Impaired fear inhibitory properties of GABA$_A$ and μ opioid receptors of the dorsal periaqueductal grey in alcohol-withdrawn rats

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The dorsal periaqueductal grey (DPAG) is a midbrain region that plays a fundamental role on the expression of the anxiety and fear-related responses. Increased fear and anxiety levels are the most frequent symptoms present in the alcohol withdrawal syndrome. This study aims to assess whether GABA and opioid receptors of the DPAG could be implicated in the expression of the conditioned fear state elicited in alcohol withdrawn-rats. For this purpose, we used the fear-potentiated startle (FPS) procedure. Drugs used were the selective GABA$_A$ agonist muscimol (1 nmol/0.2 μl) and the predominantly μ opiate receptors agonist morphine (10 nmol/0.2 μl). Exposure to aversive cues of the elevated-plus-maze (EPM) was used in order to validate the influence of alcohol withdrawal on emotionality. Data from FPS pointed out to an anxiogenic-like profile of withdrawal, a result sustained by the data collected from the EPM test. Muscimol and morphine showed their well-known anxiolytic-like profile, decreasing the fear-potentiated startle (FPS) amplitude in control subjects. However, the drugs had no effect on the levels of fear evoked in rats pre-treated with and withdrawn from alcohol. It is suggested that this lack of effect was possibly due to the desensitization of the GABA$_A$ receptors, as well as by the decrease on the responsiveness of the functions of μ opioid receptors, resulting from chronic administration of ethyl alcohol. These findings shed light on some aspects of the anxiety-like behavior elicited during alcohol withdrawal bringing new information on the influence of GABA and opioid receptors of the DPAG on the expression of unconditioned and conditioned fear responses.

Key words: alcohol withdrawal, DPAG, GABA, opiates, fear-conditioning

INTRODUCTION

Chronic alcohol consumption leads to tolerance and dependence (Fadda and Rossetti 1998, Koob 1998). Withdrawal from acute treatment with high alcohol doses (hangover) induces behaviors that resemble those observed on withdrawal from chronic alcohol exposure (Varlinskaya and Spear 2004, Doremus-Fitzwater and Spear 2007). In both conditions, the abrupt cessation from alcohol intake elicits a multitude of physical and emotional symptoms, particularly high levels of anxiety in humans or anxiety-like behavior in rodents (Kushner et al. 1990, Schuckit and Hesselbrock

1994, Kliethermes 2005). In addition, exaggerated anxiety-like behavior in alcohol-withdrawn animals when exposed to a mild stress that does not induce behavioral disturbances in control animals has been noted (Valdez et al. 2002).

Changes in GABA levels, the primary inhibitory neurotransmitter in the brain and the major target of alcohol, underlie the expression of dependence and withdrawal from alcohol. Consumption of alcohol leads to central nervous system depression as a consequence of enhancing GABAergic neurotransmission (Chastain 2006). The action of continued alcohol use allows a series of neurobiological alterations to occur in order to compensate for its depressant action (Grant and Lovinger 1995, Grobin et al. 1998, Chandler 2003). Alcohol intake also interferes with endogenous opioid mechanisms, promoting a dose-dependent increase in

the firing of dopaminergic neurons in the mesolimbic pathway (Gessa et al. 1985 , Gainoulakis 1996). Lower doses of morphine increase and higher doses of the opiate decrease alcohol consumption (Ulm et al. 1995). Moreover, opioid antagonists such as naloxone and naltrexone have been shown to decrease alcohol consumption under various experimental conditions (Brown and Holtzman 1979, Altshuler et al. 1980, Reid and Hunter 1984, Doyle and Samson 1985). In fact, clinical studies have shown the opioid receptor antagonist naltrexone to be effective in the treatment of alcohol dependence (Latt et al. 2002). The cross interaction between alcohol and opioids can also be demonstrated by medical interventions in alcoholic opioid-dependent patients, in whom the addition of low doses of naltrexone to methadone taper was able to reduce withdrawal symptoms (Mannelli et al. 2011).

Neural activation of the dorsal aspects of the periaqueductal grey matter (DPAG) elicits several behavioral and somatic manifestations characteristic of high fear states (Brandao et al. 1999, Vianna et al. 2001a). These changes seem very much like those observed in animals facing predators or dangerous environmental stimuli (Brandao et al. 1999, Vianna et al. 2001a,b, Graeff 2004) and even those reported during alcohol withdrawal (Johnston et al. 1991, Munafo et al. 2005). Direct injections of high doses of morphine or GABA inhibitors such as bicuculline or semicarbazide into this midbrain region elicit a fearful hyperactivity. On the other hand, local injections of low doses of morphine or the GABA$_A$ agonist muscimol have the opposite effects, i.e. inhibitory effects on this defense reaction (Jenck et al. 1983, Brandao et al. 1985, 2005, Anseloni et al. 1999). In our laboratory we have consistently demonstrated that rats under withdrawal from drugs of abuse, including ethyl alcohol, presented increased neural midbrain activation (Cabral et al. 2006, Fontanesi et al. 2007, Avila et al. 2008, Ferreira et al. 2010). However, despite the effects of alcohol withdrawal on anxiety having been well investigated relatively few studies have examined the influence of the neural substrates of the midbrain on defensive behavior related to fear/anxiety-like symptoms following abstinence from chronic alcohol intake.

