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Prior Multiple Ethanol Withdrawals Enhance Stress-Induced Anxiety-Like Behavior: Inhibition by CRF_1- and Benzodiazepine-Receptor Antagonists and a 5-HT_{1a}-Receptor Agonist

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Repeated withdrawals from chronic ethanol induce a persistent adaptive change. Further, stress substitutes for the initial two withdrawals of a multiple-withdrawal protocol to sensitize rats to withdrawal-induced anxiety-like behavior ('anxiety'). Therefore, it was tested whether the persistent adaptation induced by multiple-withdrawal exposures allows stress to elicit anxiety after a period of abstinence. Social interaction was used to assess the degree of anxiety induced by 45 min of restraint stress 3, 7, or 14 days after rats were exposed to multiple withdrawals from a chronic 4.5% ethanol diet. Restraint stress reduced social interaction (ie anxiety-like behavior) at 3, but not at 7 or 14 days, after the multiple withdrawals. No anxiety response was observed in animals that received multiple withdrawals without stress or in animals that received stress when exposed only to control liquid diet. Drugs (ie a CRF_1-receptor antagonist, a benzodiazepine receptor antagonist, and a 5-HT_{1A}-receptor agonist) previously demonstrated to block the cumulative adaptation, when administered during repeated withdrawals, prevented stress-induced anxiety-like behavior during abstinence. Additionally, these drugs applied prior to stress in the rats previously exposed to the repeated withdrawal protocol, likewise, minimized stress-induced anxiety. The anxiety following stress during abstinence from previous chronic ethanol exposure is indicative of an interaction of stress with the persistent adaptive change caused by repeated withdrawals. Stress eliciting anxiety-like behavior during abstinence from previous ethanol exposures in rats is consistent with stress inducing anxiety during recovery (sobriety) in the alcoholic, a circumstance that can facilitate craving and relapse.

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INTRODUCTION

Rasmussen *et al* (2001) observed anxiety-like behavior in the elevated-plus maze 4 weeks after removal from a 4-week exposure to chronic ethanol diet. Likewise, Valdez *et al* (2002) found that an extended chronic ethanol vapor exposure followed by repeated bouts of ethanol self-administration produced a persistent deficit in the elevated-plus maze after protracted absence from chronic ethanol. Overstreet *et al* (2002) found that repeated withdrawals from chronic ethanol reduced social interaction—an established measure of anxiety-like behavior in rats (File, 1980; File and Hyde, 1978; File and Seth, 2003). These observations are consistent with chronic ethanol inducing adaptive changes that have a persistent influence on brain function.

Breese *et al* (2004) found that stress substituted for multiple withdrawal-induced facilitation of the anxiety-like response when rats were withdrawn from a single chronic ethanol exposure that would not otherwise induce this behavioral change. Furthermore, upon re-exposure to a short-term exposure to ethanol at a later time, the prior repeated stress/withdrawal protocol enhanced withdrawal-induced anxiety-like behavior (Breese *et al*, 2004). This persistent effect on anxiety-like behavior following the repeated stress/withdrawal protocol provided convincing evidence for stress producing neural adaptations similar to those of withdrawal (Breese *et al*, 2004).

An anxiety-like emotional state can be present during extended abstinence in the alcoholic (Adinoff *et al*, 1995;

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Willinger *et al*, 2002; Begleiter and Porjesz, 1979). Therefore, since repeated stresses substitute for the repeated withdrawals (Breese *et al*, 2004), it was presumed that exposure to stress during a period of protracted abstinence in the alcoholic may contribute to their negative emotional state during periods of sobriety (Begleiter and Porjesz, 1979). To explore this view, animals were stressed in the present investigation after being previously exposed to withdrawal from multiple chronic ethanol exposures (repeated withdrawal protocol). Based upon the persisting adaptation induced by the repeated withdrawal protocol, it was predicted that the stress challenge would induce anxiety-like behavior in the rats previously exposed to this protocol, just as was observed following withdrawal from a 5-day re-exposure to chronic ethanol—a measure of persistent adaptation (Overstreet *et al*, 2002; Breese *et al*, 2004).

