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Aversive learning as a mechanism for lack of repeated anxiolytic-like effect in the elevated plus-maze

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ABSTRACT

Rodents re-exposed to the elevated plus-maze no longer respond to anxiolytic-like drugs, such as benzodiazepines. This phenomenon is thought to be due to retrieval of aversive learning associated with the initial exploration of this potentially dangerous environment. Based on this assumption, one might expect the maintenance of the drug's anxiolytic-like effect in rodents already experienced in the elevated plus-maze if the acquisition and/or consolidation of this learning were impaired. Using male Wistar rats, we investigated whether the systemic administration of propranolol, at putative learning-impairing doses, prior to or immediately after the first (Trial 1) elevated plus-maze exposure would retain the midazolam anxiolytic-like effect on the second (Trial 2) exposure to this apparatus. There was an anxiolytic-like effect, characterized by an increase in the open-arms exploration, in response to 0.25 mg/kg of midazolam on Trial 2 only in rats administered with 20 mg/kg of propranolol before Trial 1. Although propranolol had a dose-dependent and behaviorally-selective anti-anxiety effect (significant at 20 mg/kg) on Trial 1, further minute-by-minute analysis confirmed the propranolol-induced learning acquisition deficit in this group on Trial 2. The knowledge of the environment actually contributes to the unresponsiveness to anxiolytic-like drugs observed in rats re-exposed to the elevated plus-maze.

1. Introduction

The consequence of prior (Trial 1) elevated plus-maze exposure on the anxiolytic-like effect of drugs has attracted attention since the characteristic increase in the open-arms exploration observed after their administration is no longer found in rats and mice re-exposed (Trial 2) to this apparatus (File et al., 1990; Rodgers et al., 1996). This phenomenon is pertinent to compounds which augment the GABA-A receptor-complex activity, such as benzodiazepines, barbiturates and ethanol, reduce the NMDA/glycine-B-receptor-complex activity, such as MK-801, HA-966 and memantine, as well as to those that either activate or antagonize the serotonin type 1A receptor (for a review see Carobrez and Bertoglio, 2005).

Several hypotheses have been proposed to explain this lack of anxiolysis in elevated plus-maze experienced rodents. One of them attributes this phenomenon to the retrieval of an aversive learning, which is acquired throughout the initial exploration of this potentially dangerous environment (File, 1993; Bertoglio and Carobrez, 2000; Lamprea et al., 2000; Dal-Cól et al., 2003; Vargas et al., 2006). The following results have corroborated this proposal: 1) the temporary deactivation with lidocaine of the dorsal hippocampus prior to Trial 1, of the basolateral amygdala immediately after Trial 1, or of the dorsomedial hypothalamus and the dorsolateral periaqueductal gray prior to Trial 2, retains the anxiolytic-like effect of benzodiazepines, such as chlordiazepoxide and midazolam, on Trial 2 (File et al., 1998, 1999; Bertoglio et al., 2005, 2006). These findings are interpreted as an impairment of acquisition (hippocampus), consolidation (amygdala) and retrieval (hypothalamus and periaqueductal) of information gathered during Trial 1; and 2) the administration of an amnesic dose (10-fold the anxiolytic-like dose) of chlordiazepoxide (File et al., 1990) or scopolamine (Bertoglio and Carobrez, 2004) prior to Trial 1 prevents the occurrence of this phenomenon in elevated plus-maze experienced rats. On the other hand, the enhancement of memory retention, by systemically administering either amphetamine or pentylenetetrazole post-Trial 1, facilitates its development (Vargas et al., 2006).

In this context, noradrenergic mechanisms have been implicated in learning and memory processes for emotional events (Chamberlain et al., 2006). Of particular relevance to the present work are those findings showing that propranolol, a beta-noradrenergic antagonist, impairs the stage of learning acquisition in a variety of tasks in humans (Cahill et al., 1994; van Stegeren et al., 1998) and laboratory animals (Gold et al., 1986; Lennartz et al., 1996).

