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The blockade of AMPA-kainate and NMDA receptors in the dorsal periaqueductal gray reduces the effects of diazepam withdrawal in rats

Luiz F.S. Souza-Pinto, Vanessa M. Castilho, Marcus Lira Brandão, Manoel Jorge Nobre *

Instituto de Neurociências & Comportamento — INeC, Campus USP, Av. Bandeirantes 3900, Monte Alegre, Ribeirão Preto-SP, 14040-901, Brazil

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Abstract

It is well established that the most persistent sign of withdrawal from chronic benzodiazepine use in humans is anxiety. In contrast to other types of drugs of abuse, the emergence of this anxiety does not seem to be linked directly to alterations in the levels of dopamine in the mesolimbic system. Some studies have proposed that fear-like behaviors elicited by benzodiazepine withdrawal could be the result either of alterations in the sensitivity of $GABA_A$ receptors or in the neuronal hyperexcitability that results from neuroadaptative responses to chronic treatment, probably mediated by glutamate. The increased fear-like behaviors induced by benzodiazepine withdrawal are similar to the defense reaction displayed by animals exposed to dangerous situations or submitted to electrical or chemical stimulation of the dorsal periaqueductal gray (dPAG), a key structure of the brain aversive system. However, the involvement of the dPAG in drug abuse has been investigated only in the context of the physical effects of drug dependence. Thus, in this study we investigated the effects of injections into the dPAG of the glutamic acid diethyl ester (GDEE) and 2-amino-7-phosphonoheptanoate (AP-7) (AMPA-kainate and NMDA receptors antagonists, respectively) on fear-like behaviors promoted by benzodiazepine withdrawal in rats submitted to aversive events (foot-shocks) immediately before chronic diazepam administration in a conditioning place-preference paradigm, using a light–dark box. Our results showed that inhibition of the glutamatergic neurotransmission in the dPAG reduces the consequence of the diazepam withdrawal in rats, implicating the excitatory amino acids of the dPAG in the modulation of the aversive state induced by benzodiazepine drugs withdrawal.

Keywords: Diazepam withdrawal; Fear; Anxiety; Periaqueductal gray; Glutamate

1. Introduction

Many studies in the literature show that with the abstinence from several types of drugs of abuse such as benzodiazepines (Bzp), psycostimulants and opioids emerges a series of affective symptoms such as dysphoria, irritability and anxiety accompanied by sensory and autonomic alterations (Markou et al., 1998; Koob and Le Moal, 2001).

It is well established that the most persistent sign of withdrawal from chronic Bzp use in humans is anxiety (Marks, 1978). Behavioral changes indicating anxiety have been found in animals tested after withdrawal from diazepam (Dzp) treatment of 21–28 days and even only 7–14 days (Emmett-Oglesby et al., 1983; File et al., 1987; Baldwin and File, 1988, 1989; File, 1990; File and Andrews, 1991).

Drugs of abuse produce their effects through enhancing the efflux of dopamine from mesolimbic dopaminergic neurons (Di Chiara and Imperato, 1988; Carboni et al., 1989; Lingford-Hughes and Nutt, 2003), and the decrease verified after withdrawal accounts for the appearance of the withdrawal symptoms. On the other hand, Bzp substances seem to promote its rewarding effects (and the aversive ones observed during withdrawal) probably acting on other brain systems, as this class of drugs has a peculiar characteristic of reducing the basal dopamine levels in the nucleus accumbens of rats challenged with an acute dose and even after 48-hour withdrawal of chronic treatment (Finlay et al., 1992).

Some studies have proposed that fear-like behaviors elicited by Bzp withdrawal could be the result of alterations in the sensitivity of $GABA_A$ receptors. This is because GABA mechanisms play a

* Corresponding author. Tel.: +55 1636023788; fax: +55 1636024830.
E-mail address: mjnes@usp.br (M.J. Nobre).

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functional role in the modulation of the pharmacological action of the Bzp (Impagnatiello et al., 1996) and in the anxiety-related disorders (Biggio et al., 1990). Other studies have suggested that withdrawal from Bzp may reflect a certain type of neuronal hyperexcitability that results from neuroadaptative responses to chronic treatment (Tsuda et al., 1999), probably mediated by glutamate (Pratt et al., 1998). However, the first experimental evidence for the involvement of the glutamate receptors in the withdrawal effects of Bzp showed the involvement of the AMPA-kainate and NMDA receptors in the production and in the maintenance, respectively, of the abstinence symptoms to these drugs (Steppuhn and Turski, 1993).