METHODS

Animals

Male Sprague–Dawley rats (Charles-River, Raleigh, NC) approaching 6 weeks of age (180–200 g) were initially housed in groups of three or four for several days to adapt to the animal facility conditions (light:dark cycle of 12:12, with lights on between 0900 and 2100) before being individually housed and exposed to control and ethanol-containing diets. The Institutional Animal Care & Use Committee (IACUC) at the University of North Carolina has approved all procedures described dealing with chronic ethanol exposures and stress.

Ethanol and Control Diets

After placement in individual cages, rats received a lactalbumin/dextrose-based nutritionally complete liquid diet (with concentrations of vitamins, minerals, and other nutrients derived from ICN Research Diets) (eg Frye *et al*, 1983; Overstreet *et al*, 2002). Dextrose calories in the control diet were equated with calories for ethanol in the ethanol-containing diet. After 3 days of control diet, a portion of the rats received either a 4.5% (w/v) ethanol diet administered for three cycles of 5 days interspersed with 2 days of control diet. These 2 days of control diet constituted the 'withdrawal' phase of the multiple-withdrawal protocol (Overstreet *et al*, 2002). The other rats received control liquid diet continuously. A modified pair-feeding design was used, which involved providing a volume of control diet equivalent to the average volume consumed the previous day by the rats maintained on ethanol diet. Rats were weighed at weekly intervals to establish similar body weights in the groups. In a portion of the animals exposed to the control diet or to the repeated ethanol diet, restraint stress (see below) was applied at 3, 7, or 14 days after removal from ethanol diet.

Stress Application

In order to evaluate the effect of stress on social interaction after exposure to the repeated withdrawal protocol, some animals were restrained in either a wire mesh conical tube (20 cm in length and 9 cm at the opening) or decapicone® disposable rodent restrainers (Braintree Scientific Inc., Braintree MA) for 45 min 3, 7, or 14 days after the final exposure to the ethanol or control diets. Social interaction was also measured in animals that received exposures to a chronic ethanol diet or the control liquid diet without exposure to restraint stress.

Measurement of Social Interaction

The time rats spent in social interaction (as a measure of anxiety-like behavior) and the number of line crosses (as a measure of general activity level) were assessed for all animal groups. The social interaction test, first introduced by File and Hyde (1978), has been validated as a measure of anxiety-like behavior in many investigations (File, 1980; Guy and Gardner, 1985; Irvine *et al*, 2001; see review by File and Seth, 2003). Testing was carried out under relatively low lighting in a square open field (60 cm × 60 cm, with 16 squares marked out on the floor); rats were unfamiliar with this environment (Overstreet *et al*, 2002). Assessment of social interaction was made in the various groupings of rats (ie that received the multiple-withdrawal or the control diet protocols) with and without prior exposure to stress. Experienced observers who were 'blind' to treatment conditions made the social interaction determination, which was assessed from the amount of time animals actively interacted (ie grooming, sniffing, boxing, or crawling over/under each other) during a 5-min session was measured. Social interaction was determined utilizing data from individual rats in the pair, a modification of the standard social interaction test (File, 1980; File and Seth, 2003). Statistical analyses of data from single animals in the pair in another data set revealed that the measures in individual rats provide the same statistical outcome as treating the social interaction scores of the rat pair as a unit (Overstreet *et al*, 2003b; Breese *et al*, 2004). Thus, this approach reduces the number of animals needed for testing (Overstreet *et al*, 2002). Social interaction was measured 30 min after the stress exposure. Each squad of 40 rats was tested in subgroups of 20, with balanced numbers of rats in each treatment group. Rat pairs were matched on the basis of ethanol intakes, body weights, and treatment conditions and placed simultaneously in the open field (Overstreet *et al*, 2002). This behavioral testing after exposure to stress was conducted at 3, 7, or 14 days after removal from the repeated withdrawal protocol.

While measuring social interaction, there was simultaneous recording of line crosses (by two forepaws), which served as a measure of locomotor activity independent of social interaction (File, 1980). In a previous study of 25 pairs of rats maintained on control diet and 25 on ethanol diet, the social interaction and locomotor activity were essentially independent behaviors, as there was no significant correlation between these measures in either group (Overstreet *et al*, 2002).

