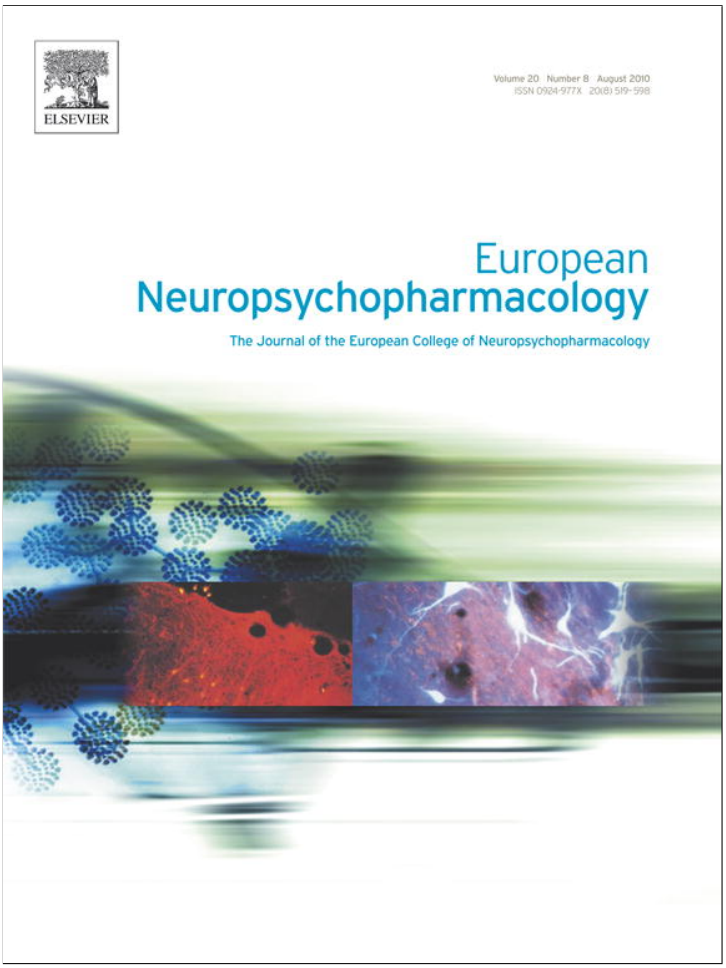

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Increased voluntary ethanol consumption and c-Fos expression in selected brain areas induced by fear memory retrieval in ethanol withdrawn rats

María Eugenia Bertotto, Daniela Fernanda Bussolino, Víctor Alejandro Molina, Irene Delia Martijena*

IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5016 Córdoba, Argentina

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Abstract

Withdrawal from chronic ethanol administration facilitated the formation of contextual fear memory. The effect of fear memory retrieval on subsequent ethanol consumption, by employing a two-bottle free-choice procedure with either water or ethanol (2–8% v/v), was investigated in ethanol withdrawn rats. The effect of fear memory extinction with or without D-cycloserine (DCS, 5 mg/kg i.p.) on subsequent ethanol consumption was also evaluated. In addition, we examined c-Fos expression in different brain areas following the fear memory recall. The retrieval of such fear memory induced a significant increase in ethanol consumption in ethanol withdrawn but not in control animals. Regardless of DCS treatment, this increase was attenuated by extinction training. In ethanol withdrawn rats, context-dependent memory retrieval was accompanied by an increased c-Fos expression in the basolateral amygdala, ventrolateral periaqueductal gray, dentate gyrus and dorsomedial periaqueductal gray. Among these brain areas suggested to be implicated in the modulation of motivation and of emotional states, the basolateral amygdala has a crucial role in the emergence of negative affective state during ethanol withdrawal. These data suggest that retrieval of fear memory in ethanol withdrawn rats affected ethanol consumption and that amygdala may be involved in this effect.

1. Introduction

It is well documented that the prolonged consumption of alcohol leads to the development of tolerance and dependence in both animals and humans (Sellers and Kalant, 1976). Alcohol dependence is revealed by the emergence of a withdrawal syndrome after the cessation of alcohol administration. This

* Corresponding author. IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria. 5016 Córdoba, Argentina. Tel.: +54 0351 4334437; fax: +54 0351 4334420.
E-mail address: imartije@fcq.unc.edu.ar (I.D. Martijena).

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syndrome is characterized by both physical signs and negative affective states, including dysphoria, irritability, increased anxiety and depressed mood (de Witte et al., 2003; Markou et al., 1998). Affective disturbances and the occurrence of negative emotions following ethanol withdrawal can persist for considerable periods of time, and are still present in alcoholics long after the recovery of acute somatic and autonomic symptoms (de Witte et al., 2003). These disturbances in affective states can play an important role in the maintenance of alcohol addiction (Koob et al., 1998).

Distinctive behavioral alterations in animal models of acute and protracted ethanol withdrawal have been suggested to reflect the excessive anxiety and psychological discomfort shown by humans following withdrawal (Kliethermes, 2005; van Erp and Miczek, 2001; Koob et al., 1998).

In addition, stressful life events are critical factors known to increase the risk of relapse in abstinent alcoholics and in animal models of drug addiction (Breese et al., 2005; Pohorecky, 1990; Sinha, 2001; Smith and Aston-Jones, 2008). Increased behavioral responsiveness to stress (Holter et al., 2000; Möller et al., 1997; Rassnick et al., 1993, Rasmussen et al., 2001; Valdez et al., 2003) and enhanced ethanol self-administration during early and protracted ethanol withdrawal have been reported in ethanol-dependent rats (Roberts et al., 2000; Sommer et al., 2008; Valdez et al., 2002, 2004).

We have recently reported that withdrawal from chronic ethanol administration facilitated the formation of contextual fear memory, when anxiety induced by abrupt ethanol discontinuation was no longer evident (Bertotto et al., 2006). This effect of ethanol withdrawal lasted up to 2 weeks, thus supporting the viewpoint that the vulnerability to exaggerated fear acquisition is a long lasting phenomenon. Taking into account that stressful life events and a maladaptive response to stress influence alcohol drinking, a long lasting hyperreactivity to stress and the facilitation to generate fear memories observed in ethanol withdrawn animals might be a predisposing factor for ethanol consumption. Therefore, the main goal of the present study was to investigate whether the retrieval of fear memory influences subsequent ethanol consumption in a two-bottle free-choice paradigm in ethanol withdrawn rats.

The expression of the immediate early gene, c-Fos, has been extensively used as a marker of neuronal activation induced by a variety of experimental procedures (Cullinan et al., 1995; Dragunow and Faull, 1989) including withdrawal from drugs of abuse such as ethanol (Knapp et al., 1998; Matsumoto et al., 1993; Moy et al., 2000). In addition, c-Fos may play a role in the neuronal modification underlying memory formation (Guzowski et al., 2001; Herrera and Robertson, 1996). Therefore, the expression of c-Fos protein or c-fos mRNA have also been used to map functional circuitry underlying both contextual and cued fear conditioning (Campeau et al., 1997; Milanovic et al., 1998; Radulovic et al., 1998; Rosen et al., 1998). Previous experiments have focused on the c-Fos expression after fear acquisition (Fujisaki et al., 2004; Milanovic et al., 1998; Stanciu et al., 2001) or during learning retrieval (Fujisaki et al., 2004; Strekalova et al., 2003). Although the neural substrates involved both in Pavlovian fear conditioning and ethanol withdrawal have been extensively studied; the brain areas involved in the exaggerated emotional reaction displayed by ethanol withdrawn rats remain largely unknown. Therefore, an additional purpose of the present study was to identify the potential neural substrates involved in the increased contextual fear response in ethanol withdrawn rats. To address this goal, we examined c-Fos immunoreactivity in different brain areas related to stress, fear and anxiety after the retention test for contextual fear memory in ethanol withdrawn animals.

2. Experimental procedures

2.1. Subjects

Male Wistar rats from our breeding stock, weighing 250–270 g, were housed in groups of 3 per cage (Meert and Huysmans, 1994) with food and water ad libitum except when detailed otherwise in the protocol. Animals were maintained in a 12-h light–dark cycle (lights on at 0700) at a room temperature of 21–22 °C. Animals were acclimatized to the animal housing facilities for at least 1 week before starting the experiments, in which, all testing took place between 0900 and 1700 h. Separate groups of rats were used in each experiment. In these experiments, the person who performed the behavioral assessment was blind to the experimental conditions of each animal. The protocols used were approved by the Animal Care Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, and are consistent with the standards for the care and use of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory animals. In all the experimental procedures involving animals, all efforts were made to minimize pain and the number of animals used.