Based on these facts, the following hypothesis was formulated: if an aversive learning occurs during Trial 1, then its impairment should retain the drug anxiolytic-like effect in rodents re-exposed to the elevated plus-maze. In the present study we addressed this issue by investigating whether the administration of propranolol prior to Trial 1, or immediately after Trial 1, would prevent the lack of the midazolam anxiolytic-like effect in elevated plus-maze experienced rats.

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2. Materials and methods

2.1. Animals

The subjects were male Wistar rats weighing 300–350 g, aged 14–16 weeks at the time of testing, housed in groups of five per cage (50×30×15 cm) in a temperature-controlled room (22±1 °C), under standard laboratory conditions with free access to food and water, and with a 12 h light/12 h dark cycle (lights on at 07:00 h a.m.).

2.2. Drugs

(S)-(-)-propranolol hydrochloride (Sigma-Aldrich, USA) and midazolam (Roche, Brazil) were dissolved in 0.9% NaCl (saline), which, alone, served as a vehicle control. The solutions were administered intraperitoneally in an injection volume of 1.0 ml/kg. Propranolol and midazolam dose selection was based on previous studies published elsewhere (Gold et al., 1986; Cruz et al., 1994; Lennartz et al., 1996; Bertoglio et al., 2005).

2.3. Apparatus

The elevated plus-maze apparatus was made of wood and consisted of two opposite open-arms, 50×10 cm (surrounded by a 1 cm high Plexiglas ledge), and two enclosed-arms, 50×10×40 cm, set up 50 cm above the floor. The junction area of the four arms (central platform) measured 10×10 cm (Treit et al., 1993; Carobrez and Bertoglio, 2005). In order to avoid urine impregnation the floor of the apparatus was painted with impermeable epoxy resin.

2.4. Experimental design

All procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals, which are in compliance with international laws and politics. The local Ethical Committee also approved (069/CEUA/PRPe/2007) the current protocol.

Behavioral tests were carried out in a low illumination (40-lux) condition room, during the diurnal phase (between 13:00 and 17:00 h). Elevated plus-maze sessions last for 5 min, and were recorded by a video camera while a monitor and a DVD-recording system were installed in an adjacent room. An observer, which was blind to the treatment condition described below, scored the behavioral parameters from the DVD, with an agreement between repeated analyses (inter-observer reliability) of the same test of ≥90%. After each session, the apparatus was cleaned with 10% ethanol solution (v/v) and dry towels.

2.4.1. Experiment 1: effects of midazolam on Trial 2 of rats treated with propranolol prior to Trial 1

Rats were randomly assigned for one of the four groups according to the drug treatment given 30 min before the first elevated plus-maze exposure: saline (n=21), propranolol 5.0 mg/kg (n=21), propranolol 10 mg/kg (n=18), or propranolol 20 mg/kg (n=25). Twenty-four hours later, each group was randomly divided in two groups (n=9–13 per group) based on the drug treatment – saline or 0.25 mg/kg of midazolam – given 30 min prior to the second elevated plus-maze exposure. In each situation the animals were tested in a counterbalanced order for treatment condition.

2.4.2. Experiment 2: effects of midazolam on Trial 2 of rats treated with propranolol post-Trial 1

Rats were randomly assigned for one of the two groups according to the drug treatment given immediately after the first elevated plus-maze exposure: saline (n=16) or propranolol 20 mg/kg (n=17). Twenty-four hours later, each group was randomly divided in two groups (n=8–9 per group) based on the drug treatment – saline or 0.25 mg/kg of midazolam – given 30 min prior to the second elevated plus-maze exposure. The animals were tested in a counterbalanced order for treatment condition in each situation.

