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Modulation of Ethanol Withdrawal–Induced Anxiety-Like Behavior During Later Withdrawals by Treatment of Early Withdrawals With Benzodiazepine/γ-Aminobutyric Acid Ligands

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Abstract

Background—Anxiety states, including those arising during acute or protracted withdrawal periods, may be precipitating factors in alcoholic relapse. Given the cyclical nature of ethanol withdrawal associated with repeated cycles of ethanol intake and abstinence in a pattern that often spans years, meaningful attempts to model ethanol withdrawal–associated anxiety should incorporate cycled ethanol treatments. The studies reported herein examined the effects of γ-aminobutyric acid–modulating drugs on social interaction behavior—an established model of anxiety—in rats exposed to repeated cycles of ethanol treatment and withdrawal.

Methods—Rats were exposed to 8 to 12 g/kg/day ethanol during three 7-day dietary cycles (5 days on ethanol diet followed by 2 days on control diet). Ethanol was administered either at hour 4 of withdrawal after cessation of each of the first 2 ethanol cycles or during the final withdrawal only. In other groups, the early withdrawals were treated with alphaxalone, diazepam, PK11159, or flumazenil to block anxiety-like behavior during an untreated later (third) withdrawal. The benzodiazepine inverse agonist DMCM (methyl–6, 7–dymerhoxy–4–ethyl–beta–carboline–3–carboxylate) was also given repeatedly to determine whether it would sensitize anxiety-like behavior during a future withdrawal. Finally, the effects of all drugs on deficits in locomotor behavior were assessed.

Results—Pretreatment of earlier withdrawals with alphaxalone, diazepam, ethanol, or flumazenil reduced social interaction deficits during a later withdrawal, but pretreatment with PK11195 did not. In contrast, DMCM administered in lieu of early withdrawals increased social interaction deficits during an untreated later withdrawal. Locomotor deficits were significantly reversed only by the acute ethanol and diazepam treatment during the final withdrawal.

Conclusions—Single-dose administration of drugs that enhance or diminish activity at benzodiazepine–γ-aminobutyric acid- receptors during earlier withdrawals reduced or potentiated, respectively, anxiety-like behavior during later, drug-free withdrawals. These results support the potential of the novel strategy of using prophylactic therapy administered during early withdrawals to ameliorate symptoms of later withdrawals.

Keywords

Repeated Withdrawal; Anxiety; Flumazenil; Diazepam; Alphaxalone

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One of the important features of ethanol exposure in humans is its cyclical nature, whereby alcoholics undergo repeated cycles of intoxication and withdrawal over years (Ballenger and Post, 1978; Brown et al., 1988; Malcolm et al., 2000). Most animal studies modeling ethanol withdrawal entail a single cycle of ethanol treatment and withdrawal and do not reflect its cyclical aspect as observed in humans. The few studies that have been conducted with repeated-cycle models of ethanol withdrawal demonstrate that repeated cycles of treatment and withdrawal can significantly exacerbate seizure symptoms (e.g., Becker and Hale, 1993; McCown and Breese, 1990) and that in some models, such effects may be persistent (Kokka et al., 1993; McCown and Breese, 1990). Furthermore, psychological/emotional withdrawal symptoms such as anxiety may also worsen in the repeated-cycle model (Overstreet et al., 2002, 2003) or in the related chronic intermittent ethanol model (Cagetti et al., 2003).

Anxiety is a prominent and common symptom of withdrawal from chronic ethanol in humans (Koob and Le Moal, 1997; Meyer, 1986; Naranjo and Sellers, 1985) and animals (Baldwin et al., 1991; Criswell and Breese, 1993; File et al., 1989; Knapp et al., 1998; Moy et al., 1997, 2000). Anxiety states, including those arising during acute or protracted withdrawal periods, may be precipitating factors in alcoholic relapse (Driessen et al., 2001; Sinha, 2001). An understanding of the conditions in which anxiety arises is important in delineating its neurochemical mechanisms and in developing ameliorative treatments. Given the cyclical nature of ethanol withdrawal as encountered in the clinical setting, meaningful attempts to model and to pharmacologically manipulate ethanol withdrawal–associated anxiety should incorporate cycled ethanol treatments.

The studies reported herein examined the pharmacology of social interaction behavior—an established model of anxiety (File et al., 1989, 1993, 1996, 1999; Overstreet et al., 2003)—in rats exposed to repeated cycles of ethanol treatment and withdrawal (Overstreet et al., 2002). Based in part on hypotheses of underactive γ-aminobutyric acid (GABA) systems during ethanol withdrawal (e.g., Allan and Harris, 1987; Crews et al., 1996; Frye et al., 1986), the current studies were conducted primarily to examine benzodiazepine (BZD)–GABAergic influences on this novel effect of cycling on anxiety-like behavior. Drugs predicted to mitigate withdrawal symptoms based on their reported ability to enhance GABA activity via actions including BZD receptor stimulation and neurosteroid receptor stimulation were chosen. Flumazenil, generally considered a BZD receptor antagonist, was also tested based on previous work demonstrating a paradoxical anxiolytic action during withdrawal from other chronic ethanol treatment protocols (e.g., Criswell and Breese, 1993; Knapp et al., 2004; Moy et al., 1997, 2000). Importantly, some groups were specifically chosen to receive drug treatments only during the first two withdrawals of the three-withdrawal protocol. Therefore, behavioral testing in these animals was conducted in a drug-free state.

Because other agents known to reduce anxiety-like symptoms in alcohol-withdrawn rats (Knapp et al., 2004) also counteract the anxiety when given as pretreatments (Overstreet et al., 2003, 2004), it was anticipated that BZD-GABA receptor modulating ligands such as flumazenil and the BZD receptor agonist diazepam would counteract ethanol withdrawal–induced anxiety-like behavior in rats subjected to multiple cycles of ethanol exposure.

MATERIALS AND METHODS

Animals

For 5 days before dietary treatments, groups of 40 adult male Sprague Dawley® rats (160–180 g at the start of each experiment) from Charles River (Raleigh, NC) had ad libitum access to food and water in a temperature (22°C)– and humidity (40%)–controlled environment on a normal 12:12-hr light cycle with lights on at 0900 hr. For each group of 40 rats, 8 received control diet throughout, and 32 received an ethanol-containing diet as described below. Animal

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use was approved by the Institutional Animal Care and Use Committee at the University of North Carolina as per the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Drug Treatments

Ethanol (from 95% stock, Aaper, Shelbyville, KY) was administered via a nutritionally complete liquid diet as previously described (Criswell and Breese, 1993; Frye et al., 1981; Knapp et al., 1998). Briefly, group-housed rats were presented with the diet without ethanol for 3 days to acclimate the animals to this food source. All animals were then singly housed and exposed to either control diet or repeated cycles of ethanol diet (7% w/v; Frye et al., 1983; Moy et al., 1997; Overstreet et al., 2002). The ethanol diet was nutritionally complete (with concentrations of vitamins, minerals, lactalbumin, and other nutrients derived from ICN Research Diets (Costa Mesa, CA) and calorically balanced (with dextrose) across ethanol-treated animals and controls. The control diet rats were pair fed based on the average volume of ethanol diet consumed. After 5 days on the ethanol diet, during which time the rats consumed 8 to 12 g/kg/day, rats were withdrawn for the sixth and seventh days of treatment by replacing their ethanol diet with control diet. This 7-day cycle was repeated for 2 cycles, and then a final 5-day exposure to ethanol diet was given before behavioral testing (Fig. 1). The focus on this cycled treatment strategy for all animals was based in part on the parallels to human alcoholism and in part on the previous observation (Overstreet et al., 2002) that rats receiving cycling of 7% ethanol diet demonstrated hyperresponsivity to a future subthreshold chronic ethanol treatment even if the animals had up to 32 days of “recovery” from the earlier treatment cycle. In contrast, animals that had received similar amounts of ethanol in a continuous fashion did not demonstrate this persistence. All groups readily gained weight during the ethanol exposure period (Table 1).

