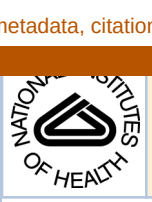

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The Alcohol Deprivation Effect (ADE) in C57BL/6J mice is observed using operant self-administration procedures and is modulated by CRF-1 receptor signaling

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Abstract

Background—The alcohol deprivation effect (ADE) is characterized by transient excessive alcohol consumption upon reinstatement of ethanol following a period of ethanol deprivation. While this phenomenon has been observed in rats using both bottle drinking (consummatory behavior) and operant self-administration (consummatory and appetitive “ethanol-seeking” behavior) procedures, ADE studies in mice have primarily relied on bottle drinking measures. Furthermore, the neurochemical pathways that modulate the ADE are not well understood. Therefore, we determined whether the ADE can be observed in C57BL/6J mice using operant self-administration procedures and if expression of the ADE is modulated by the corticotropin releasing factor-1 (CRF-1) receptor.

Methods—C57BL/6J mice were trained in a 2-hour operant self-administration paradigm to lever press for 10% ethanol or water on separate response keys. Between operant sessions, mice had access to ethanol in their homecage. Once stable responding occurred, mice were deprived of ethanol for 4-days, and were then retested with ethanol in the operant paradigm for 3 consecutive days. Next, to assess the role of the CRF-1 receptor, mice were given intraperitoneal (i.p.) injection (0, 10, or 20 mg/kg) of the CRF-1 receptor antagonist CP-154,526 30-minutes before ADE testing. Additional experiments assessed 1) ADE responding in which the alternate response lever was inactive, 2) the effects of CP-154,526 on self-administration of a 1% sucrose solution following 4-days of deprivation, and 3) ADE responding in which mice did not received i.p. injections throughout the experiment.

Results—Mice exhibited a significant increase in post-deprivation lever responding for ethanol with either a water reinforced or inactive alternate lever. Interestingly, i.p. injection of a 10 mg/kg dose of CP-154,526 protected against the ADE while not affecting lever responding for a sucrose solution. Finally, baseline and deprivation-induced increases of ethanol reinforced lever responding were greater in mice not given i.p. injections.

Conclusions—The ADE in C57BL/6J mice can be modeled using the operant self-administration paradigm and increased ethanol self-administration associated with the ADE is modulated by CRF-1 receptor signaling.

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Keywords

C57BL/6J Mice; Alcohol Deprivation Effect (ADE); Two-Bottle Consumption; Operant Self-Administration; Corticotropin Releasing Factor

INTRODUCTION

Alcohol relapse is a major problem in the treatment of alcoholism. Approximately 60–80% of abstinent alcoholics will relapse at one point in their lifetime (Barrick and Connors, 2002; Chiauzzi, 1991). Thus, understanding the neurobiology of relapse and associated behaviors is a critical step towards the development of drugs aimed at treating alcoholism. Relapse after long periods of abstinence is frequently associated with excessive, or uncontrolled, ethanol drinking (Holter et al., 2000). Recent procedures have been developed and validated as animal models of this uncontrolled ethanol drinking. One procedure involves periodic deprivation from ethanol after which animals consume significantly more ethanol than they had consumed prior to the deprivation period. This phenomenon has been labeled the alcohol deprivation effect (ADE) and is thought to model compulsive uncontrolled relapse drinking characteristic of alcohol dependent humans (Spanagel and Holter, 1999).

The ADE is a robust phenomenon evident in rats (Backstrom et al., 2004b; Bell et al., 2004; Colombo et al., 2003; Dayas et al., 2004; Fullgrabe et al., 2007; Funk et al., 2004; Heyser et al., 1997; Holter et al., 2000; McKinzie et al., 1998; Oster et al., 2006; Rodd-Henricks et al., 2001; Rodd-Henricks et al., 2000a; Rodd-Henricks et al., 2000b; Rodd et al., 2003; Rodd et al., 2006; Serra et al., 2003; Vengeliene et al., 2005; Vengeliene et al., 2006; Wolffgramm and Heyne, 1995), mice (Cowen et al., 2003a; Cowen et al., 2003b; Khisti et al., 2006; Melendez et al., 2006; Sanchis-Segura et al., 2006; Zghoul et al., 2007), monkeys (Kornet et al., 1990; Sinclair, 1971) and humans (Burish et al., 1981; Mello, 1972). The ADE can be seen at ethanol deprivation intervals as short as 12-hours (Sinclair et al., 1989) or as long as 75-days (Sinclair, 1973), and has been shown to increase in magnitude and duration following multiple cycles of ethanol deprivation in alcohol preferring (P) rats and high alcohol drinking (HAD) rats (Breese et al., 2004; McKinzie et al., 1998; Rodd-Henricks et al., 2001; 2002a; b; Rodd et al., 2003). Importantly, ADE ethanol drinking appears to be truly “uncontrolled” as rats will continue to drink increased amounts of ethanol that are adulterated with aversive tastes such as quinine (Spanagel et al., 1996).

Ingestive behavior (i.e., feeding and drinking) is complex and may be divided into at least two components. Appetitive behaviors are those used to locate and acquire stimuli (e.g., food and water) in the environment while consummatory behaviors are those used to directly consume the stimuli once they have been obtained (Samson and Hodge, 1995). Previous experiments evaluating the ADE in mice have primarily measured consummatory behavior, that is, the mice engaged in simple consumption of the ethanol solution from a sipper tube that extended into the cage after a period of imposed ethanol abstinence. Operant procedures allow for the analysis of consummatory behavior as well as appetitive or “seeking” responses (i.e., lever pressing is required to gain access to the ethanol solution). The distinction between appetitive and consummatory behavior has a useful clinical application. Some human alcoholics report a subjective “craving” component toward alcohol (Jellinek, 1955) which may ultimately drive intentional behaviors involved in obtaining access to alcohol (i.e., the appetitive component). Additionally, alcoholism is thought to entail loss of control over ethanol drinking (Marlatt and George, 1984) once consumption has been initiated (i.e., the consummatory component). Furthermore, drugs acting on dopamine or glutamatergic receptors have been found to uniquely influence consummatory or appetitive behaviors associated with ethanol ingestion (Czachowski et al., 2001a; Czachowski et al.,

2001b; Czachowski et al., 2002). Because different neuronal pathways appear to modulate appetitive versus consummatory behaviors during ethanol self-administration, and because ADE studies in mice have relied on bottle drinking procedures, one goal of the present project was to determine if a reliable ADE could be observed in C57BL/6J mice using operant self-administration procedures.