2.2. Ethanol liquid diet procedure

Ethanol was administered as the sole source of nutrients in a chocolate flavored nutritionally complete liquid diet (ABBOTT LABORATORIES B.V.) to rats in their home cages. Animals were randomly allocated to two treatment groups, one receiving a non-ethanol-containing liquid diet (CON group) and the other one chronically exposed to ethanol via an ethanol-containing liquid diet (ETOH group). All rats received the control liquid diet for an initial 3-day period. Ethanol-treated rats then received a diet containing ethanol (6% v/v) for 14 days with water available ad libitum. The liquid diet was freely available to the ethanol-treated groups and the mean amount of diet consumed was recorded daily. Control animals were pair-fed with the same diet, but with dextrose substituted isocalorically for ethanol. The volume of diet given to each control-treated group was equal to the mean volume of diet consumed by the ethanol-treated rats. Diets were prepared and administered at the onset of the dark cycle with the liquid diet being removed at 0700 h on the day of withdrawal and the animals fed with laboratory chow for the rest of the experiments. This method of ethanol administration has been previously shown to reliably induce ethanol dependence (Baldwin et al., 1991; Bertotto et al., 2006; Devaud et al., 1996; Weiss et al., 1996) and has typically produced blood ethanol concentrations (BACs) ranging from 80 to 132 mg% with comparable weight gains in both control and ethanol groups (Bertotto et al., 2006). In fact, the interruption of a similar chronic protocol of ethanol administration as that used in the current study resulted in increased anxiety observed at 8 h on the elevated plus maze, but not 48 or 72 h after withdrawal (Bertotto et al., 2006). In addition, our previous data also showed that vulnerability to exaggerated fear responses is a long lasting phenomenon that can persist for at least up to two weeks following ethanol discontinuation. Collectively, these findings indicate that the current procedure of ethanol administration, which produced moderate blood ethanol concentrations, was effective in modeling important aspects of ethanol dependence such as an increase of anxiety and of

the behavioral sensitivity to stress upon discontinuation of ethanol administration.

2.3. Contextual fear conditioning

2.3.1. Apparatus

In experiments designed to evaluate contextual fear learning, distinctive chambers were placed in acoustically isolated separate rooms maintained at a constant temperature of 21 ± 1 °C. These chambers varied according to location, size, color, illumination, floor and walls. The conditioning chamber was connected to a scrambled shocker (Ugo Basile Biological Research Apparatus, Italy) and was made of gray acrylic (20×23×20 cm) with a transparent lid. The grid floor consisted of 10 parallel stainless steel grid bars, each measuring 4 mm in diameter and spaced 1.5 cm apart (center to center). The second chamber was made of wood (33×25×33 cm) with black walls, a black rubber floor and a lid of transparent plastic. This context was made as different as possible from the one originally used during training, thereby maximizing the possibility of obtaining different levels of expression of the acquired memory. Obviously, differences in the expression of learning in the present circumstances might correspond to contextual changes and/or changes regarding the discrete stimuli that define the context. The chambers were located in different rooms illuminated by a white fluorescent tube located on the ceiling. Background noise was supplied by ventilation fans and shock scramblers. Both chambers were cleaned with a 10% aqueous ethanol solution before and after each session. The training and test sessions were conducted between 0900 and 1100 h.

2.3.2. Procedure

On the day of conditioning, rats were moved from their housing room and individually placed in the conditioning chamber. Animals were then left undisturbed for a 3 min acclimatization period (pre-shock period), which was followed by 3 unsignaled scrambled footshocks (0.4 mA, 3 s duration and 30 s intershock interval). Animals remained in the chamber for an additional 2 min (post-shock period), before being immediately brought back to their home cages and returned to the colony room.

Testing for contextual fear conditioning was performed 24 h after training with rats being randomly assigned to two subgroups. Half of the animals were reintroduced into the conditioned context (same context) for a 10 min period without shock delivery, and the other half exposed for the same period of time to the second chamber (different context). The behavior of each rat was continuously videotaped during the 3 min pre-shock period, the 2 min post-shock period, and during the entire 10 min testing period. Freezing – a commonly used index of fear in rats – was defined as a total absence of body or head movement except for that associated with breathing (Blanchard and Blanchard, 1969; Bolles and Collier, 1976). Freezing behavior was scored at the end of the experiment by a person who was blind to the experimental condition of each animal. The measure of fear was quantified (in seconds) using a stop-watch and expressed as the percentage of total time.

2.4. Two-bottle choice procedure

The voluntary intake of alcohol was determined using a standard two-bottle choice test between water and increasing concentrations of ethanol, similar to that used in a previous study by Martijena et al. (2001). The concentration of ethanol (v/v) was increased every 4 days as follows: 2, 4, 6, and then 8% for the final 8 days. Fluids were always available to the animals for 2 h (1500–1700 h) each day in two 50 ml graduated tubes. The position of the two bottles was randomly interchanged each day to prevent the development of a positional habit. Ethanol was freshly prepared each day by diluting 96% ETOH (Porta, Argentina) in tap water. During all test sessions, animals were transferred to individual plastic cages with standard lab chow. Body weights were recorded every fourth day immediately before starting the test. The amount of fluids consumed was measured and the intake of ethanol was calculated in terms of g/ethanol/kg body weight/2 h. Water and total fluid intake were calculated in terms of ml/kg/2 h. At the end of the drinking session, animals were returned to their home cage with food but no fluid available until the next session.

2.5. Drug administration

D-cycloserine (DCS, Sigma-Aldrich, St. Louis, MO, USA), a partial agonist of NMDA receptor, was freshly dissolved in sterile isotonic saline (SAL) (0.9%) and injected i.p. at a volume of 1.0 ml/kg. SAL was used for CON injections.

2.6. c-Fos immunocytochemistry

Animals were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) 90 min after the end of the test and perfused transcardially with saline followed by a solution of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Once this procedure was completed, the brains were removed, and left in the same fixative solution overnight at 4 °C, before being placed in 30% sucrose solution in PBS. Subsequently, the brains were sectioned in a cryostat into 30 μm thick coronal slices and these sections immersed in 0.1 M PBS. Free-floating sections were incubated for an hour in a solution of 10% methanol, 3% hydrogen peroxide in PBS to eliminate the endogenous peroxidase activity. After being placed for 1 h in a blocking solution (5% bovine-serum albumin (BSA) and 0.3% tritón X-100 in 0.1 M PBS), tissue sections were incubated for 48 h at 4 °C with polyclonal c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:5000 in 0.1 M PBS containing 1% BSA. Sections were washed in 0.1 M PBS and then incubated for 1 h at room temperature with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA, diluted 1:200 in 0.1 M PBS containing 1% BSA) followed by an avidin–biotin–peroxidase (ABC) complex (Vectastain ABC kit, Vector Laboratories) for 1 h at room temperature. For visualization purposes, the peroxidase reaction end product, 3′-diamino-benzidine tetrahydrochloride (DAB Sigma) was used as a chromogen (Sigma fast tablet set) and sections were incubated for 5 min with a solution containing 0.05% of DAB and 0.0006% of hydrogen peroxide. Brain sections were mounted onto slice glass, dehydrated and cover slipped prior to viewing with light microscopy.

2.7. Quantification

The positive c-Fos cells were identified using light field microscopy (Zeiss Axioplan) at a magnification of 200× and images were acquired using MetaMorph software and then, the quantification was performed using Scion Image software. This quantification was carried out by a person not involved in the experiment and performed using an identically sized area (0.16 mm2) of the same shape for each brain region. The number of c-Fos positive cells was obtained from each area of interest, with a constant background intensity being maintained across different areas.

The anteroposterior (AP) coordinates from bregma of sections included for detailed analysis were: basolateral amygdala (BLA): −2.30 to −3.30, central amygdala (CeA): −1.80 to −2.80; lateral amygdala (LA): −2.56 to −3.60; cingulate cortex area 1 (Cg1), infralimbic cortex (IL), prelimbic cortex (PrL) and secondary motor cortex (M2): 3.20 to 2.20; nucleus accumbens core (NAcc core), nucleus accumbens shell (NAcc shell) and striatum (CPU): 1.60 to 0.70; field CA1 of hippocampus (CA1), field CA2 of hippocampus (CA2), field CA3 of hippocampus (CA3) and dentate gyrus (DG): −3.14 to −3.60; lateral septal nucleus, ventral part (LSV): 0.48 to −0.30; ventral tegmental area (VTA) −5.08 to −6.04, dorsomedial periaqueductal gray (DMPAG) and ventrolateral periaqueductal gray

(VLPAG): −7.64 to −8.30. The locations of the areas used were taken from Paxinos and Watson, 1997.