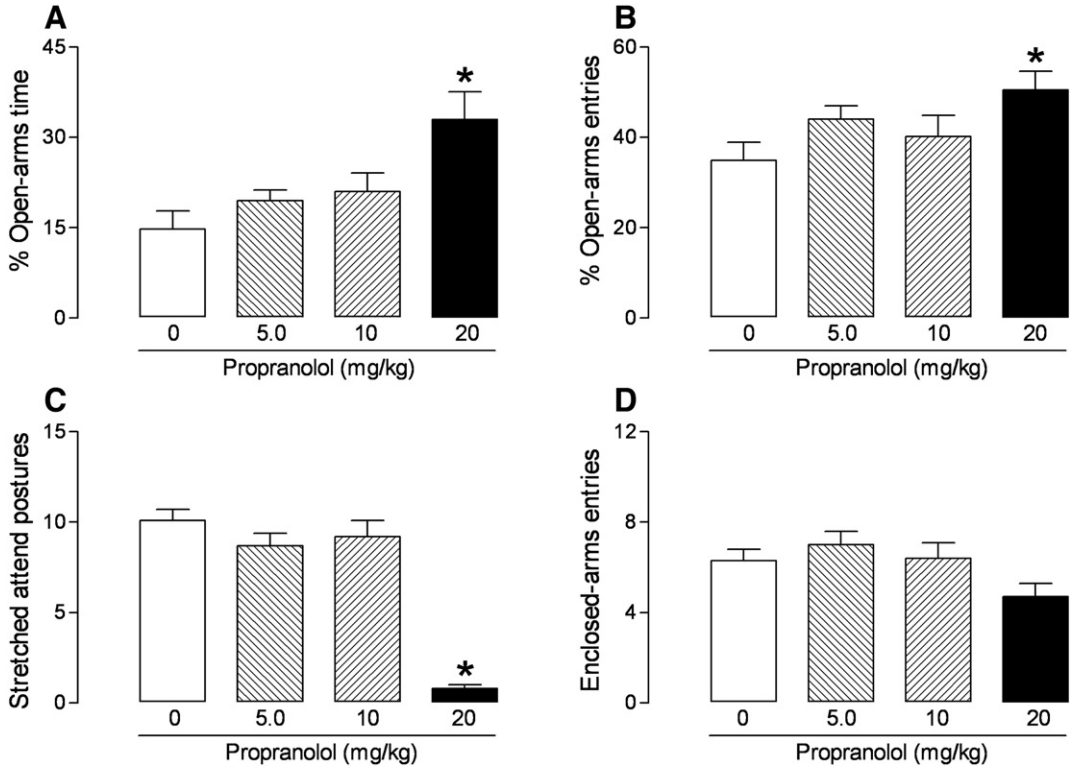

Fig. 1. Effects of propranolol (5.0–20 mg/kg) in rats submitted to the first elevated plus-maze exposure (n=9–13). Bars represent the mean+SEM. Asterisk indicates a significant difference from saline-treated group (p<0.05).

