

Reduction of ethanol intake by corticotropin-releasing factor receptor-1 antagonist in “heavy-drinking” mice in a free-choice paradigm

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Abstract

Rationale We hypothesized that the corticotropin-releasing factor (CRF) system is hyperresponsive in animals with high ethanol intake, which exhibits a reduction of ethanol intake when administered with a CRF₁ receptor antagonist.

Methods Outbred Swiss mice were subjected to a long-term, three-bottle, free-choice paradigm (5 and 10 % [v/v] ethanol and water) that consisted of four phases: acquisition (AC; 10 weeks), withdrawal (W; 2 weeks), reexposure (RE; 2 weeks), and quinine-adulteration (AD; 2 weeks). Based on individual ethanol intake, the mice were classified into three groups: A group, preference for ethanol and persistently high consumption during AD phase; B group, preference for ethanol and a reduction of ethanol intake in the AD phase; and C group; preference for water during all phases. A control group only had access to water. CRF₁ receptor messenger RNA (mRNA) levels in the amygdala and the effect of the CRF₁ receptor antagonist CP-154,526 on ethanol and water intake in the subgroups were studied.

Results CRF₁ transcript levels were higher in the B group than in the control group. The highest dose of CP-154,526 reduced ethanol intake and preference, with no changes in water consumption, in the A group compared with vehicle. The B group exhibited a reduction of both ethanol and water intake, with no changes in preference. The C group exhibited no changes in response to the CRF₁ antagonist.

Conclusions CRF₁ receptors appear to be involved in ethanol consumption in mice with high ethanol consumption, and CRF system-mediated neuroadaptations depend on drinking profiles.

Keywords Addiction · Ethanol · CRF · CP-154,526 · Free-choice paradigm

Introduction

Alcohol addiction (i.e., alcoholism) has been hypothesized as a cycle composed of three stages interacting with each other: *binge/intoxication*, *withdrawal/negative affect*, and *preoccupation/anticipation*. Impulsivity, driven by positive reinforcement, dominates the early stages of alcoholism and compulsivity, driven by negative reinforcement, dominates during later stages. Compulsive alcohol use during later stages is mediated by a loss of reward system function and recruitment of brain stress systems (Koob and Le Moal 1997; 2001; Koob and Volkow 2010). These latter systems include corticotropin-releasing factor (CRF), its two receptor subtypes CRF-R1 and CRF-R2, and norepinephrine in the extended amygdala (Koob 2008).

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In animal models, CRF release is increased in the central nucleus of the amygdala (CeA) by acute ethanol administration (Lam and Gianoulakis 2011) and during ethanol withdrawal in the CeA and bed nucleus of stria terminalis in rats exposed to ethanol vapor (Merlo-Pich et al. 1995; Olive et al. 2002). Sommer et al. (2008) showed that rats exposed to ethanol vapors for 7 weeks and then withdrawn for 3 weeks had increased CRF messenger RNA (mRNA) in the CeA and increased CRF₁ mRNA in the basolateral and medial nuclei of the amygdala. Roberto et al. (2010) reported that dependent rats exhibited heightened sensitivity to the effects of CRF and CRF₁ antagonists on GABA release and increased CRF and CRF₁ expression in the CeA.

Using the drinking-in-the-dark (DID) model (Rhodes et al. 2005), a reduction in binge drinking was observed in mice when the following treatments were given: CRF₁ receptor antagonist CP-154,526 (Sparta et al. 2008); non-selective CRF antagonist α -helical CRF_{9,41} and CRF₂ receptor agonist urocortin 3 (Lowery et al. 2010). Nonselective CRF antagonists like D-Phe-CRF₁₂₋₄₁ (Valdez et al. 2002), CRF₁ receptor antagonists, such as MTIP (Gehlert et al. 2007), MPZP (Gilpin et al. 2008), antalarmin, MJL-1-109-2, and R121919 (Funk et al. 2007) all reduced ethanol self-administration in animals that had been previously exposed to ethanol vapor. Moreover, urocortin 1, which has equal activity at CRF₁ and CRF₂ receptors, was injected directly into the lateral septum and attenuated alcohol self-administration during both the acquisition and expression phases of a limited-access alcohol drinking paradigm (Ryabinin et al. 2008). Injections of D-Phe-CRF₁₂₋₄₁ in the CeA were able to replicate the behavioral results observed in studies using i.p. injections, while injections in the nucleus accumbens shell or in the bed nucleus of the stria terminalis were not (Funk et al. 2006a). Lowery-Gionta et al. (2012) also reported the role of the CeA but not basolateral nucleus of the amygdala in the CRF₁ antagonist-induced reduction of binge-like ethanol drinking. Other authors reported a role for CRF₁ receptors in other brain areas, such as the ventral tegmental area, dorsal raphe nucleus, and hypothalamus (Hwa et al. 2013; Sparta et al. 2013; Simms et al. 2014).

