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Dependence-induced increases in ethanol self-administration in mice are blocked by the CRF_1 receptor antagonist antalarmin and by CRF_1 receptor knockout

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Abstract

Models of dependence-induced increases in ethanol self-administration will be critical in increasing our understanding of the processes of addiction and relapse, underlying mechanisms, and potential therapeutics. One system that has received considerable attention recently is the CRF_1 system that may mediate the link between anxiety states and relapse drinking. C57BL/6J mice were trained to lever press for ethanol, were made dependent and then were allowed to self-administer ethanol following a period of abstinence. The effect of the CRF_1 antagonist, antalarmin, was examined on this abstinence-induced self-administration in a separate group of mice. Finally, dependence-induced changes in ethanol self-administration were examined in CRF_1 knockout and wild type mice. The results indicated that ethanol self-administration was increased following the induction of dependence, but only after a period of abstinence. This increase in ethanol self-administration was blocked by antalarmin. Furthermore, CRF_1 knockout mice did not display this increased ethanol self-administration following dependence and abstinence. These studies, using both a pharmacological and genetic approach, support a critical role for the CRF_1 system in ethanol self-administration following dependence. In addition, a model is presented that may be useful for studies examining underlying mechanisms of the ethanol addiction process as well as for testing potential therapeutics.

Keywords: Mouse; Dependence; Abstinence; Ethanol self-administration; Corticotropin-releasing factor; CRF_1

1. Introduction

There is a growing necessity to produce models of ethanol intake in dependent animals in order to investigate the changes that occur through the process of addiction that lead to excessive ethanol consumption, loss of control and relapse drinking. Several rat models have been developed in which increased ethanol self-administration is observed following dependence induction (Roberts et al., 1996, 2000a, Rimondini et al., 2003; O'Dell et al., 2004). More recently, several groups have been developing mouse models of dependence-induced drinking. C57BL/6J mice exposed to repeated cycles of ethanol vapor increased ethanol drinking following withdrawal (Becker and Lopez, 2004; Lopez and Becker, 2005; Finn et al., 2007).

One system that appears to play a critical role in the enhanced ethanol self-administration subsequent to dependence involves the stress neuropeptide, corticotropin-releasing factor (CRF). This is not surprising considering that the affective signs of ethanol withdrawal and abstinence such as anxiety, increased responsiveness to stressors, and depressed mood, appear to be critically important in relapse to drinking in alcoholics (Hershon, 1977; Mossberg et al., 1985; De Soto et al., 1989; Parsons et al., 1990; Miller and Harris, 2000) and ethanol withdrawal is associated with disruptions in CRF functioning (Wilkins et al., 1992; Pich et al., 1995; Adinoff et al., 1996; Ehrenreich et al., 1997; Kreek and Koob, 1998; Olive et al., 2002; Valdez et al., 2003). The CRF receptor antagonist, d-Phe-CRF(12–41), attenuated dependence-induced increases in

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ethanol self-administration without affecting ethanol self-administration in non-dependent rats (Valdez et al., 2002).

The CRF_1 receptor appears to mediate anxiety responses and also behavioral consequences of ethanol withdrawal. CRF_1 knockout mice display decreased anxiety-like behavior (Smith et al., 1998; Contarino et al., 1999; Timpl et al., 1998) and may be less sensitive to the anxiogenic-like effects of ethanol withdrawal (Timpl et al., 1998). CRF_1 antagonists have been shown to decrease anxiety-like behavior of rats undergoing repeated ethanol withdrawal (Overstreet et al., 2004, 2005) and specifically decrease ethanol self-administration associated with withdrawal in rats (Funk et al., 2007). There is some suggestion that CRF_1 also mediates the association between anxiety and ethanol consumption. The blood–brain barrier penetrating CRF_1 antagonist, antalarmin, decreased voluntary ethanol consumption in isolation-reared Fawn-Hooded rats (Lodge and Lawrence, 2003). In contrast, non-dependent mice lacking CRF_1 receptors displayed increases in ethanol drinking following repeated exposures to stressors (Sillaber et al., 2002). These findings suggest that the effects of decreased CRF_1 functioning on ethanol consumption may depend on the stress and/or dependence state of the animal.

The purpose of this study was to examine the role of CRF_1 in dependence-induced increases in ethanol self-administration using both a pharmacologic and a genetic approach. First, a model of ethanol intake in dependent mice using an operant self-administration paradigm is presented. This is an important contribution to the existing mouse models as the operant procedure involves both consummatory and appetitive/motivational aspects of ethanol self-administration, whereas the more traditionally employed bottle drinking studies (for example two bottle choice) primarily focus on the consummatory aspects of ethanol. Second, the effect of a CRF_1 antagonist on baseline and dependence-induced increases in operant ethanol self-administration was assessed. Finally, ethanol self-administration was investigated in CRF_1 knockout mice before and after dependence induction.