The expression of the increased fear-like behaviors induced by Bzp withdrawal is similar to the defense reaction displayed by animals exposed to dangerous situations or submitted to electrical or chemical stimulation of the dorsal periaqueductal gray (dPAG), a key structure of the brain aversive system (Brandão et al., 1999; Nobre et al., 2000, 2002, 2003, 2004; Brandão et al., 2005). The electrical or chemical (e.g. kainic acid) stimulation of the dPAG produces marked flight responses, increased defensive reactions (upright postures, locomotion, jumping and freezing behavior), ultrasound vocalization (22-kHz calls) and cardiovascular and respiratory alterations (Behbehani, 1995). However, the involvement of the dPAG in drug abuse has been investigated only in the context of the physical effects of drug dependence (Nestler and Aghajanian, 1997). An enhancement of the glutamatergic neurotransmission in this structure produces a flight reaction (Fanselow, 1991). However, whereas it is well established that excitatory amino acids (EAA) play a crucial role in the defense reaction generated and elaborated in the dPAG, nothing has been done to examine their participation in the withdrawal of the Bzp.

Thus, the present study looked at the role of the glutamatergic neurons of the dPAG in producing the fear-like behaviors of the Bzp withdrawal. Two experiments were conducted using a conditioning place-preference (CPP) paradigm to evaluate: (1) whether the negative reinforcing effects of the abstinence of Dzp elicit preference for the conditioning side wherein a concurrent aversive conditioning took place, and (2) whether the EAA mechanisms of the dPAG play a role in the expression of the fear-like behaviors promoting conditioned place-preference in diazepam-withdrawn rats.

2. Methods

2.1. Animals

The animals used were Wistar rats, weighing 100–110 g at the beginning of the treatment, from the animal house of the Campus of Ribeirão Preto, University of São Paulo. They were housed in groups of four in Plexiglass-walled cages, lined with wood shavings changed every 3 days, maintained in a 12:12 dark/light cycle (lights on 07:00 h) at 24±1 °C, and given free access to food and water. Before the beginning of the treatments, the animals passed a three-day habituation period to accustom them to the lodging conditions. The experiments reported in this article were performed in compliance with the recommendations of SBNeC (Brazilian Society of Neuroscience and Behavior), which are in accordance with the rules of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Surgery

Surgeries were performed on day 14 (four days before the end of treatments) since long post-surgery periods may cause problems such as loose prosthesis, infections, etc. The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and mounted in a digital stereotaxic frame (Insight, Brazil). A cannula made by stainless steel needle (24G) was directed to the dPAG. The upper incisor bar was set at 2.5 mm below the interaural line so that the skull was horizontal between bregma and lambda. The cannula was introduced vertically using the following coordinates with the bregma serving as the reference for each plane: AP −7.0 mm; ML 0.6 mm; and DV −2.0 mm (Paxinos and Watson, 1997). The cannula was fixed to the skull by means of acrylic resin and three stainless steel screws. At the end of the surgery each animal received an intramuscular injection (0.2 ml) of a veterinary pentabiotic (penicillin — 240.000 UI, plus 100 mg of dihydrostreptomycin sulfate and streptomycin sulfate by ml) followed by an injection of the anti-inflammatory and analgesic banamine (flunixin meglumine, 2.5 mg/kg).

2.3. Diazepam withdrawal

The animals were given Dzp (10 mg/kg) or saline intraperitoneally (i.p.), once a day, for eighteen days. The treatment regimen used in this experiment was adapted from other studies in the literature (Gibson et al., 1996; Schleimer et al., 2005). Dzp (Roche, USA) was dissolved in saline (0.9%) with added propilenoglycol (5%). Other drugs used were glutamic acid diethyl ester (GDEE) (160 nmol/0.2 µl, Sigma, USA) and 2-amino-7-phosphonoheptanoate (AP-7) (10 nmol/0.2 µl, Sigma, USA) that were dissolved in phosphate-buffered saline (0.1 M) shortly before being microinjected to the dPAG. The pH of the solutions after preparation was 7.2. Phosphate-buffered saline also served as vehicle control. Systemic injections of saline (plus propilenoglycol, 5%) were given in a volume of 1 ml/kg. GDEE and AP-7 were microinjected 10 and 5 min before the test, respectively. The doses of the antagonists were chosen based on previous studies (Brandão et al., 1999; Nobre et al., 2004; Brandão et al., 2005). Forty-eight hours (day 20) after the last i.p. injection (withdrawal phase), the animals were submitted to the light–dark test.

