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## Blockade of the Corticotropin Releasing Factor Type Receptor Attenuates Elevated Ethanol Drinking Associated With Drinking in the Dark Procedures

Dennis R. Sparta, Angela M. Sparrow, Emily G. Lowery, Jon R. Fee, Darin J. Knapp, and Todd E. Thiele

From the Department of Psychology (DRS, AMS, EGL, JRF, TET), Bowles Center for Alcohol Studies (DJK, TET), and Department of Psychiatry (DJK), University of North Carolina, Chapel Hill, North Carolina

### Abstract

**Background**—Drinking in the dark (DID) procedures have recently been developed to induce high levels of ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations (BECs) reaching levels that have measurable effects on physiology and/or behavior. The present experiments determined whether the increased ethanol drinking caused by DID procedures can be attenuated by pretreatment with CP-154,526; a corticotropin releasing factor type-1 (CRF<sub>1</sub>) receptor antagonist.

**Methods**—In Experiment 1, male C57BL/6J mice received ethanol (20% v/v) in place of water for 4 hours, beginning with 3 hours into the dark cycle. On the fourth day, mice were given an intraperitoneal injection of one of the 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before receiving their ethanol bottle. In Experiment 2, C57BL/6J mice had 2 hours of access to the 20% ethanol solution, beginning with 3 hours into the dark cycle on days 1 to 3, and 4 hours of access to the ethanol bottle on day 4 of DID procedures. Mice were given an intraperitoneal injection of one of the 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before receiving their ethanol bottle on day 4. Tail blood samples were collected immediately after the 4-hour ethanol access period on the fourth day of each experiment. Additional control experiments assessed the effects of CP-154,526 on 4-hour consumption of a 10% (w/v) sucrose solution and open-field locomotor activity.

**Results**—In Experiment 1, the vehicle-treated group consumed approximately 4.0 g/kg/4 h of ethanol and achieved BECs of approximately 30 mg%. Furthermore, pretreatment with the CRF<sub>1</sub> receptor antagonist did not alter ethanol consumption. On the other hand, procedures used in Experiment 2 resulted in vehicle-treated mice consuming approximately 6.0 g/kg/4 h of ethanol with BECs of about 80 mg%. Additionally, the 10 mg/kg dose of CP-154,526 significantly reduced ethanol consumption and BECs to approximately 3.0 g/kg/4 h and 27 mg%, respectively, relative to vehicle-treated mice. Importantly, the 10 mg/kg dose of the CRF<sub>1</sub>R antagonist did not significantly alter 4-hour sucrose consumption or locomotor activity.

**Conclusions**—These data indicate that CRF<sub>1</sub>R signaling modulates high, but not moderate, levels of ethanol drinking associated with DID procedures.

### Keywords

C57BL/6J Mice; Drinking in the Dark; Corticotropin Releasing Factor; CRF<sub>1</sub> Receptor; CP-154,526

Rodent models of alcoholism, including inbred and selectively bred strains have been useful tools for identifying the genetic and neurobiological factors that underlie this disease. However, in many cases, rodents do not consume enough alcohol to reach the point of behavioral and/or pharmacological intoxication (Spanagel, 2000). Recently, “drinking in the dark” (DID) procedures have been developed to induce excessive ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations (BECs) reaching levels that have measurable effects on physiology and/or behavior (Rhodes et al., 2005, 2007). With these procedures, C57BL/6J mice are given access to a 20% ethanol solution for 2 to 4 hours, starting with 3 hours into their dark cycle. C57BL/6J can achieve BECs of >100 mg% and exhibit signs of behavioral intoxication as measured by motor deficits on the rotarod and balance beam (Rhodes et al., 2005, 2007). It has been argued that the DID model has predictive validity for testing potential pharmacological targets aimed at treating alcohol abuse disorders as naltrexone, an opioid receptor antagonist which is currently used to treat alcoholism, dose dependently attenuates high levels of ethanol drinking induced by DID procedures (Kamdar et al., 2007).