In an initial experiment to ensure that the anxiety-like responding that occurred during withdrawal depended on the withdrawal (rather than the chronic ethanol exposure per se), ethanol in the form of acute injections was returned to three groups of withdrawn animals before behavioral testing on the final withdrawal day. All remaining receptor antagonist or agonist treatments were given on the first day of the 2 days of withdrawal after removal of chronic ethanol diet treatment (days 6 and 13). Given that the maximal anxiety-like behavioral responses resulting from this procedure occur during hours 5 to 7 after removal of the ethanol diet from the animals’ cages (Criswell and Breese, 1993; Knapp et al., 1998; Moy et al., 2000), drugs were administered intraperitoneally at hour 4 of the first and second withdrawals. In other experiments, BZD-GABA drugs including ethanol (Aaper Alcohol & Chemical Co.), flumazenil (RO15-1788, Hoffman-La Roche Inc., Nutley, NJ), PK11195 (which blocks the peripheral BZD receptor; Sigma, St. Louis, MO), and diazepam (Hoffman-La Roche Inc.) were administered during the third withdrawal in attempts to acutely block withdrawal-associated behavioral changes.

In addition to these drugs anticipated to reduce anxiety-like behavior, the BZD receptor inverse agonist DMCM was administered in an attempt to potentiate ethanol withdrawal–associated anxiety-like behavior in two ethanol exposure conditions that do not normally induce anxiety-like behavior—a 5-day exposure to 7% ethanol or a 15-day exposure to 4.5% ethanol (Overstreet et al., 2002). In addition, to confirm the hypothesized opposite modulatory effects of the BZD receptor antagonist flumazenil and the BZD receptor inverse agonist DMCM on withdrawal-associated anxiety-related behavior, a final study exposed rats to one or three 5-day cycles of 4.5% ethanol. Rats exposed to only 5 days of ethanol were pretreated with vehicle or 0.5 mg/kg DMCM 6 and 11 days before withdrawal from ethanol. Rats that received three cycles were pretreated with vehicle or 5 mg/kg flumazenil at hour 4 of withdrawal after the

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first and second cycles. The GABAergic neurosteroid receptor agonist alphaxalone (Steroids Inc., Newport, RI) was also examined.

Ethanol for injection (0.5–1.5 g/kg) was prepared as a 10% w/v solution in saline while flumazenil (5 or 10 mg/kg), alphaxalone (3 or 9 mg/kg), PK11195 (10 mg/kg), and diazepam (1 or 3 mg/kg) were prepared as a sonicated suspension in 0.5% carboxymethylcellulose. Except for the groups treated acutely with ethanol (0.5–1.5 g/kg), flumazenil (5 mg/kg), diazepam (1 mg/kg), or alphaxalone (3 mg/kg) on the final withdrawal day (30 min before testing, 7 min for flumazenil), all groups received drug injections on the first two withdrawals only (days 6 and 13) at hour 4 after withdrawal. Vehicle injections on the behavioral test day (day 20) were administered 30 min before testing.

Behavioral Testing

The social interaction test was used to assess anxiety-like behavior (Duxon et al., 1997; File et al., 1996, 1999; Gonzalez et al., 1996) between hours 5 and 7 after removal of the ethanol diet from the rats' cages. Rats were placed in a square open field (60 × 60 cm, with sixteen 15 × 15-cm squares marked on the floor) to which they were naive. Two rats that had similar treatment histories and were approximately equal in body weight were placed simultaneously into the test box for a 5-min period, and the time the animals spent engaged in social interaction (conspecific grooming, sniffing, following, crawling over/under) was recorded by an observer blind to the treatment conditions. Locomotor activity, expressed as the number of squares entered during the 5-min session, was also recorded. Pairs of rats experiencing ethanol withdrawal consistently reduce their social interaction time relative to pairs of controls (Knapp et al., 2004; Overstreet et al. 2002, 2003). The relative independence of social interaction behavior from forward locomotion behavior as well as the relative independence between members of a pair of social interaction behaviors has been described in detail (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2002, 2003, 2004). Given the documented independence of social interactions between members of a pair, data from individual animals rather than the average performance of the pair were quantified.

Statistical Analysis

Time (in seconds) spent in social interaction and the number of squares entered are normally distributed scores. Therefore, data were analyzed with ANOVA. When significant, Tukey tests were used to compare pairs of groups (n = 8–10).

RESULTS

Acute Ethanol Treatment During Final (Third) Withdrawal

To confirm that the anxiety-like behavioral profile of ethanol-withdrawn rats was dependent on the loss (metabolic elimination) of ethanol, ethanol in the form of acute injections of 0.5, 1.0, or 1.5 g/kg ethanol was returned to subgroups of withdrawing rats (Fig. 2, top). Ethanol injections produced a dose-dependent increase in time spent in social interaction [$F(3,36) = 7.48$, $p < 0.001$] because the social interaction level of withdrawing animals acutely treated with either 1.0 or 1.5 g/kg ethanol did not differ from that of control diet-exposed animals. A similar pattern was observed for locomotor activity [$F(3,36) = 3.25$, $p < 0.01$; (Fig. 2, bottom].

The next experiment examined the effects of pretreating rats with acute doses of ethanol during early withdrawals on anxiety-like behavior during the final withdrawal (Fig. 3, top). There were significant group effects [$F(3,36) = 11.89$, $p < 0.0001$], with the vehicle-treated ethanol-withdrawn group spending less time in social interaction than the rats exposed to control diet. Acute ethanol pretreatment during the first two withdrawals partially blocked the anxiety-like behavior during the untreated final withdrawal, whereas the single acute treatment during the

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third withdrawal again significantly attenuated social interaction deficits (Figs. 2 and 3, top). However, although the locomotor activity deficits were also blocked in the groups receiving acute ethanol injections during the final withdrawal (Figs. 2 and 3, bottom), no such blockade was seen among the group effects [$F(3,36) = 11.06$, $p < 0.001$] for animals that had received the ethanol pretreatment during the early withdrawals (Fig. 3, bottom).

Effects of Diazepam Pretreatment on Multiple Withdrawal-Induced Anxiety-Like Behavior

Overall, diazepam reduced withdrawal-induced anxiety-like behavior [$F(3,28) = 10.58$, $p < 0.001$]. Acutely injected diazepam counteracted the withdrawal-associated reduction in social interaction (Fig. 4, top). Diazepam also counteracted the anxiety-like behavior after the final withdrawal when given as a pretreatment during the first and second withdrawals (Fig. 4, top). Although there were also significant group differences in the locomotor activity scores [$F(3,28) = 10.61$, $p < 0.0001$], the pattern of effects on activity scores differed from those on social interaction. Only acutely administered diazepam increased the low activity of ethanol-withdrawn rats (Fig. 4, bottom).

Effects of Flumazenil Pretreatment on Multiple Withdrawal-Induced Anxiety-Like Behavior

Overall, flumazenil reduced withdrawal-induced anxiety-like behavior [$F(4,48) = 17.05$, $p < 0.0001$; Fig. 5, top]. Flumazenil was as effective in counteracting the anxiety-like behavior when given as a pretreatment during the first and second withdrawals as it was when given acutely (Fig. 5, top). There were also clear deficits in locomotor activity in the ethanol-withdrawn rats [$F(4,48) = 12.70$, $p < 0.0001$], but flumazenil did not modify this measure regardless of mode of treatment (Fig. 5, bottom).

Effects of PK11195 Pretreatment on Multiple Withdrawal-Induced Anxiety-Like Behavior

In the experiment with PK11195, a significant group effect was observed [$F(3,28) = 85.72$, $p < 0.001$], but all of the ethanol-withdrawn groups exhibited anxiety-like behavior (Fig. 6, top). PK11195 did not counteract anxiety-like behavior in ethanol-withdrawn rats regardless of mode of treatment (Fig. 6, top). A similar pattern was observed for locomotor activity [$F(3,28) = 83.14$, $p < 0.001$; Fig. 6, bottom].