2.4. Apparatus and procedure

The behavioral tests were conducted in the light phase of the cycle (09:00 to 17:00 h). All experiments were carried out in an acrylic box (light–dark test) composed of two different compartments: a light side (white walls with transparent roof) and a dark side (opaque black walls and roof). The floor of both sides was made by stainless steel bars (2 mm thick) 15 mm

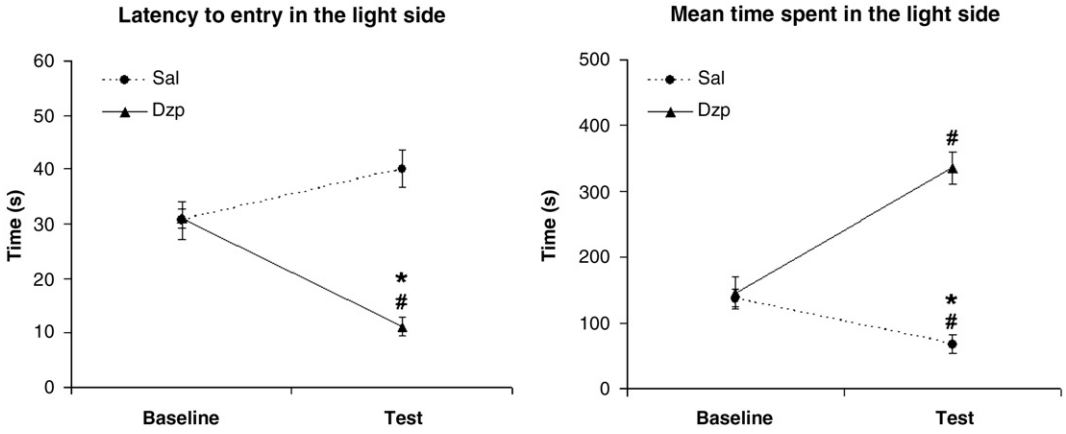

Fig. 1. Effects of the interruption of the chronic i.p. treatment (No-Shock groups) with saline (Sal) or diazepam (Dzp) on the latency for entry (left) and the time spent in the light side (right) of the test box of rats tested 48 h after the last injection (withdrawal), when compared with its baseline values. Vertical bars represent SEM. *Significant difference between Dzp and Sal groups within the same condition (Baseline or Test). #Significant difference between Baseline and Test conditions within the same group (Sal or Dzp). Two-way RM ANOVA, post-hoc Newman–Keuls; $p<0.05$.

apart. The test box was situated inside a wood chamber with external acoustic and light isolation. An incandescent white bulb (20 lx) was placed inside the chamber, 50 cm above the experimental box, and a micro-camera, located 50 cm above the light side, was linked to an external video-camera system to monitor the behavior of the animals during the sessions (baseline, conditioning, test, and dPAG treatment). In the first experiment (No-Shock groups) we measured the place preference induced by chronic Dzp treatment in rats conditioned in an aversive context (test × baseline conditions). In the second experiment (Shock groups) we evaluated the effects of EAA receptor antagonists injected into the dPAG of rats under Dzp withdrawal placed in the CPP paradigm, as described below.

2.4.1. Groups

The animals were randomly assigned to two main groups named No-Shock (i.p. chronic injections) or Shock groups (i.p. chronic injections × dPAG microinjections). The first group was divided into two: control or diazepam withdrawal ($n=8$ in each group). The second group was divided into six: saline/saline, saline/GDEE, saline/AP-7 ($n=10$ by group), diazepam/saline ($n=10$), diazepam/GDEE ($n=11$) and diazepam/AP-7 ($n=10$).

2.4.2. Baseline

Before the animals were submitted to the drug regimen a baseline session was conducted. Briefly, the animals were put in the dark side and allowed to explore both sides of the box for 10 min. Afterwards, the latency in crossing from the dark to the light compartment and the time of permanence in the light or dark side were recorded for 10 min. All animals stayed in the dark compartment much longer than in the light compartment. Therefore, here we report that we utilized a biased baseline procedure.

2.4.3. Conditioning

Twenty-four hours after the baseline measures and until the end of the treatment, all animals received a daily i.p. injection of saline or Dzp and soon afterwards were confined in the light side of the test box for 30 min. On days 6, 12 and 18, the animals of the Shock groups were confined in the light side and received 10 foot-shocks in variable 30 s intervals, while we registered the length of time of "freezing" induced by these aversive stimulations. Soon after the end of the sessions, the animals received an i.p. injection of Dzp, and were confined for 30 additional minutes in this side of the test box.

2.4.4. Testing

Forty-eight hours after the interruption of the treatments (day 20) the rats were submitted to testing sessions similar to the baseline sessions.

2.4.5. dPAG treatment

Three hours after the test session the effects of the glutamate receptor antagonists AP-7 or GDEE were examined. Five or ten minutes after the injections of AP-7 or GDEE into dPAG, respectively, the animals were placed again in the dark side of the test box and the behavioral measurements were redetermined.