A second goal of the present report was to further characterize the neurochemical substrate involved in modulating the ADE. Previous studies utilizing pharmacological approaches have implicated the dopamine D3 (Vengeliene et al., 2006), glutamate (Backstrom et al., 2004a; Holter and Spanagel, 1999; Rodd et al., 2006; Sanchis-Segura et al., 2006; Spanagel et al., 1996; Vengeliene et al., 2005)] and opioid (Holter et al., 2000) receptors in signaling of ADE drinking. Another interesting target is corticotropin releasing factor (CRF), a 41 amino acid polypeptide with high concentrations in the hypothalamus, the brainstem, and the amygdala (Swanson et al., 1983). Both acute and chronic ethanol exposure activate central CRF pathways (Koob et al., 1993; Rasmussen et al., 2000; Rivier et al., 1984). Increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo Pich et al., 1995) and the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993). Of critical interest, administration of CRF receptor antagonists have been shown to attenuate excessive ethanol drinking in ethanol-dependent rodents without influencing ethanol intake by non-dependent animals (Chu et al., 2007; Finn et al., 2007; Funk and Koob, 2007; Funk et al., 2006; Funk et al., 2007; Gehlert et al., 2007; Overstreet et al., 2007; Valdez et al., 2002). These observations suggest that central CRF receptor signaling modulates increased ethanol drinking in dependent animals and thus make CRF a possible candidate in the modulation of ADE drinking. To address this question, we studied the expression of ADE behavior in C57BL/6J mice following administration of the CRF-1 receptor antagonist, CP-154,526. Data from the present report suggest that the ADE can be modeled in C57BL/6J mice using an operant self-administration paradigm, and that expression of the ADE is modulated by CRF-1 receptor signaling.

METHODS

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used in all experiments. Mice were 6–8 weeks old and weighed between 25–30 g at the start of all experiments and were single housed in polypropylene cages with corncob bedding and ad *libitum* access to food and water. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except were noted. The vivarium rooms were maintained at an ambient temperature of 22° C with a 12-hour/12-hour light-dark cycle. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (Council, 1996).

Drugs

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF-1 receptor (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Peripheral administration of CP-154,526 crosses the blood-brain barrier and reaches peak brain concentrations 20 minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Importantly, previous research found that systemic administration of a 10 mg/kg dose of CP-154,526

effectively reduced anxiety-like behavior in mice (Griebel et al., 1998). During operant training (see below), mice received daily intraperitoneal (i.p.) injections of 0.5% CMC (5 ml/kg) 30-minutes before operant sessions to habituate them to injection procedures (except in Experiment 4). Injection site was alternated between sides (left or right) daily to minimize tissue damage.

Operant Training

Self-administration experiments were conducted in 16 modular mouse operant chambers (Med Associates, Georgia, VT) with dimensions of 21.6 × 17.8 × 12.7 cm and a stainless steel grid floor. All chambers were housed in a sound-attenuating shell with a ventilation fan. Liquid receptacles were located in the center of the right and left chamber walls and a stainless steel response lever was to the right of each receptacle. Liquid solutions (primary lever produced sucrose or ethanol and a second lever produced water or was inactive) were infused using 10 ml plastic syringes which were mounted on a programmable pump (PHM-100, 3.33 rpm). The pump delivered 0.01 ml of solution per activation. A yellow stimulus light and tone (80 dB) were activated when the primary lever (sucrose/ethanol reinforced) was depressed (except in Experiment 3 below). No stimulus light or tone occurred when the second lever (water reinforced or inactive) was pressed. A house light inside the operant chambers was on for the duration of the test. Data recorded during each 2-hour operant session included the number of sucrose/ethanol- and water-reinforced (or inactive) responses (bar presses), the number of sucrose/ethanol and water reinforcers (pump activation), and ethanol intake (g/kg body weight). The operant chambers were interfaced to an IBM computer and all data were automatically recorded using Med Associates software (MED-PC for Windows®, Version IV). All operant sessions were completed in the light phase of the light/dark cycle.

Mice were placed under a modified operant sucrose fading procedure (Samson, 1986; Schroeder et al., 2003). Briefly, mice were initially trained to respond to the levers. Responses to the primary lever resulted in the delivery of a 10% sucrose solution (w/v) and responses to the second lever caused delivery of distilled water (or was inactive). Mice were allowed to respond for 10% sucrose for 4-days in 16-hour sessions in order to strengthen lever pressing behavior. Sessions were then reduced to 2-hours per day for the remainder of the experiment. Following stable responding (i.e., no significant differences in responding over 3 consecutive days), increasing concentrations of ethanol were introduced to the 10% sucrose solution every 2-days (2, 4, 8, and 10% ethanol (v/v)). Then, the sucrose concentration was reduced every 2-days (5, 2, and 0% sucrose) until mice were responding only for 10% ethanol. From the point at which ethanol was introduced into the sucrose solution onward, mice were given access to two bottles in their homecages (one containing water and the other contain an ethanol solution). The ethanol concentration presented in the homecage matched the concentration of ethanol being tested in the operant chambers. Thus, animals had access to ethanol for 24-hours per day during the ethanol training phase to prevent ethanol deprivation. Once mice displayed stable responding for 10% ethanol (approximately 2-weeks of training), ADE sessions were initiated. Lever responding over the last 3-days of training were averaged for each mouse and served as their baseline response rate.