Corticotropin releasing factor (CRF) is a 41 amino acid neuromodulator that is widely expressed throughout the central nervous system (Bloom et al., 1982; Merchenthaler et al., 1982). CRF has been shown to modulate diverse biological functions including food intake, stress and anxiety-like behaviors, and neurobiological responses to ethanol [for reviews, see (Heilig and Koob, 2007; Valdez, 2006; Zorrilla and Koob, 2004; Zorrilla et al., 2003)]. Increases in CRF immunoreactivity (Olive et al., 2002; Zorrilla et al., 2001) and levels of extracellular CRF (Funk et al., 2006) are seen in the amygdala following ethanol withdrawal. Exposure to ethanol causes robust activation of the hypothalamic-pituitary-adrenal (HPA)-axis (Rivier, 1996; Rivier et al., 1990), which is initiated by ethanol-induced increases of CRF activity within the hypothalamus (Li et al., 2005; Rivier and Lee, 1996). Recent pharmacological and genetic evidence support the hypothesis that CRF exerts its effects on ethanol consumption through activation of the CRF<sub>1</sub> receptor (CRF<sub>1</sub>R). Blockade of the CRF<sub>1</sub>R attenuates ethanol intake in dependent, but not nondependent rodents (Funk et al., 2007; Gehlert et al., 2007). Consistently, CRF<sub>1</sub>R deficient mice failed to show increased ethanol consumption following the acquisition of ethanol dependence and a period of abstinence that was observed in wild-type mice (Chu et al., 2007). Interestingly, a genetic polymorphism at the *Crh1* locus, which encodes the CRF<sub>1</sub>R was found to be significantly linked to alcoholism (Treutlein et al., 2006).

Because CRF receptor signaling has been implicated in a wide range of neurobiological responses to ethanol, the goal of the present set of experiments was to determine whether the increased consumption of ethanol associated with DID procedures can be modulated by pretreatment with CP-154,526, a CRF<sub>1</sub>R antagonist. Specifically, because ethanol triggers HPA-axis signaling which is initiated by ethanol-induced increases of CRF activity within the hypothalamus (Li et al., 2005; Rivier and Lee, 1996), and because levels of corticosterone, a HPA-axis-associated hormone, have been shown to positively correlated with ethanol intake (Fahlke et al., 1994, 1995, 1996), we predicted that CRF<sub>1</sub>R blockade would attenuate increased ethanol drinking promoted by DID procedures.

## METHODS

### Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were 6 to 8 weeks old, weighed between 25 to 30 g at the onset of each experiment, and were single-housed in polypropylene cages with corncob bedding. Standard rodent chow (Teklad, Madison, WI) and water were available at all times, except where noted. The vivarium rooms were maintained at an ambient temperature of 22°C with a 12 h/12 h light–dark cycle. Lights came on at 10:30 pm and went off at 10:30 am. All experimental procedures were

approved by the University of North Carolina Animal Care and Use Committee (IACUC) and were in compliance with the NIH Guide for Care and Use of Laboratory Animals.

## 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<sub>1</sub>R ( $K_i < 10$  nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Importantly, peripheral administration of CP-154,526 has been shown to cross the blood-brain barrier and reach 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). Additionally, intraperitoneal (i.p.) injection of CP-154,526 in the dose range examined here appears to produce antidepressant-like and anxiolytic-like effects in rodents (Breese et al., 2004; Chen et al., 1997; Lundkvist et al., 1996; Mansbach et al., 1997), data that suggest functional central actions of this drug when it is administered peripherally. All concentrations of CP-154,526 used in the present experiment were mixed such that the final injection volume was 5 ml/kg. To habituate mice to procedures, all mice handled were given i.p. injections of CMC (5 ml/kg) daily for approximately 7 days before the initiation of the experiments. The site of injection was switched daily in an attempt to limit discomfort and tissue damage.