2. Materials and methods

2.1. General methods

2.1.1. Subjects

Mice were housed 2–4 per cage in a temperature controlled reverse light cycle room (lights off between 8:00 am and 8:00 pm). Testing occurred during the dark phase of the circadian cycle. Mice received ad libitum access to food and water throughout the experiment with the following exception. Mice tested in the operant self-administration paradigm were water restricted prior to the first 3 training sessions in order to motivate the mice to press the levers. Water bottles were removed 16 h prior to testing on these first 3 days and then replaced immediately following testing. All procedures were conducted in accordance with the guidelines established by the USDA and the National Institutes of Health in the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

2.1.2. Operant ethanol self-administration

Operant testing chambers outfitted for lever responding for liquid reinforcement were used in this study. Each of these clear Plexiglas chambers measures 14.9 × 15.2 × 18.3 cm and is housed within a larger exterior box equipped with an exhaust fan serving to ventilate the chamber and to mask background noise. One wall of each operant chamber is equipped with two levers (2.5 cm in width, 5 cm apart and 2.5 cm from the grid floor). Between the levers there are two plastic drinking cups separated by a clear Plexiglas divider (7.5 × 10 cm). A lever press requires 5 ± 1 g of downward force and results in the disruption of a photocell beam. A continuous reinforcement schedule (FR1) was used initially, whereby a single lever press resulted in the delivery of 0.01 ml of fluid into one of the two drinking cups. The FR requirement was increased on an individual mouse basis so that responding matched consumption (i.e. no ethanol fluid was left in the drinking cups at the end of the sessions) up to a maximum of FR4. Fluid delivery and recording of operant responses (photocell beam breaks) were controlled by microcomputer. Mice were trained in daily 30-min sessions, 5 days per week. Test sessions were extended to 60 min following the training phase of the experiment.

A saccharin fading procedure used previously in mice (Roberts et al., 2000b) to establish ethanol as a reinforcer was employed. Both levers were available and responding on one lever resulted in the delivery of saccharin/ethanol and responding on the other resulted in the delivery of nothing or water. The progression of saccharin fading training was as follows: 7 days of saccharin vs. nothing (first 3 days following water restriction), 3 days of 5% ethanol + saccharin vs. nothing, 3 days of 5% ethanol + saccharin vs. water, 3 days of 5% ethanol vs. water, 4 days of 8% ethanol + saccharin vs. water, 4 days of 8% ethanol vs. water, and 6 days of 10% ethanol + saccharin vs. water. For the final 20 days prior to ethanol or control vapor exposure, unsweetened 10% ethanol and water were available. Throughout operant training, the lever associated with saccharin/ethanol and the lever associated with nothing/water were kept constant.

Ethanol dilutions (5, 8, and 10% w/v) were made using 95% ethyl alcohol and water. Sodium saccharin (Sigma Chemical Co., St. Louis, MO, USA) was added to water or the ethanol solutions to achieve a final concentration of 0.2%.

2.1.3. Blood alcohol determination

Approximately 40 μl of blood was obtained by cutting 0.5 mm from the tip of each mouse's tail with a clean razor blade. With repeated sampling, the scabs were nicked in lieu of cutting additional tail. Blood was collected in capillary tubes and emptied into Eppendorf tubes containing evaporated heparin and kept on ice. Samples were centrifuged and serum decanted into fresh Eppendorf tubes. The serum was injected into an oxygen-rate alcohol analyzer (Microstat GM7, Analox Instruments, Inc., Lunenburg, MA) for blood alcohol determination.

2.2. Dependence induction and abstinence testing

Following ethanol self-administration training, mice were separated into two groups based on equal responding across the

final 5 operant sessions. One group of mice was made dependent on ethanol, while the other group was treated identically, but without ethanol exposure. Previous studies have suggested that intermittent exposure to ethanol during dependence induction results in more robust increases in ethanol consumption post-withdrawal (O'Dell et al., 2004; Lopez and Becker, 2005). Both ethanol vapor exposure and ethanol liquid diet exposure can be used to accomplish this. The ethanol vapor can be scheduled very precisely with the use of automatic timers; whereas ethanol liquid diet periodicity is assumed to reflect circadian rhythmicity in consummatory behavior. It was our intention to induce dependence in all mice in these studies using ethanol vapor chambers for greater control; however, because of a deleterious reaction in the CRF_1 knockout and wild type mice, a liquid diet protocol ultimately was employed in these strains, as described below (Experiment 3).