Experiment 1: Effect of Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever

Immediately following baseline sessions, mice (n = 32) were not run in the operant chambers and homecage ethanol was removed for a 4-day ethanol deprivation period. Following the deprivation period, mice were tested in daily 2-hour operant sessions over 3 consecutive days and given access to 10% ethanol in their homecages immediately after the

first post-deprivation operant session. Ethanol deprivation and access were repeated, and mice were then used in Experiment 2.

Experiment 2: Effect of CP-154,526 Administration on Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever

Following 4-days of baseline responding, mice were deprived of ethanol for 4-days (no operant sessions and no homecage ethanol access). Mice were distributed to 3 groups matched for baseline lever responding and given i.p. injection of 0, 10, or 20 mg/kg doses of CP-154,526 30-minutes before the test session which immediately followed the 4-day deprivation period. The effects of CP-154,526 on deprivation-induced ethanol reinforced lever pressing was then assessed over the 2-hour operant test session. After a second 4-day deprivation period, mice previously injected with the 0 and 10 mg/kg doses of CP-154,526 (with the exception of two mice that became sick) were injected with the other dose 30-minutes before a second 2-hour ADE test session. Mice previously injected with the 20 mg/kg dose were not tested a second time due to the apparent aversive effects induced by this high dose of CP-154,526. At the end of the study, the sample size for the 0, 10, and 20 mg/kg groups were n = 25, 27, and 10, respectively.

Experiment 3: Ethanol Deprivation-Induced Lever Responding with Inactive Alternate Response Lever

To further characterize the ADE using operant procedures, male C57BL/6J mice (n = 14) were trained as described above with two exceptions: the second lever was inactive such that responses were not reinforced, and no tone or light were presented when the primary lever was activated. Following baseline responding, mice were deprived of ethanol for 4-days (no operant procedures and no home cage ethanol access). Mice were then tested in 2-hour operant sessions over 3 consecutive days along with homecage access to ethanol.

Experiment 4: Effect of CP-154,526 Administration on Sucrose Deprivation Testing with Water Reinforced Alternate Response Lever

Male C57BL/6J mice (n = 15) were tested to determine if a 4-day deprivation and pretreatment with CP-154,526 would alter lever pressing reinforced with 1% (w/v mixed in tap water) sucrose. The 1% sucrose solution was chosen because it promoted similar levels of lever pressing obtained with 10% ethanol reinforcement during 2-h test sessions. Responding to the primary lever was reinforced with 1% sucrose and the second lever was reinforced with water. During training, mice were also given 1% sucrose in addition to water in their home cages. After a stable baseline for sucrose responding was established, mice were distributed into two groups matched for baseline sucrose responding. Following a 4-day sucrose deprivation period (no operant procedures and no home cage sucrose access), mice were injected with 0 (n = 7) or a 10 mg/kg dose of CP-154,526 (n = 8) 30-minutes before a 2-h operant test session.

Experiment 5: Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever in Mice not given i.p. Injections

To determine if the stress associated with daily i.p. injections in Experiment 1-4 may have altered the overall level of lever pressing, mice were tested in the operant self-administration paradigm in the absence of i.p. injections. Briefly, male C57BL/6J mice (n = 32) were trained to press levers for ethanol or water reinforcement as described above except i.p. injections were never administered. Once stable responding occurred for the 10% ethanol solution, mice were not run in the operant chambers and homecage ethanol was removed for a 4-day break. Mice were then tested in 2-hour operant sessions over 3 consecutive days with access to 10% ethanol in their homecages as described above.

Data Analysis

All data in this report are presented as means ± SEM. We used analyses of variance (ANOVA) to analyze data from each experiment. When significant effects were obtained, we performed planned comparisons with paired or independent t-tests (Winer et al., 1991). Significance was accepted at $p < 0.05$ (two-tailed).

RESULTS

Experiment 1: Effect of Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever

Fig. 1a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (BL; last three sessions before the first ethanol deprivation cycle) and during the three sessions of post-deprivation responding following the first and a repeated deprivation session. A two-way mixed-factor ANOVA run on 10% ethanol lever response data indicated a significant main effect of session [$F(3,186) = 22.42$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 3.80$, $p = 0.01$]. Following the 1st ethanol deprivation cycle, planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first post-deprivation session relative to baseline ethanol lever responding [$t = 3.49$, $p < 0.01$]. Following the repeated ethanol deprivation cycle, the rate of ethanol lever pressing on the first, second, and third post-deprivation sessions were significantly higher when compared to the baseline ethanol lever responding [$t = 6.68$, $p < 0.01$; $t = 4.24$, $p < 0.01$; $t = 3.17$, $p = 0.03$, respectively]. Mean lever responses for water at baseline and during the 2-hour post-deprivation sessions are shown in Fig. 1b. A two-way mixed-factor ANOVA run on water data indicated a significant main effect of session [$F(3,186) = 16.95$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 5.24$, $p < 0.01$]. Following the 1st ethanol deprivation cycle, planned comparisons revealed that water lever pressing on the first session of operant testing was significantly higher than the baseline water response rate [$t = 4.67$, $p < 0.01$]. Following the repeated ethanol deprivation cycle, lever pressing for water on the first, second, and third post-deprivation sessions were significantly higher when compared to the baseline water lever responding [$t = 4.96$, $p < 0.01$; $t = 2.98$, $p = 0.01$; $t = 2.82$, $p = 0.01$, respectively].