### Experiment 1: DID After Administration of CP-154,526 With 4-Hour Training Sessions

All mice ( $n = 39$ ) underwent a modified DID protocol (Rhodes et al., 2005). Briefly, all homecage water bottles were replaced with a single bottle of 20% (v/v) ethanol, 3 hours into the start of the dark phase. The 20% ethanol solution remained on the homecage for 4 hours. All mice had ad libitum access to food during this time. After the 4 hour session, the 20% ethanol bottle was replaced with a bottle containing water. On the first 3 days of this procedure, mice were given an i.p. injection of CMC, 30 minutes prior to the presentation of the ethanol bottle. Mice were then distributed into 4 groups, matched for an average ethanol consumption that occurred over the first 3 days of the experiment (that is, the mice were distributed so that the baseline level of ethanol consumption was approximately equal between the groups). On the fourth day, mice were given an i.p. injection of one of 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) mixed in CMC, 30 minutes prior to the application of the ethanol bottle. Immediately following the 4 hour test session, tail blood (6  $\mu$ l) was collected from mice to determine BECs.

### Experiment 2: DID After Administration of CP-154,526 With 2-Hour Training Sessions

Procedures for this experiment were similar to those used in Experiment 1 except that mice ( $n = 40$ ) had access to the ethanol bottle for 2 hours (rather than 4 hours) during days 1 to 3. As above, mice were given an i.p. injection of CMC, 30 minutes before access to ethanol, and mice were distributed to 4 groups matched for average ethanol consumption that occurred over the first 3 days of testing. On the fourth day, mice were injected with one of 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) mixed in CMC, 30 minutes prior to the application of the ethanol bottle. Immediately following the 4 hour test session, tail blood (6  $\mu$ l) was collected from mice to determine BECs. This alternate DID procedure was used because Rhodes et al. (2005) found that shortening the length of ethanol access during the first 3 days of training led to greater ethanol consumption and greater BECs on the fourth day of access.

### Experiment 3: Open-Field Locomotor Activity After Administration of CP-154,526

To determine whether CP-154,526 could impair locomotor activity, naïve male C57BL/6J mice ( $n = 20$ ) were tested in an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena measured  $40.64 \times 40.64$

× 30.48 cm and was made of clear Plexiglass. Several cms of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. C57BL/6J mice were handled and injected with CMC daily for 7 days before activity testing. CMC or CP-154,526 (10 mg/kg) was administered to mice ( $n = 10$  per group) and then 30 minutes later, mice were placed in the center of the locomotor activity chamber. All mice were tested beginning 3 hours into the dark cycle to match DID procedures. Horizontal distance traveled (in centimeters) was recorded as an index of motor function during the 4-hour test session.

#### **Experiment 4: Sucrose DID After Administration of CP-154,526 With 2-Hour Training Sessions**

To determine if a 10 mg/kg dose of CP-154,526 had a general suppressive effect on consummatory behavior, male C57BL/6J mice ( $n = 20$ ) were tested with procedures similar to those used in Experiment 2 except that the solution used for each 2-hour training session and the 4-hour test session was a 10% (w/v) sucrose solution. Mice were habituated to i.p. injections with CMC over 7 days and were also given i.p. injections of CMC on days 1 to 3. Mice were distributed to 2 groups matched for average sucrose consumption that occurred over the first 3 days, and were injected with CMC or a 10 mg/kg dose of CP-154,526 ( $n = 10$  per group), 30-minutes prior to the 4-hour test on day 4.