Drug Testing

The involvement of specific neural systems in any change in stress-induced anxiety-like behavior during abstinence from the repeated withdrawals was established by admin-

istering animals intraperitoneally with either a CRF_1-receptor antagonist (CP154,526; 10 mg/kg; Pfizer Inc., Groton, CT), a benzodiazepine receptor antagonist (flumazenil; 5 mg/kg; Roche, Basel, Switzerland), or a 5-HT_{1A}-receptor agonist (buspirone; 0.6 mg/kg; RBI-Sigma, St Louis, MO) 4 h after the removal of the ethanol diet during each of the initial two cycles. Drug doses were chosen based upon effectiveness in previous studies to diminish the adaptive change induced by the repeated withdrawal protocol (Overstreet *et al*, 2003a, b, 2004a, b) or the adaptive change induced by the multiple stress/withdrawal protocol (Breese *et al*, 2004). Buspirone was dissolved in saline. The flumazenil and CP154,526 were suspended in 0.5% carboxy-methylcellulose. Animals not treated with drugs were given 0.5% carboxy-methylcellulose to control for any effect of vehicle. Subsequently, controls and drug-treated groups were challenged with stress 3 days after the final removal from multiple-withdrawal protocol. In another experiment, the rats were exposed to the repeated withdrawal protocol and these drugs were administered 30 min prior to stress application.

Data Analysis

Statistical analyses were carried out using the GBStat software package. The data were initially analyzed by ANOVAs, with one- or two-way ANOVAs carried out depending upon the research design. If the main and/or interaction effects were statistically significant, *post hoc* analyses were performed using Tukey's protected t tests (see Breese *et al*, 2004; Knapp *et al*, 2004b; Overstreet *et al*, 2003b).

RESULTS

Effect of Stress Duration on Social Interaction

The initial experiment determined the length of time necessary for restraint stress to induce anxiety-like behavior in control rats. Stress was applied for 15, 30, 45, or 60 min prior to testing for social interaction 30 min after stress exposure. As shown in Figure 1, only the 60 min exposure to restraint stress reduced social interaction. Therefore, in the future experiments, the 45 min exposure to stress was used to evaluate the effect of the adaptation to repeated withdrawals on the ability of stress to induce anxiety-like behavior.

Effect of Stress on Social Interaction Following the Repeated Withdrawal Protocol

At 3 days after exposure to the repeated withdrawal protocol, a 45 min exposure to restraint stress significantly reduced social interaction 30 min later (Figure 2). This restraint stress did not alter social interaction in animals that received only control liquid diet (Control Diet Stress). Likewise, no change in social interaction was observed 3 days after exposure to only the multiple-withdrawal protocol (Ethanol Diet No stress; Figure 2).

During the measurement of social interaction, locomotor activity scores 30 min after the stress for the various groups were not significantly affected (see Figure 2 legend).

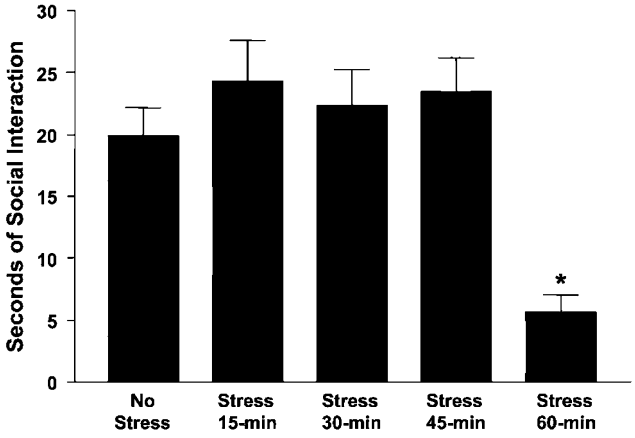

Figure 1 Duration of restraint stress to reduce social interaction. Restraint stress was applied to rats (8–10/group) for 15, 30, 45, or 60 min, and social interaction, a measure of anxiety-like behavior, was assessed 30 min after the stress exposure. Only the group exposed to 60 min of restraint stress exhibited significant reduction in social interaction ($F(4,33) = 6.73$). *$p < 0.001$ compared to the groups that received no stress (No Stress). No change in locomotor activity was recorded after any of the applications of stress ($F(4,33) = 1.48$; $p > 0.1$).