2.5. Microinjection procedures

Three hours after the testing condition (48-hour withdrawal) the animals of the Shock Group were gently wrapped in a cloth, hand-held and a thin dental needle (o.d. 0.3 mm) was introduced through the guide-cannula until its lower end was 3 mm below its tip. The injection needle was linked to a 5-μl syringe pump (Insight, Brazil) by means of polyethylene tubing (PE-10) and a volume of 0.2 μl was injected during 30 s. The displacement of an air bubble inside the polyethylene was used to monitor the microinjection.

2.6. Histology

Upon completion of the experiments, the animals were deeply anesthetized with urethane and perfused intracardially with saline 0.9% followed by formalin solution (4%). Three

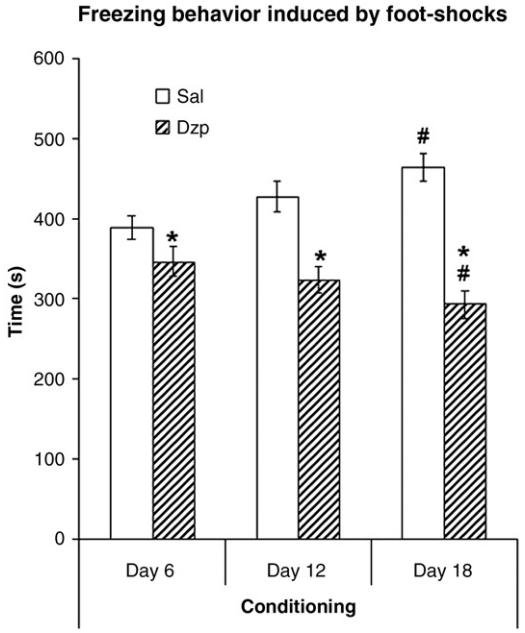

Fig. 2. Mean time of freezing induced by foot-shocks across the three aversive conditioning sessions of rats submitted to chronic i.p. injection of saline (Sal) or diazepam (Dzp). Columns represent the mean and vertical bars the SEM. *Significant difference between Dzp and Sal groups within the same trial (day of conditioning). #Significant difference between the third day of conditioning when compared with the first one, within the same group (Sal or Dzp). Two-way RM ANOVA, post-hoc Newman–Keuls; $p<0.05$.

hours later the brains were immersed in a sucrose solution (30%). Seven days later the brains were frozen. Serial 60-μm brain sections were cut using a cryostat (Leyca-Germany) and stained with neutral red in order to localize the positions of the microinjection sites according to Paxinos and Watson's (1997) atlas.

2.7. Analysis of results

Data are reported as mean+SEM. For each experimental situation the latency to enter and the time of permanence in the light compartment scored for the baseline and testing (No-Shock groups) and for the baseline, testing and dPAG sessions (saline-dPAG, GDEE-dPAG or AP-7-dPAG) were subjected to two-way analyses of variance (ANOVA) (groups × conditions) with repeated measures. Factor groups refer to saline or diazepam i.p. injected animals, and the factor condition refers to baseline, test or dPAG-treatment sessions. In the case of the freezing behavior, statistical comparisons were made using a two-way RM ANOVA being the sessions on days 6, 12 and 18 the repeated measures. Newman–Keuls' post-hoc comparisons were carried out whenever significant overall F-values were obtained. In all cases a probability level of $p<0.05$ was considered to be significant.

3. Results

Fig. 1 shows the effects of the interruption of the chronic diazepam regimen in rats of the No-Shock groups on the mean latency of entry (left), and the mean time spent in the light side (right) of the light–dark box. Two-way ANOVA with repeated measures (groups × condition) revealed significant differences between groups [latency $F_{1,14}=15.85$, time $F_{1,14}=79.73$; $p<0.05$], condition [time $F_{1,14}=6.87$; $p<0.05$] and interaction