2.8. Statistical analyses

All results are expressed as the means ± SEM with behavioral results analyzed by ANOVAS. The source of the main significant effects or interactions was determined by Fisher's protected least significant difference (PLSD) post-hoc multiple comparisons test. The significance level used for all statistical analyses was set at $p<0.05$.

2.9. Experimental design

2.9.1. Experiment 1

The goal of this experiment was to investigate the effect of fear memory retrieval on ETOH consumption by employing a free-choice paradigm between water and increasing ETOH concentrations in ETOH withdrawn rats.

Animals were randomly assigned to CON or to ETOH groups. During days 2–4 of ethanol withdrawal, animals were placed in individual plastic cages with only water available in both tubes. Animals were deprived of water 18 h prior to the first water intake test session, and subsequent drinking sessions were conducted following 22 h of water restriction. All animals were subjected to contextual fear conditioning training on the 4th day of withdrawal as previously described. Twenty-four hours after training, the animals from the CON and ETOH groups were divided into two groups and the freezing response was recorded in the training context (same) or in a different context from which they were trained (different). The experimental design resulted in the following groups: CON-same ($n=12$); CON-different ($n=11$); ETOH-same ($n=12$); and ETOH-different ($n=12$). The free choice between ethanol and water began approximately 4 h after the end of the fear memory test, and water was made available in one tube and an ethanol solution in the other tube for the duration of the study.

In addition, animals from the CON ($n=11$) and ETOH groups ($n=11$) were placed in the conditioning chamber and kept there for the same time but without being giving any shock experience. These groups were studied for the possible effect of withdrawal from ethanol *per se* on subsequent ethanol intake in rats subjected to a free-choice test.

Due to the fact that CON and ETOH withdrawn rats had different freezing responses during the memory test (Bertotto et al., 2006), we considered the possibility that solely the strength of the conditioned memory might have a differential impact on subsequent ethanol consumption. In order to explore this possibility, CON rats were trained using a strong fear conditioning procedure, in order to induce a freezing response similar to that exhibited by the ETOH group. A total of 30 CON rats were divided into 3 groups: one was fear conditioned as previously described ($n=9$), and the other two were fear conditioned with either 3 shocks of 0.7 mA ($n=12$) or 5 shocks of 0.4 mA ($n=9$).

Finally, we assessed whether extinction of fear memory affected the subsequent ethanol intake in ETOH rats. In addition, the effect of D-cycloserine (DCS) in conjunction with extinction training on subsequent ethanol consumption was also evaluated. We selected a dose of 5 mg/kg of DCS based on our previous findings which showed that this dose facilitated the fear memory extinction in ETOH rats (Bertotto et al., 2006). A total of 27 ETOH rats were subjected to the fear conditioning procedure as previously described. One day later, the freezing response to the trained context was evaluated in 18 animals for 10 min. Immediately after this memory test (test 1 or first extinction trial), half of the animals were injected with saline (EXT-SAL) and the other half with DCS (5 mg/kg, EXT-DCS). The remaining trained rats were left undisturbed in their home cages (NO-EXT). The following day, all rats were re-exposed to the training context and the freezing response was evaluated for 10 min (test 2, first extinction trial for the NO-EXT group and second extinction trial for the EXT-SAL and EXT-DCS groups). The free choice between ethanol and water began approximately 4 h after the end of this memory test.

2.9.2. Experiment 2

In this experiment, we examined c-Fos expression after contextual fear memory retrieval in ETOH withdrawn rats. On the 4th day of withdrawal, rats were fear conditioned and the freezing response was recorded in the training context or in the different context 24 h later as previously described. The experimental design resulted in the following groups: CON-same ($n=10$); CON-different ($n=10$); ETOH-same ($n=10$); and ETOH-different ($n=10$). Then, the animals were returned to their home cages and sacrificed to determine c-Fos expression 90 min after the end of the test. The c-Fos analysis was conducted in brain areas of 7–8 animals, randomly chosen from each of the CON and ETOH withdrawn groups.

3. Results

3.1. Experiment 1

Fig. 1 displays the mean percentage time of freezing for animals that were submitted to fear conditioning and tested in the same or in a different context of training. As expected, animals tested in the same context, regardless of diet treatment, displayed more time freezing than animals tested in the different context. In addition, the increase in the percentage time of freezing in the same context compared to the different context was greater in the ETOH withdrawn group than in the CON group, confirming previous results from our laboratory (Bertotto et al., 2006). ANOVA showed a

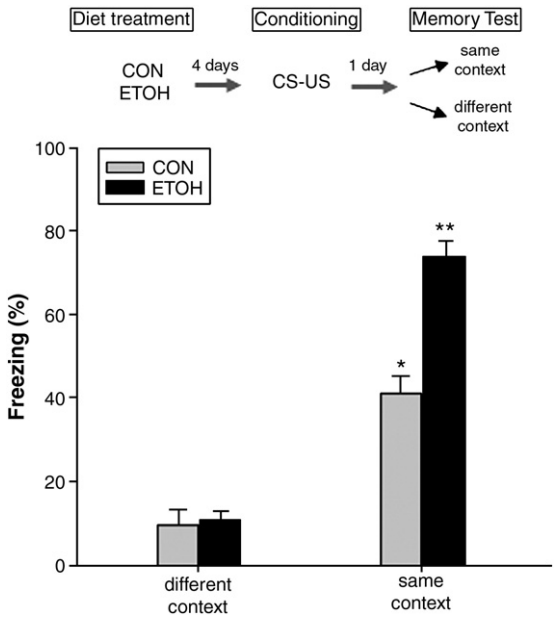


Figure 1 Effect of ETOH withdrawal on fear learning. Rats were treated for 14 days with ethanol (6% v/v) or control liquid diet. On the 4th day of withdrawal, all animals were subjected to a contextual fear conditioning and were evaluated 24 h later in the training context (same) or in a different context (different). Values represent the means ± S.E.M. of the percentage of time spent freezing during the 10 min test period. *$p<0.01$ vs. the respective different group. **$p<0.01$ vs. all other groups.

significant effect of diet treatment [$F(1,43)=36.32$; $p<0.001$], test context [$F(1,43)=294.30$; $p<0.001$] and of diet treatment × test context interaction [$F(1,43)=32.78$; $p<0.001$]. Furthermore, the post-hoc test revealed significant differences between the same and different context groups for both CON and ETOH withdrawal ($p<0.01$). The analysis also showed that the percentage time of freezing of ETOH withdrawn rats in the same context was higher compared with all remaining groups ($p<0.01$). The percentage time of freezing found in the different context was not significantly different between CON and ETOH groups with no other significant differences being detected, between these groups. An analysis of the time spent freezing during the pre-shock or post-shock periods also revealed no significant differences between the experimental groups. The means ± S.E.M. for the pre-shock period were: 5 ± 1 s for CON and 7 ± 2 s for ETOH withdrawn groups, with the means ± S.E.M. for the post-shock period being 105 ± 2 s for CON and 107 ± 3 s for ETOH withdrawn groups.

Next, we examined the effects of fear memory retrieval on subsequent voluntary ethanol consumption. Fig. 2A shows the intake of ethanol following the test for memory retrieval in CON and ETOH withdrawn rats. A significant increase in ethanol drinking was induced by the recall of conditioned aversive memory, but only in ETOH withdrawn rats. Regardless of the diet treatment, exposure to the different context did not modify subsequent ethanol drinking. A three-way ANOVA revealed a significant effect of context exposure [$F(1,43)=21.08$; $p<0.001$], diet treatment × context exposure interaction [$F(1,43)=9.73$; $p=0.003$], ethanol concentration as a repeated measure [$F(3,129)=20.13$; $p<0.001$], ethanol concentration × context interaction [$F(3,129)=5.89$; $p<0.001$] and diet × context exposure × ethanol concentration [$F(3,129)=2,75$; $p=0.045$]. However, no other effects or interactions were detected. Further post-hoc comparisons indicated that ETOH withdrawn rats tested in the same context drank more ethanol in comparison with the other groups. This difference was statistically significant when animals were offered ethanol concentrations of 4 to 8% v/v ($p<0.01$).

The water and total fluid intake are shown in Fig. 2B and C, respectively. ETOH withdrawn rats tested in the same context drank less water than the other group of animals. This effect was statistically significant from 4 to 8% v/v ethanol concentrations ($p<0.01$). A three-way ANOVA for water intake revealed a significant effect of context [$F(1,43)=20.93$; $p<0.001$], diet treatment × context exposure interaction [$F(1,43)=4.98$; $p=0.031$], ethanol concentration as a repeated measure [$F(3,129)=9.57$; $p<0.001$], and context exposure × ethanol concentration interaction [$F(3,129)=0.40$; $p=0.751$]. No other effects or interactions were noted. However, the ANOVA for total fluid intake only indicated a significant effect of ethanol concentration [$F(3,129)=3.84$; $p=0.011$].