Although the DPAG is linked to the generation and expression of unconditioned fear, as noted elsewhere, there is some evidence of its involvement in fear conditioning (Brandao et al. 1999, 2008, Reimer et al. 2012). This is an important factor on the field of alcohol abuse since withdrawal from chronic alcohol administration eases the formation of contextual fear memory and this facilitation might be a predisposing factor for alcohol consumption (Bertotto et al. 2006).

Based on this evidence, the present study aims to assess whether the GABA$_A$ and opiate mechanisms of the DPAG are implicate in the increased negative emotional states elicited by punctuated cues (light) in rats going through ethyl alcohol withdrawal. For this purpose, we used the potentiated startle test, a behavioral procedure that allowed us to assess the unconditioned (USR) and conditioned startle (CSR), and the fear-potentiated startle response (FPS) as well. In this study, we assume the FPS amplitude as the best index of fear elicited by alcohol withdrawal. Given that the DPAG has a fundamental role on the defensive response elicited by fear and anxiety-related stimuli, we hypothesized that withdrawal from alcohol, which increases the levels of anxiety and fear-related behaviors, would promote a consequent increase in the amplitude of fear-potentiated startle (FPS). This increase will be susceptible to the inhibitory effects on fear-like behaviors of intra-DPAG injections of the GABA$_A$ agonist muscimol or low doses of the opiate morphine.

METHODS

Subjects

Ninety-four male Wistar rats weighing 250±20 g (from the campus of Ribeirão Preto, University of São Paulo) were group-housed (4 rats/cage) in Plexiglas-walled cages (45×35×15 cm). They were maintained on a 12-h light/dark cycle (lights on at 07:00 AM, 24±1°C) with food and water available ad libitum for the duration of the experiments.

Ethical statement

We hereby declare that the protocol of animal experimentation described in this manuscript received formal approval from the Committee on Animal Research and Ethics (CEUA) of the University of São Paulo (process 08.1.1547.53.3). In addition, we follow the recommendations of the Brazilian Society for Neuroscience and Behavior, which are in accordance with the U. S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011). The number of animals used was the

Table I

Main statistical differences between saline and alcohol withdrawal groups according to the performance in the EPM

	Mean ± SEM			
	Saline (*n*=10)	Alcohol (*n*=10)	*t*	Sig.
Closed-arms entry	8.70±0.58	8.20±0.61	0.48	*P*> 0.05
% Open-arms time	25 .54±1.36	12.40±0.62	7.19	*P*<0.001*
% Open-arms entry	45.12±3.16	21.67±2.73	4.50	*P*<0.001*

Variables in study (number of closed-arm entries, and percentage of entries and time spent in the open-arms) passed by student *t*-test analysis. *Significant differences at the level of $P \leq 0.05$.

minimum necessary to ensure the reliability of the results. All necessary precautions were taken in order to minimize animal suffering.

Surgery

After 7 days of habituation to the living conditions of the animal house, the animals were anesthetized with an intraperitoneal (IP) injection of 0.1 ml ketamine hydrochloride + 0.1 ml xylazine mixture (60/10 mg/kg), and mounted in a digital stereotaxic frame (Insight, São Paulo, Brazil). A cannula made from a stainless steel needle (24 gauge, 14 mm length) was introduced directly in the DPAG, taking into account the coordinates of the Paxinos and Watson's Atlas (2008), using the line of bregma as the reference point (anterior/posterior: −6.96 mm; medial/lateral: ±0.2 mm, dorsal/ventral: −2.0 mm). To anchor the prosthesis cannulae were fixed to the skull by acrylic resin and three stainless steel screws. Following the surgery, each animal received an intramuscular injection of a veterinary pentabiotic (120 000 UI, 0.2 ml) and an injection of the anti-inflammatory and analgesic drug Banamine (flumixin meglumine, 2.5 mg/kg). The guide cannula was sealed with a stainless steel wire to protect it from clogging. After surgery, the animals were undisturbed for 5 days for recovery.

Variables

In our study, three main levels of analysis were used and two dependent variables were recorded, as follows: the latency and amplitude of USR (a reflex response in nature, highly influenced by muscle contraction), the latency and amplitude of CSR (a fore-

seeable, cue-evoked startle), and the amplitude of FPS (an index of fear showed as the percentage of the amplitude of startle achieved between noise-alone and light-noise trials). FPS were calculated as follows: FPS = (light/noise − noise alone) / (noise alone × 100). The raw amplitude of CSR recorded during light-noise trials does not reflect the real nature of aversive emotion, as startle is highly influenced by obvious floor and ceiling effects (Davis 2001). Thus, we consider the amplitude of FPS (percentage obtained between USR/CSR responses) as the best score to analyze the emotional component of fear, provided that in this condition the effects above described are absent.