#### **Blood Ethanol Concentrations After Administration of CP-154,526**

Blood ethanol samples were analyzed with gas chromatographic methods described elsewhere (Knapp et al., 1993; Navarro et al., 2003). Tail blood (6  $\mu$ l) and standards (6  $\mu$ l; 0 to 300 mg/100 ml) were mixed with 375  $\mu$ l of distilled water and 0.5 g of NaCl in 12 × 75 mm borosilicate glass culture tubes. The tubes were capped and then heated at 55°C for 10 minutes in a water bath, at which point 1.5 ml of headspace gas was removed with a plastic 3.0 ml syringe and injected directly into an SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA) equipped with an external syringe adapter and a 1.0 ml external loading loop. The oven temperature was isothermal at 140°C and contained a Hayesep D column and a flame ionization detector. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/min, respectively. Peak retention time was 2 minutes, and the areas under the curve were analyzed with SRI PeakSimple software (SRI Instruments) for Windows running on a Dell (Dell, Round Rock, TX) Inspiron 3500 laptop computer.

#### **Data Analysis**

All data in this report are presented as means  $\pm$  SEM. One-way analyses of variance (ANOVA) were used to analyze data from Experiments 1 and 2. When significant main effects were obtained, Tukey's HSD post hoc tests were performed for group comparisons (Winer et al., 1991). For Experiment 3, a repeated measures ANOVA was used to analyze locomotor activity data over the 4-hour session. For Experiment 4, an independent student's t-test was performed to assess sucrose consumption data. Significance was accepted at  $p < 0.05$  (two-tailed).

## **RESULTS**

#### **Experiment 1: DID After Administration of CP-154,526 With 4-Hour Training Sessions**

The volume of ethanol consumed (g/kg) and BECs achieved following 4 hours of access to ethanol on day 4 of Experiment 1 are presented in Fig. 1A and 1B, respectively. Mice pretreated with the 0, 1, 3, and 10 mg/kg doses of CP-154, 526 drank  $26.13 \pm 3.35$ ,  $27.11 \pm 3.47$ ,  $31.31 \pm 2.85$ , and  $28.21 \pm 2.92$  ml/kg/4 h of ethanol, respectively. One-way ANOVAs performed on these data revealed no significant effects of pre-treatment with CP-154,526 on the amount of ethanol consumed [ $F(3, 35) = 0.504$ ,  $p = 0.682$ ] or BECs [ $F(3, 35) = 0.829$ ,  $p = 0.487$ ].

### Experiment 2: DID After Administration of CP-154,526 With 2-Hour Training Sessions

The volume of ethanol consumed (g/kg) and BECs achieved following 4 hours of access to ethanol on day 4 of Experiment 2 are presented in Fig. 2A and 2B, respectively. Mice pretreated with the 0, 1, 3, and 10 mg/kg doses of CP-154, 526 drank  $37.89 \pm 3.23$ ,  $40.03 \pm 5.14$ ,  $32.79 \pm 4.73$ , and  $17.81 \pm 4.64$  ml/kg/4 h of ethanol, respectively. A one-way ANOVA performed on ethanol consumption data was significant [ $F(3, 36) = 4.961$ ,  $p = 0.006$ ]. Tukey's HSD post hoc tests revealed that the 10 mg/kg dose of CP-154,526 significantly reduced ethanol consumption relative to the control group. Neither 1 nor 3 mg/kg doses of CP-154,526 significantly altered ethanol consumption relative to the CMC-treated group. A one-way ANOVA performed on BEC data was significant [ $F(3, 36) = 4.493$ ,  $p = 0.009$ ], and Tukey's HSD post hoc tests showed that the group treated with the 10 mg/kg dose of CP-154,526 displayed significantly lower BECs relative to the CMC-treated group. Groups pretreated with the 1 or 3 mg/kg doses of CP-154,526 did not display BECs that were significantly different from the CMC treated group.

### Experiment 3: Open-Field Locomotor Activity After Administration of CP-154,526

Data representing 4 hour locomotor activity following i.p. injection of CMC or a 10 mg/kg dose of CP-154,526 are presented in Fig. 3. A  $2 \times 4$  (dose  $\times$  hours) repeated measures ANOVA run on the locomotor activity data revealed a main effect of hour [ $F(3, 54) = 67.614$ ,  $p = 0.001$ ]. However, neither pretreatment with CP-154,526 (dose) nor the interaction between dose and hours were statistically significant.