Figs. 1c and d present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. A two-way mixed-factor ANOVA run on ethanol consumption data revealed a significant main effect of session [$F(3,186) = 21.11$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 3.38$, $p = 0.02$]. Following the 1st ethanol deprivation cycle, mice consumed significantly more ethanol relative to baseline following the first post-deprivation session [$t = 3.39$, $p < 0.01$]. Following the repeated ethanol deprivation cycle, mice consumed more ethanol relative to baseline during each of the three post-deprivation sessions [$t = 6.34$, $p < 0.01$; $t = 4.07$, $p < 0.01$; $t = 2.90$, $p < 0.01$]. Similarly, a two-way mixed-factor ANOVA run on water consumption data revealed a significant main effect of session [$F(3,186) = 16.81$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 4.81$, $p < 0.01$]. Following the first deprivation cycle, mice showed elevated water consumption relative to baseline during the first post-deprivation session [$t = 4.66$, $p < 0.01$], and following the repeated deprivation cycle water consumption was significantly elevated above baseline levels during each of the three sessions [$t = 4.48$, $p < 0.01$; $t = 2.76$, $p = 0.01$; $t = 2.55$, $p = 0.02$].

Experiment 2: Effect of CP-154,526 Administration on Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever

To determine if CRF-1 receptor signaling modulates deprivation-induced increases of ethanol-reinforced lever pressing, mice were pretreated with the CRF-1 receptor antagonist CP-154,526 30-minutes before testing. Fig. 2a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice during baseline and on the post-deprivation session in which mice were administered CP-154,526 (0, 10, 20 mg/kg) 30-minutes before operant testing. A one-way ANOVA comparing each of the four conditions was significant [$F(3,90) = 6.044$, $p = 0.001$]. Consistent with the ADE, mice showed significantly greater post-deprivation lever responding following administration of the vehicle when compared to their baseline ethanol lever response rate [$t = 2.07$, $p = 0.044$]. Importantly, there was no significant difference between baseline ethanol responding and post-deprivation ethanol responding when mice were administered the 10 mg/kg dose of CP-154,526. However, the 20 mg/kg dose of CP-154,526 significantly reduced 10% ethanol lever responding relative to baseline [$t = 2.458$, $p = 0.018$]. Fig. 2b depicts the mean lever responses for water during baseline and on the post-deprivation session following administration of CP-154,526 (0, 10, 20 mg/kg). A one-way ANOVA run on the data was significant [$F(3,90) = 4.94$, $p = 0.003$]. The vehicle treated group had a significantly greater number of water lever responses when compared to the baseline water lever response rate [$t = 2.18$, $p = 0.034$]. Relative to baseline, there was no significant difference in water responding following treatment with the 10 mg/kg dose of CP-154,526, and the 20 mg/kg dose of CP-154,526 significantly reduced water lever responding relative to baseline [$t = 3.424$, $p = 0.001$].

Fig. 2c depicts the mean consumption of 10% ethanol (g/kg/2-hour session) by C57BL/6J. A one-way ANOVA comparing each of the four conditions was significant [$F(3,90) = 4.903$, $p < 0.003$]. Planned comparisons with two-tailed t-tests revealed that groups treated with vehicle or the 10 mg/kg dose of CP-154,526 did not significantly differ in post-deprivation ethanol consumption relative to baseline intake. Since we predicted a significant increase of ethanol consumption following ethanol deprivation, we performed a directional one-tailed t-test and found that the vehicle treated group showed a significant deprivation-induced increase of ethanol consumption relative to baseline levels [$t = 1.719$, $p = 0.0456$]. The 20 mg/kg dose of CP-154,526 significantly reduced 10% ethanol intake relative to baseline [$t = 2.458$, $p = 0.018$]. Fig. 2d depicts water consumption (ml/kg/2-hour session) by the C57BL6J mice. A one-way ANOVA run on the data was significant [$F(3,90) = 4.903$ $p = 0.003$]. The only significant planned comparison showed that the 20 mg/kg dose of CP-154,526 significantly reduced water consumption relative to baseline [$t = 3.503$, $p = 0.001$].

Experiment 3: Ethanol Deprivation-Induced Lever Responding with Inactive Alternate Response Lever

Unexpectedly, there was a deprivation-induced increase of water-reinforced lever responding in Experiments 1 and 2. To determine if increased responding on the water-reinforced lever may have resulted from a general non-specific increase in activity following the deprivation sessions, Experiment 3 examined the ADE using operant procedures but with the secondary lever inactive (non-reinforced). We reasoned that non-specific increases of activity resulting from a deprivation period should also promote increased responding to a non-reinforced lever. Fig. 3a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following the 4-day deprivation. A repeated measures ANOVA comparing baseline responding and the 3-days of post-deprivation responding was significant [$F(3,39) = 3.671$, $p = 0.020$]. Planned

comparisons revealed that mice performed significantly more responses for 10% ethanol on the first and second (but not third) post-deprivation session relative to baseline ethanol lever responding [$t = 2.434$, $p = 0.030$; $t = -2.902$, $p = 0.012$]. Fig. 3b shows mean ethanol consumption (g/kg/2-h) during this study. A repeated measures ANOVA comparing baseline ethanol intake and the 3-days of post-deprivation ethanol consumption was significant [$F(3,39) = 3.920$, $p = 0.015$], and planned comparisons revealed that mice consumed significantly more 10% ethanol on the first and second (but not third) post-deprivation session relative to baseline ethanol intake [$t = 2.481$, $p = 0.028$; $t = 3.009$, $p = 0.010$]. Fig. 3c shows mean responses to the inactive lever during each 2-hour session. A repeated measures ANOVA performed on these data did not achieve statistical significance [$F(3,39) = 1.145$, $p = 0.343$].

Experiment 4: Effect of CP-154,526 Administration on Sucrose Deprivation Testing with Water Reinforced Alternate Response Lever

To determine if the ability of CP-154,526 to attenuate deprivation-induced lever responding was specific to ethanol reinforcement, we determined if the 10 mg/kg dose of this CRF-1 receptor antagonist would attenuate lever responding reinforced with 1% sucrose solution following a 4-day deprivation period. Fig. 4a shows mean sucrose reinforced lever pressing (over 2-hours) during baseline and on the session immediately after the 4-day sucrose deprivation period, while Fig. 4b shows mean water reinforced responding on the second lever during the same sessions. Two-way mixed factor ANOVAs performed on ethanol- and water-reinforced lever responding data failed to show significant main effects of session (baseline versus post-deprivation) or CP-154,526 dose (0 or 10 mg/kg) or significant interaction effects. Figs. 4c and 4d show mean sucrose and water consumption during the test, respectively. Similar to lever responding data, two-way mixed factor ANOVAs performed on consumption data failed to achieve statistical significance.