Before beginning the second CRF_1 mouse study we performed a test experiment in which we exposed 7 C57BL/6J mice to ethanol liquid diet to confirm its intermittent nature. When the mice had been exposed for 12 days, blood was sampled 4 h into the dark phase and then 12 h later (4 h into the dark phase). Blood alcohol levels taken in the dark phase averaged 198.4±28.6 mg% and during the light phase were 25.8±11.4. This suggests that the liquid diet procedure does produce a cyclical exposure pattern and is similar to the vapor exposure paradigm in this regard.

2.3. Experiment 1. Operant responding for ethanol during and after dependence induction in C57BL/6J mice

Twenty four 8-week-old male C57BL6J mice were obtained from the The Scripps Research Institute breeding colony. The mice were trained to lever press for ethanol and then half were exposed to ethanol vapor for 3 weeks. Operant self-administration continued throughout this time, 5 days per week. This exposure to self-administered ethanol during daily "mini-withdrawals" was shown in pilot studies to be important for establishing an association between withdrawal and its alleviation by ethanol. Following the vapor exposure phase, mice were removed from the chambers, sampled for blood alcohol levels a final time, and allowed a one week period of abstinence before operant ethanol self-administration sessions were resumed for an additional 13 days.

2.3.1. Ethanol vapor exposure

Mice were exposed to ethanol vapor for 14 h per day (14 h on, 10 h off) for 21 days or were housed in identical cages in the same room, but not exposed to ethanol vapor. Throughout this vapor exposure phase of the experiment all mice were tested in 1 hour self-administration sessions 5 days/week (16 sessions total). The timing of self-administration was 8 h into the 10 hour "vapor off" period (10:00 am, lights off) to minimize the influence of residual blood alcohol levels on responding.

Mice were exposed to ethanol vapor in La Jolla Alcohol Research chambers. Ethanol vapor, created by dripping 95% ethanol into a glass receptacle on a warming plate, was independently introduced into each sealed chamber consisting of a standard mouse cage fitted with a water delivery system. The ethanol drip rate was controlled in order to obtain blood alcohol levels initially around 100 mg% and then progressively increasing to about 200 mg% across the exposure time. Mice were sampled for blood alcohol levels every 2–4 days of vapor exposure (total of 6 samples taken per mouse).

2.4. Experiment 2. Effect of antalarmin on self-administration following dependence induction in C57BL/6J mice

Sixteen 8-week-old male C57BL6J mice were obtained from the The Scripps Research Institute breeding colony. The mice were trained to lever press for ethanol and then half were exposed to ethanol vapor for 3 weeks. Training and testing were identical to those of Experiment 1. On day 10 of abstinence, 4 control and 4 ethanol vapor mice were injected with either vehicle or 30 mg/kg antalarmin. Antalarmin (*N*-butyl-*N*-ethyl-[2,5,6-trimethyl-7-(2,4,6)trimethylphenyl)-7*H*-pyrrolo[2,3-*d*] pyrimidin-4-yl) was synthesized according to the procedure described by Y.L. Chen and A.A. Fossa, Eur. Pat. Appl. 773023 A1 970514 (Pfizer Inc., USA, June 29, 1998) and was generously provided by Joachim Nozulak (Nervous System Research, Novartis Pharma AG, Basel, Switzerland). Antalarmin has a very steep dose–response function and requires doses in this range to reduce ethanol self-administration in rats undergoing ethanol withdrawal (Funk et al., 2007) as well as for its anxiolytic-like effects (Zorrilla and Koob, 2004).

A within subjects design was employed whereby the groups were injected with the alternate solution one week later, following an additional abstinence period, for a final group size of 8 mice per dose. The vehicle used was 0.5% carboxymethylcellulose (pH 4.5) and antalarmin was suspended in this at a concentration of 1 mg/ml. Mice were injected intraperitoneally a volume of 0.03 ml/g body weight. Methylcellulose is a safe (LD50 in mice ~250 g/kg) intraperitoneal vehicle for lipophilic drugs (e.g. Akiyama et al., 1999; Kanematsu et al., 2002; O'Connor et al., 2000). The injections occurred 1 h prior to operant self-administration testing.

2.5. Experiment 3. Ethanol self-administration in CRF_1 knockout mice following dependence induction

In our initial experiment, we sought to use the same vapor induction method as previously used in C57BL/6 mice, but found that the CRF_1 knockout and wild type mice did not tolerate the ethanol vapor procedure. Three mice (2 knockout and 1 wild type) became very ill during the first day of vapor exposure, another three mice (2 knockout and 1 wild type) became ill during day 2 of vapor exposure, and another two (1 knockout and 1 wild type) the next day. At this point, we terminated the study. This was very peculiar as blood alcohol levels of the surviving mice on day 1 averaged about 22 mg%, and then about 42 mg% on day 2 and 49 mg% on day 3 (we intentionally started a bit more slowly than usual as we were concerned that the knockout mice would be less robust in the face of stressor exposure). We exposed the remaining mice to ethanol-containing liquid diet which they tolerated well even

when achieving much higher blood alcohol levels. Therefore, we started training a new cohort of mice to self-administer ethanol and used the liquid diet approach to dependence induction.