In humans, genetic studies of polymorphisms of the *crhr1* gene (Blomeyer et al. 2008) and CRF-binding protein gene (Tartter and Ray 2012) found associations between genotypes and binge drinking, lifetime prevalence of alcohol intake, and lifetime prevalence of drunkenness.

Although the vast majority of studies have shown that CRF antagonists reduce ethanol consumption, some studies found no such effect (Ji et al. 2008; Sabino et al. 2013). The wide variation of the effects of CRF in brain circuitry underscores the need to study this system using several drinking models that focus on different components of the human disorder. The present used model yielded three

drinking profiles in outbred mice being based on a three-bottle (water and two different ethanol concentrations, 5 and 10 % v/v), free-choice paradigm. This model has been shown to exhibit face validity (i.e., long-term high ethanol intake, heightened anxiety during ethanol withdrawal, and persistent intake despite quinine-adulterated ethanol solutions), predictive validity when tested with naltrexone (Fachin-Scheit et al. 2006), and reliability (the model has been replicated in several studies; Ribeiro et al. 2008, 2012; Correia et al. 2009; Villas Boas et al. 2012). Moreover, Wollfgramm and Heyne (1995), and recently Vendruscolo et al. (2012), proposed that persistent ethanol drinking despite the bitter taste of quinine-adulterated solutions is a measure of compulsive behavior (i.e., loss of control over intake, a hallmark of alcohol use disorder).

The present study assessed the effect of a CRF₁ receptor antagonist and the transcription level of the CRF₁ receptor gene in the three classified groups of mice. Our hypothesis was that extrahypothalamic CRF transmission and CRF₁ signaling are hyperresponsive in animals with high ethanol intake and that these individuals will exhibit a reduction of ethanol intake when administered with CRF₁ antagonist, and they will have higher CRF₁ gene transcript levels.

Methods

Animals

One hundred forty naive Swiss male mice locally bred that were 6-week-old weighing 20–30 g in the beginning of experiments, and 27–57 g in the end of experiments, were housed individually (20×30×20 cm), under a 12-h/12-h light/dark cycle, 22±2 °C, and ad libitum access to food (Purina Laboratories, Brazil). All of the procedures were performed during the light cycle. The protocol (no. 391) was approved by the Ethics Committee of the UFPR.

Drugs

Ethanol solutions (5 and 10 %, v/v) were prepared by diluting ethanol 95 % (Vetec Laboratories, USA) with tap water every other day. In adulteration phase (AD), ethanol solutions were added 0.005 g/L quinine hydrochloride. CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine; donated by Pfizer) was prepared for intraperitoneal administration by suspending it in 0.5 % carboxymethyl cellulose (CMC, 0.1 mL/10 g). The CRF₁ antagonist doses were chosen based on Lowery et al. (2010) and Pastor et al. (2008).

Experimental design

Extended ethanol intake

Two groups of mice ($n=60$ per group) were exposed to the free choice paradigm: one for the molecular analysis (experiment 1) and the other for the CP-154,526 treatment (experiment 2). Two control groups ($n=10$ per group) consisted of mice which only had access to water during all phases of the paradigm. Figure 1 represents the experimental design of the paradigm which ran for 16 weeks and was divided into four phases. Over the course of a 70-day period (acquisition [AC] phase), the experimental mice had a free choice between water, 5 % ethanol, and 10 % ethanol. Then, the ethanol solutions were removed for 14 days (withdrawal [W] phase), and the mice had access only to water. Approximately 5 h after ethanol withdrawal, in the afternoon, the animals were subjected to the elevated plus maze in a drug-free condition to measure anxiety-like behavior (percent open arm time [%OT]). Ethanol was then offered again for 14 days (reexposure [RE1] phase). In the final 14-day phase (adulteration [AD] phase), the ethanol solutions were adulterated with quinine hydrochloride. Control animals had access only to water. After the last day of AD phase, the mice had 7-day access to non-adulterated ethanol solutions and water (RE2) to allow a return of their previous intake profiles. To classify each mouse into each group, we considered (i) individual preference between total ethanol intake (5 % plus 10 % ethanol in milliliter) and water intake (in milliliter) and (ii) individual ethanol consumption in g/kg/day during each phase: A group, preference for ethanol during all phases and persistent consumption in AD phase; B group, preference for ethanol in AC phase and an intake reduction in the AD phase; C group, preference for water during all phases. The first and second experiments'

protocols are shown, respectively, in the upper and lower parts of Fig. 1. In experiment 1, 50 μ L microcapillary tubes were used to collect retro-orbital blood samples during the light cycle to determine blood ethanol concentrations (BEC, Ethanol Assay Kit, GenWay Biotech, USA). BECs were evaluated in mice (seven C, six B, and eight A) that were used for the mRNA analysis.