Experiment 5: Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever in Mice not given i.p. Injections

While we observed a deprivation-induced increase of ethanol self-administration in Experiment 1, the amount of ethanol consumed after repeated deprivations was only about 1.0 g/kg over the 2-hour test. Since mice were periodically given i.p. injections in the experiments above, the present experiment determined if the level of ethanol-reinforced responding and ethanol intake would be higher in mice that did not experience injections during the study. Fig. 5a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following the 4-day deprivation period. A one-way repeated measures ANOVA performed on 10% ethanol lever responding data was significant [$F(3,93) = 8.786$, $p < 0.001$]. Planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first post-deprivation session relative to baseline ethanol lever responding [$t = 4.449$, $p < 0.001$]. Mean lever responses for water at baseline and during the 2-hour post-deprivation sessions are shown in Fig. 5b. A one-way repeated measures ANOVA performed on water lever responding data was significant [$F(3,93) = 6.460$, $p = 0.001$] Water lever pressing on the first post-deprivation session of operant testing was significantly higher than the baseline water response rate [$t = 3.595$, $p = 0.001$].

Figs. 5c and d present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. A repeated measures ANOVA performed on ethanol consumption data was significant [$F(3,93) = 8.736$, $p < 0.001$], and planned comparisons showed that mice consumed significantly more ethanol relative to baseline following the first post-deprivation session [$t = 4.065$ $p < 0.001$]. Here, mice consumed approximately 2.0 g/kg of ethanol

during the first 2-hour post-deprivation test session. Similarly, a repeated measures one-way ANOVA performed on water consumption data revealed a significant effect [$F(3,93) = 5.975$, $p = 0.001$] and a planned comparison showed elevated water consumption relative to baseline during the first post-deprivation session [$t = 3.344$, $p = 0.002$].

DISCUSSION

The present investigation shows that the ADE can be achieved with C57BL/6J mice using operant self-administration procedures. These observations add to the literature by showing that the ADE is associated with increased appetitive ethanol-seeking behavior (i.e., lever pressing to gain access to ethanol reinforcement) as well as increased consummatory behavior (ethanol consumption) when ethanol is returned after a period of forced abstinence in C57BL/6J mice. These findings are consistent with the rat literature in which the ADE has been observed using both bottle drinking and operant self-administration procedures (e.g., (Overstreet et al., 2007; Rodd-Henricks et al., 2001; Rodd-Henricks et al., 2000a; Rodd et al., 2003; Toalston et al., 2008)). Secondly, we show that pretreatment with a CRF-1 receptor antagonist protects against deprivation-induced increases of ethanol self-administration, an outcome evidenced by the observation the mice pre-treated with the 10 mg/kg dose of CP-154,526 showed levels of ethanol-reinforced lever pressing after 4-days of ethanol deprivation that were similar to baseline levels (Experiment 2). These results suggest that CRF-1 receptor signaling modulates the ADE in C57BL/6J mice.

One surprising observation in the present set of experiments was that the deprivation procedure caused an increase of water-reinforced lever pressing that paralleled ethanol-reinforced responding. This observation may suggest that the deprivation procedure employed here promoted a general increase in activity when mice were returned to the operant chambers, or that the deprivation procedure non-specifically enhanced the reinforcing value of both ethanol and water. Both of these possibilities are unlikely for two reasons. First, when the second operant lever was inactive (non-reinforced) in Experiment 3, mice displayed deprivation-induced increases of ethanol-reinforced lever responding but no significant increase of responding to the inactive key. If the deprivation procedure caused a general increase of activity when mice were returned to the operant chambers, inactive lever pressing would be expected to significantly increase, which did not happen (although there was a modest non-significant elevation on post-deprivation days 1 and 2). Second, the deprivation procedure did not lead to increased sucrose-reinforced lever pressing, a finding indicating that deprivation does not promote a non-specific enhancement of reinforcer value. The sucrose study also provides additional evidence against deprivation-induced increases of general activity. One likely explanation for deprivation-induced increases of water-reinforced responding is that since ethanol is a diuretic agent, the increased motivation to gain access to water in mice with elevated ethanol self-administration may be due to thirst resulting from dehydration. Consistent with this argument, deprivation-induced increase of water-reinforced responding only occurred when ethanol served as the reinforcer for the primary lever (Fig. 1, Fig. 2, and Fig. 5), but not when sucrose was used as the reinforcer on the primary lever (Fig. 4).

In addition to demonstrating that the present ADE procedures do not promote deprivation-induced increases of sucrose-reinforced behavior, the sucrose control study (Experiment 4) also demonstrates that the effects of the 10 mg/kg dose of CP-154,526 were specific to responding for ethanol. Thus, after a 4-day deprivation, mice pre-treated with the 10 mg/kg dose of CP-154,526 showed levels of ethanol-reinforced responding that were similar to pre-deprivation levels, while vehicle treated mice demonstrated the characteristic ADE. On the other hand, the 10 mg/kg dose did not significantly alter sucrose-reinforced responding after a 4- day deprivation period. Furthermore, we have previously shown that the 10 mg/kg dose

of CP-154,526 does not alter open-field locomotor activity in C57BL/6J mice over a 4-hour test (Sparta et al., 2008). These observations provide novel evidence suggesting that CRF-1 receptor signaling selectively modulates deprivation-induced increases of ethanol-seeking behavior rather than affecting ongoing behavior in general. However, since mice had experienced the ADE prior to the test with the CRF-1 receptor antagonist (in Experiment 1), it is unclear if CRF-1 receptor blockade would attenuate the ADE after an initial ethanol deprivation. It should be noted that the 20 mg/kg dose of CP-154,526 likely produced non-specific behavioral side-effects as this dose reduced ethanol-reinforced responding below levels observed in vehicle treated mice and below pre-deprivation baseline levels.