Ten male CRF_1 knockout and 10 wild type littermate control mice on a mixed C57BL/6J × 129SvJ background (Smith et al., 1998) bred at The Scripps Research Institute were used in this experiment. The mice were trained to lever press for ethanol and then all mice were made dependent on ethanol. As mentioned above, an ethanol liquid diet procedure was employed in this study to induce dependence because the CRF_1 knockout mice as well as their wild type controls had a negative response to ethanol vapor exposure. Mice were not tested in the self-administration boxes during the liquid diet exposure period because in this paradigm mice can self-administer the ethanol-containing diet at any time and, therefore, can develop an association between withdrawal symptoms and their alleviation by ethanol. Following the 2 week ethanol liquid diet phase, mice were sampled for blood alcohol levels a final time, and allowed a one week period of abstinence before operant ethanol self-administration sessions were resumed for an additional 10 days.

2.5.1. Ethanol liquid diet treatment

CRF_1 knockout and wild type mice were made dependent on ethanol using an adapted liquid diet method (Rassnick et al., 1992). Mice received all of their calories from the liquid diet, but did have access to water bottles in addition to the liquid diet throughout the 2 week exposure period. The ethanol diet was prepared fresh daily (8:00 am) by supplementing chocolate-flavored Boost High Protein, a nutritionally complete liquid food (Mead Johnson, Inc.) with ethanol (95% w/v), a vitamin/mineral mixture (ICN Nutritional Biomedicals) and water. The ethanol concentration was progressively increased during the chronic treatment and was 2% w/v on days 1–3, 3% w/v on days 4–6, 4% w/v on days 7–9, and 5% w/v on days 10–14. Mice were sampled for blood alcohol levels every 2–4 days of liquid diet exposure (total of 5 samples taken per mouse).

2.5.2. Data analysis

Operant ethanol self-administration and preference ratios were analyzed separately across the baseline, vapor exposure (as applicable), and abstinence phases using 2-way repeated measures ANOVA with group (control vs. ethanol dependent) or genotype (wild type vs. knockout) and sessions as the variables. In addition, average responses across the baseline and abstinence sessions for each mouse were used to examine within-subject changes in ethanol self-administration and preference ratios associated with dependence induction. Thus the groups were compared to each other within each phase and groups also were examined across phases. The effect of antalarmin was analyzed using a 2-way repeated measures ANOVA with group and dose (vehicle vs 30 mg/kg antalarmin) as the factors. Blood alcohol levels were compared between wild type and knockout mice using a 2-way repeated measures ANOVA with genotype and sample as the variables. Student's *t*-tests were used for post hoc comparisons. For all analyses, statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Experiment 1

Two ethanol vapor mice displayed signs of over-intoxication (loss of righting reflex, weight loss and blood alcohol levels significantly above the target range) while being exposed to the vapor and were removed from the experiment. See Table 1 for the blood alcohol levels of the remaining 10 vapor-exposed mice.

Responding for ethanol across the last 10 days of training (Baseline), throughout the vapor exposure period (Vapor Exposure), and following 1 week of abstinence (Abstinence) is shown in Fig. 1A. There was no significant effect of group in the Baseline period ($F(1,20)=0.24$, $p>0.05$) or during Vapor Exposure ($F(1,20)=0.11$, $p>0.05$). The former was expected as these values were used in the subsequent group selection. In contrast, ethanol dependent mice responded for significantly more ethanol than control mice across the Abstinence test sessions (main effect of group: $F(1,20)=5.5$, $p<0.05$). In fact, on the final day, ethanol intakes in control mice were 0.6 ± 0.1 g/kg, while ethanol vapor-exposed mice responded for 1.1 ± 0.2 g/kg ethanol. There were significant effects of sessions across the Baseline ($F(9,180)=3.2$, $p<0.01$), Vapor Exposure ($F(15,300)=2.1$, $p<0.01$), and Abstinence ($F(12,240)=2.7$, $p<0.01$) periods due to fluctuations in response rates. However, these fluctuations appeared to be group-independent as there were no significant interactions involving group and session in any case.