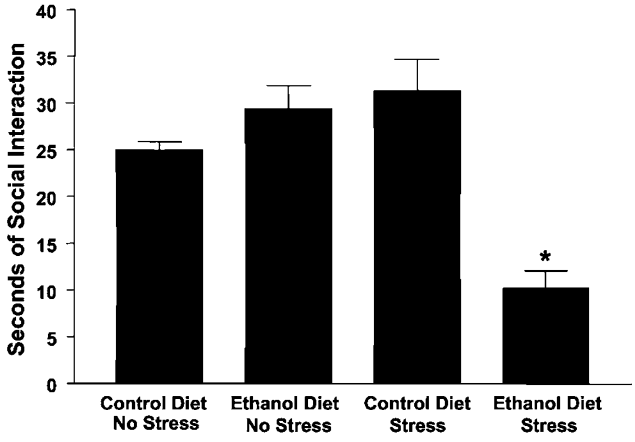

Figure 2 Effect of stress on anxiety-like behavior in animals previously exposed to multiple withdrawals. Rats (8–10/group) were exposed to restraint stress for 45 min 3 days after receiving control diet (Control Diet Stress) or multiple withdrawals from 4.5% ethanol diet (Ethanol Diet Stress). A control diet without stress group (Control Diet, No Stress) or a multiple withdrawal without stress group (Ethanol Diet, No Stress) were included for comparison. Social interaction was assessed 30 min after receiving restraint stress ($F(3,27) = 3.83$). *$p < 0.05$ when Ethanol Diet Stress group is compared to the other treatments. No significant change ($F(3,27) = 2.28$; $p > 0.1$) in locomotor activity was observed in the repeatedly withdrawn rats 30 min after stress application—the time social interaction was measured.

Therefore, it is apparent that activity was not a complicating factor for interpretation of the effect of stress on social interaction.

Time Course of Stress-Induced Anxiety-Like Behavior after Repeated Withdrawals

In order to determine the duration of influence the adaptation elicited by the repeated withdrawals had on

stress induction of anxiety-like behavior, animals were stressed 3, 7, or 14 days after the final withdrawal. As shown in Figure 3, the 3-day exposure to stress (Ethanol Diet 3-Day Stress) reduced social interaction as noted previously (Figure 2), whereas stress applied at 7 or 14 days after removal from the repeated withdrawals did not reduce social interaction.

Effect of Selected Drugs Administered during Repeated Withdrawals on the Stress-Induced Anxiety-Like Behavior during Abstinence

A 5-HT_{1A}-receptor agonist, buspirone, the benzodiazepine receptor antagonist, flumazenil, or the CRF_1-receptor antagonist, CP154,526, were administered during the initial two withdrawals, but not the third, of the repeated withdrawal protocol. These treatments previously blocked the reduced social interaction seen following this repeated ethanol cycling (Overstreet *et al*, 2003a, b, 2004b; Knapp *et al*, 2004b, 2005). Treatment with the these drugs during the initial repeated withdrawal cycles prevented the stress-induced reduction in social interaction induced 3 days after removal from the multiple-withdrawal protocol (Figure 4). As noted in the Figure 4 legend, locomotor activity was not altered during the measurement of social interaction.

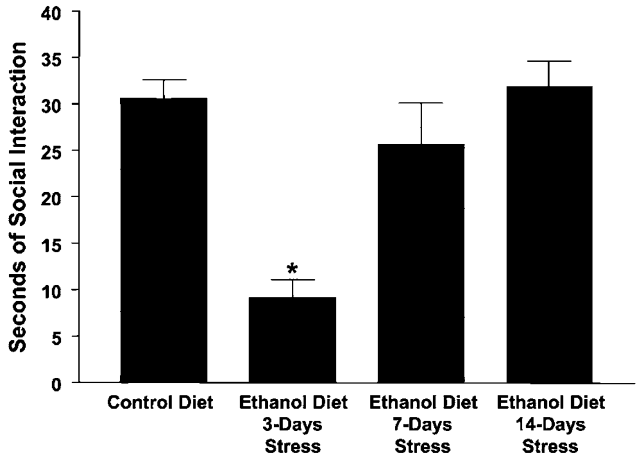

Figure 3 Effect of stress on social interaction in animals 3, 7, and 14 days after the repeated withdrawal protocol. Animals were tested for social interaction 30 min after being stressed for 45 min at 3, 7, or 14 days after removal of the ethanol diet. *$p<0.0001$ ($F(3,34)=14.9$) compared to control diet (Control Diet, No Stress).