With the present procedures, activation of the primary lever caused a brief activation of light and tone conditioned stimuli (CSs) that occurred concurrently with reinforcer presentation. One potential concern is that these CSs may have acquired conditioned secondary reinforcer value, and thus deprivation-induced increases of lever pressing may have been driven by CS reinforcement rather than increased motivation to gain access to ethanol. There are two observations that argue against this possibility. First, no light/tone CSs were used in Experiment 3, yet deprivation-induced increases of ethanol-reinforced lever pressing were observed. On the other hand, the CSs were used in the sucrose control study (Experiment 4) where there were no observed increases of sucrose-reinforced lever pressing following deprivation. Thus, a role for the CSs in modulating deprivation-induced increases of ethanol-reinforced lever pressing seems unlikely.

In Experiment 1, mice consumed a little more than 1 g/kg/2-hours during the first day of post-deprivation testing after repeated deprivation. To determine if the regular i.p. injections given to mice may have led to an overall reduction of ethanol-seeking behavior and consumption, mice were tested using the ADE procedures but without any i.p. injections in Experiment 5. Consistent with an inhibitory effect of injections on ethanol-reinforced responding, mice in Experiment 5 consumed approximately 2 g/kg/2-hours of ethanol during the first test after the 4-day deprivation period. Based on previous research in which C57BL/6J mice consumed approximately 2 g/kg of ethanol over a 2-hour test (Rhodes et al., 2005), mice in Experiment 5 would have achieved blood ethanol levels of approximately 55 mg/ml following the first day of post-deprivation testing, although caution is necessary with respect to this blood ethanol estimate given the procedural differences between the Rhodes et al. work and the present study. Nonetheless, based on the previous bottle drinking studies (Rhodes et al., 2005), we speculate that longer test sessions (e.g., 4-hours) and testing within the animal's dark cycle would further increase deprivation-induced increases of ethanol-reinforced responding.

While procedures involving bottle drinking allow for the analysis of factors that modulate consummatory behavior, it has been suggested that operant procedures allow for the analysis of appetitive or "ethanol-seeking" behavior (measured by lever pressing) as well as consummatory behavior (Samson and Hodge, 1995). Previous work with mice using bottle drinking procedures demonstrate that deprivation-induced increases of ethanol intake results from increased levels of consummatory behavior (Cowen et al., 2003a; Cowen et al., 2003b; Khisti et al., 2006; Melendez et al., 2006; Sanchis-Segura et al., 2006; Zghoul et al., 2007). Using operant self-administration procedures, here we show that the ADE also involves increased appetitive "ethanol-seeking" behavior. Because different neuronal pathways appear to modulate appetitive versus consummatory behaviors during ethanol self-administration (Czachowski et al., 2001a; Czachowski et al., 2001b; Czachowski et al., 2002; Ford et al., 2007), the combined use of bottle drinking and operant self-administration procedures will allow for a more complete characterization of the neurobiological mechanisms underlying the ADE. Here we show that CRF-1 receptor signaling modulates appetitive components of the ADE. It will be interesting to determine if CRF-1 receptor

antagonists also modulate deprivation-induced increases of ethanol intake using bottle drinking procedures.

The present findings are consistent with previous data that have revealed a role for CRF receptor signaling in neurobiological responses to ethanol. First, increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo Pich et al., 1995) while the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993). Second, antagonism of CRF receptors attenuates increased ethanol drinking in rodents made dependent to ethanol by exposure to ethanol diet or ethanol vapor, but has no effect on moderate levels of ethanol consumption in non-dependent rodents (Chu et al., 2007; Finn et al., 2007; Funk and Koob, 2007; Funk et al., 2006; Funk et al., 2007; Gehlert et al., 2007; Valdez et al., 2002). Recently, we found that pre-treatment with CP-154,526 prevents binge-like ethanol drinking in C57BL/6J mice (Sparta et al., 2008). Third, stress-induced reinstatement of operant ethanol self-administration (an animal model of ethanol relapse) is blocked by administration of a CRF receptor antagonist and increased by central infusion of CRF (Le et al., 2000; Liu and Weiss, 2002; Stewart, 2004). Taken together with the present results, a picture emerges such that CRF receptor signaling appears to be part of a dynamic mechanism that is involved with the development of ethanol dependence stemming from repeated ethanol exposure and withdrawal, a mechanism illustrated by the recently proposed allostasis and “kindling”/stress models of drug dependence (Breese et al., 2005; Koob, 2003; Koob and Le Moal, 2001).

In conclusion, we show here that the ADE in male C57BL/6J mice is observed using operant self-administration procedures. Importantly, we provide novel evidence that expression of the ADE in C57BL/6J mice may be modulated by the CRF-1 receptor. It will be important to determine if the CRF-1 receptor modulates the ADE using bottle drinking procedures, or if CRF-1 receptor signaling selectively modulates appetitive ethanol-seeking behaviors associated with the ADE as revealed in the present study. The present work adds to a growing body of literature implicating the CRF system in modulating neurobiological responses to ethanol, observations that together suggesting a possible therapeutic role for CRF-1 receptor antagonists in the treatment of alcoholism and the prevention of relapse.

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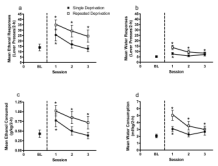

Fig. 1.

Lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test sessions following the first 4-day ethanol deprivation cycle (deprivation 1) and after a repeated ethanol deprivation cycle (repeated deprivation). Consumption of 10% (v/v) ethanol (g/kg) (c) and water (ml/kg) (d) during the 2-hour test sessions following the first and repeated ethanol deprivation cycles. Baseline (BL) refers to the average of the last three sessions before ADE procedures were introduced. All values are means ± SEM. *$p < 0.05$ relative to baseline measures.