Fig. 1B shows preference ratios (ethanol responding/total responding) across Baseline, Vapor Exposure, and Abstinence sessions. There was no difference between control and ethanol dependent mice in Baseline preference ($F(1,20)=0.01$, $p>0.05$). There were significant main effects of group, however, across the Vapor Exposure ($F(1,20)=5.1$, $p<0.05$) and Abstinence ($F(1,20)<12.0$, $p=0.01$) phases with higher preference ratios in vapor exposed mice. There were no effects of session or significant interactions between group and session across the Baseline and Vapor Exposure periods; however, there was a significant effect of session in the Abstinence period ($F(12,240)=2.3$, $p<0.01$), but no significant group by session interaction, suggesting a group-independent fluctuation in preference ratio across sessions.

Averaged Baseline and Abstinence responding and preference were analyzed across the two groups in order to determine whether the mice showed evidence of increased self-administration following dependence induction. For example, control mice responded for an average of 27.8 ± 2.9 ethanol reinforcers during the Baseline phase and 26.1 ± 2.1 following Abstinence; whereas ethanol vapor-treated mice responded for an average of 25.6 ± 3.5 reinforcers during the Baseline phase and 37.0 ± 4.4 following Abstinence. Results of the 2-way repeated measures ANOVA indicated no significant overall effect of group or phase; however, the group by phase interaction was significant ($F(1,20)=5.6$, $p=0.02$). Further analysis indicated that ethanol responding was not different across these phases in control mice ($F(1,11)=0.19$, $p=0.7$), but that Abstinence responding was

Table 1
Blood alcohol levels (BAL) throughout the dependence induction phase of each experiment

	Day 2	Day 5	Day 9	Day 12	Day 14	Day 16	Day 19	Day 21
Experiment 1	100.3±4.8	111.4±3.8	132.3±6.4	151.0±5.4	NA	168.2±5.3	202.3±7.2	207.9±7.4
Experiment 2	115.3±11.5	127.5±10.0	143.5±9.2	161.7±9.9	NA	177.4±11.1	190.5±11.2	219.6±10.9
Experiment 3								
°Wild type	87.9±28.7	128.2±22.7	146.4±10.3	171.3±7.4	189.1±13.1	NA	NA	NA
°CRF_1 knockout	98.5±26.7	144.5±35.8	120.4±14.7	157.7±17.1	180.4±15.1	NA	NA	NA

significantly higher in ethanol dependent mice relative to Baseline ($F(1,9)=7.9$, $p=0.02$). In terms of preference, there was an overall effect of group ($F(1,20)=5.8$, $p=0.03$), with ethanol dependent mice having higher preference ratios, and a significant group by phase interaction ($F(1,20)=9.6$, $p<0.01$). Again, ethanol dependent mice showed a change in preference ratios across these time periods, with increases evident in the Abstinence phase relative to the Baseline phase ($F(1,9)=7.2$, $p=0.03$). This effect was not observed in the control group ($F(1,11)=3.9$, $p=0.07$).

Collectively, these results suggest that ethanol self-administration and its relative motivational capacity are increased in mice made dependent upon ethanol and allowed a period of abstinence.

3.2. Experiment 2

The blood alcohol levels of the 8 vapor-exposed mice are shown in Table 1. The effect of the CRF_1 receptor antagonist, antalarmin, on ethanol self-administration following abstinence is shown in Fig. 2. While there were no significant main effects of ethanol dependence ($F(1,14)=0.6$, $p=0.4$) or antalarmin administration ($F(1,14)=4.1$, $p=0.06$), there was a significant group × dose interaction ($F(1,14)=5.5$, $p=0.03$). Further analysis revealed a moderately significant difference between vehicle treated control and ethanol dependent mice ($p=0.05$), supporting an increasing effect of dependence induction on ethanol self-administration. There was no effect of antalarmin administration in control mice ($p=0.8$); however, antalarmin

