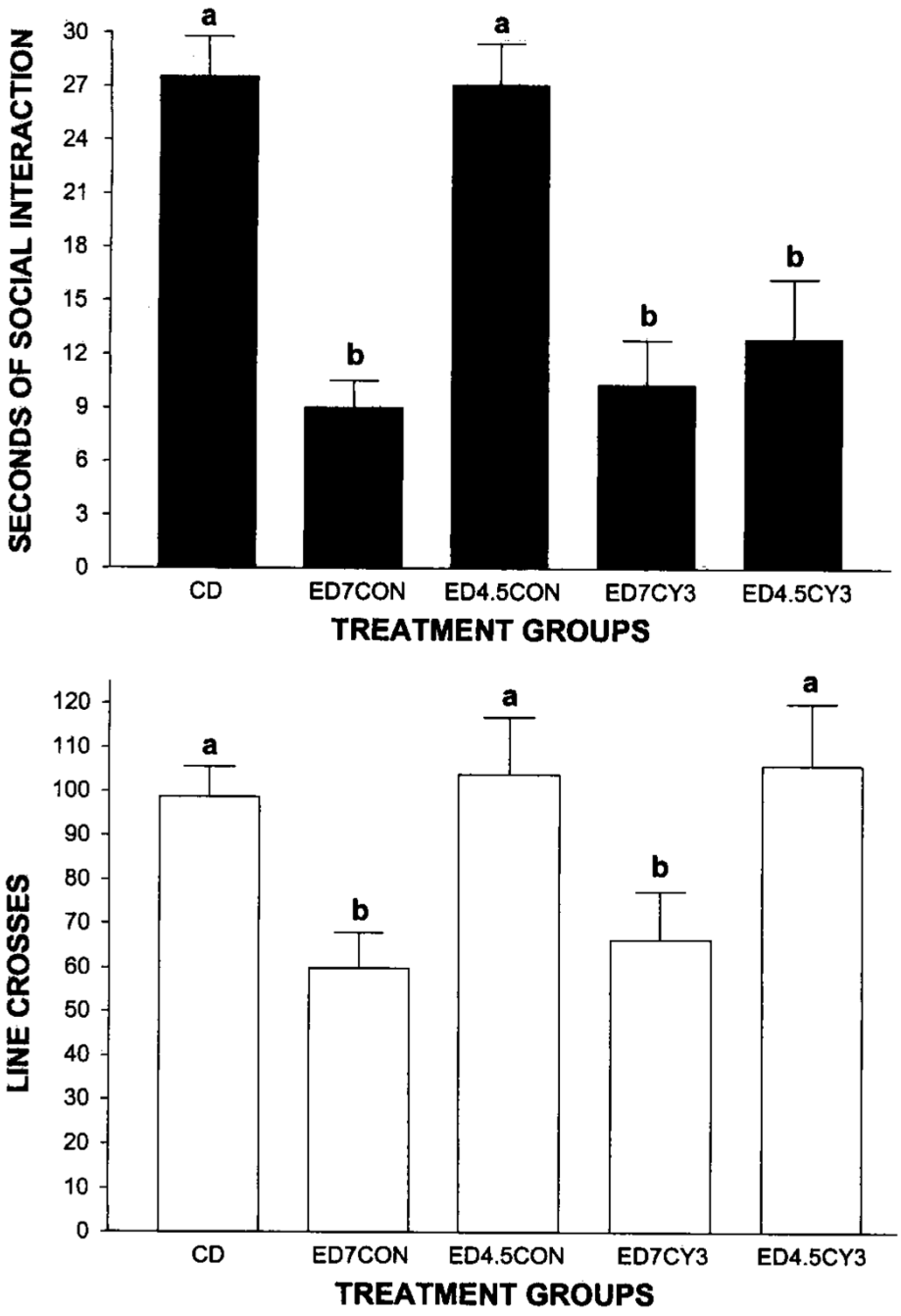

Fig. 4.

Effects of continuous versus cyclic exposure to 4.5% or 7% ethanol diets on behavior of male rats in the social interaction test. Experimental rats were continuously exposed to 4.5% (ED4.5CON) or 7% (ED7CON) ethanol diets or received three cycles of 4.5% (ED4.5CY3) or 7% (ED7CY3) and were compared with rats exposed to control diet (CD). The social Interaction test was conducted between 5 and 6 hr after removal of ethanol. Data represent mean ± SEM for 8 to 10 rats. Groups with different letters are significantly different, according to Tukey's tests.

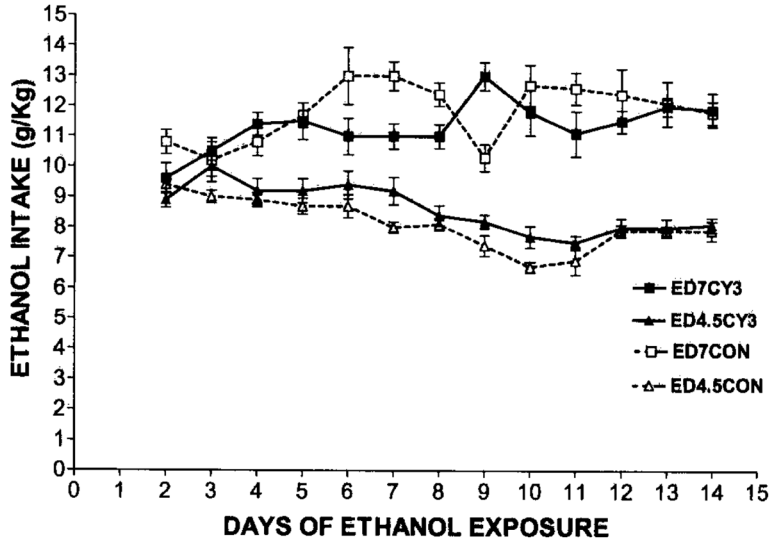

Fig. 5.