Effect of Selected Drugs on the Stress-Induced Anxiety-Like Behavior during Abstinence

In an additional experiment, it was determined whether pretreatment with the CP154,526, buspirone, or flumazenil 30 min prior to application of restraint stress to rats previously exposed to the repeated withdrawal protocol would block the anxiety-like response to this challenge. As shown in Figure 5, these drugs prevented the stress-induced reduction in social interaction. In agreement with earlier results that these drugs do not have an effect on social interaction when administered to control animals (Knapp *et al*, 2004b), administration of these drugs 3 days after repeated withdrawals had no effect on social interaction 30 min after each of the drugs when compared to vehicle (see Table 1).

While buspirone and CP154,526 did not affect locomotor activity during the assessment of social interaction following stress (see procedure for locomotor activity in Methods), the group treated with flumazenil prior to stress exhibited a significant reduction in locomotion (Figure 5 legend). However, social interaction in the flumazenil multiple-withdrawn group returned to the level observed in control diet group (Figure 5), an observation that

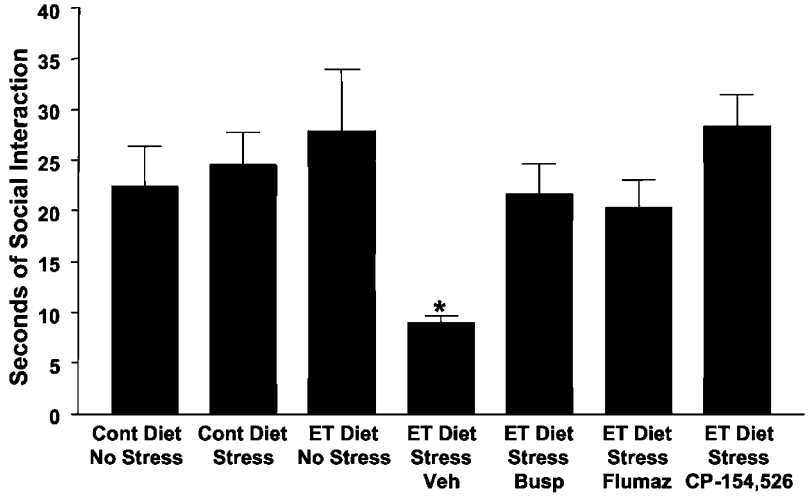

Figure 4 Effect of drug treatments during repeated withdrawals on the reduction in social interaction induced by restraint stress. The 5-HT_{1A} agonist, buspirone, (Busp; 0.6 mg/kg), flumazenil (Flumaz; 5 mg/kg), a benzodiazepine antagonist, or the CRF_1-receptor antagonist, CP154,526 (10 mg/kg), were administered during the first two withdrawal periods, but not the final withdrawal, of the multiple-withdrawal protocol. Vehicle was given to the group that received ethanol diet and stress (ET Diet Stress Veh). All drug treatments significantly prevented the reduction in social interaction induced by stress applied 3 days following the repeated withdrawals. *$p<0.05$ ($F(6,48)=2.77$) compared to Ethanol Diet No Stress, Control Diet Stress, and Control Diet No stress groups and all drug groups stressed (Ethanol Diet Stress—buspirone (Busp), flumazenil (Flumaz), and CP-154,526 groups). Locomotor activity was not reduced following stress in any of the drug treatment groups compared to Ethanol No Stress ($F(6,48)=2.00$; $p>0.05$).