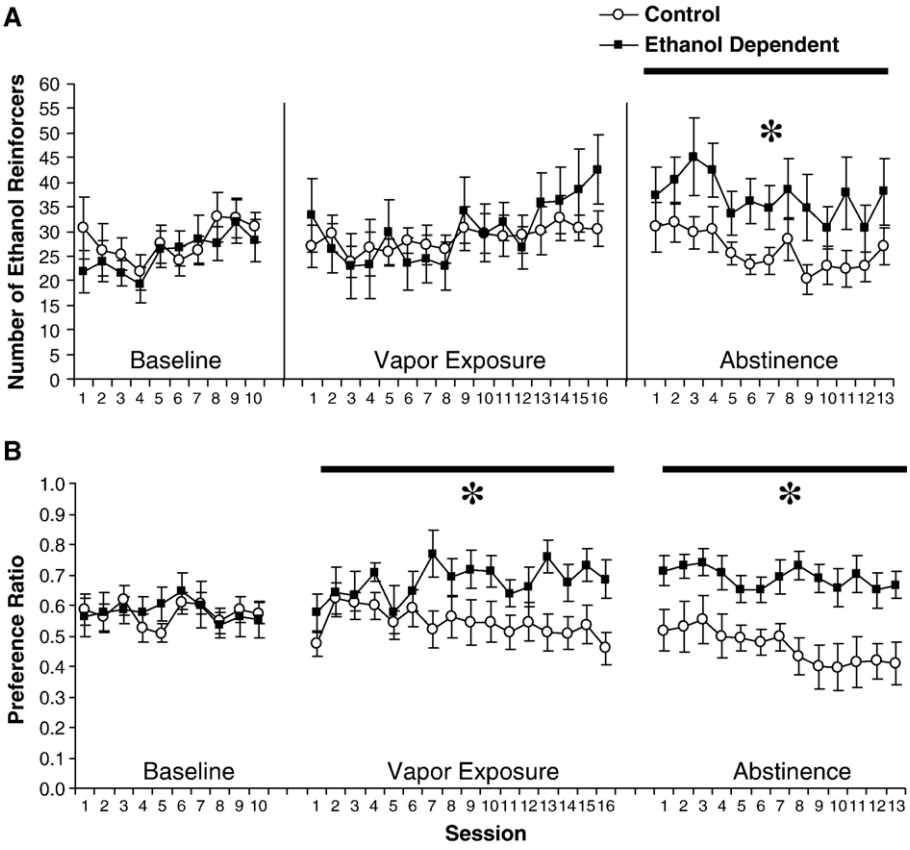

Fig. 1. Self-administration of unadulterated 10% ethanol by male C57BL/6J mice across the final ten days of training (Baseline), during ethanol vapor (or air) exposure (Vapor Exposure), and following a 1 week period of no ethanol exposure (Abstinence). A. Number of ethanol reinforcers received during 1 hour sessions. B. Preference ratios calculated as the number of ethanol reinforcers divided by the total number of reinforcers (ethanol + water) received in each session. Data presented are means ± SEM. * indicates significant main effect of group (Control vs. Ethanol Dependent).

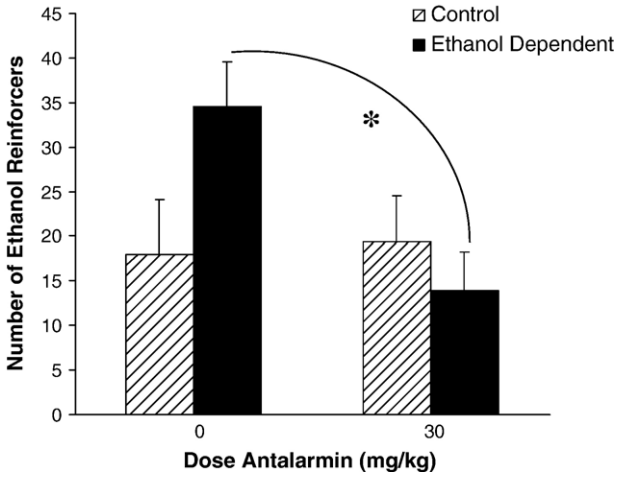

Fig. 2. Effects of the CRF_1 antagonist, antalarmin, on self-administration of unadulterated 10% ethanol by dependent and control male C57BL/6J mice following a period of abstinence. Data presented are means±SEM. * indicates significant difference between Control and Ethanol Dependent.

significantly decreased responding in ethanol vapor treated mice ($p=0.02$). These results indicate that antalarmin decreased ethanol self-administration in abstinent mice, but not in non-dependent control mice.

3.3. Experiment 3

There was no difference between CRF_1 knockout and wild type mice in blood alcohol levels across the liquid diet exposure (Table 1; $F(1,18)=0.05$, $p>0.05$). Responding for ethanol across the last 10 days of training (Baseline) and following 1 week of abstinence (Abstinence) is shown in Fig. 3A. There was no difference between wild type and CRF_1 knockout mice in the Baseline phase (main effector of group: $F(1,18)=0.4$, $p>0.05$), nor were there significant main effects of session ($F(9,162)=1.8$, $p>0.05$), or genotype by session interaction ($F(9,162)=1.7$, $p>0.05$) in this phase. However there was a significant group difference in the Abstinence phase (main effect: $F(1,18)=4.0$, $p=0.05$) with wild type mice responding for significantly more ethanol than control mice. In addition, during the Abstinence phase there was a significant effect of session ($F(9,162)=3.9$, $p<0.01$), but no genotype by session interaction ($F(9,162)=0.8$, $p>0.05$), suggesting a genotype-independent fluctuation across time, but with wild type mice responding more across the entire phase.