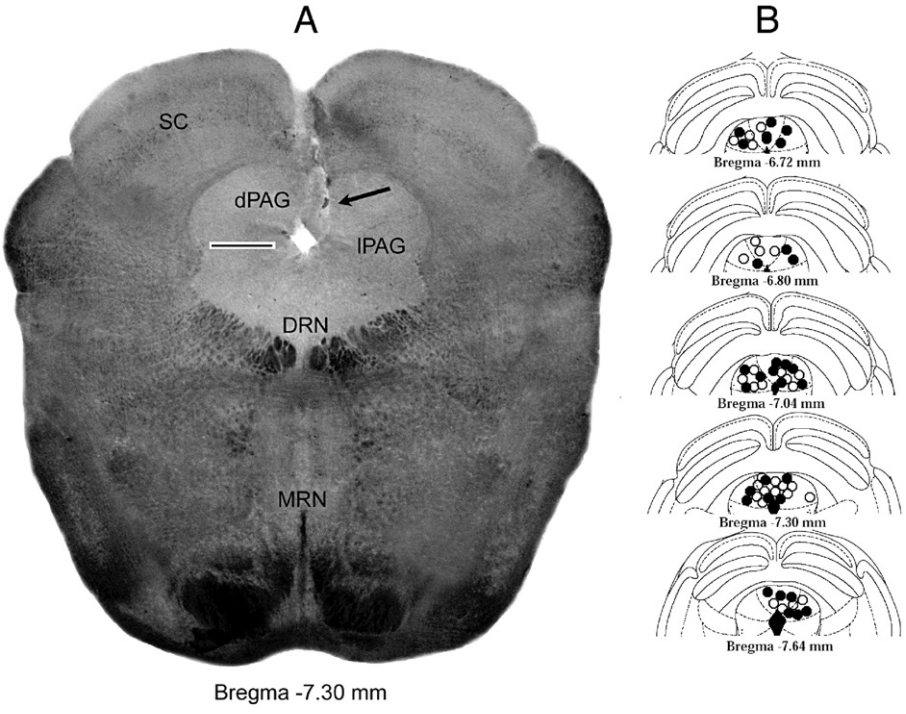

Fig. 3. (A) Representative photomicrograph of a site of dPAG injection. Scale bar represents 500 μm. (B) Location of the tips of the dPAG injections. The figures in millimeters indicate the coordinates of the Paxinos and Watson's (1997) atlas.

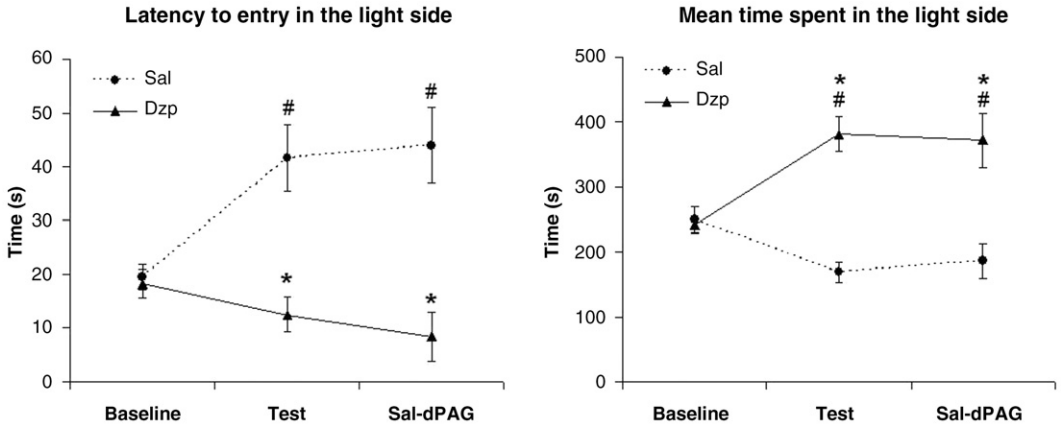

Fig. 4. Effects of the interruption of the chronic treatment with saline (Sal) or diazepam (Dzp) on the latency for entry (left) and the time spent in the light side (right) of rats submitted to the saline injections into dPAG 3 h after the test. Vertical bars represent SEM. *Significant difference between Dzp and Sal groups within the same condition (Baseline, test, dPAG treatment). #Significant difference between Baseline, Test and dPAG treatment within the same group (Sal or Dzp). Two-way RM ANOVA, post-hoc Newman–Keuls; $p<0.05$.

between factors [latency $F_{1,14}=27.24$, time $F_{1,14}=32.49$; $p<0.05$]. The Newman–Keuls' post-hoc analysis showed that, in contrast with the vehicle-treated animals, diazepam withdrawal in rats significantly reduced the latency to get in the light side and increased the time of permanence in the conditioned side (light compartment). These data show a place aversion in the saline group and a place preference in the diazepam group to the light side of the box across the testing sessions.

The results obtained with the Shock groups during conditioning showed that chronic Dzp regimen was effective in reducing the time of freezing behavior elicited by foot-shocks. Two-way ANOVA with repeated measures detected significant differences between the treatments ($F_{1,59}=30.06$; $p<0.05$), no significant effects on condition ($F_{2,118}=0.33$; $p>0.05$) and significant interaction between the factors ($F_{1,118}=11.16$; $p<0.05$). Post-hoc Newman–Keuls showed a gradual reduction in the time of freezing of Dzp-treated animals in relation to the control group, across the three aversive conditioning sessions (Fig. 2).