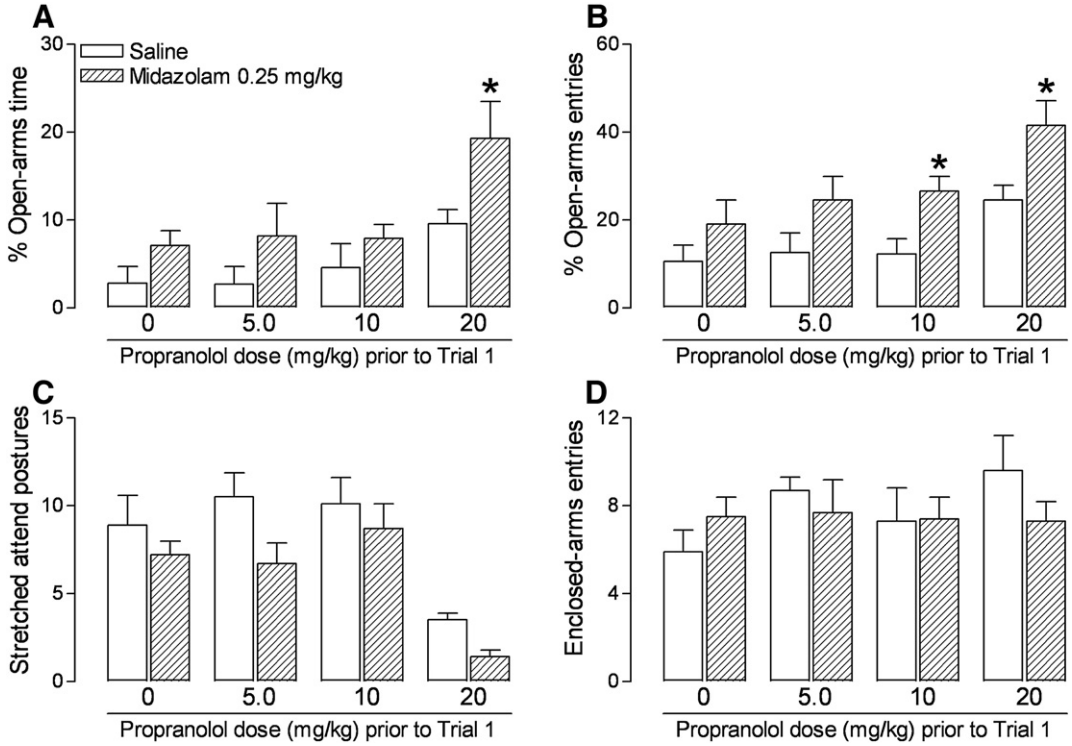

Fig. 2. Effects of midazolam (0.25 mg/kg) on Trial 2 of rats administered with propranolol (5.0–20 mg/kg) prior to Trial 1 (n=9–13). Bars represent the mean+SEM. Asterisk indicates a significant difference from respective saline-treated group (p<0.05).

2.5. Behavioral measures

The following parameters were scored from the DVD: the number of open- and enclosed-arms entries (EAE) with the four paws, and the time spent in the central platform, open- and enclosed-arms. These data were used to calculate the percentage of open-arm entries {% OAE; [open-entries/(open+enclosed-entries)]×100} and the percentage of time spent in the open-arms {%OAT; [(open time/300)×100]}. The number of stretched-attend postures (SAPs), defined as an exploratory posture where the animal stretches forward and then retracts to its original position, performed by rats from the central platform or enclosed-arms towards open-arms, was also recorded.

2.6. Statistics

Data from full elevated plus-maze sessions were analyzed by one- (treatment) or two-way (pretreatment and treatment) analysis of variance (ANOVA). Minute-by-minute data for the %OAT parameter were analyzed by three-way (pretreatment, treatment and time bin) repeated measures ANOVA. When variances among groups were not homogenous, the raw data were log transformed, and then rechecked. In all situations in which this approach was required, the homogeneity of variance was ensured. The Duncan test was used for post-hoc comparisons when appropriated. The significance level was set at P<0.05.

3. Results

3.1. Experiment 1: effects of midazolam on Trial 2 of rats treated with propranolol prior to Trial 1

There was an increase in %OAT [$F_{3,81}$=5.10, P<0.01] and in %OAE [$F_{3,81}$=2.91, P<0.05], as well as a reduction in SAPs [$F_{3,81}$=59.5, P<0.00001], in rats exposed to the elevated plus-maze after the treatment with 20 mg/kg of propranolol (Fig. 1A–C). At the dose range

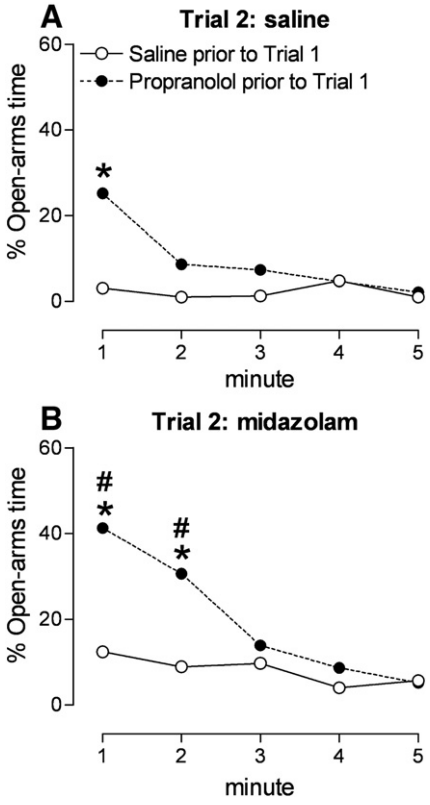

Fig. 3. Minute-by-minute effect of midazolam (0.25 mg/kg) on the percentage of time spent in the open-arms on Trial 2 of rats treated with 20 mg/kg of propranolol prior to Trial 1 (n=9–13). Lines represent the mean. Asterisk indicates a significant difference from respective saline-treated group (p<0.05) while the hash symbol (#) indicates a significant difference from propranolol-saline group (p<0.05).

