

Role of beta-adrenergic receptors in the ventromedial prefrontal cortex during contextual fear extinction in rats

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ARTICLE INFO

Article history:

Received 25 January 2010

Revised 11 July 2010

Accepted 20 July 2010

Available online 25 July 2010

Keywords:

Noradrenergic system

Beta-adrenoceptor

Beta-blocker

Contextual fear

Ventromedial prefrontal cortex

Extinction

ABSTRACT

It has been reported that stress-related activation of the noradrenergic system strengthens the formation of aversive memories and that beta-adrenergic receptors seem to be involved in this emotional memory processing. In this study, the effects of beta-adrenergic compounds on the extinction of contextual conditioned fear responses were evaluated. Rats were trained with footshock in a conditioning box. In the 3 days following the training, the animals were re-exposed to the apparatus and received either a single or repeated intraperitoneal injections of the beta-adrenergic antagonist propranolol, the beta-adrenergic agonist isoproterenol, or saline 30 min before (acquisition of extinction) or immediately after (consolidation of extinction) the extinction sessions. A drug-free session was performed on the last day. While repeated isoproterenol treatment facilitated the consolidation of contextual fear extinction, repeated propranolol administration impaired the acquisition and the consolidation of this process. Further, the role of ventromedial prefrontal cortex (vmPFC) in the extinction of contextual conditioned fear was tested with an immunohistochemistry assay. Our results show a reduction in Fos-protein expression between the first and the last extinction session. In a follow-up experiment, intra-vmPFC microinjection of isoproterenol before the first extinction session facilitated the extinction of contextual fear. This facilitation was antagonized by pre-treatment with atenolol, suggesting that this change is mediated by beta-1-adrenergic activity. Our results reinforce the role of the vmPFC in fear extinction mechanisms, suggesting that vmPFC-beta-1-adrenergic receptor activation underlies part of the facilitation of the fear extinction processes.

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1. Introduction

Several reports have shown that traumatic events, such as civil wars, acts of terrorism, and natural catastrophes, could augment the risk for the development of anxiety disorders (Bonne, Grillon, Vythilingam, Neumeister, & Charney, 2004; Feldner, Monson, & Friedman, 2007). Following extreme stress situations, some individuals will show defensive behavioral responses in non-stressful situations as the result of long-lasting recurrence of the trauma consolidated as an aversive memory (Nemeroff et al., 2006). Most of the common therapeutic techniques for the attenuation of anxiety disorders in humans are essentially extinction procedures involving repeated exposure to the feared object or situation in the absence of any clear danger (Cloitre, 2009; Foa, 2006). Insights into the neuropharmacological aspects of post-traumatic recurrent memories in humans have been obtained through pharmacologi-

cal, neurochemical and behavioral studies using rats to study the extinction of Pavlovian fear conditioning (Quirk & Mueller, 2008).

Contextual conditioned fear has been widely used as an important behavioral paradigm for studying the fear extinction processes in laboratory animals. The conditioned context contains a large set of physical elements or features (odor, floor texture, sound level, illumination, shape, size) that can simulate the human pathological situation (Ji & Maren, 2007; Rudy, Huff, & Matus-Amat, 2004). In this model, a neutral stimulus (e.g., a new context) acquires aversive properties after association with a biologically relevant stimulus, such as a footshock. The repeated presentation of the conditioned stimulus in the absence of aversive consequences results in a reduced occurrence of defensive behaviors, a phenomenon referred to as extinction (Myers & Davis, 2007; Tronson & Taylor, 2007).

It has long been known that fearful or threatening experiences are accompanied by an enhancement in noradrenergic transmission, which seems to be responsible for the strength and persistence of the aversive memories (McGaugh & Roozendaal, 2002; Orr et al., 2006). In fact, many studies have illustrated the key role

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of noradrenergic receptors in fear memory process by using the beta-adrenoceptor blockade to impair the acquisition (Do Monte, Canteras, Fernandes, Assreuy, & Carobrez, 2008; Gold, Vogt, & Hall, 1986; Stern, Carobrez, & Bertoglio, 2008), consolidation (Cahill, Pham, & Setlow, 2000; Ferry & McGaugh, 1999; Kroon & Carobrez, 2009), retrieval (Murchinson et al., 2004; Ouyang & Thomas, 2005; Rodriguez-Romaguera, Sotres-Bayon, Mueller, & Quirk, 2009) and reconsolidation (Abrari, Rashidy-Pour, Semnani, & Fathollahi, 2008; Debiec & LeDoux, 2004; Przybylski, Roulet, & Sara, 1999) of fear memories in different experimental protocols. Recently, the mechanisms underlying conditioned fear extinction have been suggested as a new form of inhibitory learning (Bouton, Westbrook, Corcoran, & Maren, 2006). Thus, it is possible that the increased systemic beta-adrenergic activation could facilitate these extinction mechanisms and that a reduction in beta-adrenergic activity could impair this process.