As revealed in the Fig. 3, all the tips of the injection cannulae were located in the dPAG. Figs. 4–6 showed that the same pattern of effects obtained with the No-Shock groups was observed with the animals of the Shock groups injected with saline before the test sessions. However, 48 h after the interruption of the chronic treatment with Dzp the animals still displayed a preference for the light side of the box. The two-way ANOVA with repeated measures performed on the data obtained with injections of saline (Fig. 4), GDEE (Fig. 5) or AP-7 (Fig. 6) into the dPAG of rats under saline or Dzp chronic regimen showed significant effects of the treatment (saline: latency $F_{1,18}=20.05$, time $F_{1,18}=33.15$, GDEE: latency $F_{1,19}=4.97$, time $F_{1,19}=7.68$, AP-7: latency $F_{1,18}=13.23$, time $F_{1,18}=12.53$; $p<0.05$), no effects of condition on saline (latency $F_{2,36}=2.64$; time $F_{2,36}=1.12$; $p>0.05$), significant

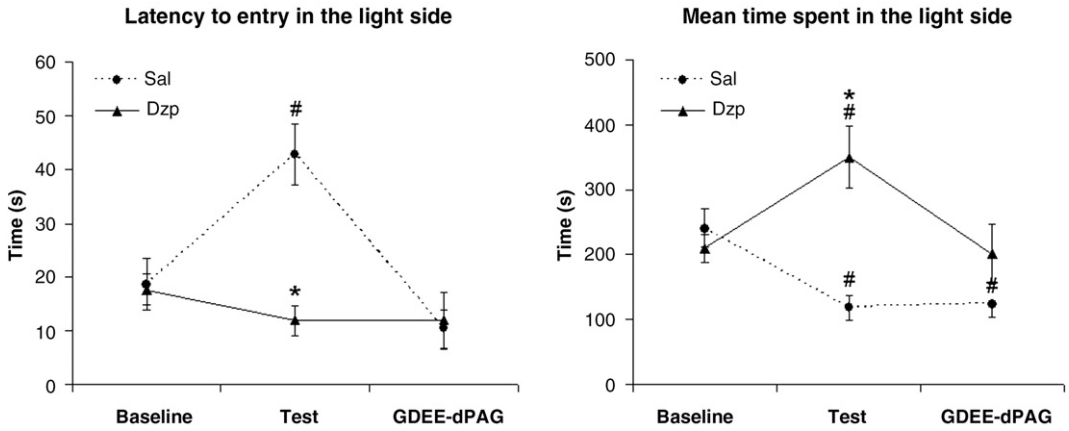

Fig. 5. Effects of the interruption of the chronic treatment with saline (Sal) or diazepam (Dzp) on the latency for entry (left) and the time spent in the light side (right) of rats submitted to the GDEE injections into dPAG 3 h after the test. Vertical bars represent SEM. *Significant difference between Dzp and Sal groups within the same condition (Baseline, Test, dPAG treatment). #Significant difference between Baseline, Test and dPAG treatment within the same group (Sal or Dzp). Two-way RM ANOVA, post-hoc Newman–Keuls; $p<0.05$.

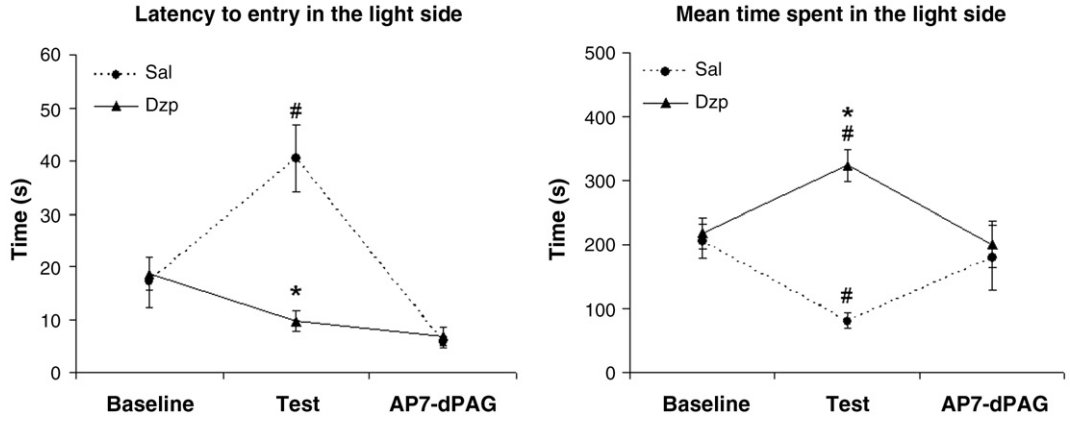

Fig. 6. Effects of the interruption of the chronic treatment with saline (Sal) or diazepam (Dzp) on the latency for entry (left) and the time spent in the light side (right) of rats submitted to the AP-7 injections into dPAG 3 h after the test. Vertical bars represent SEM. *Significant difference between Dzp and Sal groups within the same condition (Baseline, Test, dPAG treatment). #Significant difference between Baseline, Test and dPAG treatment within the same group (Sal or Dzp). Two-way RM ANOVA, post-hoc Newman–Keuls; $p<0.05$.