Experiment 1: gene expression assessed by real-time polymerase chain reaction

Mice were euthanized by decapitation. The brains were rapidly dissected on ice, and bilateral amygdala punches were obtained according to Paxinos and Franklin (2001): -0.94 to -1.22 mm from bregma, ± 2 to ± 3 mm from midline, and -4 to -5 mm from dura. Total RNA was extracted using TRizol according to the manufacturer's protocol (Invitrogen, Brazil). The samples were quantified using a microplate reader (Eon, Biotek, USA), and RNA integrity was visualized in 1 % agarose gel stained with ethidium bromide. The minimum acceptable 260/280 nm ratio was 1.7. Reverse transcription was performed in a total volume of 20 μ L using 1 μ g of total RNA and oligo (dT20) primers (Exxtend, Brazil). SuperScriptIII (Invitrogen, Brazil) was used according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed in a StepOnePlus system that utilized SYBR Green PCR Master Mix (Applied Biosystems, Brazil). PCR amplification was performed without the extension step (95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s). Fluorescence acquisition was measured during the last step of each cycle (60 °C). The data were analyzed using StepOnePlus software v2.3 and Microsoft Excel. The minimum acceptable correlation coefficient was 0.90. In all of the reactions, a negative control was used that

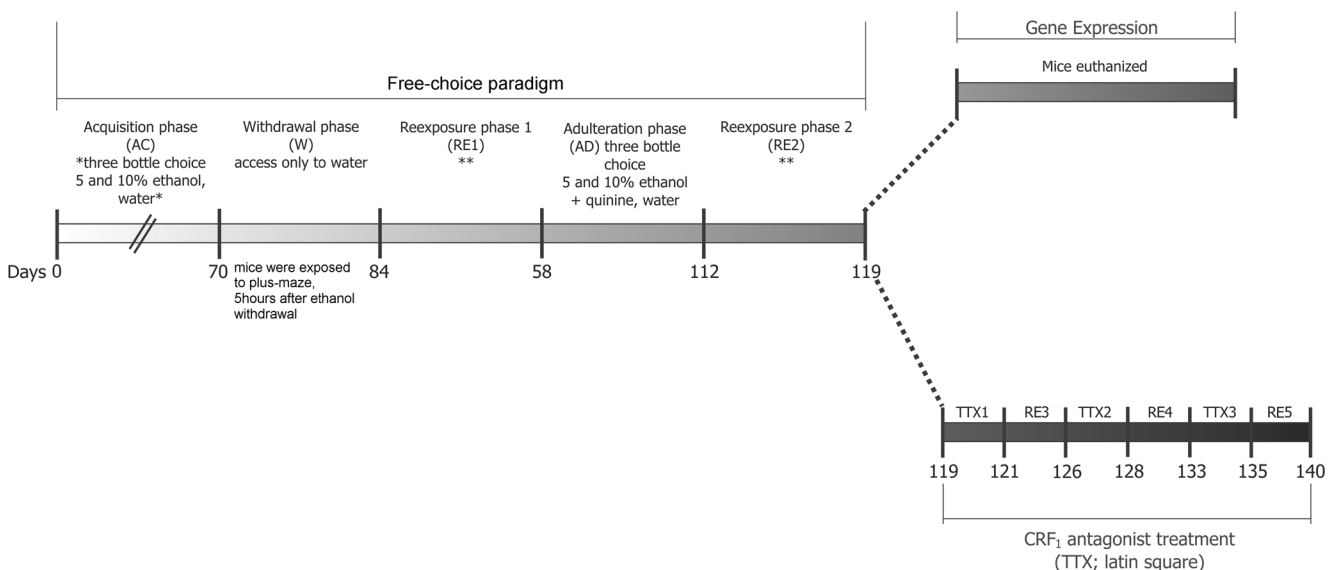


Fig. 1 Experimental design of the free-choice paradigm and CRF₁ gene expression (*upper*) and CRF₁ antagonist treatment (*lower*)

contained no tissue sample. Melting curves were examined to guarantee the absence of any spurious products. To quantify mRNA levels, we used the geometric mean of two reference genes (*Ppia* [peptidylprolyl isomerase A or cyclophilin A] and *Hprt* [hypoxanthine-guanine phosphoribosyl transferase]) to calculate the normalization factor (Vandesompele et al. 2002). The relative quantity of CRF₁ mRNA was divided by the normalization factor. The primer sequences were obtained from the PrimerBank database (<http://pga.mgh.harvard.edu/primerbank/index.html>; accessed April 15, 2013, Spandidos et al. 2010). The *crhr1* primer (PrimerBank ID 6681013a1) had the following sequences: forward, 5'-GGAACCTCATCTCGGCTTTCA-3'; reverse, 5'-GTTACGTGGAAGTAGTTGTAGGC-3'. For the reference genes, the primer sequences were designed and tested as described by Bibancos et al. (2007).