Effects of Alphaxalone Pretreatment on Multiple Withdrawal-Induced Anxiety-Like Behavior

Alphaxalone had a unique profile on social interaction behavior of ethanol-withdrawn rats (Fig. 7, top). Although there were significant group differences [$F(3,28) = 10.08$, $p < 0.001$], the rats treated acutely with alphaxalone did not exhibit an anxiolytic response (increase in social interaction), whereas the rats pretreated with alphaxalone during the first and second withdrawals did (Fig. 7, top). Neither pretreatment nor acute treatment with alphaxalone counteracted the reduced activity induced by ethanol withdrawal [$F(3,28) = 9.44$, $p < 0.001$; Fig. 7, bottom].

Potentiation of Anxiety-Like Behavior in Ethanol-Withdrawn Rats by DMCM

A significant group effect on time spent in social interaction was observed in the DMCM experiment [$F(4,48) = 24.78$, $p < 0.001$], but the two exposure conditions that were given vehicle did not differ significantly from the group exposed to control diet (Fig. 8, top). Instead, the two groups that received pretreatment with DMCM 11 and 6 days before the withdrawal episode exhibited significant reductions in social interaction behavior (Fig. 8, top). This treatment protocol also potentiated ethanol withdrawal-associated deficits in locomotor activity [$F(4,48) = 15.20$, $p < 0.001$; Fig. 8, bottom]. Pretreatment with DMCM reduced locomotor activity even in animals exposed to 4.5% ethanol, a condition not typically associated with locomotor deficits (Fig. 8, bottom). In the experiment conducted to investigate hypothesized opposite actions of DMCM and flumazenil on anxiety-like behavior, significant

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group differences in anxiety-related behavior were observed [$F(4,35) = 12.24$, $p < 0.001$; Fig. 9, top]. The lowest times spent in social interaction were exhibited by the group given 1 cycle and pretreated with DMCM or 3 cycles and pretreated with vehicle, whereas the groups given 1 cycle and pretreated with vehicle or 3 cycles and pretreated with flumazenil had significantly higher scores (Fig. 9, top). Therefore, the potentiation of ethanol-withdrawal anxiety-like behavior by DMCM and its blockade by pretreatment with flumazenil were confirmed in rats exposed to a lower concentration of ethanol. Although there were also significant group differences for locomotor activity [$F(4,35) = 13.03$, $p < 0.001$], there were no differences as a consequence of pretreatment (Fig. 9, bottom). Rats that received three 5-day cycles of 4.5% ethanol exposure were more active than rats exposed to just one cycle, but there were no differences between the drug- and vehicle-treated groups in either condition.

Ethanol Intake and Body Weights

Each group consumed approximately 8 to 12 g/kg/day ethanol throughout the course of the treatments, and each group gained weight over this time period. There were no significant group differences in body weights or alcohol intakes (Table 1). A previous report demonstrated that blood ethanol levels obtained from the cycled protocol averaged around 100 mg/100 ml with a nearly complete elimination by hour 4 of withdrawal (Overstreet et al., 2002).

DISCUSSION

The results of this series of studies extend previous findings (e.g., Buck et al., 1991; File et al., 1989; Knapp et al., 1998, 2004; Moy et al., 1997, 2000) by demonstrating that acute treatment of ethanol withdrawal by agents that alter GABA receptor function after initial cycles of ethanol exposure and withdrawal reduces the expression of anxiety-like behavior during a later withdrawal episode. This novel demonstration of drug efficacy in counteracting the anxiety-like behavior during a future withdrawal constitutes a prophylactic effect that provides further evidence for an adaptive process that accrues from one withdrawal to the next and mediates the sensitization of withdrawal during repeated withdrawal cycling. Furthermore, the current studies show that locomotor activity, which is also reduced in ethanol-withdrawn rats, is less amenable than anxiety-like behavior to the counteracting effects of these agents.

The fact that ethanol and diazepam counteracted the anxiety-like behavior when given acutely 30 min before the behavioral test is consistent with the view that the withdrawal-induced anxiety is a consequence of ethanol withdrawal and involves BZD receptors. However, the fact that the BZD receptor antagonist flumazenil also counteracted the anxiety-related behavior, whereas peripheral-type BZD receptor antagonist PK11195 (Bidder et al., 1992; File and Pellow, 1985; Syapin and Alkana, 1988) did not, argues for the selective involvement of central BZD receptors. The data for PK11195 are also consistent with previous findings that the drug does not block anxiety in other contexts (File and Pellow, 1985).

The lack of an anxiolytic effect of acutely administered alphaxalone even though prophylactic treatment was partially effective may be dose related. Higher doses of this neuroactive steroid could not be given in the acute protocol because of its sedative effects. The dose of 3 mg/kg used in the acute study was at the low end of doses reported by others to have anxiolytic effects (Britton et al., 1991, 1992). Another factor is the possibility that rats may have been hyposensitive to the effects of alphaxalone, as has been reported by Cagetti et al. (2003), who used a different protocol of ethanol exposure. Nevertheless, prophylactic treatment with alphaxalone was effective in partially counteracting alcohol-withdrawal anxiety as were flumazenil and diazepam. This action of alphaxalone is consistent with the idea that endogenous neurosteroid actions in ethanol-treated animals may reflect altered affinities of BZDs and other GABA modulators for their respective receptors (e.g., Olsen et al., 2004)—an effect that might influence the action of flumazenil. It remains to be determined whether

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the various pretreatments given in the current studies prevent the adaptive changes in GABA type A ($GABA_A$) receptor subtypes induced by chronic alcohol (Grobin et al., 1998), chronic intermittent alcohol (e.g., Cagetti et al., 2003), or the current repeated-cycle model.

The role of $GABA_A$ receptors and their modulation by BZDs in the expression of ethanol withdrawal anxiety has been consistently shown in behavioral experiments (e.g., Criswell and Breese, 1993; File et al., 1992; Jung et al., 2000; Moy et al., 1997, 2000). However, a general agonist action at BZD receptors may insufficiently account for BZD-related mechanisms relevant to ethanol withdrawal. Because the anxiolytic action of BZD receptor agonists is most likely related to their ability to enhance GABA function in key brain regions, the BZD antagonist flumazenil might be predicted to have no effect, or perhaps to exacerbate, withdrawal anxiety. However, in this study and others (Criswell and Breese, 1993; File et al., 1989, 1992; Moy et al., 1997, 2000), flumazenil blocked anxiety arising in a variety of ethanol withdrawal paradigms. These findings argue for a second mechanism of action perhaps related to the blockade of endogenous BZD receptor inverse agonists that may be present after chronic ethanol exposure (Buck et al., 1991; File et al., 1989; Moy et al., 1997, 2000). This effect is interesting in light of the fact that flumazenil has a half-life of 16 min (Lister et al., 1984) and yet can reduce the anxiety of ethanol withdrawal when given many hours or days before a single withdrawal (Buck et al., 1991; File et al., 1989) or during multiple withdrawals before a final withdrawal (Fig. 4A). Such actions of flumazenil are reminiscent of the work of Gonsalves and Gallager (1988), which showed that flumazenil can rapidly and persistently reverse tolerance to chronic BZD exposure. These persistent effects of flumazenil in the absence of drug underscore the likelihood that flumazenil recruits cellular or molecular processes that persist beyond the drug's presence and that these processes may constitute a basis for an important and persistent ethanol-dependent phenomenon.