effects of condition on GDEE and AP-7 groups (GDEE: latency $F_{2.38}=5.10$, time $F_{2,38}=3.38$, AP-7: latency $F_{2,36}=10.68$; $p<0.05$, time $F_{2,36}=0.20$; $p>0.05$), and significant interaction between the factors (saline: latency $F_{2,36}=10.99$, time $F_{2,36}=12.51$, GDEE: latency $F_{2,38}=6.29$, time $F_{2,38}=10.06$, AP-7: latency $F_{2,36}=10.29$, time $F_{2,36}=7.89$; $p<0.05$ for all groups). Post-hoc analysis showed that saline injections into the dPAG did not change the place aversion to the light side in the saline or the place preference in Dzp-injected animals (Fig. 4). The microinjection of the AMPA antagonist GDEE decreased the latency (Fig. 5, left) but did not change the time spent in the conditioned side in control animals (Fig. 5, right). In the same way, the microinjection of the NMDA antagonist AP-7 into the dPAG produced similar antiaversive effects on the latency (Fig. 6, left) and in the time spent in the light compartment in saline-treated animals (Fig. 6, right). On the other hand, the local injections into the dPAG of both glutamatergic antagonists GDEE or AP-7 in spite of not changing the latency, decreased the time spent in the preferred side, blocking the effects of the Dzp withdrawal.

4. Discussion

A factor common to all dependency-producing drugs is that they drive the organism to initiate a complex pattern of drug-seeking behavior that is controlled by a number of different processes, including its positively reinforcing properties (producing pleasurable effects) and negatively reinforcing properties (removal of unpleasant effects elicited by withdrawal, after the drug reinstatement). In our study, 48 h of Dzp withdrawal (that increases the fear levels of rats submitted to many behavioral procedures) decreases the latency and increases the time spent in the drug-paired side of the light–dark box, showing the development of CPP (Fig. 1). In this case, it is likely that this CPP, which supposedly reflects a certain type of drug-seeking behavior, is an attempt to eliminate the negative-affective state developed during withdrawal. This negative-affective state seems to be powerful enough to drive the animal towards the drug-delivery environment, so that not even the presentation of a second strong aversive stimulus immediately before the drug (foot-shocks) blocked the CPP. Control rats tested 48 h after the last injection of saline showed the expected aversion for the luminous and open space of the light compartment of the experimental box, represented by the increase in the latency to cross towards and the decrease of the time of permanence in the light side. In this case, it is possible that the aversion promoted by the daily injection and manipulation of the animals also accounts for this effect.

The negative reinforcing effects of the withdrawal of a drug of abuse can be assessed if we measure the strength of the withdrawal effects in rats placed in a Pavlovian conditioning paradigm, associating the drug positive effects with a punishment. In this study, foot-shocks applied in the three sessions of the conditioning phase added to the light compartment as unconditioned aversive stimulus. Fig. 2 shows an increase in the time of freezing of control animals across the three aversive conditioning sessions and a decrease of this measure in the Dzp-withdrawn animals. In this case, the increase in the time of freezing in the vehicle-treated rats appears to be consequence of a sum of the aversive conditioning effects generated by the light, foot-shocks, open space, and context, which were attenuated in animals chronically treated with Dzp.

Our results replicate, in an animal model, one of the main concepts related to drug dependence reported in DSM-IV (American Psychiatric Association, 2000), that is, the subject has an extremely high motivation to engage in activities focused on the procurement and consumption of the drug, despite the risk of harmful consequences involved. Actually, this finding was first reported by Deroche-Gamonet et al. (2004) through measuring the persistence of rats in responding for the drug when drug delivery was associated with a concomitant punishment.

Much evidence points to the critical role of the EAA in the integration of the somatic and autonomic responses of the defensive reaction, particularly for that elicited through the stimulation of the dPAG (Brandão et al., 1999). Additionally, many studies have demonstrated the importance of the

glutamate receptors in the physical dependence caused by the chronic consumption of alcohol, barbiturates and opiates (for a revision see Stephens, 1995). The first experimental evidence suggesting the importance of the glutamate receptors in the production of the symptoms of Bzp withdrawal was the demonstration that the intracerebral injection of AMPA or NMDA receptors antagonists reduces the appearance of these signs in mice (Steppuhn and Turski, 1993). However, although in this experiment little anxiety was recorded between the first and third days of withdrawal, many other studies have shown that even 24 or 48 h of withdrawal are sufficient to elicit high levels of anxiety in rats (File et al., 1991; Martijena et al., 1996; Andrews et al., 1997; Allison and Pratt, 2006) and mice (Pokk and Zharkovsky, 1998; VonVoigtlander and Lewis, 1991).