Table 1
Trial 1 elevated plus-maze scores of undrugged rats administered with saline or 20 mg/kg of propranolol immediately after Trial 1 and with saline or 0.25 mg/kg of midazolam before Trial 2 (n=8–9)

Behavioral measure	% Open-arm time	% Open-arm entries	Stretched-attend postures	Enclosed-arm entries
Saline–saline	26.6±7.9	46.1±6.9	12.1±1.3	4.9±0.6
Saline–midazolam	27.5±6.4	47.9±4.1	10.1±2.3	6.4±0.5
Propranolol–saline	27.6±5.0	44.9±5.0	11.3±1.3	7.2±0.7
Propranolol–midazolam	18.8±4.5	36.1±4.5	11.9±1.6	6.3±0.9

Data are presented as Mean±S.E.M.

tested here, however, propranolol did not significantly [$F_{3,81}$=1.84, P<0.12] interfere with EAE (Fig. 1D).

Fig. 2 shows the elevated plus-maze Trial 2 scores produced by midazolam in groups treated with saline or propranolol prior to Trial 1. There was a significant pretreatment and a treatment effect for %OAT [$F_{3,77}$=7.71, P<0.0001 and $F_{1,77}$=15.2, P<0.001, respectively], %OAE [$F_{3,77}$=6.29, P<0.001 and $F_{1,77}$=27.7, P<0.00001, respectively], and SAPs [$F_{3,77}$=20.8, P<0.00001 and $F_{1,77}$=13.0, P<0.001, respectively]. Further pair-wise comparisons revealed an increase in open-arms exploration by midazolam in the group administered with 20 mg/kg of propranolol (Fig. 2A–B). Midazolam also increased the %OAE in the group administered with 10 mg/kg of propranolol prior to Trial 1 (Fig. 2B). With regard to EAE (Fig. 2D), neither pretreatment [$F_{3,77}$=0.69, P<0.56] nor treatment [$F_{1,77}$=0.04, P<0.85] effects were statistically significant. The pretreatment versus treatment interaction was statistically insignificant for any behavioral measures scored on Trial 2 (%OAT [$F_{3,77}$=0.62, P<0.60], %OAE [$F_{3,77}$=0.05, P<0.98], SAPs [$F_{3,77}$=1.36, P<0.27], and EAE [$F_{3,77}$=0.54, P<0.66]). Moreover, prior experience in the elevated plus-maze significantly decreased %OAT and %OAE on Trial 2 of rats injected with saline prior to Trial 1 and prior to Trial 2 (Figs. 1 and 2), and eliminated the midazolam anxiolytic-like effect in the group treated with saline before Trial 1 and with midazolam before Trial 2 (Fig. 2A–B).

Fig. 3 shows the Trial 2 minute-by-minute %OAT scores produced by midazolam in groups treated with saline or 20 mg/kg of propranolol before Trial 1. There were significant pretreatment versus time bin [$F_{4,168}$=5.72, P<0.001] and treatment versus time bin [$F_{4,168}$=2.73, P<0.05] interactions. However, neither the pretreatment versus treatment [$F_{1,42}$=0.43, P<0.52] nor the pretreatment versus treatment versus time bin [$F_{4,168}$=0.58, P<0.68] interactions were statistically significant. Subsequent pair-wise comparisons showed an increase in %OAT on the first minute of Trial 2 in saline-treated rats that had been injected with propranolol before Trial 1, as compared to those rats administered with saline either prior to Trial 1 or prior to Trial 2 (Fig. 3A). The %OAT was also augmented by midazolam on the first and second minutes of Trial 2 in rats treated with propranolol prior to Trial 1, as compared to those rats administered with saline prior to Trial 1 and midazolam prior to Trial 2 (Fig. 3B). The magnitude of propranolol's effect was statistically significantly higher in midazolam-treated group (Fig. 3).