### Experiment 4: Sucrose DID After Administration of CP-154,526 With 2-Hour Training Sessions

The volume of sucrose consumed (ml/kg) following 4 hours of access to ethanol on day 4 of Experiment 1 are presented in Fig. 4. An independent t-test performed on these data did not achieve statistical significance [ $t(18) = 1.330$ ,  $p = 0.205$ ].

## DISCUSSION

Here, we demonstrate that i.p. injection of a 10 mg/kg dose of CP-154,526, a selective CRF<sub>1</sub>R antagonist, significantly attenuated ethanol consumption and BECs in C57BL/6J mice when DID procedures that promoted high levels of ethanol consumption (approximately 6.0 g/kg/4 h) were employed (Experiment 2). Interestingly, CP-154,526 had no effect on ethanol consumption or BECs, when DID procedures that promoted more moderate levels ethanol consumption (approximately 4.0 g/kg/4 h) were employed (Experiment 1). These observations suggest high, but not moderate levels of ethanol consumption induced by specific DID procedures are modulated by CRF<sub>1</sub>R signaling.

It was possible that the 10 mg/kg dose of CP-154,526 reduced ethanol consumption because of non-specific effects, such as impairment of motor function or general reductions of consummatory behavior. To determine the effects of CP-154,526 on motor function, a control experiment (Experiment 3) was performed to assess the effects of the 10 mg/kg dose of CP-154,526 on locomotor activity over a 4-hour test 3 hours into the dark cycle. The 10 mg/kg dose of CP-154,526 did not significantly alter 4-hour open-field locomotor activity, thus the ability of this dose of CP-154,526 to reduce ethanol drinking in Experiment 2 was probably not related to effects of this drug on motor function. Importantly, pre-treatment with the 10 mg/kg dose of CP-154,526 did not influence high level of consumption of 10% sucrose over 4 hours when access began 3 hours into the dark cycle (Experiment 4), and this dose did not alter moderate ethanol consumption in Experiment 1. Both observations suggest that reduced ethanol drinking induced by pretreatment with CP-154,526 in Experiment 2 is probably not

related to nonspecific effects of this drug on consummatory behavior. Rather, it appears that CP-154,526 specifically modulates ethanol drinking when consumption levels are elevated.

These data present novel evidence suggesting that CRF<sub>1</sub>R signaling is involved with modulating high or excessive binge-like ethanol consumption in C57BL/6J mice that are induced by specific DID procedures. Interestingly, these observations parallel previous data, where antagonism of CRF receptors attenuated increased ethanol drinking in rodents made dependent to ethanol by exposure to ethanol diet or ethanol vapor, but had no effect on moderate levels of ethanol consumption in nondependent rodents (Finn et al., 2007; Sabino et al., 2006; Valdez et al., 2002). While ethanol drinking associated with DID procedures is unlikely to promote ethanol dependence to the degree achieved by exposure to ethanol vapor or ethanol-containing diets, the present findings, in tandem with previous work, suggest that CRF<sub>1</sub>R signaling modulates increased ethanol drinking induced by a variety of rodent models. Ethanol exposure induces rapid activation (within 15 minutes) of HPA-axis signaling (Rivier, 1996; Rivier et al., 1990), an effect which is attenuated by pretreatment with CRF receptor antagonists (Rivier and Lee, 1996). Because CP-154,526 has been shown to attenuate stress-induced activation of HPA-axis activity (Arborelius et al., 2000; Xu et al., 2005), it is tempting to speculate that increased ethanol drinking associated with DID procedures is mediated, in part, by an up-regulation of HPA-axis activity, an effect which may be prevented by pretreatment with the CRF<sub>1</sub>R antagonist. Consistently, treatment with corticosterone (a hormone that is secreted with HPA-axis activation) increases daily ethanol drinking by rodents, while inhibition of endogenous corticosterone synthesis or adrenalectomy suppress ethanol consumption (Fahlke et al., 1994, 1995, 1996). However, it should be noted that corticosterone pretreatment blocked the acquisition ethanol-induced conditioned place preference (CPP) in the TO strain of mice (Brooks et al., 2004), while a corticosterone synthesis inhibitor did not alter the expression or acquisition of CPP in DBA/2J mice (Chester and Cunningham, 1998), suggesting that corticosterone may not modulate ethanol's reinforcing properties. The possible role of HPA-axis activity in the modulation of increased ethanol drinking with DID procedures, or extrahypothalamic CRF signaling if involved, will be the topic of future research.