The argument that withdrawal-induced anxiety-like behavior may be attributed in part to the release of endogenous BZD receptor inverse agonist-like substances (Buck et al., 1991; File et al., 1989; Moy et al., 1997, 2000) is supported by the results with the BZD receptor inverse agonist DMCM in the current studies. Pretreatment with this compound before exposure to a single 5-day episode of 7% ethanol or during a 15-day episode of 4.5% ethanol led to a withdrawal-induced reduction in social interaction behavior. This anxiety-like behavior of rats pretreated with DMCM and given a single 5-day exposure to 4.5% ethanol closely resembled that of rats that received three 5-day cycles of 4.5% ethanol (Fig. 9A). Therefore, ethanol withdrawal-induced anxiety-like behavior is potentiated in DMCM-pretreated rats just as in cycled rats (Overstreet et al., 2002). One group of endogenous substances reported to have BDZ inverse agonist properties is represented by diazepam-binding inhibitor (DBI) and its active metabolites octadecaneuropeptide (ODN) and triakontatetraneuropeptide (e.g., Costa and Guidotti, 1991; Guidotti, 1991). ODN reportedly induces anxiety that is blocked by diazepam and flumazenil (De Mateos-Verchere et al., 1998). Although electrophysiological evidence (reviewed in Bormann, 1991) also suggests that DBI can reduce the action of GABA, a more cautious interpretation of the current data might be that the actions of DMCM and ethanol withdrawal on potentiation of anxiety could occur through relatively independent mechanisms. Consequently, the potential role in withdrawal-induced anxiety of endogenous substances with BDZ inverse agonist properties warrants further investigation.

Another possible explanation by which the BZD antagonist flumazenil may have actions that mimic an agonist is that it takes on agonist-like properties in ethanol-exposed tissue. Relatedly, flumazenil may act on a specific type of $GABA_A$ receptor complex containing specific combinations of subunits regardless of ethanol history (e.g., Barnard et al., 1998; Wafford et al., 1993; Weiss et al., 2002) or flumazenil may, with specific doses and conditions, have behavioral actions suggesting intrinsic activities other than antagonism (e.g., Danzter and Pério, 1982; File et al., 1986; Little et al., 1984). Of relevance to the ethanol field is the work

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of Devaud et al. (1995) where, for example, the α_1 subunit decreases while the α_4 subunit increases in specific brain areas after chronic ethanol exposure in vivo. Furthermore, persistent effects of this type on $GABA_A$ receptors have been noted by Cagetti et al. (2003) after the chronic intermittent ethanol treatment paradigm: "A specific GABA-A receptor subtype that may predict novel partial agonist-like actions of flumazenil include the combination $\alpha_4\beta_1\gamma_2$ (Wafford et al., 1996), thus an increase in the levels of this receptor by chronic ethanol exposure and/or withdrawal (Devaud et al., 1995; Sanna et al., 2003), might predispose the animal to respond to flumazenil at least in part as it would to diazepam." Therefore, given the potential differential actions of flumazenil that depend on dose or a specific receptor subtype, it might be useful to know whether site-specific changes in $GABA_A$ receptor structure mediate phenotypically specific actions of flumazenil in the current and related noncontinuous ethanol exposure paradigms.

A final possible explanation for the apparent BZD agonist action of flumazenil is that this drug in some way influences neurosteroid synthesis. Both DBI and StAR protein are reportedly involved in the steroidogenesis (Do-Rego et al., 2001; Hauet et al., 2002; Stocco, 2000), and acute ethanol treatment alters StAR messenger RNA in brain (Kim et al., 2003), whereas chronic ethanol exposure can increase DBI in brain (e.g., Katsura et al., 1998). Furthermore, Do-Rego et al. (2001) reported that ODN stimulation of neurosteroid synthesis through activation of central BZD receptors is blocked by flumazenil. The reduction of multiple withdrawal induced anxiety sensitization by alphaxalone (current study) is consistent with this view. On the other hand, PK11195, a blocker of the peripheral BZD receptor involved in steroid synthesis, did not block the adaptive mechanism associated with repeated withdrawals. This finding seems to argue against flumazenil's indirectly affecting a neurosteroid mechanism. Regardless, this potential mechanism for involvement of neurosteroid involvement in the action of flumazenil seems worthy of further attention.

Social interaction behavior was consistently modulated by the various BZD-GABA receptor ligands, whereas these drugs much less markedly and consistently affected locomotor activity. Although acutely administered ethanol and diazepam increased locomotor activity in ethanol-withdrawn rats, flumazenil, PK11195, and alphaxalone did not. Furthermore, almost no pretreatment significantly increased activity. These findings are consistent with other recent reports suggesting that anxiety-like behavior associated with ethanol withdrawal is more amenable to treatment than is the reduced activity (e.g., Breese et al., 2004; Knapp et al., 2004; Moy et al., 1997; Overstreet et al., 2003). Furthermore, the changes in locomotor activity induced by ethanol exposure and withdrawal must involve mechanisms other than those related to BZD-GABA, serotonin (Overstreet et al., 2003), or corticotropin releasing factor (Breese et al., 2004; Overstreet et al., 2004). Therefore, although acutely administered drugs can alter the locomotor deficit induced by the multiple withdrawals, the fact that the chronic (prophylactic) treatment did not have this action suggests that the motor areas might be less adaptable to GABA modulation than the limbic areas.

The fact that the BZD receptor agonist diazepam and the BZD receptor antagonist flumazenil both counteract the anxiety-like behavior in rats withdrawn from their final ethanol exposure when given as prophylactic suggests that the BZD-GABA receptor complex is intimately involved in the adaptive changes occurring during the multiple withdrawal protocol. It has been argued that alcoholic patients that have undergone multiple detoxifications are more difficult to treat (e.g., Brown et al., 1988). Therefore, the data from the current studies suggest the intriguing possibility that previous treatments with BZDs, flumazenil, or a neurosteroid may protect against treatment refractoriness associated with multiple detoxifications. Because of its short half-life, flumazenil might not be predicted to have efficacy in the clinic to reduce the anxiety symptoms in alcoholics. However, the persistent action of flumazenil in animal models suggests that considerations beyond its half-life may determine its efficacy and that this drug

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may be particularly relevant to the clinic. The short half-life of flumazenil notwithstanding, the data from the current studies indicate that flumazenil has robust effects when given as a pretreatment during multiple withdrawal cycling. Flumazenil also possesses little if any of the abuse liability of the BZD agonists. Relevant clinical studies with neurosteroids have not been reported, but the results of the current investigation suggest further consideration of such an effort is also warranted. Similarly, further clinical studies with flumazenil in this context may be fruitful.

In summary, this research shows that single-dose administration of drugs that enhance or diminish activity at BZD-GABA receptors during earlier withdrawals reduced or potentiated, respectively, anxiety-like behavior during later, drug-free withdrawals. The findings support the rationale for continued efforts to develop symptom-specific pharmacotherapies for acute treatment of ethanol withdrawal. Moreover, the results support the potential of the novel strategy of using prophylactic therapy administered during early withdrawals to ameliorate symptoms of later withdrawals.

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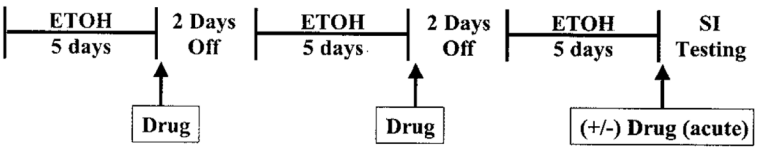

Fig. 1.

Repeated ethanol (ETOH) treatment and withdrawal strategy used in these studies. Rats were pretreated with drugs at hour 4 of the first day of the 2-day withdrawal period between cycles or acutely at hour 4.5 after the third withdrawal. Behavioral testing for anxiety occurred approximately 5 hr after the third withdrawal.