Fig. 3 shows the 4-day average intake of ethanol for each concentration of ethanol in CON and ETOH withdrawn rats that were only exposed to the fear conditioning apparatus. As can be seen in this figure, the intake of ethanol (g/kg) was not significantly different between the two groups. Although there was no effect of diet treatment, the ethanol intake depended on the ethanol concentration [$F(3,60)=7.58$; $p<0.001$] even though no significant interaction between diet treatment and

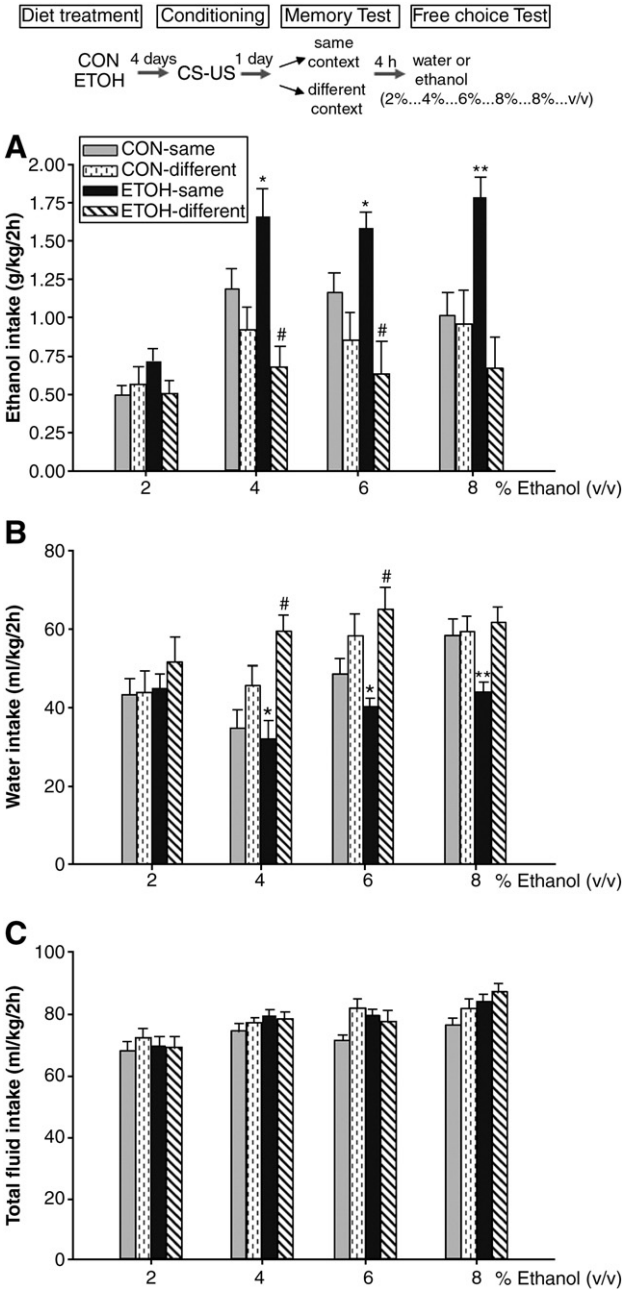


Figure 2 Effect of contextual fear memory retrieval on subsequent ethanol intake in a free-choice paradigm between water and increasing ethanol concentrations (v/v) in ETOH withdrawn rats. Rats were treated for 14 days with ethanol (6% v/v) or control liquid diet. On the 4th day of withdrawal, all animals were subjected to a contextual fear conditioning and were evaluated 24 h later in the training context (same) or in a different context (different). The free choice began 4 h after the end of the fear memory test. Each bar represents ethanol intake (A), water intake (B) and total fluid intake (C) collapsed over days for each ethanol concentration (4 days from 2% to 6% and 8 days at 8%) during a two-hour limited access to a two-bottle choice. Data are the means ± S.E.M. of ethanol intake, expressed in grams of ethanol per kilogram of body weight; water intake, expressed in ml of water per kilogram of body weight and total fluid intake, expressed in ml of fluids per kilogram of body weight. *$p<0.05$ vs. the groups tested in the different context. **$p<0.05$ significantly different from all other groups at this ethanol concentration. #$p<0.05$ vs. CON-same.

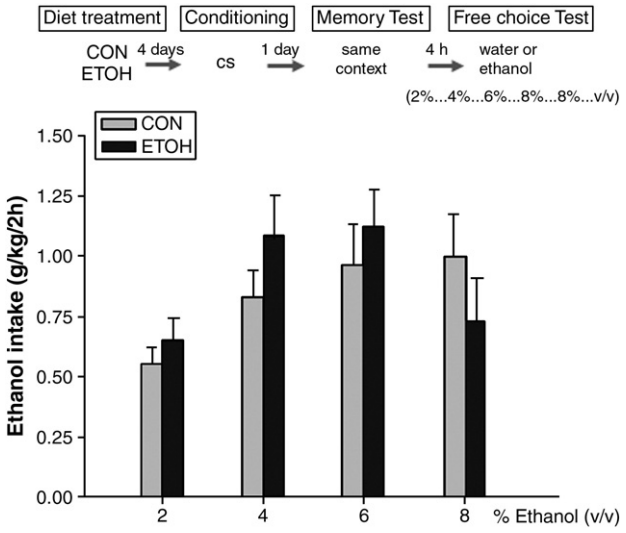


Figure 3 Effect of ethanol withdrawal on subsequent ethanol intake in a free-choice paradigm between water and increasing ethanol concentrations (v/v). Rats were treated for 14 days with ethanol (6% v/v) or control liquid diet. On the 4th day of withdrawal, all animals were exposed to the conditioning chamber without shock delivery. Twenty-four hours later, rats were re-exposed to the same chamber and the free choice began, 4 h after. Data are the means ± S.E.M. of ethanol intake, expressed in grams of ethanol per kilogram of body weight. Each bar represents ethanol intake collapsed over days within each ethanol concentration (4 days from 2% to 6% and 8 days at 8%) during a two-hour limited access to a two-bottle choice.

ethanol concentration was detected. The ANOVA for water intake only indicated a significant effect for ethanol concentration [$F(3,60)=7.92$; $p<0.001$], with no differences between both groups being noted when total fluid intake was analyzed (data not shown).

A progressive increase in body weight was observed within 20 days of the free-choice procedure. No differences in body weight gain were found between CON and ETOH withdrawn rats (the average group body weight gains were 33 ± 2, 29 ± 1 and 29 ± 3 for CON non-fear trained, CON-same and CON-different, respectively and, 30 ± 2, 33 ± 3 and 34 ± 2 for ETOH non-fear trained, ETOH-same and ETOH-different).

In an additional experiment, we examined if an intense fear reaction could promote increased ethanol consumption in CON animals. Fig. 4 shows the mean percentage of time spent freezing in CON animals that were subjected to different schedules of shock intensity during contextual fear conditioning. A one-way ANOVA indicated a significant effect of the intensity of fear conditioning [$F(2,27)=8.26$; $p=0.002$] with post-hoc comparisons ($p<0.05$) revealing that both stronger fear conditioning trainings (3 shocks of 0.7 mA and 5 shocks of 0.4 mA) promoted a higher freezing response in comparison to that induced by 3 shocks of 0.4 mA in CON rats. Moreover, the freezing levels for the group of animals subjected to a strong conditioning did not differ from each other, and were equivalent to those displayed by ETOH withdrawn rats in the same context. In order to maintain the same time exposure to the context during fear conditioning training, CON animals were trained using 3 shocks of 0.7 mA to examine the possible effect of an enhanced fear reaction on subsequent ethanol intake.

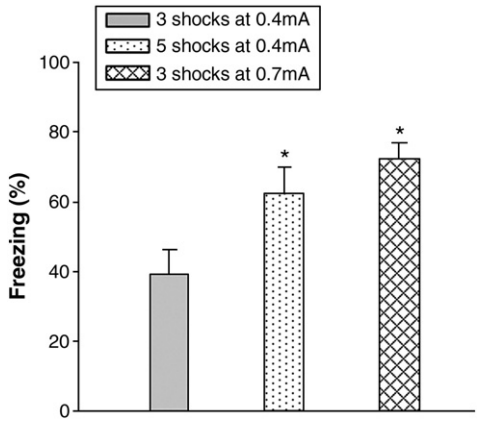


Figure 4 Effect of strong fear conditioning procedure on fear learning in CON rats. Rats were treated with control liquid diet. On the 4th day after liquid diet removal, one group of rats was fear conditioned as previously described (3 shocks of 0.4 mA) and the other two received either 3 shocks of 0.7 mA or 5 shocks of 0.4 mA. Twenty-four hours later, all animals were evaluated in the training context. Values represent the means ± S.E.M. of the percentage of time spent freezing during the 10 min test period. *$p<0.05$ vs. the group conditioned with 3 shocks of 0.4 mA.