Time course of ethanol intake in male rats exposed to 4.5% or 7% ethanol diets continuously or in three cycles. Rats were continuously exposed to 4.5% (ED4.5CON) or 7% (ED7CON) ethanol diets or received three cycles of 4.5% (ED4.5CY3) or 7% (ED7CY3). Data represent mean ± SEM for 8 to 10 rats. Symbols for statistical significance are omitted for clarity. See text for details.

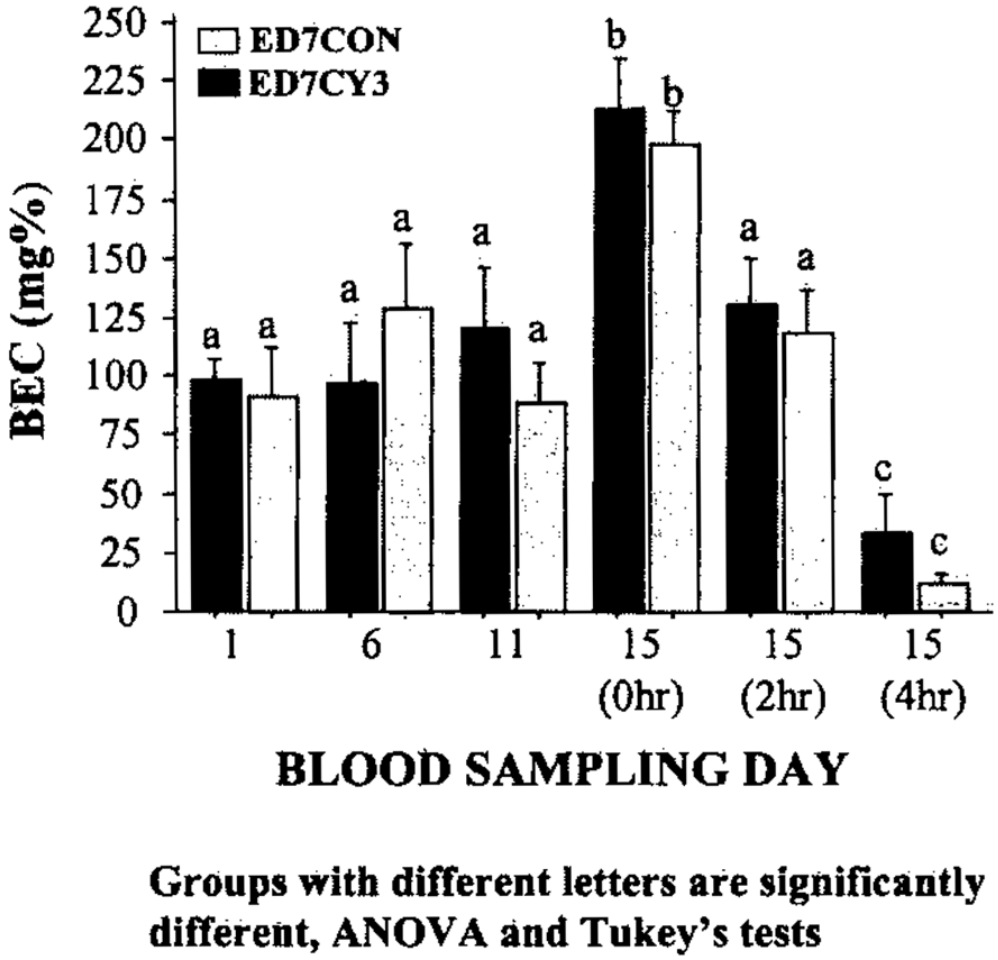

Fig. 6.

BEC in male rats exposed to 7% ethanol continuously or in three cycles. Rats were continuously exposed to 7% ethanol for 15 days (ED7CON) or to three 5-day cycles of 7% ethanol (ED7CY3) with 2-day withdrawal periods between the 5th and 6th days and the 10th and 11th days of exposure. Blood was taken from the tip of the tail during the last hour of darkness on days 1, 6, 11, and 15 arid also at 2 and 4 hr after removal of the ethanol on day 15. An ANOVA revealed significant differences among the sampling times but no significant differences between the ethanol-exposed groups.

Table 1

Social Interaction Behavior During Ethanol Access and Subsequent Withdrawal

	Experimental condition	
Treatment group	**During diet, day 14**	**Withdrawal from diet, day 15**
Control diet	15.5 ± 1.9	28.8 ± 3.4
Continuous ethanol diet	18.2 ± 3.0	13.4 ± 3.1*
Cycled ethanol diet	19.2 ± 1.6	11.8 ± 2.5*

Male rats were tested during the last hour of ethanol of control diet availability on day 14 or between 5 and 6 hr after removal of the 15th ethanol or control diet. Values represent the mean ± SEM seconds of social interaction for six rats.

*Significantly different, $p < 0.05$, from control diet group, Tukey's test.