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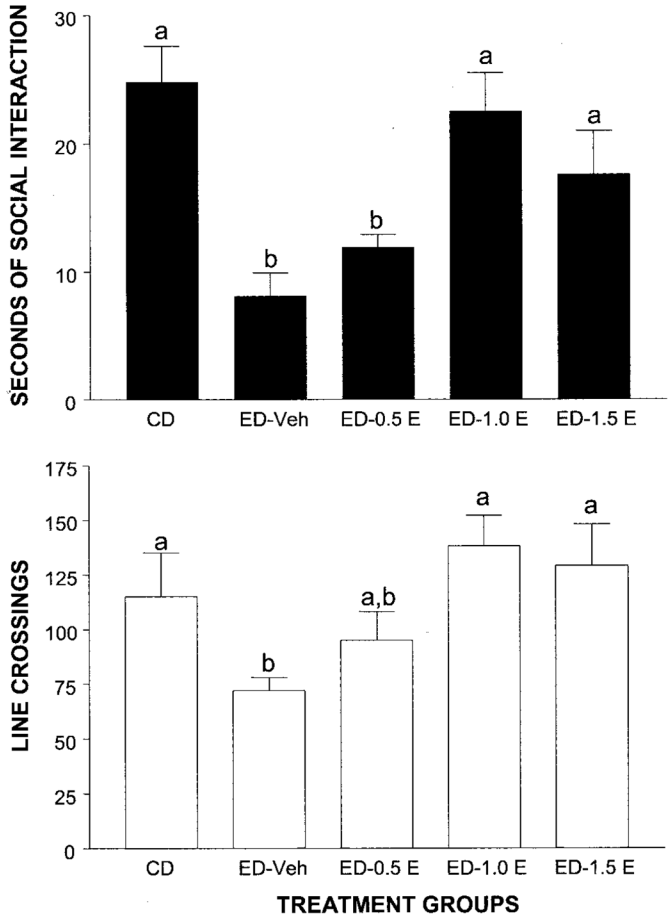

Fig. 2.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) for ethanol withdrawn rats given acute ethanol. Vehicle (Veh)– and ethanol (E; 0.5–1.5 g/kg)–treated rats exposed to ethanol diet (ED) received acute injections during the third (final) withdrawal on day 20 of the repeated withdrawal protocol and were tested for social interaction 30 min later. Means that do not share common letters are significantly different from each other, $p < 0.05$, Tukey test. CD, control diet.

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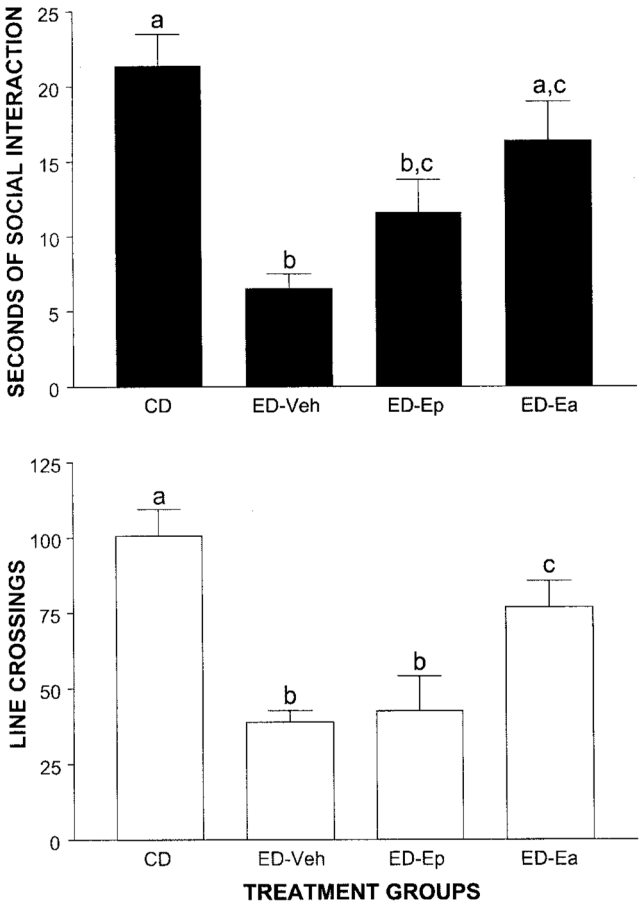

Fig. 3.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) for rats exposed to multiple ethanol withdrawals and treated with ethanol. Vehicle (Veh)–treated rats were treated either chronically (first two withdrawals) or acutely (final withdrawal only). Ethanol-treated rats were given 3 g/kg as a pretreatment during the first and second withdrawals or 1.5 g/kg acutely after the third withdrawal. Social interaction time and activity were recorded 5 hr after the third withdrawal. Means that do not share the same letters are significantly different from each other, $p < 0.05$, Tukey test. CD, control diet; Ea, acute ethanol treatment during the third withdrawal; ED, ethanol diet; Ep, ethanol pretreatment during the first two withdrawals.

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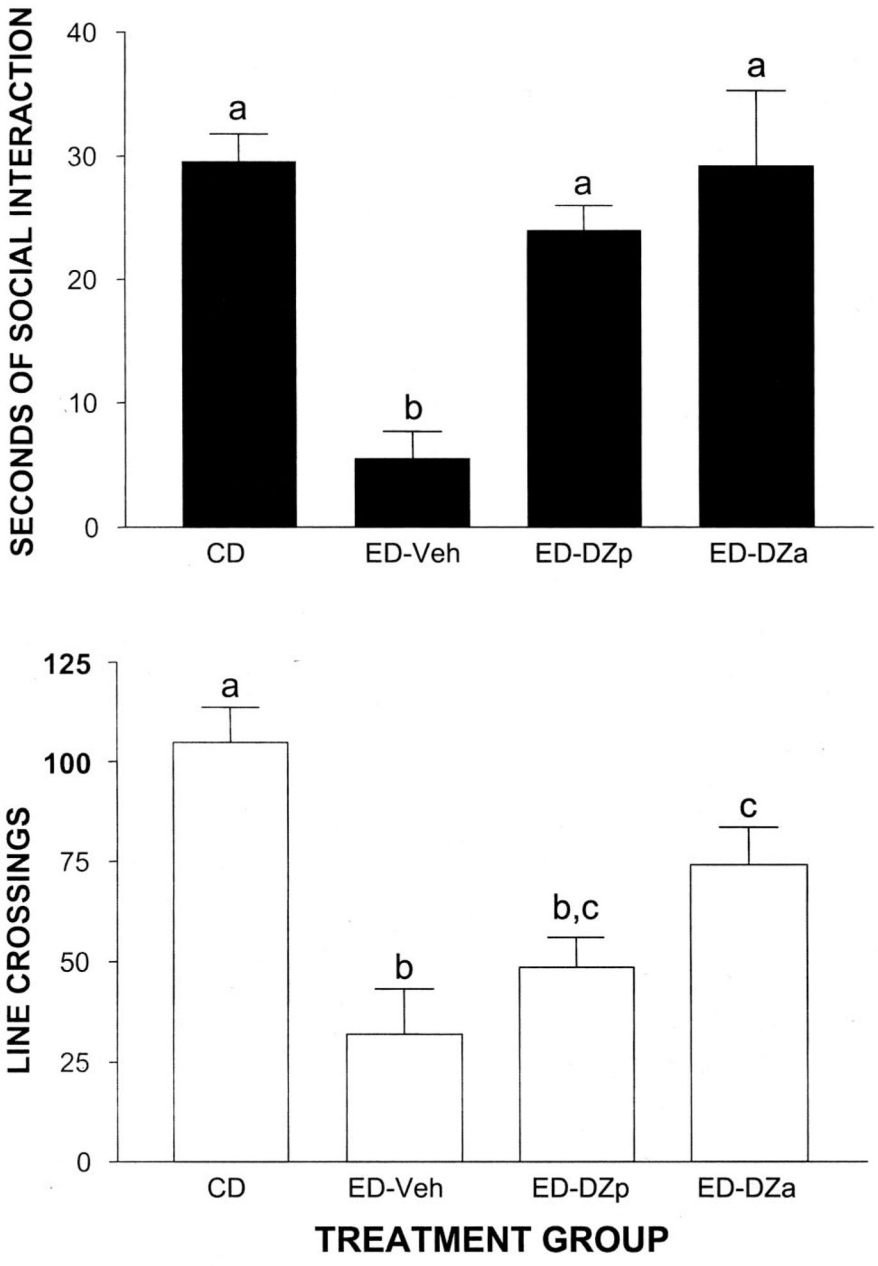

Fig. 4.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) for rats subjected to multiple ethanol withdrawals and treated with diazepam. Vehicle (Veh)–treated rats were treated either chronically (first two withdrawals) or acutely (final withdrawal only). Diazepam-treated rats were given 3 mg/kg as a pretreatment during the first and second withdrawals or 1 mg/kg acutely after the third withdrawal. Social interaction time and activity were recorded 5 hr after the third withdrawal. Means that do not share the same letters are significantly different from each other, $p < 0.05$, Tukey test. CD, control diet; DZa, diazepam treatment during the third withdrawal; DZp, diazepam treatment during the first two withdrawals; ED, ethanol diet.