Fig. 5 illustrates the effect of an intense fear response on subsequent ethanol consumption in CON rats. As can be seen, an intense fear reaction failed to promote the increase in ethanol intake as compared to that observed following a moderate fear in the CON group. A two-way ANOVA indicated a significant effect of ethanol concentration as a repeated measure [$F(3,57)=8.23$; $p<0.001$]. A two-way ANOVA for water intake revealed only a

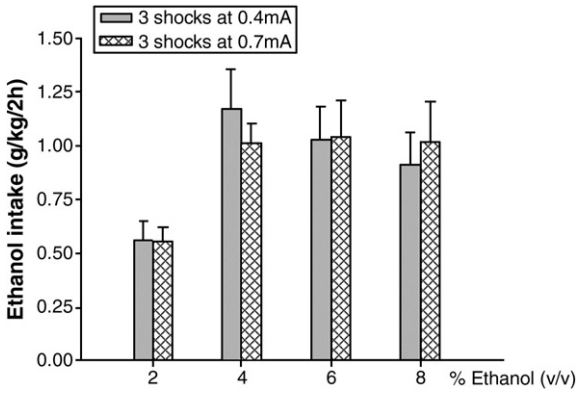


Figure 5 Effect of contextual fear memory retrieval after a strong fear conditioning on subsequent ethanol intake in a free-choice paradigm between water and increasing ethanol concentrations (v/v) in CON rats. Rats were treated with control liquid diet. On the 4th day after liquid diet removal, one group of rats was fear conditioned with 3 shocks of 0.4 mA or 3 shocks of 0.7 mA, and 24 h later evaluated in the training context. The free choice began 4 h after the end of the fear memory test. Data are the means ± S.E.M. of ethanol intake, expressed in grams of ethanol per kilogram of body weight. Each bar represents ethanol intake collapsed over days for each ethanol concentration (4 days from 2% to 6% and 8 days at 8%) during a two-hour limited access to a two-bottle choice.

significant effect for ethanol concentration [$F(3,57)=7.17$; $p<0.001$] (data not shown). No differences between these groups were noted for any ethanol concentration range when total fluid intake was analyzed (data not shown).

Fig. 6 depicts the effect of DCS (5 mg/kg) on extinction of conditioned freezing in ETOH rats. As expected, the mean percentage of time spent freezing in trial 2 was reduced in both ETOH groups compared with trial 1 indicating the emergence of fear extinction. However, the animals injected with DCS after the end of trial 1 of extinction froze less than the SAL group during the second extinction trial. A two-way ANOVA revealed significant effect of trial sessions as repeated measure [$F(1,16)=116.36$; $p<0.001$] and of trial sessions × DCS interaction [$F(1,16)=7.78$; $p=0.013$]. The post-hoc confirmed that the percentage time of freezing from both groups of rats in trial 2 were significantly lower than their respective values exhibited on the prior trial ($p<0.001$). The post-hoc also indicated that the freezing levels exhibited in trial 2 by rats injected with DCS were significantly lower compared with rats injected with SAL ($p<0.05$). The percentage time of freezing exhibited by the NO-EXT group did not differ from that shown by the EXT groups during the first extinction trial (data not shown). Importantly, the freezing levels exhibited by EXT-SAL rats during the second extinction trial were equivalent to those displayed by CON rats during the first extinction trial (see Fig. 1).

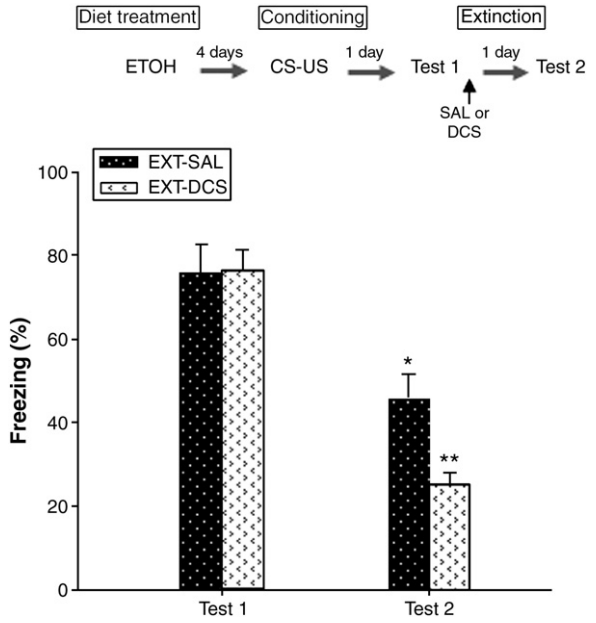


Figure 6 Effect of DCS (5 mg/kg) on extinction of conditioned freezing in ETOH withdrawn rats. Rats were treated for 14 days with ethanol (6% v/v) liquid diet. On the 4th day of withdrawal, all animals were subjected to a contextual fear conditioning and 24 h later the freezing response to the training context was evaluated for 10 min. Immediately after this memory test (test 1 or first extinction trial), half of the animals were injected with saline (EXT-SAL) and the other half with DCS 5 mg/kg (EXT-DCS). The following day, all rats were re-exposed to the training context and the freezing response was again evaluated for 10 min (test 2 or second extinction trial). Values represent the means ± S.E.M. of the percentage of time spent freezing during the 10 min test period. *$p<0.01$ vs. the respective value at test 1. **$p<0.05$ vs. all other groups.

Fig. 7 illustrates the effect of fear extinction with and without DCS administration on subsequent ethanol intake in ETOH rats in comparison with ETOH rats not subjected to extinction training. A significant increase in ethanol drinking was induced by the recall of fear memory only in the ETOH rats not subjected to extinction training. Regardless of DCS treatment, extinction training attenuated the increase in subsequent ethanol drinking induced by fear memory retrieval. A two-way ANOVA revealed a significant effect of extinction [$F(2,72)=12.69$; $p<0.001$], ethanol concentration as a repeated measure [$F(3,72)=27.57$; $p<0.001$] and ethanol concentration × extinction interaction [$F(6,72)=4.57$; $p<0.001$]. Further post-hoc indicated that NO-EXT rats drank more ethanol in comparison with the groups subjected to extinction training. This difference was statistically significant when animals were offered ethanol concentrations of 4 to 8% v/v ($p<0.05$). No differences in ethanol intake were detected between EXT-SAL and EXT-DCS groups.

3.2. Experiment 2

The main findings from experiment 1, showing enhanced contextual fear learning in ethanol withdrawn rats, were replicated in the current experiment (data not shown).

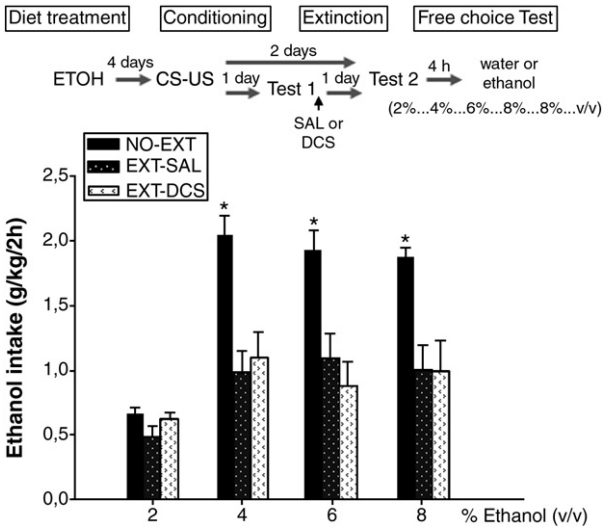


Figure 7 Effect of fear memory extinction on subsequent ethanol intake in a free-choice paradigm between water and increasing ethanol concentrations (v/v) in ETOH withdrawn rats. Rats were treated for 14 days with ethanol (6% v/v) liquid diet. On the 4th day of withdrawal, all animals were subjected to a contextual fear conditioning. Twenty-four hours later, some animals were evaluated in the training context over 10 min and immediately injected with SAL or DCS (5 mg/kg). The remaining trained rats were left undisturbed in their home cages (NO-EXT). The following day, all rats were re-exposed to the training context and the freezing response was evaluated for 10 min. The free choice between ethanol and water began approximately 4 h after the end of this memory test. Data are the means ± S.E.M. of ethanol intake, expressed in grams of ethanol per kilogram of body weight. Each bar represents ethanol intake collapsed over days for each ethanol concentration (4 days from 2% to 6% and 8 days at 8%) during a two-hour limited access to a two-bottle choice. *$p<0.05$ significantly different from all other groups at this ethanol concentration.