In our study, the microinjection of saline into the dPAG does not change the aversion promoted by the aversive conditioning (Fig. 4), but the local dPAG injection of the AMPA-kainate (GDEE) or NMDA receptors (AP-7) antagonists was effective in blocking the place aversion of Dzp-treated animals (Figs. 5 and 6), which supports the involvement of the EAA neurons of this structure in the modulation of the fear-like behaviors elicited during Dzp withdrawal. Besides, both EAA antagonists, when injected into the dPAG, did not alter the latency but reduced the time spent in the light side of the test box of abstinent animals, probably through the decrease of the high aversion levels promoted by withdrawal. With the reduction of this enhanced fear state, the anxiety-like state returned to the baseline condition. Indirect evidence of this assumption lies in the fact that both antagonists promoted a reduction in the latency to enter the light compartment in animals chronically treated with saline, indicating an antiaversive effect for the EAA receptors blocking in the dPAG. On the other hand, only the NMDA receptors antagonism was effective in changing the aversion promoted by the conditioning in vehicle-treated animals. In fact, the blockade of the EAA neurotransmission in the dPAG reduces the aversion promoted by a series of other stressors, as a consequence of the anxiolytic or even sedative effects promoted by this class of drugs on the central nervous system. However, a possible sedative effect of the drugs in the present study is discarded since the latency for transition from the dark to the light side returns to the basal condition in control animals. On the other hand, only the NMDA antagonism was effective in reducing the aversion for the light side. One of the explanations for this effect would be that both GDEE and AP-7 disrupt the aversive memory promoted by the conditioning, reducing the latency for entrance on the light side of the box. However, the exposure to the aversive environmental cues in this side promotes a reintegration of learning processes mainly due to context-dependent association. On the other hand, the inefficacy of the drugs in changing this measure in withdrawn animals could be simply due to presence of a ceiling effect. Actually, despite the absence of studies showing the memory impairment properties of the AMPA-kainate receptors antagonism, the ability of the NMDA receptors in producing learning deficits and disruption of memory abilities is well known (Benvenga and Spaulding, 1988; Danysz et al., 1988; Tang and Ho, 1988). For example, Hlinak and Krejci (2002) found that, in mice, systemic injections of MK-801, a non-competitive NMDA receptor antagonist, prolongs the latency for transition from the open to the closed arm of the plus-maze indicating some type of deficits in the "memorization" processes. In another study (Santos et al., 2006) it was demonstrated that the antagonism of the glycine-B site at NMDA receptor, through the microinjection of 7-chloro-kynurenic acid (7CK) in the dPAG, impaired the aversive memory of rats in a task of inhibitory avoidance. Regarding the absence of the effects of GDEE on the time of permanence in the light side, this could be linked to the fact that these fast-acting glutamate receptors are mainly involved in the acquisition, whereas the NMDA ones in the expression, of the fear-like behaviors (Pandóssio and Brandão, 1999). In fact, while GDEE blocks the transient freezing caused by glutamate microinjection into the inferior colliculus, AP-7 does not interfere with the occurrence of this response (Brandão et al., 2003).

All these results lead us to the conclusion that the fear elicited by Dzp withdrawal is probably due to the activation of the neural substrates of aversion in the brainstem, such as the defense reaction elicited by electrical or chemical stimulation of the dPAG (Brandão et al., 2005). Therefore, this region, mainly implicated in the production of the active responses to unconditioned dangerous stimuli could also be activated in Dzp-withdrawn animals (Fanselow, 1994). It is also likely that the decrease of the GABA inhibition promoted during withdrawal could lead to an enhancement of the excitatory tone in the dPAG that could contribute to increase the negative-emotional state, driving the animals towards the context where they where formerly reinforced by diazepam injections. The enhancement of this excitatory tone, associated with a reduction in the GABA inhibition promoted by the drug-withdrawal, could be one of the possible reasons for the appearance of the withdrawal symptoms after the interruption of the chronic treatment with Bzp and an important component of drug-seeking behavior inducing relapse. In spite of all these studies, the critical neural substrate involved in reinforcing properties and mainly the symptoms elicited during Bzp withdrawal still remain unsolved. In this content, this is the first report showing that changes in the glutamatergic neurotransmission in the dPAG might be involved in the Dzp withdrawal and relapse.

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