Experiment 2: effect of CP-154,526 on ethanol consumption

Mice received the three treatments (TTX1, TTX2, and TTX3) in a Latin-square design in a random order (vehicle, 15 mg/kg or 30 mg/kg CP-154,526), followed by a 5-day interval with no injection (RE3, RE4, and RE5), for a total of 21 days. Each dose was administered on two consecutive days in order to control any possible error of injection, with no blood accumulation of CP-154,526 considering its half-life (Keller et al. 2002). Thirty minutes after the injection of CP-154,526 or vehicle, the animals were given free-choice access to ethanol and water during the entire treatment period. Fluid consumption was quantified 24 h after each injection. The ethanol-naive control animals were randomly assigned to receive the same doses of CP-154,526 in the same schedule as the other groups and then had three-bottle, free-choice access.

Statistical analysis

The data were analyzed for distribution normality using the Kolmogorov-Smirnov test and homogeneity of variance using Levene's test. Body weight in grams and ethanol intake in milliliters were used to calculate the grams of ethanol intake per kilogram of body weight (g/kg). The preference between water and total ethanol intake (mL) during each phase was analyzed for each mouse using *t* tests. Repeated measures analysis of variance (ANOVA), followed by the Newman-Keuls test, compared the individual consumption (g/kg/day) for each mouse throughout the experiment by considering daily consumption during each phase (i.e., 14 measurements [last 2 weeks] in the AC, 14 in the RE, and 14 in the AD). Two-way repeated measures ANOVA, followed by the Newman-Keuls test was performed: (i) to compare the classified groups and the phases regarding ethanol consumption (g/kg/day), water consumption, and preference between ethanol and water, expressed as a percentage of total ethanol

intake/total fluid intake for the two replications of the paradigm; (ii) to compare the groups and the treatments regarding ethanol and water consumption during the CP-154,526 treatment period. A one-way ANOVA, followed by the Newman-Keuls test, compared the groups regarding BECs and %OT. The normalized mRNA data were compared using the Kruskal-Wallis ANOVA followed by a multiple comparison test. All of the analyses were performed using Statistica 6.1 software (StatSoft) with $p \leq 0.05$.

Results

Group classification based on individual consumption

The analysis of the individual patterns of ethanol consumption enabled us to categorize the mice into three groups. In experiment 1: A group ($n=10$), B group ($n=10$), and C group ($n=23$); 13 mice did not meet the criteria for classification and 4 mice died. In experiment 2: A group ($n=13$); B group ($n=9$); and C group ($n=24$); 12 mice did not meet the classification criteria and 2 mice died.

Intergroup differences

In experiment 1, the mean total ethanol consumption (g/kg/day, mean \pm SEM) for each group is presented in Fig. 2a for the mice that were used in the mRNA analysis (seven C mice, six B mice, and eight A mice). The ANOVA revealed significant main effects of group ($F_{2,18}=18.05$, $p<0.001$) and phase ($F_{2,36}=7.17$, $p<0.01$) and a significant group \times phase interaction ($F_{4,36}=5.32$, $p<0.01$). The post hoc analysis detected significant differences among groups and phases: the A group consumed more ethanol than the C group during all phases; B group exhibited significantly lower ethanol consumption than the A group in the RE and AD phases and higher consumption than C group in AC and RE phases. The B group exhibited a reduction of total ethanol consumption during the AD phase. No significant difference between phases was observed in the C group. The preference for ethanol and water, expressed as percentage of the mean total ethanol intake/total fluid intake (mean \pm SEM) for each group, is presented in the inset at the top right of Fig. 2a. The ANOVA revealed significant main effects of group ($F_{2,18}=78.58$, $p<0.001$) and phase ($F_{2,36}=24.03$, $p<0.001$) and a significant group \times phase interaction ($F_{4,36}=12.35$, $p<0.001$). The post hoc analysis detected significant differences among groups and phases. The A group preferred ethanol and exhibited higher percentages than the C group during all phases. The B group preferred ethanol, exhibiting higher percentages than the C group during the AC and RE phases, whereas the B group preferred water in the AD phase and exhibited a reduction of percentage of ethanol preference compared with the preceding phases and A