3.2. Experiment 2: effects of midazolam on Trial 2 of rats treated with propranolol post-Trial 1

The Trial 1 performance of undrugged rats administered with saline or 20 mg/kg of propranolol post-Trial 1 and with 0.25 mg/kg of midazolam or its vehicle before Trial 2 is shown in Table 1. All behavioral measures scored were similar in these groups (%OAT [$F_{3,29}$=0.50, P<0.68], %OAE [$F_{3,29}$=0.85, P<0.48], SAPs [$F_{3,29}$=0.22, P<0.88], and EAE [$F_{3,29}$=1.90, P<0.15]).

Fig. 4 shows the elevated plus-maze Trial 2 scores produced by midazolam in the groups treated with saline or 20 mg/kg of propranolol immediately after Trial 1. The overall ANOVA comparisons showed only statistically insignificant pretreatment or treatment effects (%OAT [$F_{1,29}$=2.42, P<0.13 and $F_{1,29}$=1.16, P<0.29, respectively], %OAE [$F_{1,29}$=1.44, P<0.24 and $F_{1,29}$=0.01, P<0.96, respectively], SAPs [$F_{1,29}$=3.12, P<0.09 and $F_{1,29}$=1.28, P<0.27, respectively], and EAE [$F_{1,29}$=0.13, P<0.72 and $F_{1,29}$=0.05, P<0.82, respectively]). The pretreatment versus treatment interaction was also not statistically significant

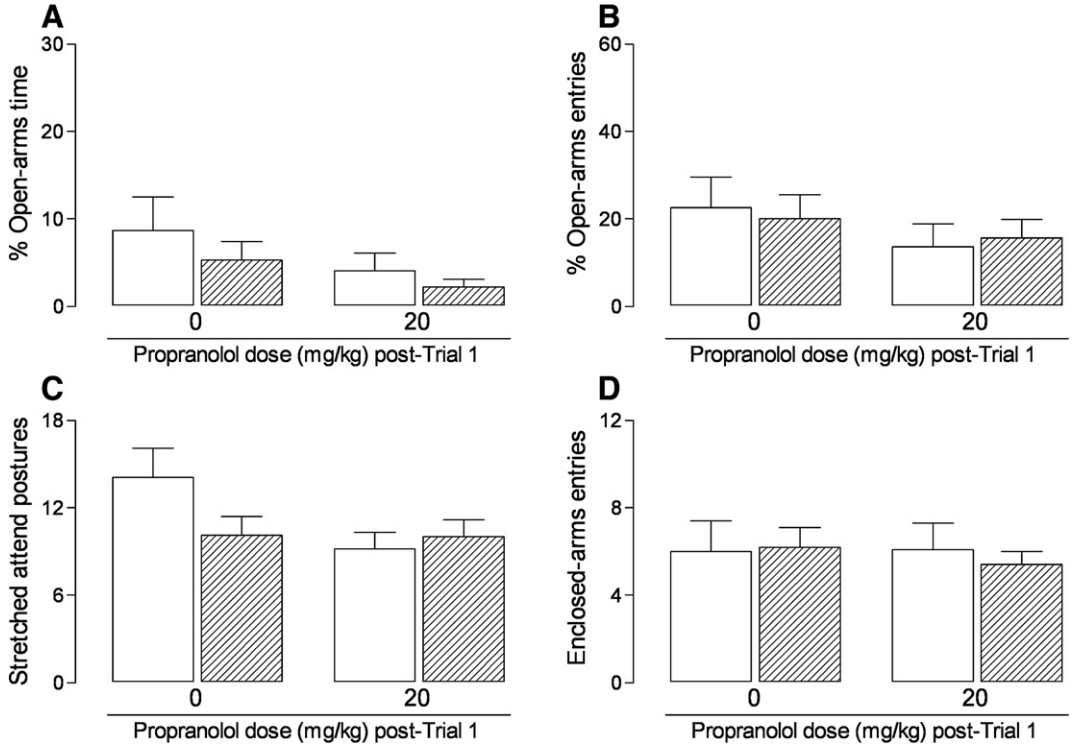

Fig. 4. Effects of midazolam (0.25 mg/kg) on Trial 2 of rats administered with 20 mg/kg of propranolol immediately after Trial 1 (n=8–9). Bars represent the mean+SEM.