Fear-potentiated startle (FPS): Matching

The FPS analysis was performed in four sound-attenuating chambers of equal dimensions (60×50×45 cm). Inside each chamber was a testing cage (16.5×7.5×7.5 cm) made of Plexiglas, with a floor consisting of six stainless-steel bars spaced 15 mm apart. Each testing cage was fixed to a stabilimeter (Insight, São Paulo, Brazil) by four thumbscrews. Inside the stabilimeter, a load-cell captured the pressure on the response platform during the startle reaction, generating analogue signals that were analyzed by Startle Reflex software (Insight, São Paulo, Brazil). A loudspeaker located 10 cm behind the testing cage delivered both the startle stimulus (50 ms burst of white noise) and continuous background noise (55 dB). The startle reaction was recorded within a time window of 200 ms after the onset of the startle stimulus. Matching sessions were conducted along with two daily sessions of startle. For each matching session the animals were

placed in the testing cage for a 5 min habituation period and afterwards were presented with a series of 30 startle-eliciting noise bursts (10 at each of three intensities, 90 dB, 95 dB, and 105 dB), with a 30 s inter-trial interval (ITI). Startle stimuli were presented in a quasi-random order with the restriction that each intensity occurred once within each successive block of three stimuli. The mean startle amplitude across the 30 test trials for each rat was used to match the animals into groups. This was in order to match the animals of the control and experimental groups in such a way that each group had the same average startle amplitude at the beginning of the experiments. Each matching session was 20 min in duration, including the habituation period.

FPS: Training

Animals were conditioned to light-CS with the testing cages localized inside a small Plexiglas chamber (35×25×25 cm) whose walls and ceiling were composed of horizontal black and white stripes (5 cm width). An opening in the rear wall allowed the presentation of the stimuli used (light). This chamber was located inside the sound-attenuating chamber already described. The animals were individually placed in the training cage for a habituation phase of 5 min. After this, each rat received ten pairings of a light (conditioned stimulus – CS, 4 s of duration, 6 W) co-terminating with a footshock (unconditioned stimuli – US, 1 s of duration, 0.6 mA). The inter-stimulus interval (ISI) varied randomly between 60 s and 180 s. The duration of each aversive training session was about 25 min, including habituation time. A interval of 24 h separated the two training sessions.

Procedure for alcohol preparation, administration and withdrawal

The method of alcohol preparation and administration was based on previous studies (Morse et al. 2000, Zhang et al. 2007). Briefly, a 99.5% solution of pure alcohol (ethanol, Vetec, São Paulo, Brazil) was diluted with distilled water. Each animal received ten IP injections (one per day) of the 20% alcohol solution, at a dose of 3.0 g/kg in a volume of 2.0 ml/100 g body weight. This dose was chosen because data from a previous study showed that withdrawal from repeat treatment with 2.0 g/kg or 3.0 g/kg of alcohol produced
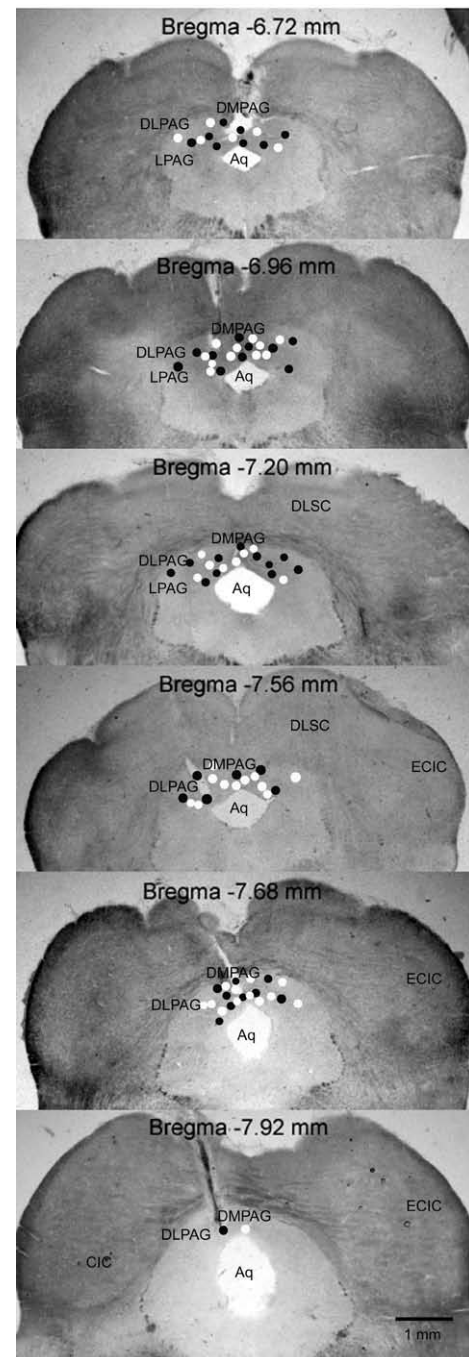