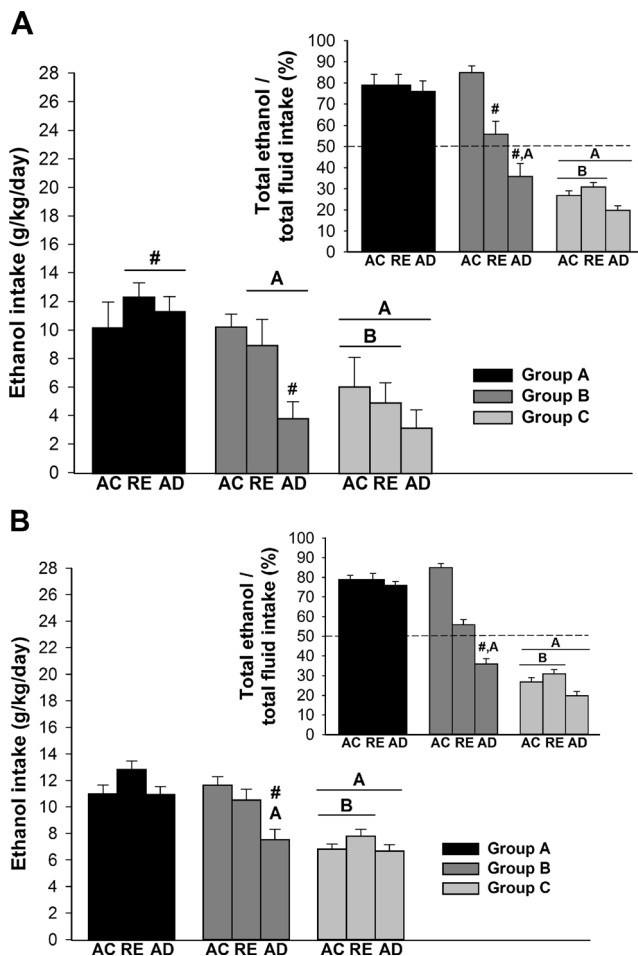


Fig. 2 Ethanol intake and preference in the two replications of the model. Analysis of ethanol intake during the experimental phases (AC acquisition phase, RE reexposure phase, AD adulteration phase) of the first experiment (a) and the second experiment (b). The data are expressed as the mean±SEM of ethanol intake (g/kg/day). (Inset) Analysis of preference expressed as percentage of ethanol intake during the same experimental phases. Symbols represent difference from the preceding phases in the same group (number sign); the A group (capital letter A); the B group (capital letter B) ($p < 0.05$)

group. The C group preferred water during all phases, with no significant differences between phases.

In experiment 2, the ANOVA of ethanol consumption (g/kg/day) revealed significant main effects of group ($F_{2,43} = 3.77$, $p < 0.05$) and phase ($F_{2,86} = 6.70$, $p < 0.005$) but no group×phase interaction ($F_{4,86} = 1.16$, $p > 0.05$). Figure 2b shows that the C group consumed less ethanol than the A group during all phases ($p < 0.001$) and less ethanol than the B group during the AC and RE phases ($p < 0.01$). The B group exhibited a decrease in ethanol intake during the AD phase compared with the AC and RE phases and compared with the A group ($p < 0.001$). The preference between ethanol and water, expressed as the mean total ethanol intake/total fluid intake (mean±SEM) for each group, is presented in the inset at the top right of Fig. 2b. The ANOVA revealed significant main effects of group ($F_{2,43} = 12.88$, $p < 0.001$) and phase ($F_{2,$

$86 = 5.40$, $p < 0.01$) but no group×phase interaction ($F_{4,86} = 1.30$, $p > 0.05$). The post hoc analysis detected significant differences among groups and phases. The A group preferred ethanol during all phases and exhibited a higher percentage than the C group during all phases. The B group preferred ethanol during the AC and RE phases, whereas the B group preferred water in the AD phase with lower preference for ethanol than the A group. The C group preferred water during all phases, with no significant differences between phases.

The BEC (mean±standard deviation) was 110 ± 28.2 mg% in the A group, 85 ± 36.1 mg% in the B group, and 45 ± 32.5 mg% in the C group. The ANOVA revealed a significant effect of treatment ($F_{2,18} = 12.89$, $p < 0.001$). The post hoc analysis showed that BEC in the A group was higher than in the B and C groups, and BEC in the B group was higher than in the C group.

The anxiety-like behavior during withdrawal phase was assessed by %OT in the elevated plus maze in experiment 2. The ANOVA revealed a significant effect of group ($F_{3,43} = 4.06$, $p < 0.02$). The post hoc test showed a reduction of %OT in the B group compared with the other groups ($p < 0.05$). The mean±SEM of %OT was 50 ± 7.7 % in the A group, 36 ± 8.6 % in the B group, 44 ± 10.2 % in the C group, and 47 ± 11.8 % in the control group.

Experiment 1: gene expression assessed by real-time polymerase chain reaction

During the 7-day period between the last day of the AD phase and sacrifice, the mice resumed their prior levels of ethanol intake (i.e., the levels in the AC phase: A group, 11.5 ± 0.8 g/kg/day; B group, 9.4 ± 1.0 g/kg/day; C group, 5.2 ± 0.6 g/kg/day).