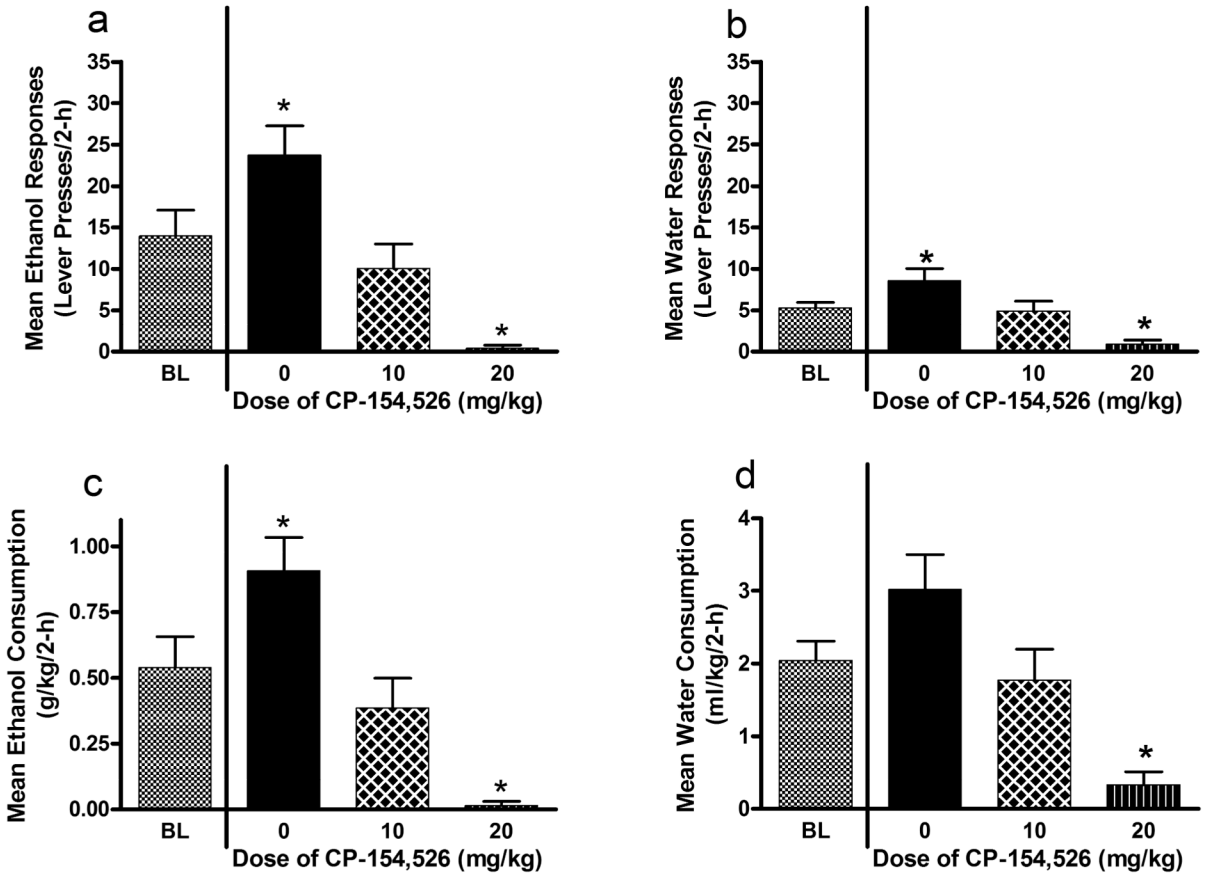

Fig. 2.

Lever responses for 10% (v/v) ethanol (a) and water (b), and consumption of ethanol (c) and water (d) during the 2-hour test immediately following 4-days of ethanol deprivation. Mice were given an intraperitoneal (i.p.) injection of the CRF-1 receptor antagonist CP-154,526 (0, 10, 20 mg/kg) 30-minutes before testing. Baseline (BL) refers to the average of the last three sessions before ADE procedures were introduced. All values are means ± SEM. *$p <$ 0.05 relative to baseline measures.

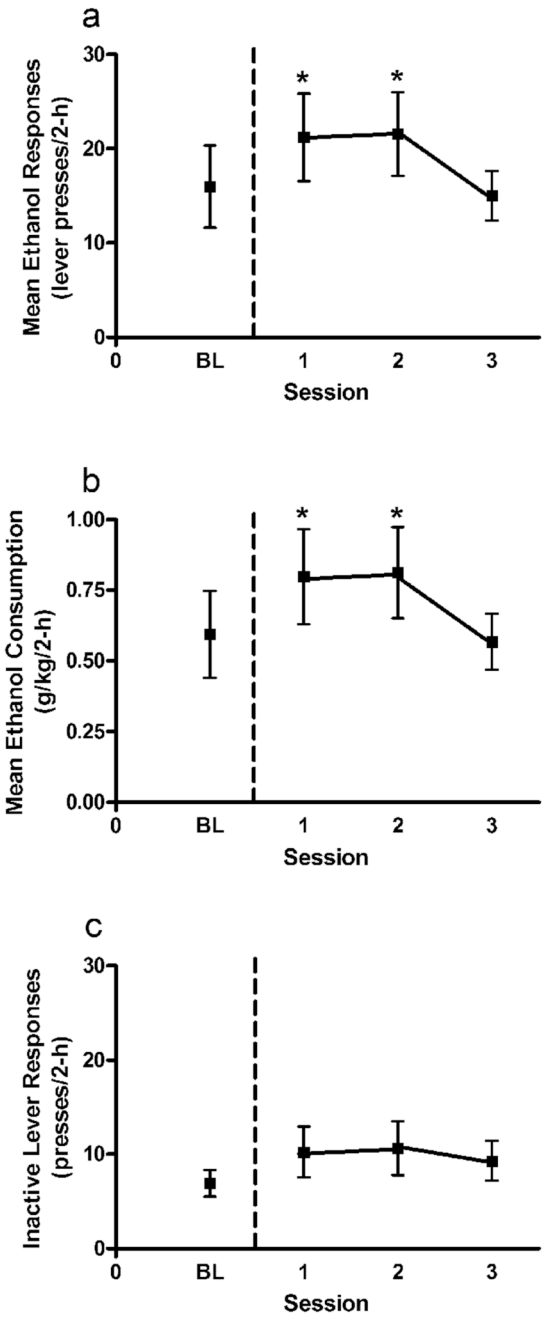

Fig. 3.