Fig. 1. Empty circles represent the sites of drug injections into the dorsal aspects of the periaqueductal grey (DPAG) of saline pre-treated rats. Black circles represent the sites of drug injections into DPAG of alcohol pre-treated rats. (DMPAG) dorsomedial periaqueductal grey column; (DLPAG) dorsolateral periaqueductal grey column; (LPAG) lateral periaqueductal grey column; (DLSC) deep layers of the superior colliculus; (ECIC) inferior colliculus external cortex; (CIC) inferior colliculus central nucleus; (Aq) Aqueduct.

an extended duration of anxiogenic-like behavior swinging from 6–15 h post-injection (Zhang et al. 2007). The volume of the solution administered, and not the alcohol concentration, was adjusted to vary the dose in order to avoid the discomfort resulting from IP of alcohol concentrations higher than 15%. Physiological saline (0.9%) was used as control solution. Twenty-four hours after the second matching sessions the animals were placed in the testing room where they were weighed and handled during 5 min. After this, the rats suffered an IP infusion of saline or alcohol solution. The injections were made on the morning of each one of the 10 treatment days, between 08:00 AM and 09:00 AM. The rats were then returned to their home cages and kept in the testing room for approximately 3 h in order to acclimate before returning to the vivarium. The same procedure was implemented for the next nine days.

FPS: Effects of alcohol withdrawal

Rats received the 10th placebo or alcohol injection 6 h before being placed into the testing cages. During these withdrawal sessions, the striped context used throughout the training were removed from the sound-attenuating chambers. Sessions were conducted without foot-shock presentation in the context in the same way as used for matching. After additional 5 min for habituation, the animals received 60 startle stimuli (20 at each of 3 intensities, 90 dB, 95 dB, and 105 dB) with a 30 s ISI. Half the startle stimuli at each intensity were presented in the absence of the CS (noise-alone trials) to provide a baseline, and the other half were presented in the presence of the CS (light-noise trials). In the light-noise trials, rats were exposed to a 4 s presentation of light-CS, co-terminating with the startle stimulus. As described above, we chose the percent of
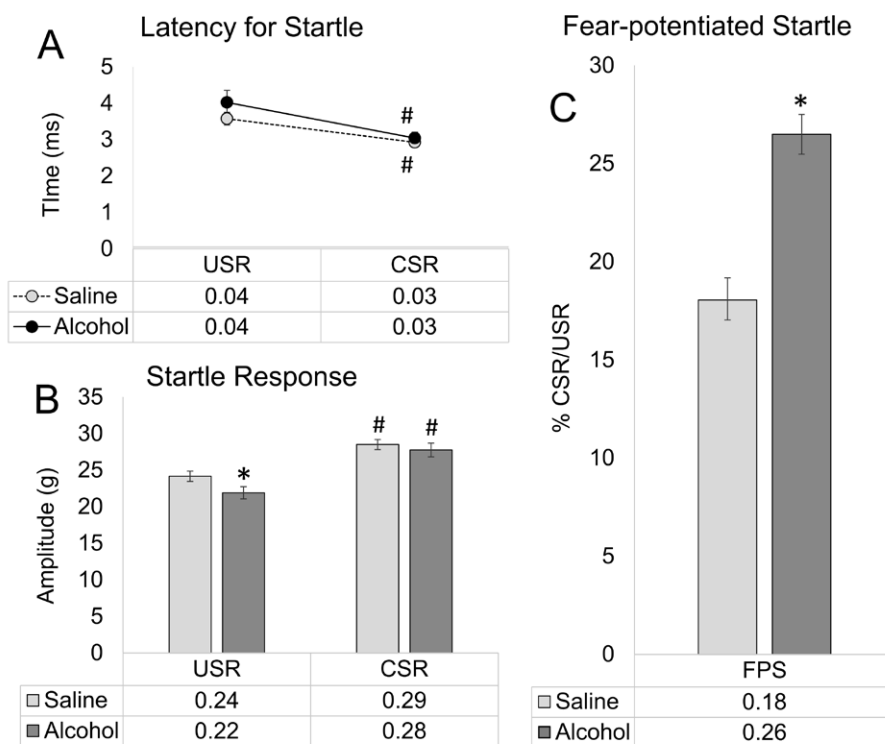

Fig. 2. Main effects of the treatments on the latency for startle (A), on the unconditioned (USR) and conditioned startle response (CSR) (B), and the amplitude of fear-potentiated startle (FPS) (C) in rats pre-treated with saline or ethyl alcohol and tested on 6 h withdrawal. Data were normalized through the use of square-root of raw data and are presented as Mean ± SEM. For this comparison we used the data collected from 95 animals (saline = 48, alcohol = 46). (A, B) *Significant difference between trials (noise alone × light/noise) within the same treatment (saline or alcohol). #Main differences between treatments (saline × alcohol withdrawal) within the same trial (noise alone or light/noise). (B) #Main differences between trials (noise alone ×light/noise) within the same treatment (saline or alcohol). A and B: Two-way RM ANOVA followed by Newman-Keuls *post-hoc*. (C) Student *t*-test for independent groups. The level of *P* was set at ≤0.05.