The Kruskal-Wallis test revealed an effect of group on the normalized transcript levels of the *crhr1* gene ($H_{3,26} = 7.54$, $p < 0.05$). The post hoc test showed that the B group had increased mRNA levels ($p < 0.05$) in relation to the control group (Fig. 3).

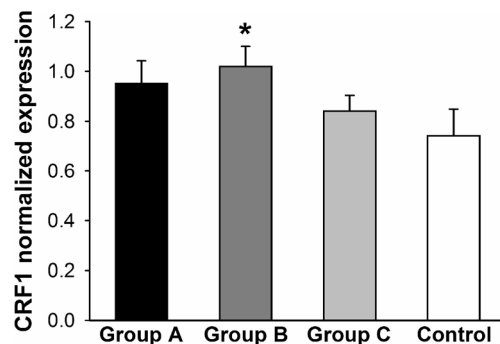


Fig. 3 CRF₁ mRNA in the amygdala in animals with different ethanol intake profiles. The data are expressed as the mean±SEM of normalized CRF₁ mRNA transcripts in each group. The control group only had access to water. * $p < 0.05$, significant difference from the control group

Experiment 2: effect of CP-154,526 on ethanol consumption

During the 7-day period between the last day of AD phase and the beginning of CP treatment, mice resumed their previous (AC) ethanol intake levels (A group: 12.5 ± 0.9 ; B group: 10.9 ± 1.1 ; C group: 7.8 ± 0.6).

The levels of ethanol consumption during each day of the 2-day treatment were similar, and thus the average consumption was used for each individual. The ANOVA of ethanol consumption before and during the CP-154,526 treatment period (named as phase here) revealed significant main effects of group ($F_{2,83}=43.34$, $p<0.001$) and phase ($F_{5,166}=67.58$, $p<0.001$) and a significant group \times phase interaction ($F_{10,166}=12.21$, $p<0.001$). Figure 4a shows that the highest dose of the antagonist significantly reduced ethanol consumption in the A and B groups compared with the vehicle. Notably, during the antagonist treatment period, the B group exhibited significant reduction in ethanol intake by returning to intake levels similar to those shown in the AC phase and the group also exhibited lower ethanol intake than the A group. The C group increased ethanol consumption during the entire antagonist treatment period with no antagonist effect. All groups also increased ethanol intake when under vehicle injection in relation to preceding phases. The ANOVA for the control group treated with the antagonist revealed no significant effects on ethanol intake ($F_{2,21}=0.94$, $p=0.41$).

The preference between ethanol and water (mean \pm SEM) for each group, before and during antagonist treatment, is presented in the inset at the top right of Fig. 4a. The ANOVA revealed significant main effects of group ($F_{2,83}=127.46$, $p<0.001$) and phase ($F_{5,166}=25.76$, $p<0.001$) and a significant group \times phase interaction ($F_{10,166}=17.49$, $p<0.001$). The post hoc analysis revealed significant differences among groups and phases. The A group preferred ethanol during all experimental phases including the antagonist treatment period. Treatment with the antagonist at both doses in the A group reduced ethanol preference compared with the AC and RE phases and with vehicle treatment. The B group preferred ethanol during the AC and RE phases but preferred water in the AD phase. With antagonist treatment, preference in the B group returned to the levels observed in the AC phase and did not change with antagonist treatment. The C group preferred water during the AC, RE, and AD phases and with vehicle treatment. During all periods of antagonist treatment, the preference for ethanol increased in the C group, with no differences between antagonist doses. The ANOVA for the control group treated with the antagonist revealed no significant effects on ethanol preference ($F_{2,21}=1.36$, $p=0.28$).

The ANOVA of water consumption before and during the CP-154,526 treatment period revealed significant main effects of group ($F_{3,111}=91.69$, $p<0.001$) and phase ($F_{5,222}=21.86$,