Next, we examined the effects of memory retrieval on c-Fos protein expression. The, cell-count data for c-Fos expression in all brain regions examined are listed in Table 1. Three general patterns of results were found:

a) regions showing a main effect of diet treatment [Cg1, PrL, IL, NAcc shell, LSV, CA1, LA, CeA and VTA]. In all nine regions, regardless of context exposure, ETOH withdrawn animals had a significantly higher number of c-Fos positive cells than CON animals.
b) regions showing significant effects of both diet treatment and test condition [BLA and VLPAG]. In these regions, ETOH withdrawn animals had a significantly higher number of c-Fos positive cells than CON animals. In addition, the c-Fos positive cell counts were significantly higher in ETOH withdrawn rats tested in the context previously paired with shock ($p<0.05$).Fig. 8 shows representative photomicrographs of c-Fos immunoreactivity in the basolateral (BLA) and central amygdaloid nucleus (CeA) after contextual fear memory test from control rats and ethanol withdrawn rats.
c) regions showing significant effects of diet treatment, test condition and diet treatment × test condition interaction [DG and DMPAG]. In these regions, ETOH withdrawn animals tested in the same context of training had significantly higher number of c-Fos positive cells than the rest of the groups. The c-Fos positive cell counts were significantly higher in ETOH withdrawn rats tested in the context previously paired with shock ($p<0.05$). In addition, c-Fos expression in CON and ETOH groups tested in the different environment did not differ from each other.

4. Discussion

In the current study, we examined whether the retrieval of contextual fear memory affected the subsequent ethanol consumption in a two-bottle choice paradigm in ETOH-dependent rats. A similar pattern of ethanol intake was found between CON and ETOH withdrawn rats that were not subjected to previous fear training. Thus, under our experimental conditions, the interruption of the chronic ethanol diet treatment had no effect *per se* on subsequent ethanol consumption. This lack of effect on ethanol intake using this withdrawal schedule was expected, since previous studies had shown that only exposure to repeated cycles of intoxication and withdrawal from ethanol or withdrawal following prolonged exposures to alcohol vapor (Sommer et al., 2008) are effective conditions to induce increased voluntary ethanol intake in genetically non-selected rats (Roberts et al., 2000; Breese et al., 2005).

The behavioral results obtained in the fear conditioning experiment confirm our previous findings showing that ethanol withdrawn animals exhibited more freezing behavior than CON rats, when exposed to the context previously paired with shock (Bertotto et al., 2006). In addition, low and comparable levels of freezing were detected between previously shocked CON and ETOH withdrawn rats that were subsequently exposed to the non-associated environment. Thus, the potentiation of freezing behavior displayed by withdrawn rats was only evident in the associated environment, suggesting that the abrupt discontinuation of ethanol ingestion facilitated the formation of contextual fear memory. Next, we examined the influence of the recall of this context-dependent fear memory on ethanol intake in ETOH withdrawn rats. The current findings in the free-choice paradigm showed that the retrieval of the aversive memory induced a persistent increase in voluntary ethanol intake in ETOH withdrawn rats compared to CON rats. The amount consumed was significantly different for the 4% ethanol solution concentration and persisted throughout the duration of the drinking test sessions.

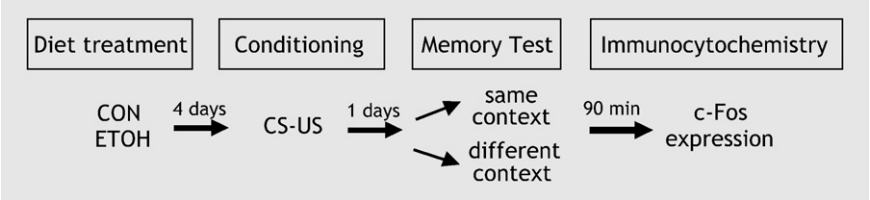
The values of ethanol consumption induced by recall in ETOH withdrawn rats were lower than those reported following stress exposure in withdrawn animals (Chester et al., 2004; Little et al., 1999; Sillaber et al., 2002; Sommer et al., 2008). However, in most of these studies, repeated exposure to stressors, such as forced swimming, footshock or restraint, was necessary to increase ethanol drinking (Breese et al., 2005; Sillaber et al., 2002; Sommer et al., 2008). Our results add to these findings, by showing that a single retrieval session of fear memory can enhance voluntary ethanol intake in ETOH withdrawn rats.

In contrast, the recall of aversive memory did not enhance voluntary ethanol consumption in CON rats. Moreover, the pattern of ethanol intake in CON rats did not differ from those exhibited by both previously fear trained CON and ETOH groups that were subsequently exposed to a non-associated environment. These results discard the possibility that the increase observed in ETOH withdrawn animals could have been caused by the prior shock treatment of these animals.

Moreover, the increased ethanol consumption induced following retrieval in withdrawn rats was not related to changes in thirst, since the total liquid intake was virtually identical among all groups of animals. Although the total food intake was not recorded, similar weight gains were observed among all groups of animals.

Control rats that had been previously subjected to a strong fear training protocol exhibited a similar freezing response to that exhibited by ETOH withdrawn rats during the retention test. However, the recall of such robust memory in CON animals did not result in an enhancement of ethanol consumption. The fact that the retrieval of a strong fear memory in CON rats does not result in increased ethanol consumption strengthens the view that enhanced ethanol intake is not merely dependent on the expression of an elevated fear response. When ETOH rats were subjected to the extinction training, they do not longer exhibit the enhanced ethanol intake. Taken together, this evidence suggests that the retrieval of fear memory only increases ethanol consumption in rats with a previous history of ethanol dependence. The facilitation of extinction or the disruption of reconsolidation of both, fear memories and drug-related memories, has been suggested as potential treatments for neuropsychiatric disorders, such as post-traumatic stress and drug addiction (Ressler et al., 2004; von der Goltz et al., 2009). Previous studies showed that extinction training combined with DCS can facilitate extinction of conditioned responses induced by a cue previously associated with drug use, indicating its potential utility as an adjunct to exposure-based therapies to prevent relapse. In the current study, when ETOH rats were subjected to extinction training, they did not exhibit the enhanced ethanol intake any longer. Although DCS facilitated the extinction of fear memory, it failed to promote a further reduction of alcohol intake in comparison to that observed by

Table 1 Cell-count data for c-Fos expression in all brain regions examined after contextual fear memory retrieval. Rats were treated for 14 days with ethanol (6% v/v) or control liquid diet. On the 4th day of withdrawal, all animals were subjected to a contextual fear conditioning and evaluated 24 h later in the training context (same) or in a different context (different) before being sacrificed to examine the c-Fos expression at 90 min after the end of the memory test. The values represent the number of c-Fos positive nuclei in 0.16 mm2 (mean ± S.E.M.). The ANOVA effects for diet treatment, test condition and diet treatment × test condition interaction are shown in the last column of the table, the significant effects are in bold.