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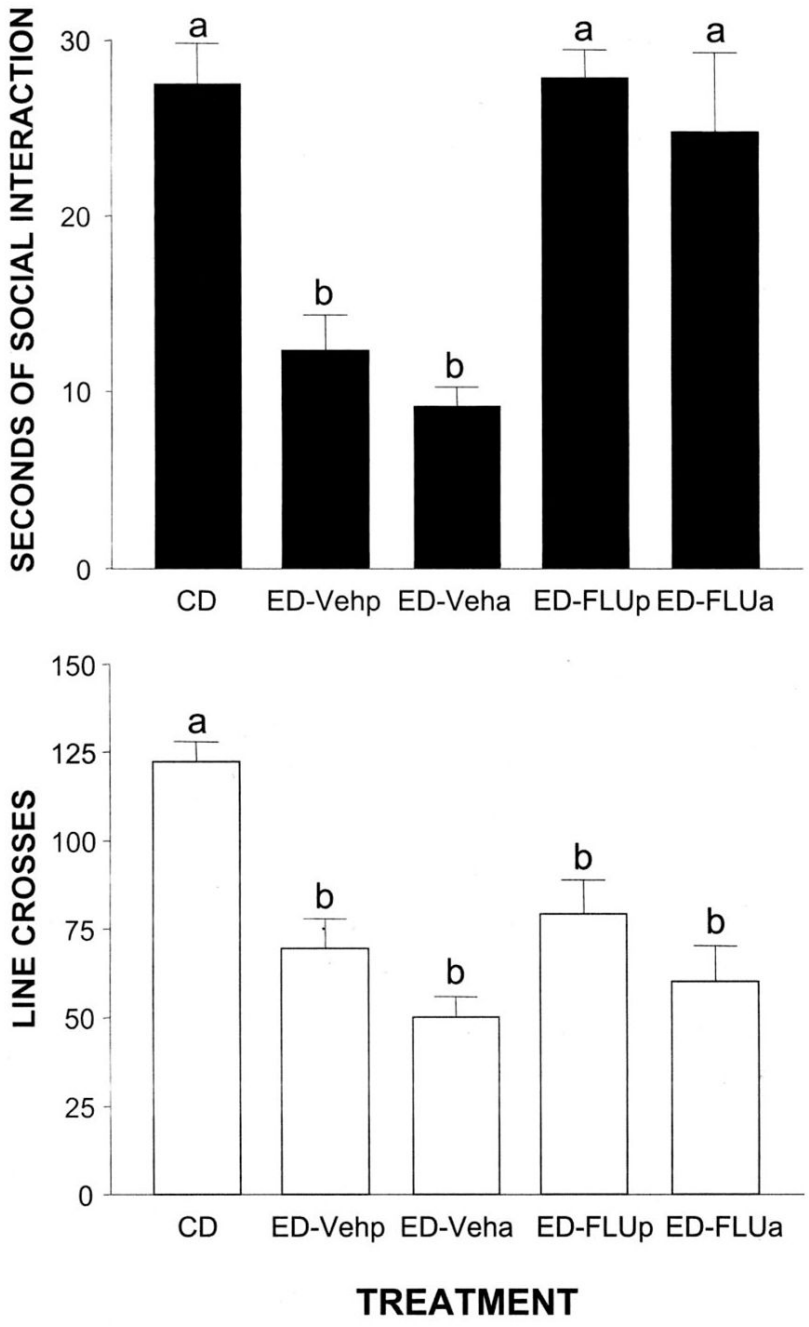

Fig. 5.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) for rats subjected to multiple ethanol withdrawals and treated with flumazenil. Vehicle-treated rats were treated either chronically (first two withdrawals) or acutely (final withdrawal only). Flumazenil-treated rats were given 10 mg/kg as a pretreatment during the first and second withdrawal periods or 5 mg/kg acutely after the third withdrawal. Groups that do not share common letters are significantly different from each other, $p < 0.05$, Tukey test. CD, control diet; ED, ethanol diet; FLUa, flumazenil treatment during the third withdrawal; FLUp, flumazenil pretreatment during the first two withdrawals; Veha, vehicle treatment during third withdrawal; Vehp, vehicle treatment during the first two withdrawals.

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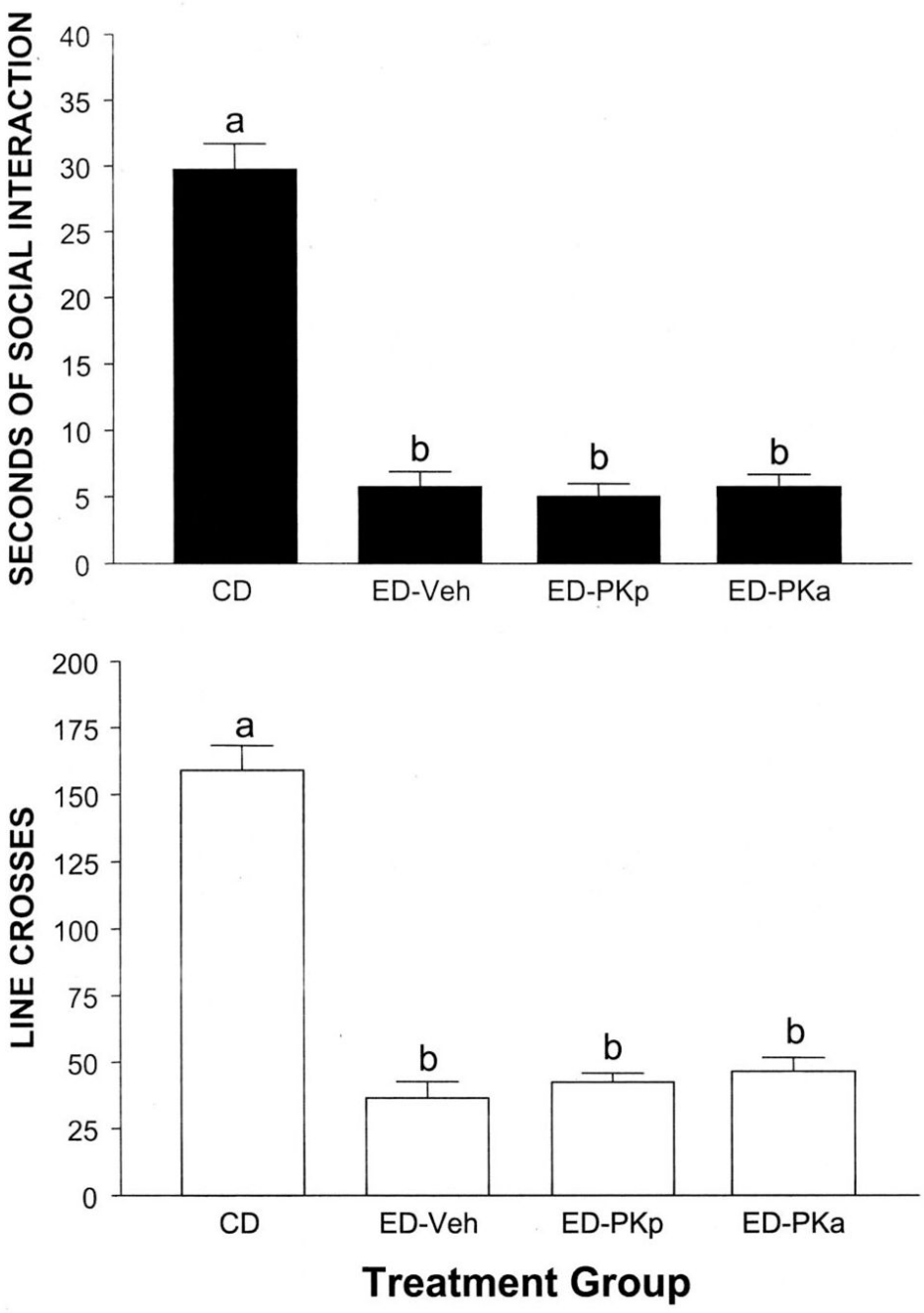