Several brain structures have been implicated in the conditioned fear extinction processes in rodents, including the amygdala (Mamiya et al., 2009), the hippocampus (Ji & Maren, 2007) and the ventromedial prefrontal cortex (vmPFC; Milad & Quirk, 2002; Peters, Kalivas, & Quirk, 2009). Interest in the vmPFC as a component of extinction circuitry has increased due to the numerous studies showing that lesion or pharmacological inactivation of this structure impairs the retrieval of extinction under various conditions (Do Monte, Allensworth, & Carobrez, 2010; Morgan & LeDoux, 1995; Quirk, Russo, Barron, & Lebron, 2000; Sierra-Mercado, Corcoran, Lebrón-Milad, & Quirk, 2006) while electrical stimulation of this structure strengthens the extinction processes (Milad, Vidal-Gonzalez, & Quirk, 2004). In addition, the medial prefrontal cortex (mPFC) receives robust afferent noradrenergic innervation from the locus coeruleus (Aston-Jones, Foote, & Segal, 1985; Robbins, 1984) and contains a high concentration of beta-1-adrenoceptors (Rainbow, Parsons, & Wolfe, 1984).

Based on these facts, the present study tested the influence of beta-adrenergic receptors in the acquisition of extinction or in the consolidation of extinction in rats using the contextual conditioned fear extinction paradigm. Following the manipulation of systemic beta-adrenergic receptors in the vmPFC, the change in Fos-immunoreactivity in response to contextual conditioned fear extinction was evaluated. Based on the suggested role of vmPFC-beta-adrenergic receptors activity in the contextual conditioned fear extinction process, the influence of vmPFC-beta-adrenergic receptors on the acquisition and consolidation of contextual conditioned fear extinction was evaluated in subjects receiving microinjections of drugs that specifically modulate beta-adrenergic receptors.

2. Materials and methods

2.1. Animals

Two hundred seventy four adult male Long-Evans hooded rats weighing 300–350 g and at 14–16 weeks of age at the time of testing were used. The animals were housed in groups of 3–4 per cage (50 × 30 × 15 cm) in standard environmental conditions (24 ± 1 °C on a 12 h/12 h dark/light cycle, light on 7:00 am) with food and water available *ad libitum*. Rats were not handled before experiments.

Experiments were performed during the middle phase of the light cycle. All animal procedures were approved by the Institutional Ethics Committee (23080.006118/2004-36/UFSC/BRAZIL) and were in accordance with NIH animal care guidelines.

2.2. Drugs

Propranolol hydrochloride or isoproterenol (Sigma–Aldrich, USA) were dissolved (10 mg/ml and 2.5 mg/ml, respectively) in

0.9% saline and administered via intraperitoneal (IP) injection at a volume of 1 ml/kg. Saline alone served as the vehicle control.

The doses of 5, 10 and 20 mg/kg IP of propranolol and 1, 2.5 and 5 mg/kg IP of isoproterenol were selected based on previous studies performed in our laboratory. The efficacy of isoproterenol as an agonist and propranolol as an antagonist at the beta-adrenoceptors were evaluated with a cardiovascular assay.

Xylazine (5 mg/kg IP, Rompun[®], Bayer, Brazil) and ketamine (75 mg/kg IP, Dopalen[®], Agribands, Brazil) were used as anesthetics. Heparin (30 IU) diluted in phosphate-buffered saline (PBS, in mM: NaCl 137, KCl 2.7, KH₂PO₄ 1.5, NaHPO₄ 8.1; pH = 7.4) was used as an anticoagulant for the cardiovascular assays.

Atenolol (RS; Atenolol, Tocris, USA) and isoproterenol (Sigma–Aldrich, USA) were dissolved in PBS, which, alone, served as the vehicle control. Subjects received a bilateral injection of atenolol (10 or 40 nmol), isoproterenol (10 or 40 nmol) or PBS in a volume of 0.2 µl.

2.3. Heart rate measurement

Under anesthesia with ketamine and xylazine, heparinized polyethylene (PE-20 and PE-50) catheters were inserted into the left femoral vein for drug injections and into the right carotid artery for recording the heart rate and mean arterial pressure. To prevent clotting, a bolus dose of heparin (30 IU) was injected immediately after vein cannulation. Heart rate and mean arterial pressure were recorded with a catheter pressure transducer (Mikro-Tip[®], Millar Instruments, Inc., Houston, Texas, USA) coupled to Powerlab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). At the end of the experiment, animals were sacrificed with an overdose of chloral hydrate.

2.4. Contextual fear conditioning apparatus

Training and testing occurred in a rectangular chamber (35 × 20 × 30 cm) with side walls constructed of aluminum and a front wall and ceiling-door made of Plexiglas. The grid floor of the chamber was made of stainless steel bars (3 mm diameter spaced 9 mm center-to-center) and was connected to a circuit board (Insight, Ch 2001) that delivered the footshocks. Before the introduction of each rat, the apparatus was cleaned with an ethanol solution (10% v/v) and dried thoroughly. All extinction sessions lasted 10 min. The percentage time the animal spent freezing (characterized by the complete absence of movements except those for breath) was recorded and used as a fear memory retrieval parameter. The extinction index represents the percentage of freezing reduction from the first to the last extinction session and was calculated with the following formula: the total time of freezing behavior during the first extinction session minus the total time of freezing behavior on the last extinction session divided by the total time of freezing behavior in the first extinction session.