Preference ratios are shown in Fig. 3B. Again, there was no significant genotypic difference in the Baseline phase ($F(1,18)$ ce:hsp sp="0.12"/>=0.12, $p>0.05$). Across the Abstinence phase there was no overall main effect of genotype ($F(1,18)=2.4$, $p>0.05$), but there was a significant genotype by session

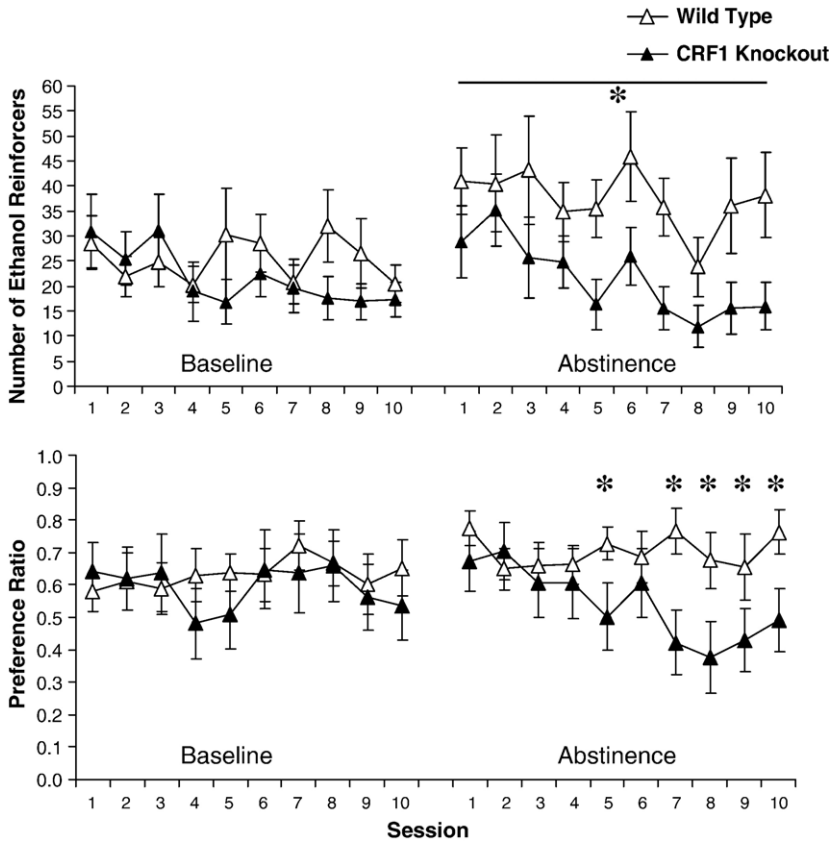

Fig. 3. Self-administration of unadulterated 10% ethanol by male CRF_1 knockout and wild type mice across the final ten days of training (Baseline) and following dependence-induction using an ethanol-containing liquid diet and a 1 week period of no ethanol exposure (Abstinence). A. Number of ethanol reinforcers received during 1 hour sessions. B. Preference ratios calculated as the number of ethanol reinforcers divided by the total number of reinforcers (ethanol+water) received in each session. Data presented are means±SEM. * indicates significant difference between Control and Ethanol Dependent.

interaction ($F(9,162)=3.2$, $p<0.01$). Further analysis revealed that wild type mice showed a greater preference for ethanol in the latter sessions of this phase (5, 7, 8, 9, and 10). There were no other significant effects except an effect of session in the Abstinence phase ($F(9,162)=2.6$, $p<0.01$).

Averaged Baseline and Abstinence responding and preference were analyzed across the two mouse strains in order to determine whether the mice showed evidence of increased self-administration following dependence induction. For example, wild type mice responded for an average of 25.4±4.2 ethanol reinforcers during the Baseline phase and 37.4±6.5 following Abstinence; whereas CRF_1 knockout mice responded for an average of 21.7±4.1 reinforcers during the Baseline phase and 21.6±4.5 following Abstinence. Results of the 2-way repeated measures ANOVA indicated no significant overall effect of genotype; however, the phase ($F(1,18)=5.9$, $p=0.03$) and group by phase interaction ($F(1,18)=6.0$, $p=0.02$) effects were significant. Further analysis indicated that ethanol responding was not different across these phases in CRF_1 knockout mice ($F(1,9)=4.0E-4$, $p=0.98$), but that Abstinence responding was significantly higher in wild type mice relative to Baseline ($F(1,9)=11.7$, $p<0.01$). In terms of preference, there were no significant effects involving group or phase.

These results indicate that knockout of the CRF_1 does not result in differences in operant ethanol self-administration in the non-dependent state, but does attenuate the increases in self-administration following dependence. There was a weaker effect on preference that appeared to only be revealed later in the abstinence period.