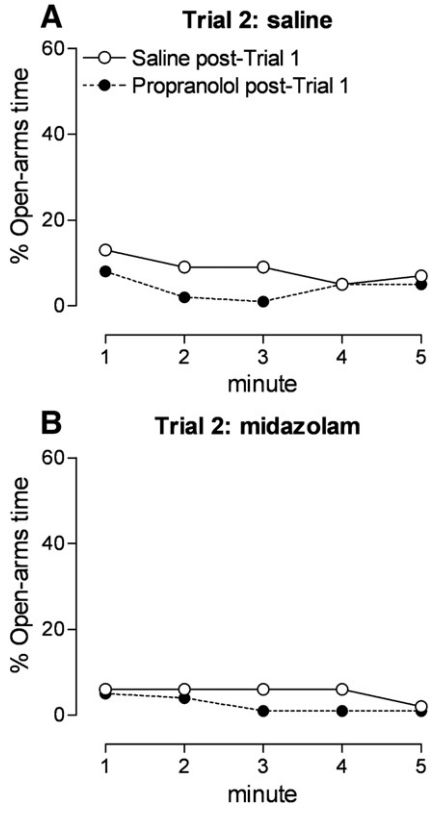

Fig. 5. Minute-by-minute effect of midazolam (0.25 mg/kg) on the percentage of time spent in the open-arms on Trial 2 of rats treated with 20 mg/kg of propranolol immediately after Trial 1 (n=8–9). Lines represent the mean.

for any behavioral parameters scored on Trial 2 (%OAT [$F_{1,29}$=0.10, P<0.75], %OAE [$F_{1,29}$=0.16, P<0.69], SAPs [$F_{1,29}$=2.82, P<0.10], and EAE [$F_{1,29}$=0.21, P<0.65]). However, prior experience in the elevated plus-maze significantly decreased %OAT and %OAE on Trial 2 of rats injected with saline immediately after Trial 1 and prior to Trial 2 (Table 1 and Fig. 4), and eliminated the midazolam anxiolytic-like effect in the group treated with saline post-Trial 1 and with midazolam before Trial 2 (Fig. 4A–B).

Fig. 5 shows the Trial 2 minute-by-minute %OAT scores produced by midazolam in groups treated with saline or 20 mg/kg of propranolol immediately after Trial 1. The overall ANOVA comparisons showed only statistically insignificant interactions (pretreatment versus time bin [$F_{4,116}$=0.48, P<0.75], treatment versus time bin [$F_{4,116}$=0.49, P<0.75], pretreatment versus treatment [$F_{1,29}$=0.10, P<0.75], and the pretreatment versus treatment versus time bin [$F_{4,116}$=0.76, P<0.55]). Accordingly, the min-by-min %OAT score produced by midazolam on Trial 2 in the group treated with 20 mg/kg of propranolol post-Trial 1 was similar to controls (Fig. 5).

4. Discussion

There was no increase in the open-arms exploration in response to midazolam during the Trial 2 of rats treated with saline prior to Trial 1, indicating the lack of its anxiolytic-like effect in rats re-exposed to the elevated plus-maze. In rats administered with 20 mg/kg of propranolol, however, the midazolam anxiolysis was retained since the open-arms exploration was augmented on the first minutes. In fact, the propranolol/saline-treated group also increased the exploration on the Trial 2 beginning, but the magnitude of drug effect found in propranolol/midazolam-treated rats was clearly higher. Moreover, controls (the saline–saline group) re-exposed to the elevated plus-maze demonstrated an increase in open-arms avoidance, therefore supporting an experimentally induced sensitization of behavioral indices of anxiety in this test (Treit et al., 1993; Bertoglio and Carobrez, 2000).