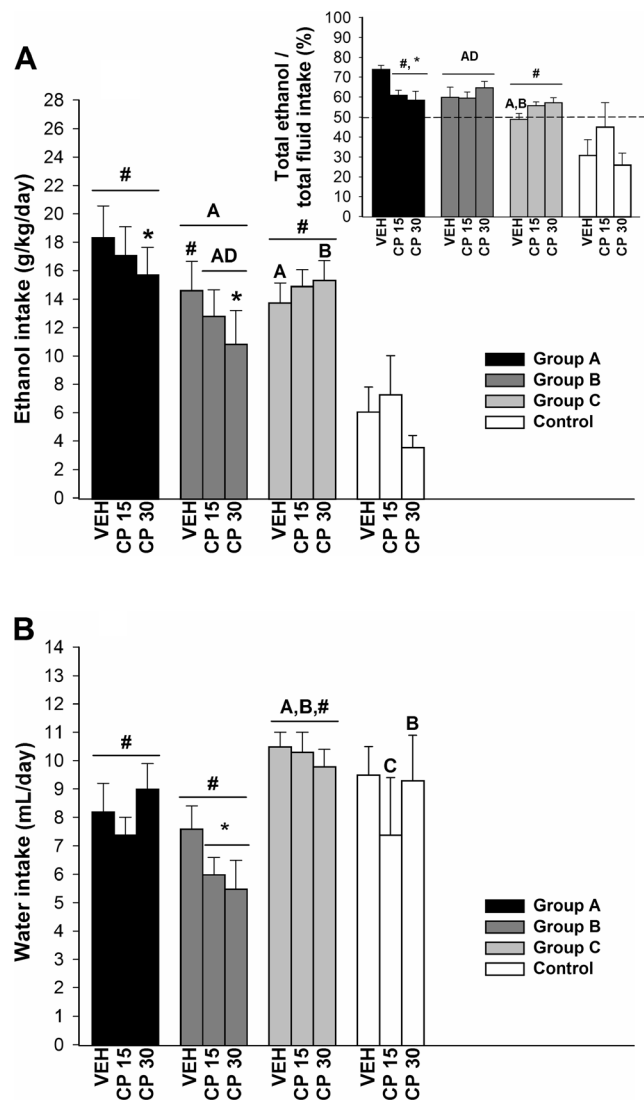


Fig. 4 Intake of and preference for ethanol or water over 24 h during CP-154,526 treatment. Data are expressed as the mean \pm SEM of ethanol intake (a) in g/kg/day and water intake (b) in mL/day in the A, B, C, and control groups during intraperitoneal CP-154,526 treatment (CP15, 15 mg/kg; CP30, 30 mg/kg of CP-154,526) or vehicle treatment (VEH). (Inset at a) Analysis of preference expressed as percentage of ethanol intake during the experimental phases. The control mice were exposed only to water during the model and had three-bottle free-choice access during the antagonist treatment period. Symbols represent difference from the A group (capital letter A); the B group (capital letter B); the C group (capital letter C); the adulteration phase in the same group (capital letters AD); vehicle treatment (asterisk); the preceding phases in the same group (number sign) (the preceding phase values are shown in Fig. 2b) ($p<0.05$)

$p<0.001$) and a significant group \times phase interaction ($F_{15,222}=4.65$, $p<0.001$). Figure 4b shows that the two doses of the antagonist reduced water consumption in the B group compared with vehicle ($p<0.05$). The A and C groups exhibited no effects of antagonist treatment compared to vehicle. All groups increased water intake with vehicle treatment compared with the preceding phases. The control group did not

exhibit any changes in water consumption throughout all the experiment.

Discussion

The main finding of this study was the confirmation of the hypothesis that only high-drinking animals (A and B mice) reduced their ethanol consumption after administration of the CRF₁ receptor antagonist. As expected, the antagonist had no effect on C mice. The effect of the antagonist was specific to ethanol intake and preference only in A animals. However, contrary to our hypotheses, CRF₁ transcript levels in the amygdala increased only in B mice.

In the present study, the mice had constant access to the ethanol solutions during the CP-154,526 treatment period, while in other studies, multiple withdrawal periods were imposed during treatment, which induced prominent negative affect. For alcohol-addicted humans, remaining completely abstinent is not the only goal of treatment (Adamson et al. 2010; Mowbray et al. 2013; DeMartini et al. 2014). Therefore, studies of new medications that use animal models should emulate the human condition. Previous research has suggested that in order to increase the validity of animal addiction models, the number of days that the animals are allowed to consume the drug and the length of time that it is available each day should be extended (Wolffgramm and Heyne 1995; Deroche-Gamonet et al. 2004; Ahmed and Koob 1998). Thus, in our model, animals were exposed to free choice over 14 weeks, 24 h a day. Indeed, the BEC determined at the end of the model (i.e., 7 days after the AD phase when the animals were again given free-choice access) reached intoxicating levels in the A and B groups, i.e., levels above 80 mg% (National Institute on Alcohol Abuse and Alcoholism 2004). Notably, in the present study, mice had only a 2-week period of abstinence during the “withdrawal phase” of the model. Although in our previous study we observed anxiety-like behavior in the elevated plus maze during withdrawal phase in groups A and B (Fachin-Scheit et al. 2006), in the present study, we observed this behavior only in group B, in accordance to what was observed in our other study (Villas Boas et al. 2012) suggesting the occurrence of negative affect predominantly on group B. The C group had similar “anxiety” levels as control group during the withdrawal phase in all replication studies and as such these mice did not experience withdrawal-related negative affect. Anyway, the lack of effect of the CRF₁ antagonist in this latter group and the occurrence of the antagonist effect for groups A and B suggest the role of CRF in high-ethanol drinkers.