startle potentiation as the main index of measuring FPS, because using this type of score avoids the bias introduced by undesirable floor and ceiling effects (Davis 2001). The noise-alone and light-noise trials were intermingled at random. The duration of the test session, including habituation time, was 37 min. In order to evaluate the validity of the method used to promote withdrawal symptoms in rats, mainly on emotionality, ten subjects randomly chosen from each group (saline and alcohol) were submitted to the elevated plus-maze (EPM) before being tested on FPS. Table I shows the data collected from EPM.

The role of DPAG GABA$_A$ and opioid receptors on the modulation of startle response of alcohol-withdrawn rats

Experimental procedure and drugs

Tests on drug effects (intra-DPAG infusions) began immediately after the end of withdrawal sessions. Drugs used were the selective GABA$_A$ agonist muscimol (1 nmol/0.2 µl; Sigma, St. Louis, MO, USA) or the opiate agonist morphine (hydrochloride, 10 nmol/0.2 µl – Cristália, São Paulo, Brazil). Drugs were dissolved in phosphate-buffered saline (PBS, pH 7.0) shortly before DPAG microinjections. PBS was used as control solution. The waiting time for test sessions after injections were 15 min for both drugs. In this study, only a single dose of muscimol and morphine was used because these doses are well-known by its fear-reducing properties in rats when injected into the DPAG (Jenck et al. 1983, Anseloni et al. 1999, Nobre et al. 2003, Borelli et al. 2006, Nobre et al. 2010). Therefore, only one dependent variable was measure at this stage, named the FPS amplitude. Each animal of each group (saline or alcohol) received only one intra-DPAG microinjection.

Microinjection procedure

The animals were kindly wrapped in a cloth and hand-held, and a thin silica capillary tubing (outside diameter, 220 µm), connected to a 5 µl syringe pump (Insight, São Paulo, Brazil), was introduced through the guide cannula until its lower end was 3 mm below its tip. A volume of 0.2 µl of PBS, muscimol, or morphine was injected over 60 seconds.

Experimental groups

We begin this study with 120 animals. At the end of the experiments, removing the subjects presenting post-surgery problems (death, prosthesis infection – 13 rats), and those in which the sites of the cannulae fell outside the DPAG (13 rats), 94 animals remained. These animals were allocated in six independent groups according to the IP injections and local drug infusions, as follows: saline IP × PBS into the DPAG (*n*=16); saline IP × muscimol into the DPAG (*n*=18); saline IP × morphine into the DPAG (*n*=14); alcohol IP × PBS into the DPAG (*n*=14); alcohol ip × muscimol into the DPAG (*n*=18) and alcohol IP × morphine into the DPAG (*n*=14).

Perfusion and histology

Completed the experiments the animals were deeply anesthetized with an IP overdose of sodium pentobarbital (60 mg/kg) and perfused intracardially with phosphate-buffered saline (pH 7.0) followed by a solution of paraformaldehyde (4%). After decapitation, their brains were removed, immersed for three days in fresh 4% formaldehyde, and then transferred to a 20% sucrose solution for cryoprotection. Coronal sections of 60 µm were cut on a freezing microtome (Leica, Germany), mounted on gelatin-coated slides, and stained with neutral-red.

Statistical analysis

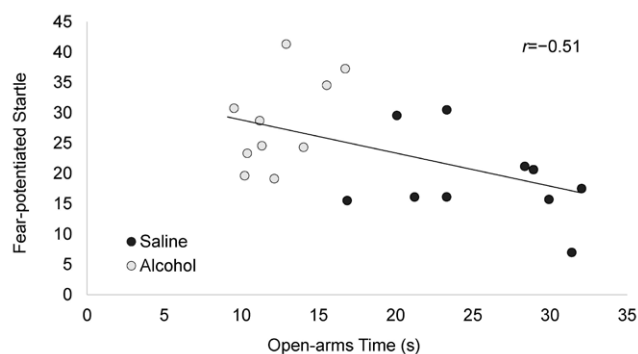For statistical analysis we used only rats with cannula tips within the boundaries of the DPAG, which

Fig. 3. Correlation between the amplitude of fear-potentiated startle (FPS) and the time spent in the open arms of the elevated plus maze of saline (*r*=−0.36) and alcohol withdrawal groups (*r*=0.53).

include the dorsomedial and dorsolateral columns. As an additional control, ten animals randomly chosen from saline or alcohol groups were exposed to the EPM before being submitted to the procedure of FPS, in order to verify the effects of alcohol withdrawal on emotionality. Statistical comparisons were conducted using the Student *t*-test for independent groups.