Area	CON-same	CON-different	ETOH-same	ETOH-different	2-way ANOVA
CG1	43 ± 7	45 ± 9	68 ± 13	64 ± 9	$F(1,25)$ - 4.37 $p=0.047$
					$F(1,25)=0.02$ $p=0.894$
					$F(1,25)=0.07$ $p=0.786$
M2	74 ± 16	58 ± 12	61 ± 18	63 ± 9	$F(1,24)=0.06$ $p=0.808$
					$F(1,24)=0.25$ $p=0.619$
					$F(1,24)=0.29$ $p=0.536$
PrL	47 ± 4	29 ± 5	74 ± 10	72 ± 15	$F(1.25)=11.52$ $p=0.002$
					$F(1,25)=0.88$ $p=0.356$
					$F(1,25)=0$ 60 $p=0.444$
IL	42 ± 7	27 ± 6	63 ± 15	58 ± 5	$F(1,25)=6.82$ $p=0.015$
					$F(1,25)=1.10$ $p=0.304$
					$F(1,25)=0.29$ $p=0.594$
N. Acc core	55 ± 15	49 ± 10	74 ± 23	55 ± 13	$F(1,24)=0.65$ $p=0.428$
					$F(1,24)=0.68$ $p=0.418$
					$F(1,24)=0.17$ $p=0.683$
N. Acc shell	45 ± 12	52 ± 11	106 ± 10	102 ± 8	$F(1,23)=28.00$ $p<0.001$
					$F(1,23)=0.03$ $p=0.867$
					$F(1,23)=0.27$ $p=0.610$
LSV	72 ± 18	62 ± 7	100 ± 11	111 ± 11	$F(1,27)=10.21$ $p=0.003$
					$F(1,27)=0.01$ $p=0.937$
					$F(1,27)=0.79$ $p=0.381$
CPU	52 ± 18	59 ± 15	49 ± 15	41 ± 11	$F(1,22)=0.54$ $p=0.468$
					$F(1,22)<0.01$ $p=0.984$
					$F(1,22)=0.23$ $p=0.620$
BLA	22 ± 4	15 ± 4	69 ± 11 *	36 ± 8	$F(1,26)=18.89$ $p<0.001$
					$F(1,26)=6.76$ $p=0.015$
					$F(1,26)=2.80$ $p=0.106$
LA	37 ± 10	28 ± 5	68 ± 11	43 ± 12	$F(1,20)=6.51$ $p=0.019$
					$F(1,20)=3.35$ $p=0.082$
					$F(1,20)=0.69$ $p=0.415$
CeA	31 ± 7	19 ± 6	87 ± 17	70 ± 19	$F(1,25)=18.12$ $p<0.001$
					$F(1,25)=1.29$ $p=0.267$
					$F(1,25)=0.06$ $p=0.815$
DG	20 ± 8	21 ± 3	64 ± 14 *	26 ± 5	$F(1,27)=9.22$ $p=0.005$
					$F(1,27)=5.30$ $p=0.029$
					$F(1,27)=5.73$ $p=0.024$
CAl	13 ± 6	17 ± 6	70 ± 24	39 ± 14	$F(1,27)=7.50$ $p=0.011$
					$F(1,27)=0.93$ $p=0.344$
					$F(1,27)=1.50$ $p=0.232$
CA2	10 ± 2	24 ± 11	35 ± 7	32 ± 20	$F(1,24)=1.70$ $p=0.205$
					$F(1,24)=0.21$ $p=0.651$
					$F(1,24)=0.38$ $p=0.541$
CA3	10 ± 3	7 ± 3	12 ± 5	21 ± 8	$F(1,27)=1.96$ $p=0.173$
					$F(1,27)=0.26$ $p=0.612$
					$F(1,27)=0.95$ $p=0.338$
VTA	19 ± 7	18 ± 8	88 ± 27	36 ± 7	$F(1,25)=11.02$ $p=0.003$

Table 1 (*continued*)

Area	CON-same	CON-different	ETOH-same	ETOH-different	2-way ANOVA
					$F(1,25)=4.10$ $p=0.054$
					$F(1,25)=3.77$ $p=0.064$
DMPAG	33±7	38±4	80±11*	36±8	$F(1,23)=8.83$ $p=0.007$
					$F(1,23)=6.71$ $p=0.016$
					$F(1,23)=10.61$ $p=0.003$
VLPAG	45±11	32±7	81±18*	46±15	$F(1,25)=4.48$ $p=0.044$
					$F(1,25)=5.09$ $p=0.033$
					$F(1,25)=0.83$ $p=0.369$

* $p<0.05$ vs. all other groups.

the behavioral intervention alone. Given that DCS was administered after the first memory test and the free choice began after the second test, the absence of effect of DCS on alcohol drinking could be explained as the result of a floor effect due to the effective extinction paradigm used. Interestingly, it has been shown that the same low dose of DCS used in the present study facilitated the extinction of alcohol-seeking behavior and subsequently reduced the resumption of an extinguished operant response (Vengeliene et al., 2008). Therefore, the potential benefits of DCS in conjunction with extinction training on the attenuation of subsequent alcohol drinking cannot be ruled out. Recently, Lee et al. (2009) have shown that DCS potentiated the reconsolidation of cue-cocaine memory with the subsequent enhancement of cocaine seeking, demonstrating that, under certain conditions, DCS can increase rather than reduce the subsequent memory expression.

In the current study, we examined the expression of c-Fos induced by the retrieval of conditioned fear, in order to investigate the neurobiological substrates of the enhanced fear reaction that could be potentially involved in the subsequent increased ethanol intake in ETOH withdrawn rats. To address this goal, all animals from both CON and ETOH withdrawn groups received the same fear conditioning

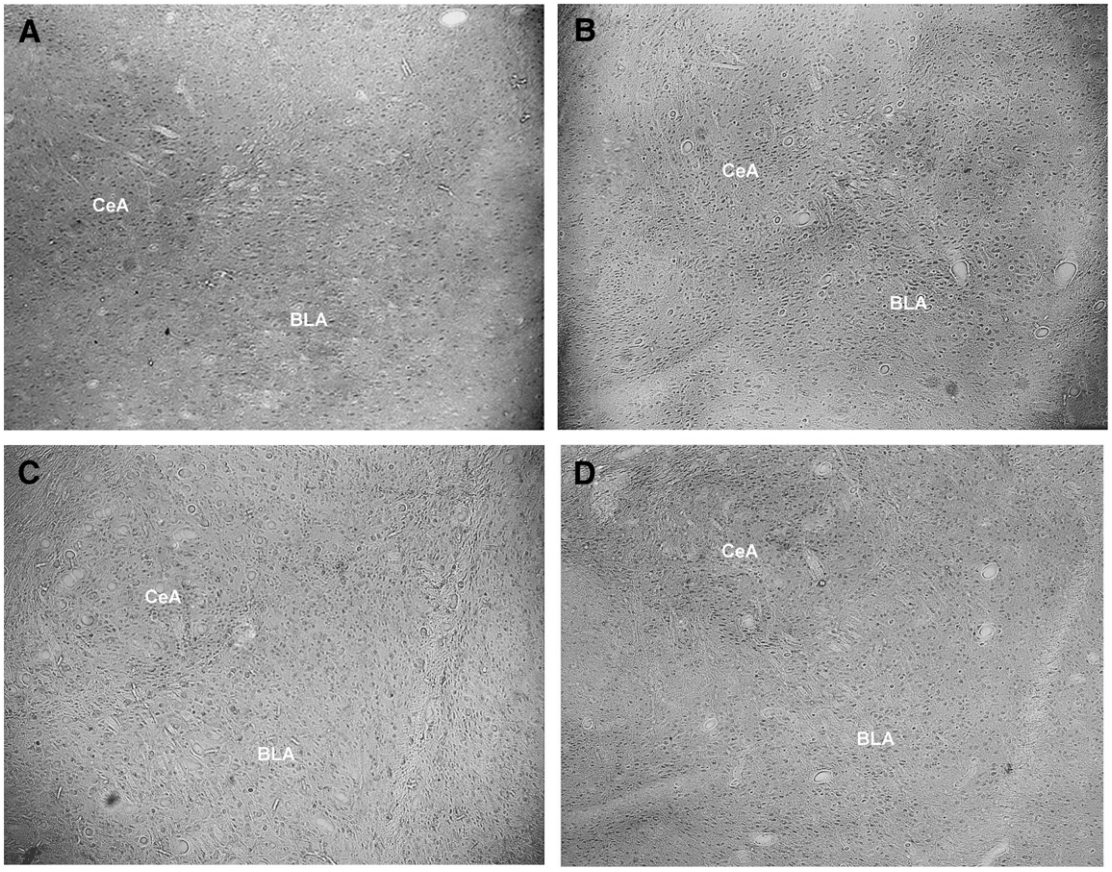


Figure 8 Representative photomicrographs (100×) of c-Fos immunoreactivity (dark dots) in the basolateral (BLA) and central amygdaloid nucleus (CeA) after contextual fear memory test from control rats tested in the same context (A) or in a different context (C) and ethanol withdrawn rats tested in the same context (B) or in a different context (D).

training, with the retention test being performed the following day in the same context or in a different context. CON and ETOH withdrawn non-fear trained groups were not included, because withdrawal from chronic ethanol did not affect *per se* subsequent ethanol intake.