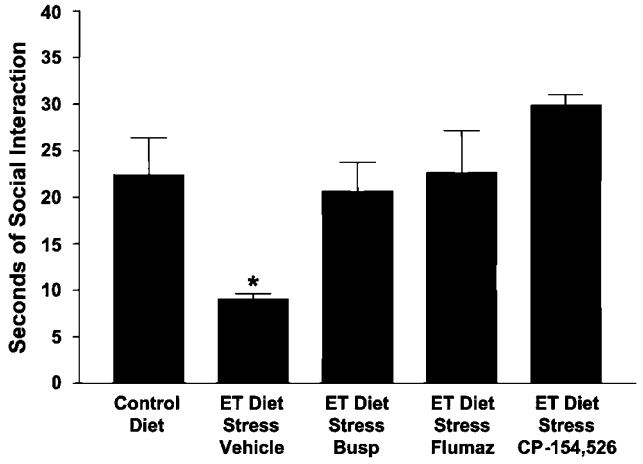

Figure 5 Effect of drug treatments prior to the stress-induced reduced social interaction during abstinence from repeated withdrawals. The buspirone, (Busp; 0.6 mg/kg), flumazenil (Flumaz; 5 mg/kg), or the CP-154,526 (10 mg/Kg) were administered 30 min prior to the application of 45 min of restraint stress applied 3 days after the repeated withdrawals. Vehicle (Veh) was given to the group that received ethanol diet and stress (ET Diet Stress Veh). Drug treatments significantly attenuated the reduction in social interaction induced by stress in the rats exposed to the repeated withdrawals. (*$p<0.01$ ($F(4,32)=5.19$) compared to Control Diet group with no stress and to all drug groups stressed (Ethanol Stress buspirone, flumazenil, and CP154,526 groups).) Locomotor activity was significantly reduced ($p<0.01$; ($F(4,33)=4.93$) following stress in the group pretreated with flumazenil, but not in any other group ($p>0.1$). As noted in Table 1, flumazenil, buspirone, and the CP-154,526 were without effect on social interaction when tested in rats 3 days after repeated withdrawals in the absence of stress.

Table 1 Lack of Action of Acutely Administered CP-154,526, Buspirone, or Flumazenil in the Social Interaction Test 3 Days after Repeated Withdrawals[a]

Treatments	Mean social interaction time (s) ± SEM
Vehicle	25.6 ± 2.8
CP-154,526	29.3 ± 7.6
Buspirone	27.3 ± 5.2
Flumazenil	29.3 ± 3.2

[a]Rats that underwent multiple withdrawals were placed into an open field 3 days after removal from ethanol diet for a 5 min period 30 min after drug or vehicle injection. There were no significant differences among groups ($F(23,25)=0.18$; $p>0.1$). $N=6$–8 rats for each group.

dissociates the locomotor change from the measure of social interaction. In the animals that received the drugs after multiple withdrawals, but were not stressed, locomotion was not affected (see legend to Table 1).

DISCUSSION

The multiple-withdrawal protocol utilized in the present studies was shown previously to result in a progressive adaptive change that sensitizes animals to the induction of anxiety-like behavior (Breese *et al*, 2004; Overstreet *et al*, 2002). However, anxiety-like behavior in animals with this history of chronic ethanol exposure returns to normal within 48 h (Overstreet *et al*, 2002). In accord with this latter observation, rats exposed to the multiple ethanol/withdrawal protocol in the present investigation did not exhibit anxiety-like behavior 3 days after the final withdrawal. However, withdrawal from an additional cycle of 5 days of 7% chronic ethanol diet exposure results in anxiety-associated response for up to 32 days following previous exposure to the multiple-withdrawal protocol (Overstreet *et al*, 2002). In other investigations in which greater amounts of ethanol were administered continuously to rats for a longer period than used for the present multiple-withdrawal protocol, rats exhibited anxiety-like behavior after an extended period (4 weeks) in the absence of further ethanol (Rasmussen *et al*, 2001; Valdez *et al*, 2002). Collectively, it can be concluded from these data that chronic ethanol exposures, which result in persistent adaptive change, enhance susceptibility for anxiety-like behavior.

Repeated restraint stress applied to animals receiving control liquid diet prior to a single withdrawal from 5 days of chronic ethanol diet also induces an anxiety-like behavioral response (Breese *et al*, 2004). This latter observation suggests that the repeated stresses substitute for the initial multiple withdrawals to induce an adaptive change. The baseline for anxiety-like behavior after the multiple stresses with the subsequent withdrawal from the 5 days of chronic ethanol exposure also returned to control levels within a short period (unpublished data). However, as seen with multiple withdrawals, a subsequent withdrawal from short-term ethanol exposure applied at least 16 days after the repeated stress/withdrawal protocol-induced anxiety-like behavior (Breese *et al*, 2004). Thus, these findings emphasize that the stress/withdrawal protocol results in a persistent adaptation comparable to that for repeated withdrawals.