Fig. 6.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) for rats subjected to multiple ethanol withdrawals and treated with PK11195. Vehicle (Veh)–treated rats were treated either chronically (first two withdrawals) or acutely (final withdrawal only), and the data were combined. PK11195-treated rats were given 10 mg/kg as a pretreatment during the first and second withdrawal periods or 5 mg/kg acutely after the third withdrawal. Groups that do not share common letters are significantly different from each other, $p < 0.05$, Tukey test. CD, control diet; ED, ethanol diet; PKa, treatment with PK11195 during the third withdrawal; PKp, treatment with PK11195 during the first two withdrawals.

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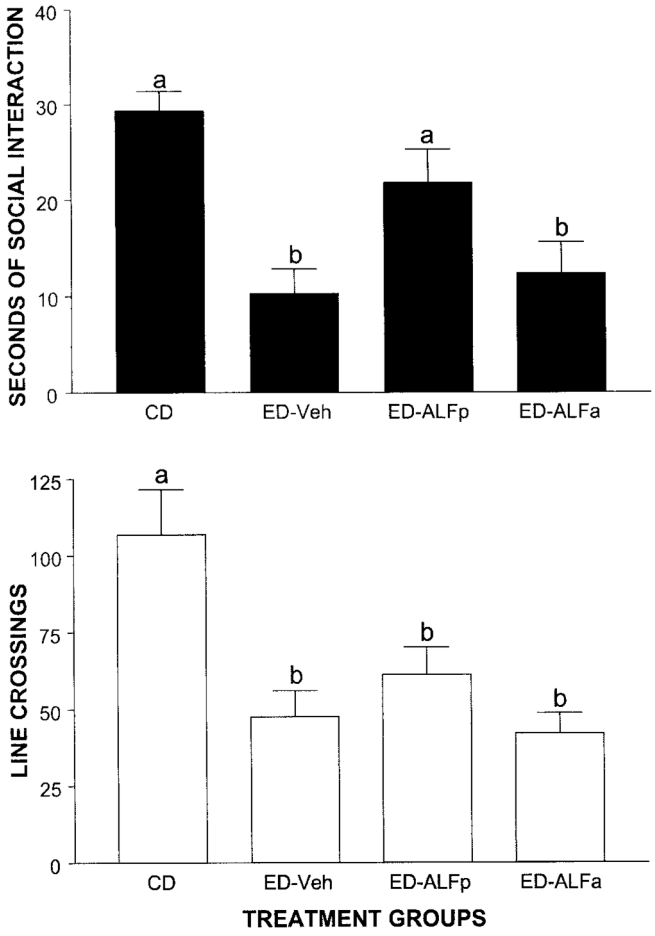

Fig. 7.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) for rats subjected to multiple ethanol withdrawals and treated with alphaxalone. Vehicle (Veh)–treated rats were treated either chronically (first two withdrawals) or acutely (final withdrawal only), and the data were combined. Alphaxalone-treated rats were given 9 mg/kg as a pretreatment during the first and second withdrawal periods or 3 mg/kg acutely after the third withdrawal. Groups that do not share common letters are significantly different from each other, $p < 0.05$, Tukey test. ALFa, alphaxalone treatment during the third withdrawal; ALFp, alphaxalone treatment during the first two withdrawals; CD, control diet; ED, ethanol diet.

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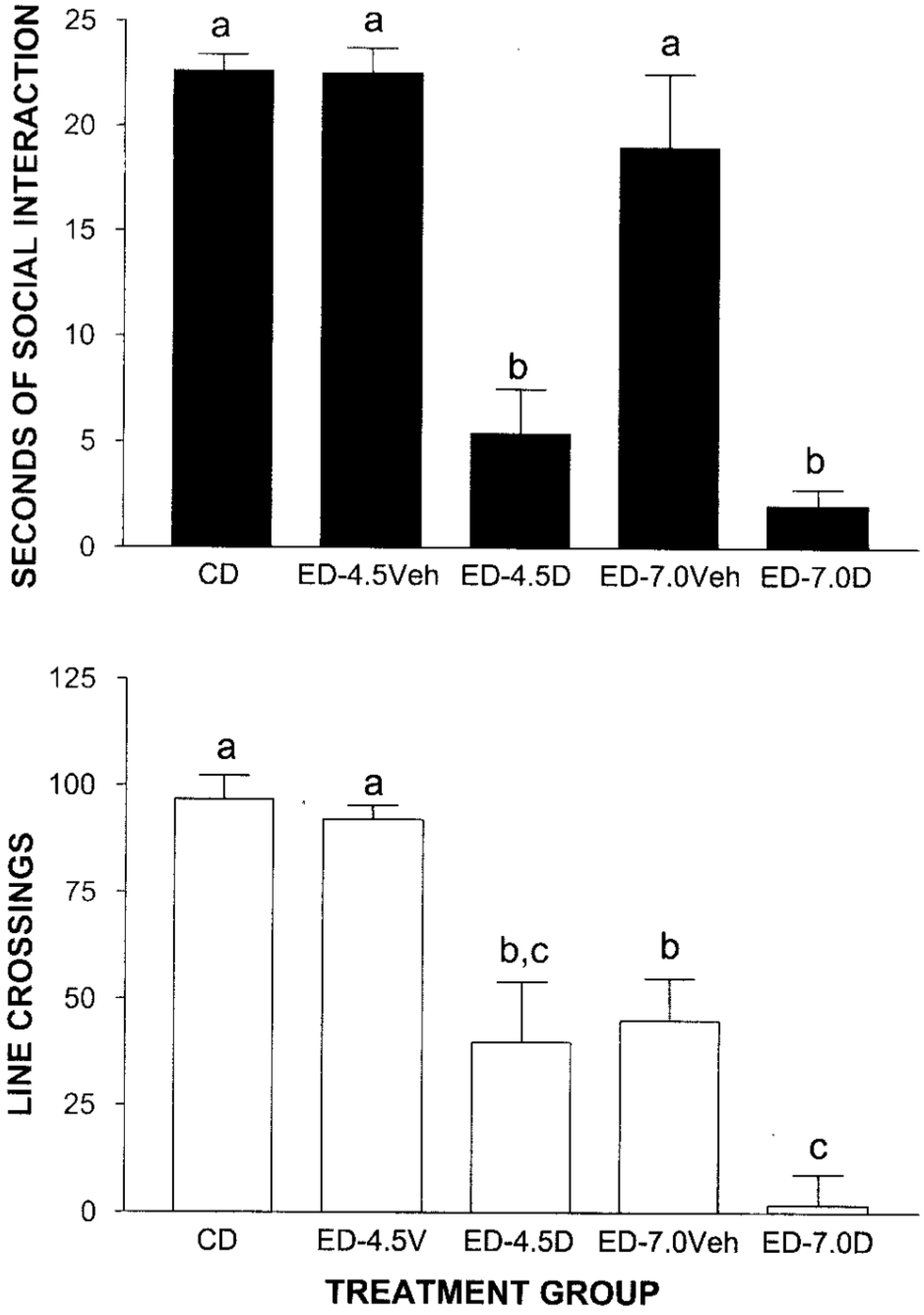

Fig. 8.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) of rats exposed to ethanol and pretreated with DMCM (D). Rats were continuously exposed to 4.5% (4.5) ethanol diet (ED) for 15 days or 7% (7.0) ED for 5 days. Rats were pretreated with either vehicle (Veh) or DMCM (0.5 mg/kg) 6 and 11 days before the ethanol was withdrawn. The social interaction test was conducted 5 hr after ethanol was withdrawn. Groups that do not share common letters are significantly different from each other, $p < 0.05$, Tukey test. CD, control diet.