It has been proposed that an aversive learning is acquired during the first elevated plus-maze exploration (File et al., 1990; Rodgers et al., 1996). The retrieval of this knowledge seems to interfere with the subsequent drug responsiveness in this test (Bertoglio and Carobrez, 2000, 2002, 2004; Dal-Cól et al., 2003; Vargas et al., 2006). The maintenance of the midazolam anxiolytic-like effect on Trial 2 implies that such learning was sufficiently impaired by 20 mg/kg of propranolol given prior to Trial 1. As the same dose of propranolol given post-Trial 1 failed to retain the midazolam anxiolysis, it is suggested that propranolol only impaired the acquisition of this knowledge related to the experience in a potentially dangerous environment. Based on the fact that propranolol *per se* reduced the aversiveness on Trial 1, it is also possible that the aversive learning was attenuated rather than impaired. A similar assumption was proposed to explain the lack of prior elevated plus-maze experience effect in animals administered with an amnesic dose of chlordiazepoxide prior to Trial 1 (File et al., 1990). It is of note that these are not mutually excluding ideas, but they do effectively provide a common denominator explanation for the apparent loss of aversive learning/memory.

Previously published studies of systemically administered propranolol have demonstrated its effects on memory acquisition. Acute pretraining administration of propranolol impaired either the retention of inhibitory avoidance in rats (Gold et al., 1986) or the memory for an emotional event in humans (Cahill et al., 1994; van Stegeren et al., 1998). The latter finding corroborates studies using animal models, and suggests that propranolol can inhibit the memory modulation. Because propranolol acts equally at both beta-1 and beta-2 noradrenergic receptors, we cannot determine from the present findings which central (van Stegeren et al., 1998) receptor subtype has contributed more to the learning acquisition deficit observed. Future experiments using beta-noradrenergic antagonists with selective actions for beta-1 and beta-2 subtypes can address this question. Furthermore, propranolol also blocks at some extent the serotonin type 1A receptor (Middlemiss, 1984). Although animal research using other beta-noradrenergic antagonists with no anti-serotonergic activity (e.g. atenolol) implicates the beta-noradrenergic receptors in memory acquisition for emotional events, it is possible that the anti-serotonergic activity could account for the propranolol effects on anxiety (Audi et al., 1989). Indeed, the systemic administration of this drug (Gorman and Dunn, 1993), and other serotonin type 1A receptor antagonists as well (Griebel et al., 2000; Carobrez and Bertoglio, 2005), produce an anxiolytic-like effect in rats exposed to the elevated plus-maze. Our results suggest a similar conclusion. After a closer look at these data (Fig. 3A), however, an alternative interpretation arises: a propranolol-induced learning acquisition deficit. The evidence is given by minute-by-minute analysis. Whereas the group treated with saline prior to Trial 1 displayed low (and stable) levels of %OAT during Trial 2, the group receiving 20 mg/kg of propranolol prior to Trial 1, but no post-Trial 1, performed differently. A similar assumption was proposed to explain conflicting results of the scopolamine on the elevated plus-maze performance (Bertoglio and Carobrez, 2004). Importantly, the level of %OAT on Trial 2 of rats treated with an anxiolytic-like dose of midazolam prior to Trial 1 was similar to controls (Carobrez and Bertoglio, 2005). Altogether, these findings reinforce the usefulness of the Trial 1 and Trial 2 protocol in detecting non-selective and/or false-positive anxiolytic-like effect of drugs (Carobrez and Bertoglio, 2005). Moreover, this approach based on targeting anxiety plus learning/memory aspects represents a valuable tool to investigate the relationship between emotion and cognition domains, which often are so integrated that they jointly contribute to behavior (Vargas et al., 2006; Kalueff and Murphy, 2007; Pessoa, 2008).

In summary, propranolol significantly impaired the acquisition, but not the consolidation, of the aversive learning which seems to be responsible for the lack of the anxiolytic-like effect of drugs in rats re-exposed to the elevated plus-maze. Alternatively, the anxiolytic-like

effect of midazolam was retained on Trial 2 because propranolol attenuated rather than impaired the aversive learning. Whatever the case, the information gathered on the initial elevated plus-maze exploration actually contributes to the occurrence of this phenomenon.

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