Regarding the startle test, some of the data collected showed a non-Gaussian distribution. Thus, data from the present experiments were normalized by using the square root of the raw data. The first comparison aimed to analyze the effects of alcohol withdrawal *per se* on the USR (noise-alone trials), CSR (light/noise trials) and FPS, used the latency and startle amplitude as the main dependent variables. For this purpose, data from saline and alcohol withdrawal of all the six groups were collapsed and submitted to a two-way ANOVA (groups × trials) with repeated measures – RM (trials). To analyze the emotional component of startle (FPS amplitude) a *t*-test was used (saline × alcohol). Additionally, to assess the strength of the predic-

tion of EPM behavior based on FPS magnitude, correlations were made between the FPS and the time spent in the open arms using Spearman's rank correlation coefficient. The effects of muscimol and morphine on the amplitude of FPS were compared, separately, with those obtained from PBS through a factorial ANOVA in a between × within design (treatments × drug effects). For all the analysis, a significant ANOVA result ($P \leq 0.05$) was followed, when appropriated, by the Newman-Keuls significant difference *post-hoc* test.

RESULTS

Figure 1 shows a photomicrograph with the sites of drug injections into the DPAG. The effects of alcohol withdrawal on the latency for startle are shown in Figure 2(A). Two-way RM ANOVA showed that trials was the only factor affected by treatments ($F_{1,92}=30.53$; $P<0.0001$). ANOVA applied on the amplitude of startle response revealed no effect of factor treatments ($F_{1,92}=2.19$; $P>0.05$), but significant difference between trials ($F_{1,92}=576.05$; $P<0.0001$) and significant interaction between treatments/trials ($F_{1,92}=8.01$; $P<0.01$) (Fig. 2B). The Student *t*-test applied to the FPS data revealed that the amplitude of startle is potentiated in rats tested under alcohol withdrawal ($t_{94}=-4.67$; $P<0.0001$) (Fig. 2C). The *post hoc* analysis demonstrated that alcohol withdrawal attenuates the USR but do not influence the aversive learning, as revealed by similar amplitude of CSR exhibited by alcohol withdrawal and saline groups. However, alcohol withdrawal increases the emotional component of startle as revealed by increased amplitude of FPS displayed by alcohol-withdrawn rats. Treatments did not change the latency for startle, but saline and alcohol groups exhibited comparable reductions in this variable during the light/noise trials. In order to verify whether increases on FPS amplitude result from changes on emotionality induced by alcohol withdrawal, Student's *t* test for independent groups was applied on the data from EPM, for comparisons of the relevant variables between saline and alcohol treatments. After *t*-test performing a correlation table was obtained for each variable. Overall, alcohol-withdrawn rats showed significant decrease on the percentage of entries and time spent in the open arms, variables linked to anxiety-like behavior in rodents, without changes on locomotor activity (Table I). In alcohol withdrawn-rats, increases in the amplitude of FPS
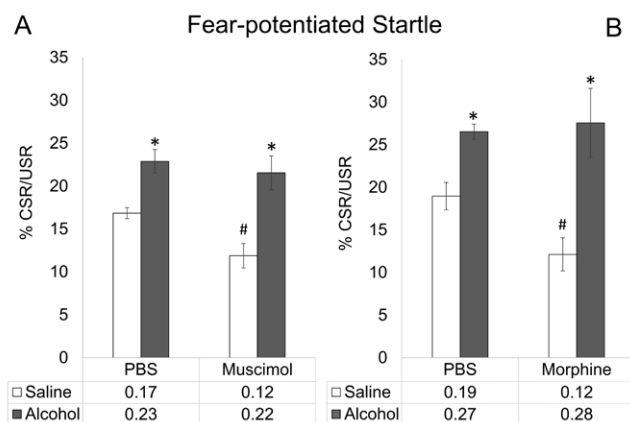

Fig. 4. Main effects of muscimol (A) and morphine (B) on the amplitude of fear-potentiated startle (FPS) of saline and alcohol-withdrawn rats. Intra-DPAG injection of phosphate-buffer saline (PBS) was used as control solution. Data were normalized through the use of square-root of raw data and are presented as Mean ± SEM. For this statistical analysis, we used the data collected from each separated group as follows: saline × PBS, *n*=16; alcohol × PBS, *n*=14; saline × muscimol, n=18; alcohol × muscimol, *n*=18; saline × morphine, *n*=14; alcohol × morphine, *n*=14. *Significant difference between treatments (saline × alcohol withdrawal) after PBS, muscimol or morphine infusions. # Significant difference between drugs (PBS × muscimol, or PBS × morphine) within the same treatment (saline or alcohol). Factorial ANOVA followed by the Newman-Keuls *post-hoc*. The level of *P* was set at ≤0.05.

were negatively correlated with the reduction in the time spent in the open arms ($r=-0.51$) (Fig. 3).