Based upon these previous findings that multiple withdrawn rats re-exposed to an additional 5-day exposure to chronic ethanol at a later time exhibit withdrawal-induced anxiety-like behavior (Overstreet *et al*, 2002) and that stress substitutes for withdrawals to facilitate adaptive change (Breese *et al*, 2004), it was predicted that a subsequent stress exposure at a later period from the initial ethanol cycling would induce anxiety-like behavior. The proposed mechanism for this action of stress would be comparable to the anxiety response seen upon an additional withdrawal from re-exposure to a short exposure to chronic ethanol in multiple withdrawn rats (Overstreet *et al*, 2002). The results of the present investigation support this prediction by demonstrating that exposure to restraint stress induces an anxiety-like response 3 days after removal from the multiple ethanol/withdrawal protocol.

These various observations indicate that the ethanol diet protocol is sufficient to induce a persistent adaptation that supports a subsequent stress induction of anxiety-like behavior during abstinence from ethanol. In this respect, Valdez *et al* (2003) demonstrated stress-induced anxiety-like behavior after 6 weeks of ethanol absence in animals with a long history of chronic ethanol exposure, but not in animals without this history. These observations indicated that stress-induced anxiety-like behavior occurs only if

animals have had previous exposure to a chronic ethanol protocol known to induce persistent adaptive change. Thus, these findings support the view that previous exposure to multiple withdrawals or a long-term exposure to ethanol presumably allows stress to emulate withdrawal during a period of abstinence.

Given earlier data demonstrating anxiety-like behavior for an extended period upon re-exposure to ethanol to rats that previously received cycling of a 7% ethanol diet (see Overstreet *et al*, 2002), it was surprising that an anxiety-associated response to restraint stress was not present beyond 3 days after removal of the repeated withdrawal protocol from the 4.5% ethanol diet. However, we have not previously reintroduced the ethanol diet at a later time in animals that underwent repeated withdrawals from the 4.5% ethanol diet to test for persistence of adaptation. Therefore, future studies should determine if an extension of the number of cycles of withdrawal from a 4.5% ethanol diet would support stress-induced anxiety-like behavior for a longer period. Additionally, it is possible that the adaptation induced by the multiple-withdrawal protocol, which allows a subsequent withdrawal from an additional 5-day cycle of chronic ethanol to elicit anxiety-like behavior, may not apply directly to stress induction of anxiety during abstinence.

Previous work demonstrated that a CRF_1-receptor antagonist, a 5-HT_{1A}-receptor agonist, and the benzodiazepine receptor antagonist administered during repeated withdrawals prevent withdrawal-induced anxiety-like behavior (Knapp *et al*, 2004b, 2005; Overstreet *et al*, 2003b, 2004a, b). This inhibitory drug action is proposed to relate to blockade of the cumulative adaptive change induced by the repeated withdrawals (Overstreet *et al*, 2003a, b, 2004a, b). Therefore, we anticipated that administration of these drugs during the repeated withdrawals would minimize the stress-induced reduction of social interaction during abstinence (see Figure 5). Documentation of an antagonism of the stress-induced anxiety-like behavior by these drugs during abstinence is consistent with the view that the effect of the stress requires an adaptation of specific neural systems.

Earlier results likewise indicate that pretreatment with a CRF_1-receptor antagonist, the 5-HT_{1A}-receptor agonist, or the benzodiazepine receptor antagonist prior to repeated stresses that precede a single 5-day exposure to ethanol diet prevents the reduction in social interaction that accompanies withdrawal from this treatment (Breese *et al*, 2004). Based upon this observation (Breese *et al*, 2004), it could be expected that drug pretreatment prior to the stress during abstinence would block the anxiety-like response, which in fact occurred. The CRF_1-receptor antagonist blockade of the stress-induced anxiety-like behavior during abstinence is comparable to that of Valdez *et al* (2003), who reported that antagonism of CRF receptor function attenuated the enhanced responsiveness to stress observed during protracted abstinence from chronic ethanol exposure. The buspirone and flumazenil inhibition of stress-induced anxiety-like behavior during abstinence from previous chronic alcohol exposure has not previously been reported.