Compared to vehicle injection, the highest dose of the CRF₁ antagonist reduced ethanol intake in the B group and reverted intake levels to that observed in the AC phase. However, a reduction of water intake was observed, with no

changes in ethanol preference. Importantly, in the A group, the antagonist had a specific effect on ethanol intake compared with vehicle treatment, with no changes in water intake. Giardino and Ryabinin (2013) reported that two CRF₁ antagonists reduced ethanol and water intake in the DID paradigm. The B group appears to present a similar drinking profile as animals with high ethanol intake in the DID paradigm. The DID paradigm was designed to use a specific mouse strain (C57BL/6 J) that is predisposed to drink large amounts of ethanol, in contrast to the heterogeneous Swiss mouse that was used in the present study. Contrary to the nonspecific effects observed in the B group that suggested changes in consummatory behavior, the A mice reduced specifically the ethanol intake and preference.

As expected by our hypothesis, higher levels in CRF₁ mRNA expression in the amygdala were found in the B group, but unexpectedly, the A group did not. Furthermore, the findings related to high anxiety-like behavior during withdrawal only in B mice supported the suggestion that the two heavy-drinker groups developed different neuroadaptations that may not be related exclusively to CRF₁ upregulation, at least in the amygdala. Furthermore, CRF systems in different brain areas, other than the amygdala, are probably modulating ethanol intake in this model. One can consider this as a limitation of the present study because we only assessed the CRF₁ mRNA in the total amygdala. Other authors (Hwa et al. 2013; Sparta et al. 2013; Simms et al. 2014) demonstrated a role for other brain areas in the CRF modulation of ethanol consumption. Thus, these areas might be involved in reducing ethanol intake when a CRF₁ antagonist is systemically administered.

The experimental protocol used during the vehicle/CRF₁ antagonist treatment period was the Latin-square design, which was used to avoid the influence of one treatment over another and to avoid the influence of habituation to handling stress (Fachin-Scheit et al. 2006; Ji et al. 2008). During the period of administration of the vehicle/antagonist, all of the mice had free-choice access to the solutions and were subjected to handling/injection stress (Gouveia and Hurst 2013). Indeed, as reviewed by George et al. (2014), behavioral phenotypes (e.g., drug intake) are modulated by the interaction between individual vulnerability and the environment. It is important to note that all of the mice were exposed to the same procedures during this period. Notably, when the mice were treated with vehicle, ethanol and water intake increased in all groups compared with the previous phases of the model. Nevertheless, the B and C groups showed lower intake than the A group. Although the C mice had similar ethanol intake to the B mice at the time of the antagonist treatment, its preference was still lower than the two other groups and the C group did not respond to the antagonist effect in a similar way. It is likely to suppose that the period of prolonged ethanol consumption, during which each group had its own history of ethanol consumption, induced differential neuroadaptations

in the three groups, leading to different responses to the antagonist. We also observed that during the 7-day period between the last day of the AD phase and the beginning of the vehicle/antagonist treatment period each group returned to the ethanol intake levels seen in AC phase. Thus, we suggest that the differential effects of the CRF₁ antagonist on ethanol intake in the A and B groups are not attributable to habituation to handling stress. Funk et al. (2006b) suggested that different types of stressors activate a different set of neuronal pathways and may justify the heterogeneity in alcohol intake responses to stress. Moreover, Sillaber et al. (2002) showed that CRF₁ receptor knockout mice did not differ from wild-type mice in baseline ethanol consumption, but when the knockout mice were submitted to repeated stress, a delayed and persistent increase in ethanol intake was observed, suggesting that changes in the CRF₁ gene modify ethanol intake only when associated with stress. Molander et al. (2012), using different knockout mouse lines, showed that CRF increased stress-induced ethanol consumption, but it is not sufficient to control baseline consumption and relapse-like drinking with a low stress load.

Our data corroborate the allostasis theory of addiction. We propose that a better understanding of the role of allostatic adaptations is needed based on different ethanol consumption profiles in different experimental protocols. Despite the important role of the CRF system in heavy-drinking animals in the present study, these animals presented different alterations of this system. The B group exhibited dysregulated CRF₁ in the amygdala, decreased ethanol and water intake with CRF₁ antagonist treatment, and significant anxiety-like behavior in the W phase. The A group decreased ethanol intake and preference with CRF₁ antagonist treatment. No effect was observed in C group. Our data suggest the importance of studying and developing drugs that modify the CRF system to effectively treat alcoholism by considering that humans also have different drinking profiles (e.g., heavy-drinking or loss of control over ethanol intake).

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Conflict of interest None

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