The second part of our analysis examined the influence of intra-DPAG injection of muscimol or morphine on the effects of alcohol withdrawal in the FPS amplitude. Statistical analysis was performed separately for each drug. With regard to the effects of muscimol, ANOVA showed significant influence of treatments ($F_{1,68}=29.89$; $P<0.0001$) and drugs ($F_{1,68}=4.83$; $P<0.05$), but no significant interaction between factors. *Post-hoc* Newman-Keuls revealed that muscimol was able to decrease the FPS in control rats in spite of showed no influence on alcohol withdrawal group. Regarding the influence of morphine on FPS, ANOVA point out significant effects for treatments ($F_{1,52}=20.51$; $P<0.0001$) only. Post-hoc analysis revealed that alcohol withdrawn-rats were unresponsive to morphine treatments. On the other hand, similar to muscimol, morphine depress the amplitude of FPS in control rats.

DISCUSSION

The data obtained in the first part of our study aiming to analyze the effects of alcohol withdrawal on startle showed that withdrawal reduces the USR without changing the aversive conditioned response (CSR). However, this reduction does not seem to reflect an attenuation of fear since the latency for startle response decreased equally in control and experimental groups. In other words, the animals startled faster when facing the light/noise trials. This means that the reduction on the USR does not imply less fear but, instead, an increased in fear-like behavior. Indeed, the variable we assume as the main index of fear in this test, the amplitude of FPS, increases significantly during alcohol withdrawal. In addition, alcohol-withdrawn rats were significantly more sensitive to the aversive cues of the EPM than the saline control group. In fact, the decrease in the time spent in the open arms correlates negatively with the increases in the amplitude of FPS. In our study, the use of the EPM test for evaluating the aversive state of the animals was based on the fact that anxiety and fear-related behaviors are known as one of the main symptoms of alcohol withdrawal syndrome (Wilson et al. 1998, Devaud et al. 1999). Animals even in only 6 h of alcohol deprivation spent more time inside the close arms and entered fewer times into the open arms of the EPM when compared to controls, displaying a typical "anxiogenic-like" profile for this test (Zhang et al. 2007).

Overall, the present findings match the study of Ripley and coauthors (2003) who showed that rats trained in fear conditioning prior to alcohol exposure and withdrawal showed no impairment in the expression of the conditioned fear response. However, the question of why alcohol withdrawal increases the negative emotional component of alcohol, but at the same time weakens the behavioral response related, is an issue that needs to be clarified. The argument of the non-monotonic effect of startle could be used to explain such discrepancy. With this in mind, it is conceivable that multiple episodes of alcohol hangover acquire the ability of a higher stressor, eliciting an intense aversive state, characteristic of alcohol withdrawal (Wills et al. 2009). In our study, the intense emotional disturbances prompted by alcohol withdrawal, as revealed by the EPM test, might switch the alcohol-withdrawn rats to a different mode of defensive response that does not include the USR as a component behavior (Walker et al. 1997). These pro-aversive effects produced by alcohol withdrawal, as showed by increased amplitude of FPS and reduced open-arms exploration on the EPM test, are a non-reinforced unconditioned response, and could well be due to neural activation of the DPAG, the main outflow of the unconditioned fear response (Brandao et al. 2003), as will be discussed below.

The modulation and expression of unconditioned fear reactions has been attributed to a set of brain regions among which stands out the DPAG, a limbic structure that is considered to be the final pathway of the stress reaction (Carrive 1993, Brandao et al. 2003, 2005). Injections into the DPAG of GABA inhibitors or GABA$_A$ agonists have opposed excitatory and inhibitory effects, respectively (Brandao et al. 2005). Attenuation of the aversive consequences in rats facing dangerous unconditioned stimuli can also be achieved by local DPAG infusion of low doses of μ-opiate receptors agonists, such as morphine (Jenck et al. 1983, Brandao et al. 1985, Anseloni et al. 1999). The DPAG has also been implicated in conditioned fear, although to a lesser extent than it has in unconditioned fear (Zanoveli et al. 2007, Reimer et al. 2008), and in modulation of the aversive consequences of withdrawal from drugs of abuse as morphine (Avila et al. 2008), alcohol (Cabral et al. 2006) and benzodiazepines (Fontanesi et al. 2007). As such, in the second part of our study we evaluated the role of GABA$_A$ or μ-opioid receptors of the DPAG on the modulation of FPS

through local injections of the GABA$_A$ full agonist muscimol or a low dose of the preferentially μ-opioid receptors agonist morphine.