Consistent with Rhodes et al. (2005), we show here that the level of ethanol consumption is sensitive to the specific DID procedures. Thus, the highest levels of ethanol consumption occurred when mice had 2 hours of access to ethanol during the first 3 days of the procedure and 4 hours of ethanol access on the final test day when BECs were assessed (Experiment 2). With this procedure, mice achieved BECs of approximately 80 mg%. On the other hand, when mice had access to ethanol solution for 4 hours on each of the 4 days of the experiment, mice achieved BECs of approximately 30 mg% (Experiment 1). However, despite higher levels of ethanol consumption with procedures used in Experiment 2, the level of ethanol consumption and the associated BECs were lower than those reported by Rhodes et al. (2005) using identical procedures and the same strain of mice. It is likely that subtle environmental differences between laboratories are the bases of differences in the level of ethanol consumption between the present observations and those previously reported (Rhodes et al., 2005), as environmental factors have been demonstrated to have significant impact on behavioral measures (Crabbe et al., 1999; Wahlsten et al., 2003).

In conclusion, this study demonstrates that i.p. administration of the systemically bioavailable and selective CRF<sub>1</sub>R antagonist, CP-154,526, reduces excessive ethanol consumption caused by specific DID procedures. These results are consistent with research showing that the CRF system modulates a spectrum of neurobiological responses to ethanol. A recent report found that both naltrexone and the dopamine re-uptake inhibitor GBR 12909 can attenuate increased ethanol consumption associated with DID procedures, suggesting a role for opioid and dopamine receptor signaling (Kamdar et al., 2007). The present observations add to this small but growing literature by demonstrating that CRF<sub>1</sub>R signaling selectively modulates high

ethanol drinking without altering moderate levels of ethanol consumption or sucrose drinking. Future research is needed to determine the brain regions in which CRF<sub>1</sub>R signaling modulates increased ethanol drinking associated with DID procedures and if the CRF<sub>2</sub> receptor plays a role.