4. Discussion

Results from this study suggest that mice made dependent on ethanol will increase ethanol self-administration following a period of abstinence. This is important as it appears to model the increased motivation for ethanol observed in abstinent alcoholics and therefore may be used for studies examining underlying mechanisms of the addictive process as well as for testing potential therapeutics. In fact, we have used this model in the present set of experiments to reveal an important contribution of the CRF_1 system in dependence-induced increases in ethanol self-administration. The CRF_1 antagonist, antalarmin, reversed the enhancement in ethanol self-administration in dependent C57BL/6J mice, while having no effect on self-administration in non-dependent mice. In addition, CRF_1 null mutant mice, while not differing from their littermate controls in baseline ethanol self-administration, did not display the enhanced levels of responding or preference for ethanol observed in wild type mice. These two very different studies support the hypothesis that CRF_1 plays a critical role in relapse-type ethanol drinking.

This operant model of dependence-induced ethanol self-administration in mice adds to the growing literature in this area. Until now, only bottle drinking models have been reported in mice (Becker and Lopez, 2004; Lopez and Becker, 2005; Finn et al., 2007). This operant model has the potential to tap into both the appetitive and consummatory aspects of ethanol reinforcement as it involves both an operant response (appetitive) and a drinking response (consummatory). However, in contrast to our observations in rats (Roberts et al., 1996, 2000a), increased ethanol self-administration has not been detected during acute withdrawal, throughout or immediately following the forced ethanol exposure period in mice. Indeed, Becker and Lopez reported increased ethanol drinking behavior approximately one week following removal from ethanol vapor in mice (Becker and Lopez, 2004; Lopez and Becker, 2005). While overall ethanol responding did not increase during the vapor exposure phase of Experiment 1, preference ratios diverged. This suggests that a shift occurred in the response pattern, whereby mice were perhaps more focused on ethanol, even if they did not take in more.

CRF_1 knockout mice, as well as wild type littermate control mice, did not tolerate the ethanol vapor procedure; therefore, these two experiments utilized different methods of dependence induction. Two factors appear to be important in both paradigms: intermittent ethanol exposure and the development of an association between withdrawal and its alleviation by self-administered ethanol. It has been shown that intermittent exposure to ethanol during the dependence induction phase leads to greater subsequent increases in ethanol self-administration in both mice (Lopez and Becker, 2005) and rats (O'Dell et al., 2004). Both the vapor and liquid diet approaches resulted in intermittent patterns of ethanol exposure. In addition, it has been suggested that the association between ethanol self-administration and the alleviation of withdrawal symptoms is important in the enhancement of self-administration subsequent to dependence (Roberts et al., 1996, 2000a). In the vapor experiments, operant testing continued throughout the vapor exposure period. In the case of the ethanol-containing liquid diet procedure, the bottles remained on the cages at all times so that the mice were able to self-administer ethanol at any time and also learn this association. The robustness of the increased ethanol self-administration across these two different dependence induction paradigms reveals the strength of this model of increased ethanol reinforcement following dependence. In the meantime we are exploring the possibility that there is something about the genetic background of these mice (C57BL/6J × 129/J) that has rendered them sensitive to an aspect of the vapor chamber experience unrelated to blood alcohol levels. Indeed, we are currently backcrossing the CRF_1 mutation onto a pure C57BL/6J background to explore this potential genetic background issue.

The pharmacologic and genetic components of this study support a critical role for the CRF_1 in ethanol drinking associated with dependence. Recently, it was shown that CRF_1 specific antagonists reduced ethanol self-administration in rats during withdrawal (Funk et al., 2007) and mice following abstinence (Finn et al., 2007). The present results extend this finding to an additional mouse model and add further support for this important association. CRF_1 receptors are expressed throughout the brain, but perhaps most importantly for ethanol dependence, in regions of the extended amygdala (Chen et al., 2000; Van Pett et al., 2000). CRF release is increased in the extended amygdala during ethanol withdrawal (Pich et al., 1995; Olive et al., 2002). The amygdala has been shown to mediate the anxiogenic-like effects

of chronic ethanol exposure (Pandey et al., 2004; Rassnick et al., 1993; Roberto et al., 2004). Using whole-cell recordings, it was shown that both CRF and ethanol enhanced GABAergic neurotransmission in amygdalar neurons from wild type and CRF_2 receptor knockout mice, but not CRF_1 receptor knockout mice (Nie et al., 2004). This was supported by pharmacological studies, and, taken together, indicated a role for CRF_1 receptors in ethanol/GABA interactions in this brain region. Therefore, it is hypothesized that CRF_1 receptors are an important contributor to dependence-induced enhancements in ethanol self-administration and this may be coupled with the anxiolytic-like effects of ethanol withdrawal and involve interactions with the GABAergic systems in the extended amygdala.

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