Investigations to define the neuroanatomical basis of the action of flumazenil and buspirone on the anxiety induced by repeated withdrawals has implicated the amygdala in the action of flumazenil (Knapp *et al*, 2004a) and the raphe in the action of buspirone (Overstreet *et al*, 2004a). Since Rassnick *et al* (1993) has implicated the amygdala in the action of CRF antagonism blocking withdrawal-induced anxiety, we might expect this site to be important to the CRF_1-receptor antagonist reduction of anxiety-like behavior following repeated withdrawals. Work is currently underway to define the brain sites important to the consequence of the repeated stress/withdrawal protocol (see Breese *et al*, 2004) to facilitate withdrawal-induced anxiety-like behavior.

Since stress is proposed to have a neural basis similar to that of withdrawal from chronic ethanol to induce anxiety-like behavior (see Breese *et al*, 2004), stress during abstinence is presumed to induce an emotional state similar to that of withdrawal from chronic ethanol (Breese *et al*, 2005a, b). Thus, as is seen with multiple withdrawals (Overstreet *et al*, 2002), the negative affect (ie anxiety) induced by stress during abstinence would presumably be heightened with the progression of ethanol abuse. Consistent with this view, Begleiter and Porjesz (1979) reported that during protracted abstinence in the alcoholic, there is a perception of a persistent 'subacute withdrawal syndrome'. Likewise, Roelofs (1985) described the anxiety, hyperventilation and craving during abstinence as a withdrawal-like syndrome. This perception of a negative emotional state during abstinence resembling withdrawal is proposed to heighten the risk for relapse (Cooney *et al*, 1997; Sinha, 2001, Sinha and O'Malley, 1999; Willinger *et al*, 2002). Consequently, the present findings concerning the response to stress after a history of chronic ethanol exposure may have important implications for understanding the 'subacute withdrawal syndrome' associated with abstinence in the alcoholic (Begleiter and Porjesz, 1979; Roelofs, 1985).

The emotional state observed during withdrawal in the alcoholic can result in craving (Duka *et al*, 2002; Heinz *et al*, 2003; Malcolm *et al*, 2000), because of their knowledge that symptoms would be reduced by ethanol (see Heyne *et al*, 2000; Wolffgramm *et al*, 2000). Since symptoms of withdrawal from ethanol abuse induce a desire to drink in some alcoholics (Duka *et al*, 2002), stress during abstinence is proposed to contribute to the craving alcoholics experience during this period (see Sinha, 2001; Breese *et al*, 2005a, b). Thus, an anxiety-like response to stress during abstinence is presumed to be critical for the enhanced risk of alcoholics for relapse (Brown *et al*, 1990, 1995; Pohorecky, 1991; Sinha, 2001). In this respect, anxiety during sobriety is reportedly a predictor of end-state drinking and return to alcohol abuse (Sloan *et al*, 2003; Willinger *et al*, 2002). The ability of stress to increase alcohol seeking in animals (Lê *et al*, 1998; Liu and Weiss, 2003; see a review by Lê and Shaham, 2002) is consistent with stress-enhancing craving during abstinence and increasing the probability of relapse and furthering alcohol abuse. The present protocol demonstrating a role for the adaptation induced by previous ethanol exposure in the stress-induced anxiety during abstinence provides a means to explore further the contribution of stress to the continuing abuse of alcohol.

The ability of drugs to prevent the adaptation responsible for the stress-induced anxiety-like behavior, and the action of stress to induce this behavioral response, raises the possibility that such drugs may have usefulness in treating the subacute withdrawal syndrome associated with abstinence in the alcoholic. A strategy of treating the conse-

quence of stress during abstinence has the potential of reducing relapse (see Sinha, 2001). Nonetheless, buspirone, a drug that reduced stress-induced anxiety-like behavior in this investigation, had only limited success in treating alcoholism (Fawcett *et al*, 2000). Consequently, any future preclinical pharmacological findings predictive of addressing symptoms induced by stress during abstinence will require clinical confirmation.

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