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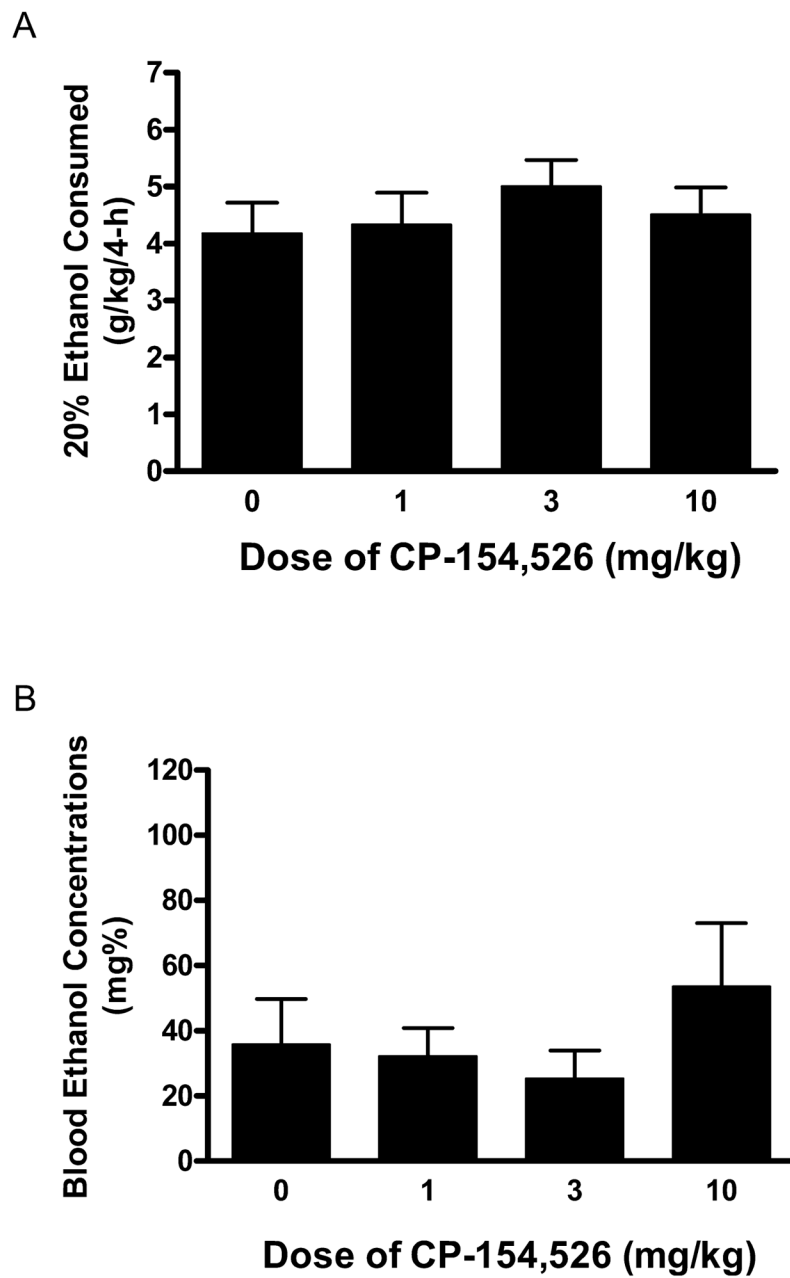
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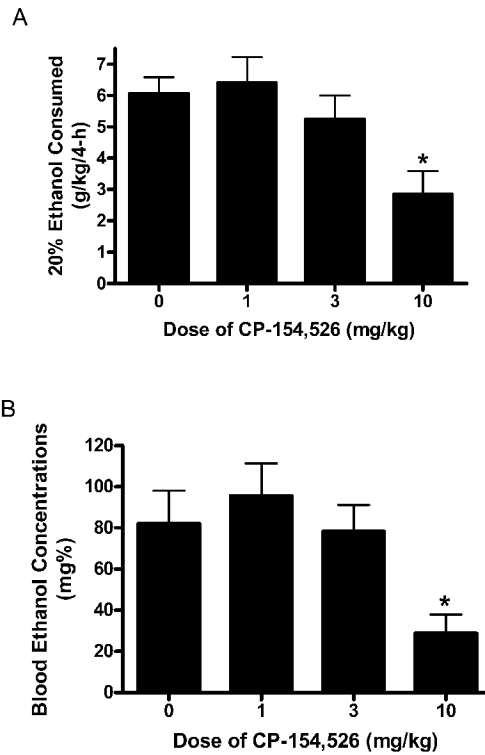
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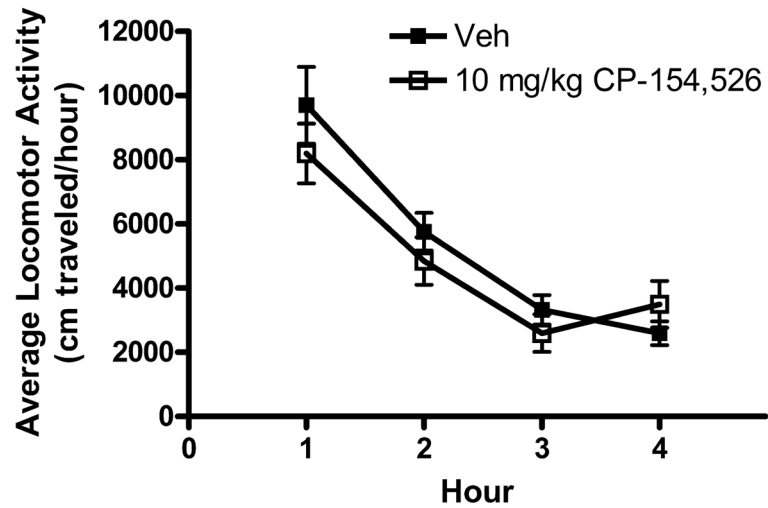
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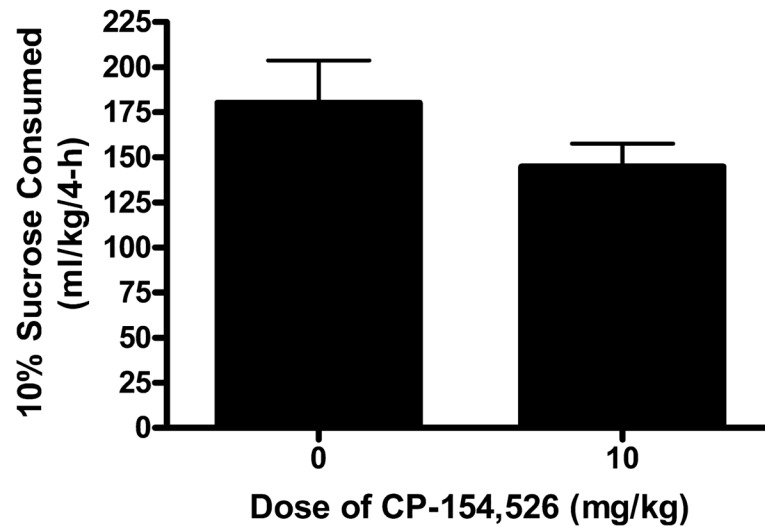
**Fig. 1.** Consumption of 20% (v/v) ethanol (**A**) and blood ethanol concentrations (BECs) (**B**) following the 4-hour ethanol consumption test on day 4 of Experiment 1. Mice were given an intraperitoneal (i.p.) injection of the CRF<sub>1</sub>R antagonist CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before access to ethanol. There were no significant differences between treatment groups. All values are means  $\pm$  SEM.



**Fig. 2.** Consumption of 20% (v/v) ethanol (**A**) and blood ethanol concentrations (BECs) (**B**) following the 4-hour ethanol consumption test on day 4 of Experiment 2. Mice were given an intraperitoneal (i.p.) injection of CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before access to ethanol. Relative to mice treated with carboxymethylcellulose (CMC), pretreatment with the 10 mg/kg dose of CP-154,526 caused a significant reduction of ethanol consumption and BECs. All values are means  $\pm$  SEM. \* $p < 0.05$  relative to the CMC treatment group.



**Fig. 3.** Open-field locomotor activity (cm/h) during the 4-hour test following intraperitoneal (i.p.) injection of CP-154,526 (10 mg/kg) or the vehicle (Veh) 30 minutes before testing. There were no significant differences between the drug pretreatment groups at any time point. All values are means  $\pm$  SEM.



**Fig. 4.** Consumption of a 10% (w/v) sucrose solution following the 4-hour sucrose consumption test on day 4 of Experiment 4. Mice were given intraperitoneal (i.p.) injection of CP-154,526 (0, 10 mg/kg), 30 minutes before access to sucrose. There was no significant difference between the two groups. All values are means  $\pm$  SEM.