Lever responses for 10% (v/v) ethanol (a) ethanol consumption (b), and responses on the inactive lever (c) during the 2-hour test sessions following the 4-day ethanol deprivation cycle. Baseline (BL) refers to the average of the last three sessions before ADE procedures were introduced. All values are means ± SEM. $p < 0.05$ relative to baseline measures.

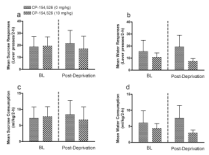

Fig. 4.

Lever responses for 1% (w/v) sucrose (a) and water (b), and consumption of sucrose (c) and water (d) during the 2-hour test session following the 4-day sucrose deprivation cycle. Baseline (BL) refers to the average of the last three sessions before the 4-day deprivation procedure. On the test day (Post-Deprivation) mice were given intraperitoneal (i.p.) injection of the CRF-1 receptor antagonist CP-154,526 (0 or 10 mg/kg) 30-minutes before testing. All values are means ± SEM.

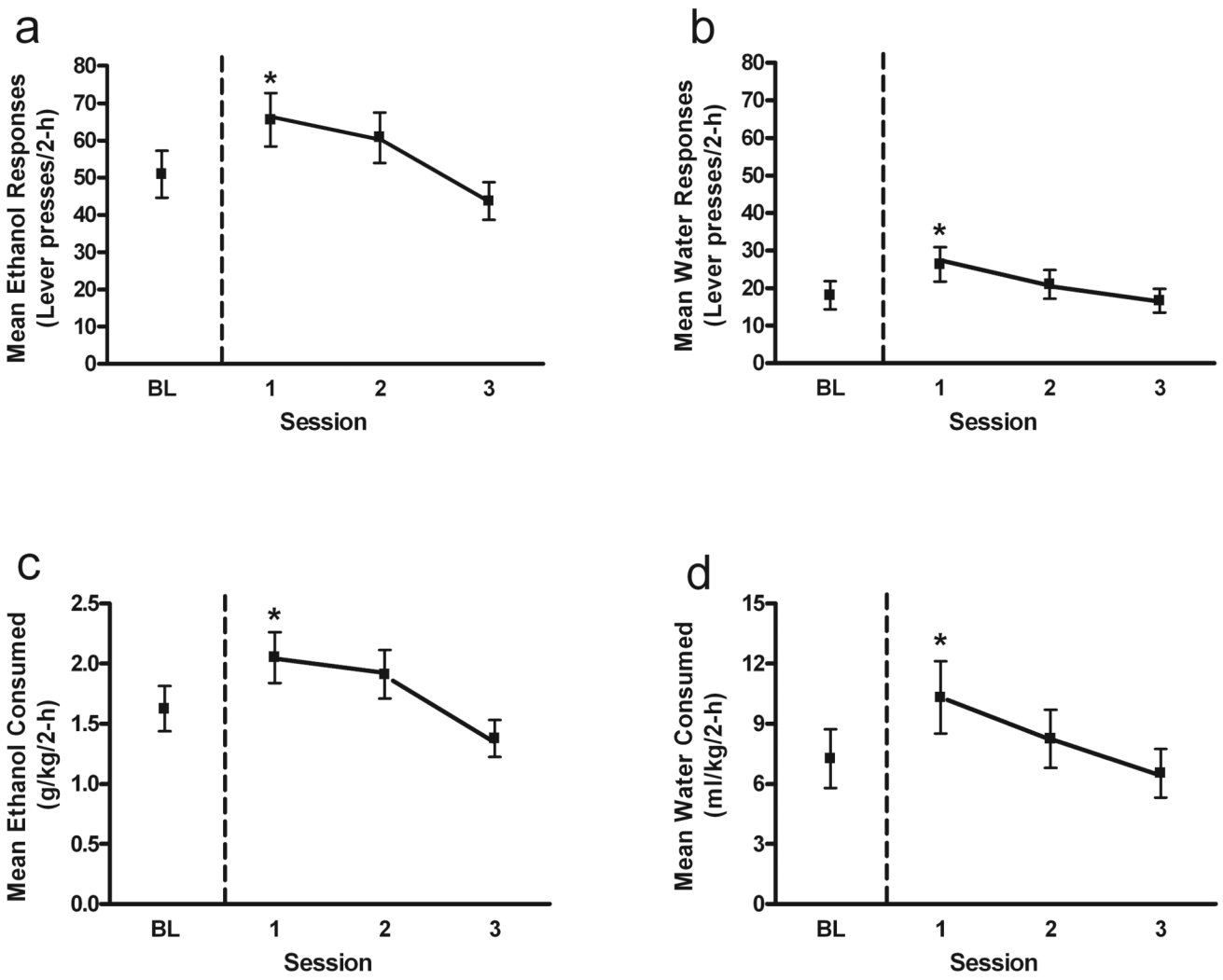

Fig. 5.

Lever responses for 10% (v/v) ethanol (a) and water (b), and consumption of ethanol (c) and water (d) during the 2-hour test sessions following the 4-day ethanol deprivation cycle. Baseline (BL) refers to the average of the last three sessions before ADE procedures were introduced. All values are means ± SEM. $p < 0.05$ relative to baseline measures.