In the present report, we decided to conceptually separately the USR, CSR and FPS taking into consideration that they are concepts that illustrate different aspects of behavior (i.e. distinct processes with distinct neurobiological substrates), being the percentage of startle the best score to analyze the emotional component of fear (Davis 2001). In line with the presumption that the DPAG is chiefly involved in the modulation/expression of unconditioned fear but in less extent in conditioned fear (Walker and Davis 1997), muscimol, when applied to this structure showed clear antiaversive effects in control animals, with no changes on the amplitude of FPS of the experimental group being noted. Overall, this is in agreement with data from previous studies in which lesions or increases in GABA inhibition in the rat DPAG impaired FPS (Fendt et al. 1996, Reimer et al. 2008). Thus, our results progress toward the main role of DPAG on control of the emotional component of startle, probably through GABA$_A$ receptors modulation. Therefore, in the present study, assuming that exposure to punctuate cues that predict the conditioned stimulus presentation increase fear not anxiety, reductions on the amplitude of FPS possibly reflects a concomitant decline in fear levels. In fact, diffuse contextual cues provided little information about the occurring of an aversive stimulation raising and, therefore, it is likely that the context by itself elicits a state of anxiety, and not fear. Giving support to our assumption, the study of Almeida and coworkers (2006) showed that an intra-DPAG administration of muscimol had no influence on the percentage of time and open entries of the EPM, a well-known index of anxiety in rodents.

Regarding the action of muscimol, one question to be elucidate is the absence of effects of alcohol withdrawal on the amplitude of FPS. One of the pharmacological targets of ethyl alcohol is the GABA$_A$ receptor. This class of GABA receptors have altered physiology and expression after chronic alcohol exposure. These changes are associated with decreases in GABA effectiveness (Faingold et al. 1998), perhaps due, in part, by receptor phosphorylation (Gyenes et al. 1994) that is thought to play an important role in GABA$_A$ receptor desensitization mechanisms (Oh and Dichter 1992).

Thus, increased phosphorylation due to protein kinases A, PKC, or protein tyrosine kinases inhibits GABA$_A$ receptor function. In addition, alcohol withdrawal increases the excitatory drive mediated by excitatory amino acids in several brain regions (Faingold et al. 1998, Long et al. 2007, Nagy 2008), including the periaqueductal grey (Long et al. 2007, Ezequiel Leite and Nobre 2012). This could explain the ineffectiveness of intra-DPAG injections of the full GABA$_A$ agonist muscimol in change the amplitude of FPS in alcohol-withdrawn rats.

The influence of morphine on antinociception is well established (McCormack et al. 1998). In addition, opioid mechanisms also underlie the expression of freezing and crouching, a behavioral repertoire always evoked in submissive rats (Archer 1973). The DPAG plays a role not only in nociception, but also in the modulation and expression of fear and anxiety-like behaviors (Bandler and Carrive 1988, Behbehani 1995, Brandao et al. 2003). The influence of opiate receptors of the DPAG on fear can be assumed taking into account that freezing induced by electric shock is reduced by naloxone treatment (Fanselow and Baackes 1982). The μ-opioid receptors have been proposed to play a fundamental role in alcohol intake (Sanchis-Segura et al. 2005). Low doses of the opiate morphine have the ability to inhibit the aversion induced by DPAG stimulation, probably through activation of μ-receptors, whereas microinjections of higher doses cause pro-aversive actions not mediated by these opioid receptors (Motta and Brandao 1993). In our study, the effects of local DPAG injections of the opiate morphine was primarily in control rats as the FPS in this group was decreased, comparing the data obtained after PBS infusions. This type of tolerance to morphine induced during alcohol withdrawal was found previously in rats consuming alcohol, but mainly to its antinociceptive effects (He and Whistler 2011). However, in the same study, it was show that the activity of μ-opioid receptors is significantly decreased in the spinal cord and the periaqueductal grey matter of alcohol pre-treated rats. Thus, the tolerance to the anti-anxiety effects of morphine occurring in rats submitted to ten daily IP injections of alcohol could result from changes in the dynamics of μ-opioid receptor function such as, for example, decreases in the responsiveness of these receptors promoted by alcohol chronic administration, as revealed in other studies (Chen and Lawrence 2000, Saland et al. 2008, He and

Whistler 2011). This phenomenon was not observed in control animals, in which morphine showed a clear and well-known fear-reducing profile.

CONCLUSIONS

In summary, our study points out that the well-known anxiolytic-like action of GABA$_A$ and μ-opioid receptors of the DPAG on fear response is impaired in alcohol pre-treated rats tested under alcohol withdrawal. This was possibly due to GABA$_A$ receptor desensitization mechanisms and from decreases in the responsiveness of μ-opioid receptor functions resulting from alcohol chronic administration. These findings shed light on some aspects of the fear-like behavior elicited during alcohol withdrawal bringing new information on the influence of GABA and opioid receptors of the DPAG on the expression of unconditioned and conditioned fear responses. Overall, these data corroborate partially our hypothesis as withdrawal from alcohol enhanced the levels of fear-like behavior but, surprisingly, showed insensitive to the fear-reducing effects of intra-DPAG injections of muscimol and morphine. Future studies on this subject should aim to investigate the possible inter-action between GABA and opioid receptors at the DPAG level on the modulation of conditioned fear, using different aspects of aversive conditioning (contextual × cue-evoked response). In addition, it is necessary to elucidate the main role of κ- and δ-opioid receptors of this brain-stem region on the modulation/expression of fear induced by alcohol withdrawal.

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