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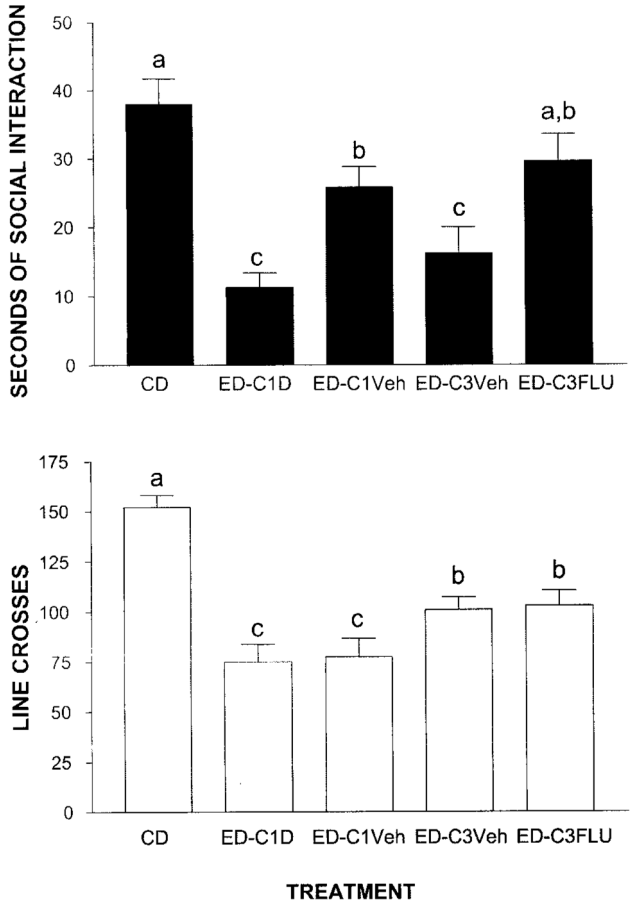

Fig. 9.

Mean social interaction time (top) and activity scores (bottom) (mean ± SEM) of rats exposed to 4.5% ethanol and pretreated with DMCM or flumazenil. Rats were exposed to a single 5-day cycle of ethanol (4.5%) exposure or three cycles of 4.5% ethanol diet. Rats were pretreated with vehicle or DMCM (0.5 mg/kg) 6 and 11 days before the 5-day exposure of ethanol was withdrawn. Other rats were pretreated with vehicle or flumazenil (5 mg/kg) at hour 4 of withdrawal after the first and second cycles of 4.5% ethanol diet. The social interaction test was conducted 5 hr after ethanol was withdrawn. Groups that do not share common letters are significantly different from each other, $p < 0.05$, Tukey test. C1D, one 5-day ethanol treatment cycle with DMCM treatments at days 6 and 11 before withdrawal; C1Veh, one 5-day ethanol treatment cycle with vehicle pre-treatments at days 6 and 11 before withdrawal; C3Veh, three 5-day ethanol treatment cycles with vehicle treatments during each of the first two withdrawals; C3FLU, three 5-day ethanol treatment cycles with flumazenil treatments during each of the first two withdrawals; CD, control diet; ED, ethanol diet.

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Table 1

Ethanol Intake and Group Weights for Rats Consuming a Nutritionally Complete and Calorically Balanced Liquid Diet Containing 0%, 4.5%, or 7% (w/v) Ethanol[a]

Experiment	Experimental Group					*F* Ratio	*df*
1	CD-Veh	ED-Veh	ED-0.5 E	ED-1.0 E	ED-1.5 E		
Ethanol (g/kg/day)	—	11.04 (0.32)	11.83 (0.25)	11.76 (0.51)	10.44 (1.06)	1.12	3,28
Weight (g)	321.6 (5.4)	332.2 (4.9)	340.8 (9.5)	336.0 (4.9)	334.6 (6.6)	1.21	4,35
2	CD-Veh	ED-Veh	ED-Ep	ED-Ea			
Ethanol (g/kg/day)	—	11.29 (0.28)	12.29 (0.30)	12.41 (0.43)		0.61	2,21
Weight (g)	318.6 (3.9)	300.1 (2.3)	303.1 (6.0)	298.3 (4.4)		1.5	3,36
3	CD-Veh	ED-Veh	ED-DZp	ED-DZa			
Ethanol (g/kg/day)	—	12.03 (0.46)	12.01 (0.50)	11.29 (0.30)		0.96	2,21
Weight (g)	254.9 (4.1)	255.1 (5.31)	262.0 (4.9)	261.0 (3.0)		0.73	3,28
4	CD-Veh	ED-Veha	ED-Vehp	ED-FLUa	ED-FLUp		
Ethanol (g/kg/day)	—	11.66 (0.35)	11.41 (0.21)	12.32 (0.32)	11.60 (0.39)	1.37	3,28
Weight (g)	254.9 (4.1)	261.8 (9.6)	257.7 (6.5)	255.3 (3.2)	261.2 (4.9)	0.65	4,35
5	CD-Veh	ED-Veh	ED-PKa	ED-PKp			
Ethanol (g/kg/day)	—	12.34 (0.39)	11.63 (0.22)	12.30 (0.21)		1.64	2,21
Weight (g)	292.4 (3.2)	288.8 (6.07)	283.4 (3.28)	284.63 (3.49)		0.9	3,39
6	CD-Veh	ED-Veh	ED-ALFa	ED-ALFp			
Ethanol (g/kg/day)	—	9.92 (0.33)	10.19 (0.40)	9.96 (0.38)		0.15	2,21
Weight (g)	311.0 (2.8)	297.0 (8.2)	298.6 (7.5)	290.3 (7.01)		1.67	3,28
7	CD-Veh	ED-4.5Veh	ED-4.5D	ED-7.0Veh	ED-7.0D		
Ethanol (g/kg/day)	—	9.06 (0.39)	8.89 (0.22)	10.85 (0.27)	9.90 (0.26)	9.45*	3,28
Weight (g)	281.5 (5.9)	299.1 (7.2)	294.0 (6.4)	253.4 (8.2)	254.5 (3.6)	11.22*	4,35
8	CD-Veh	ED-C1D	ED-C1Veh	ED-C3Veh	ED-C3FLU		
Ethanol (g/kg/day)	—	10.60 (0.46)	11.33 (0.37)	9.77 (0.39)	9.91 (0.30)	3.50**	3,28
Weight (g)	256.5 (6.1)	263.7 (6.9)	267.1 (6.6)	276.4 (5.8)	284.9 (4.6)	3.38**	4,35

[a] Experiments 1 to 8 correspond to Figures 2 to 9, respectively.

* Statistical significance only between groups receiving different concentrations of ethanol.

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** Statistical significance only between groups experiencing one *vs.* three cycles due to the natural tendency of rats to consume more ethanol diet during the first cycle.

ALF, alphaxalone; C1, one cycle; C3, three cycles; CD, control diet; D, DMCM; DZ, diazepam; 0.5 E, 0.5 g/kg ethanol injection; ED, ethanol diet; FLU, flumazenil; p, pretreatment during first two withdrawals; PK, PK11195; Veh, vehicle.