For CON rats, comparable levels of c-Fos immunoreactivity were found in all brain regions examined, regardless of the context used for memory retention. Therefore, the increased freezing response in the associated environment was not manifested by changes in c-Fos expression in any of the brain regions evaluated in CON rats. Previous studies in rodents have demonstrated that memory retrieval after contextual fear conditioning was accompanied by an increase in c-Fos expression in a number of brain structures. For instance, Beck and Fibiger (1995) showed a widespread increase in c-Fos expression in about 50 brain regions, including the cortical and subcortical areas, following the exposure to the context previously paired with shock. An increased c-Fos induction was also reported after contextual memory retrieval in the hippocampus (Milanovic et al., 1998; Strekalova et al., 2003) and in the amygdala (Milanovic et al., 1998; Scicli et al., 2004). This apparent discrepancy between our data and those previously reported may be explained by differences in the training procedures (various conditioning sessions vs. one), in the intensity and the number of footshocks, the length of time exposure during the retention test and the animal species used for fear conditioning (rats vs. mice). For instance, using a strong protocol for fear conditioning, increased c-Fos protein levels were found in the central and basolateral amygdalar nuclei (Scicli et al., 2004) following 30 but not for 8 min of reexposure to the chamber previously paired with shocks, and also in the VLPAG after the same prolonged period of time during the retention test (Carrive et al., 1997). Although CON animals displayed conditioned freezing during the 10 min retention test with the present protocol, these levels of freezing were noticeably lower than those reported in the above studies. Taken together, it could be speculated that a robust training paradigm or prolonged exposure to the conditioned context, or both, are necessary to induce increased c-Fos expression in different brain regions following the retention test in rats.

In the present study, we did not find any significant effects of memory retrieval on c-Fos expression between both ETOH withdrawn groups in the cingulate cortex area 1 (CG1), prelimbic and infralimbic cortices (PrL and IL, respectively), nucleus accumbens shell (NAc-Shell), ventro lateral septum (LSV), the CA1 field of hippocampus, lateral and central amygdaloid nucleus (LA and CeA) or the ventro tegmental area (VTA). However, c-Fos expression levels in these brain areas in ETOH rats, with or without retrieval, were significantly higher compared to all CON rats, implying that the absence of any difference between both ETOH groups with respect to the number of c-Fos positive cells showing neuronal activation in these regions cannot be attributed to the retrieval of contextual fear memory. Instead, results can be readily attributable to ETOH withdrawal combined with fear training. A similar pattern of c-Fos expression has been shown by Hansson et al. (2008) following an acute ethanol administration in alcohol naïve rats. In this same study, ethanol dependence was associated with a suppressed c-Fos response to acute ethanol challenge in brain areas involved in ethanol preference and seeking. Although these regional changes were attenuated after chronic ethanol intake, it is possible that they may account for altered stress reactivity during withdrawal. Thus, a plausible explanation for these results is based on the fact that ETOH withdrawal resulted in a sensitized response to aversive experiences, which was able to develop as a consequence of prior shock training and ETOH withdrawal. In agreement with this viewpoint, an aversive experience during ETOH withdrawal induced higher levels of c-Fos expression in the medial prefrontal, frontal and cingulate cortices than those provoked by either ethanol withdrawal or the aversive challenge alone (Knapp et al., 1998). In addition, a repeated experience of withdrawal from chronic ethanol administration did not exacerbate withdrawal-induced anxiety, but was accompanied by elevated c-Fos protein expression in the BLA and CeA nuclei of amygdala, the CA3 field of hippocampus, the nucleus accumbens core and dorsolateral PAG (Borlikova et al., 2006). All these above findings suggest that the presentation of an unconditioned stressor or conditioned freezing following the interruption of chronic ethanol administration can elicit increased neuronal activation in the brain structures that are also involved in conditioned fear responses. However, we are not able to discard the possibility that the potential increase in c-Fos protein expression induced by context-dependent memory retrieval could have been masked by the activation induced by a novel exposure in previously shocked withdrawn animals.

The most important result of the current study is the finding that context-dependent memory retrieval induced a selective increase in c-Fos expression in BLA and VLPAG only in ETOH withdrawn rats, in comparison to that induced by the exposure to the non-associated environment in dependent animals. In addition, a significant and robust c-Fos expression was induced by contextual fear memory recall in DG and in DMPAG only in ETOH withdrawn rats.

A substantial amount of evidence supports the notion that the amygdala is a key brain structure in the modulation of emotional behavior (Davis, 1992; LeDoux, 2000; Maren, 2005). In studies involving fear conditioning, the amygdala has been shown to participate in the acquisition, storage and retrieval of fear memory (Fanselow and LeDoux, 1999; Fendt and Fanselow, 1999; LeDoux et al, 1990; Miserendino et al., 1990). Furthermore, a large body of evidence supports the idea that the BLA is involved in emotional pavlovian learning, neural plasticity and storage of emotional memory. Consistent with the crucial role of BLA in learned fear, reexposure to the conditioned context was accompanied by an increased c-Fos expression in BLA in ETOH withdrawn rats. Related to this, previous research from our laboratory has demonstrated that a history of stress or withdrawal from hypnotic-sedative drugs results in the reduction of feed-back GABAergic inhibition in BLA projection neurons, leading to neuronal hyperexcitability and increased plasticity that facilitates fear learning (Isoardi et al., 2004, 2007; Rodríguez Manzanares et al., 2005). The results obtained in the present work, showing a strong activation in BLA following the retrieval test for conditioned fear, provide further evidence suggesting a role of this amygdaloid nucleus in the retrieval of emotional memories during withdrawal from ETOH.

The hippocampus has been involved in the acquisition, consolidation and temporary storage of contextual fear (Anagnostaras et al., 2001; Biedenkapp and Rudy, 2004;

Fanselow, 2000; Holland and Bouton, 1999; Maren and Holt, 2000). In the present study, a marked induction of c-Fos protein was only found in the DG from withdrawn rats. Related to this, a recent report proposed that the DG plays an important role in the encoding of memories of a psychologically stressful event (Reul and Chandramohan, 2007). Moreover, Hernández-Rabaza et al. (2008), using two classical paradigms of associative learning, demonstrated that the dorsal DG participates in the acquisition and expression of associations between contexts and internal states, with these being either hedonic or aversive. Furthermore, the DG is a region of the hippocampus that is heavily influenced by the BLA. In fact, the BLA can modulate neural plasticity in the DG, with this effect being proposed as a mechanism whereby emotional stimuli affect hippocampal memory processes (Abe, 2001; Akirav and Richter-Levin, 2002; Paré, 2003). Therefore, the increased neuronal activity seen in DG may well reflect a role of this brain area in the expression of the contextual fear response in ETOH withdrawn rats.

The periaqueductal central gray (PAG) plays a crucial role in the mediation of defensive reactions, including the freezing induced by conditioned fear (Fendt and Fanselow, 1999). However, the ventral and dorsal portions of the PAG have different roles in the defensive reactions. Whereas the VPAG seems to be involved in the occurrence of conditioned freezing behavior, the neural substrates of fear in the DPAG appears to be more associated with the active forms of defensive behavior. Conditioned fear to a context was shown to be associated with freezing and increased c-Fos expression in the PAG, with this increase being greater in the ventrolateral PAG column than in the lateral, dorsolateral or dorsomedial columns (Carrive et al., 1997). In agreement with these findings, we showed an increased c-Fos protein expression in VLPAG induced by the retrieval of fear memory in withdrawn rats. Therefore, the increased neuronal activity reflected by increased c-Fos induction in VLPAG may well reflect a role in the expression of the fear response during withdrawal from ethanol. In addition, we also detected increased c-Fos expression in the DMPAG, but only in ETOH withdrawn rats. It has been previously shown that the electrical or chemical stimulation of the dorsal half of the periaqueductal gray leads to a vigorous defensive response characterized by alertness, freezing and escape behavior (Brandão et al., 1999; Vianna et al., 2001). Moreover, it has been reported that freezing-provoking stimulation caused increases in c-Fos expression in the DMPAG, while escape-provoking stimulation led to increases at both the DMPAG and DLPAG (Vianna et al., 2003). Recently, the abrupt discontinuation of an ethanol diet treatment resulted in a decreased stimulation threshold for freezing and escape, following electrical stimulation in DPAG (Cabral et al., 2006), indicating that ethanol withdrawal sensitizes the substrates of fear at the level of dorsal periaqueductal gray.

In summary, the brain areas showing an increased c-Fos expression in the present study, following retrieval of contextual fear memory in withdrawn animals, were those suggested to be implicated in the modulation of motivation and of emotional states. Among these areas, the BLA seemed to be the key structure in gating memory formation in other brain structures, such as in the hippocampus of these animals. Finally, our study indicated that retrieval of fear memory affected ethanol consumption in ETOH withdrawn rats, and that the amygdala may have been involved in this effect.

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Contributors

M.E. Bertotto carried out most of the experiments, undertook the statistical analyses and edited the manuscript. D.F. Bussolino collaborated with the immunocytochemical experiments. I.D. Martijena designed this study, analyzed the data and wrote the manuscript. V.A. Molina contributed to the interpretation of data and to the writing of the manuscript.

Conflict of interest

The authors have no conflicts of interest to report, nor any involvement to disclose, financial or otherwise, that may bias the conduct, interpretation or presentation of this work.

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