The experiments were carried out in a soundproof room with an illumination of 100 lux. A video camera was positioned 80 cm from the front wall of the apparatus and a recording system was located in an adjacent room.

2.5. Surgery

Animals were anesthetized with xylazine (12.5 mg/kg) and ketamine (75 mg/kg) IP injections and positioned in a stereotaxic frame. Lidocaine (0.1 ml; 2 mg/ml) was subcutaneously injected in the scalp and a longitudinal incision was made. The bone was then exposed and two stainless steel guide cannulas (diameter = 0.7 mm; length = 11 mm) were stereotaxically implanted with the cannula tips aimed at the vmPFC [coordinates: anteroposterior +3.0 mm from bregma; mediolateral ±0.6 mm from midline;

dorsoventral –1.8 mm from skull surface; (Paxinos & Watson, 1997)]. The cannulas were anchored to the skull with dental cement and two stainless steel screws were positioned in the skull. After surgery, rats were allowed one week to recover before the experimental procedures.

2.6. Intracerebral injection

Intracerebral injection was carried out by inserting two 14.0 mm long stainless steel needles through the guide cannulas while the rat was gently restrained. The needles were connected to a 5 μ l Hamilton microsyringe with PE-10 tubing. An infusion machine (Insight, B12000 model) allowed for bilateral microinjections over a 20 s time period (0.6 μ l/min). The needles were kept in the cannulas for an additional 20 s after drug infusion to maximize the diffusion and prevent backflow of drug into the cannulas. Drug solutions were freshly prepared before each experiment.

2.7. Histology

To evaluate the microinjection sites, rats were deeply anesthetized with chloral hydrate (150 mg/kg IP). Evans blue dye (0.2 μ l/side; 3%) was administered with the same needle utilized in the experiments. Animals were perfused transcardially with 0.9% saline and 10% formaldehyde for 10 min each. After decapitation, the brains were removed of the skull and stored in a 10% formaldehyde solution (w/v). At least 24 h before sectioning, the brains were immersed in a 30% sucrose solution (w/v) for cryoprotection. Coronal slices (50 μ m) were cut on a cryostat (Leica CM1850) and placed on gelatin-coated slides. The sections were examined with optical microscopy in order to determine the microinjection sites stained by Evans blue dye. Only animals with microinjections bilaterally located in the vmPFC were included in the statistical analysis. It was considered vmPFC the infralimbic cortex and the ventral border of the prelimbic cortex.

2.8. Procedures

2.8.1. Experiment 1: effects of single or repeated systemic administration of propranolol or isoproterenol on heart rate

The antagonistic effects of acute propranolol treatment ($N = 4$; 10 mg/kg IP 30 min prior) on the increased heart rate induced by intravenous injections of isoproterenol (0.1; 0.3 and 1 nmol/kg) were verified in anesthetized rats. This study was conducted to test whether the dose and the time interval of propranolol injection were adequate regimens for following behavioral experiments.

The long-term cardiac effects of propranolol ($N = 5$; 20 mg/kg IP), isoproterenol ($N = 5$; 5 mg/kg IP) or saline ($N = 5$) administered for three consecutive days were evaluated on the fourth day. In addition, to investigate the possibility of residual drug presence, rats also received repeated infusions of propranolol ($N = 5$; 20 mg/kg IP) for three consecutive days and were tested on the fourth day with increasing doses of isoproterenol.

2.8.2. Experiment 2: effects of a single systemic administration of propranolol, isoproterenol or saline on the acquisition of contextual conditioned fear extinction

Rats were placed in an adjacent room for at least 30 min before the beginning of experiments. After this period, they were transferred to the experimental room and placed in the conditioning chamber. Subjects were allowed 1 min of exploration in the conditioning chamber. After this, the subjects received one footshock (1 mA, 2 s) and were kept in the apparatus for an additional minute. Twenty-four hours later, they were returned to the conditioning chamber for the first extinction session after being randomly assigned to groups that received an IP injection of saline, isopro-

terenol or propranolol 30 min prior to the first extinction session (acquisition of extinction). On the following 3 days, rats were returned to the apparatus without drug administration. The percentage of time spent freezing was recorded during each extinction session.

2.8.3. Experiment 3: effects of repeated systemic administrations of propranolol, isoproterenol or saline on the acquisition or consolidation of contextual conditioned fear extinction

Rats were contextually fear conditioned as described for Experiment 2. On the next day, subjects were randomly divided into groups that received propranolol, isoproterenol or saline 30 min before (acquisition of extinction) or immediately after (consolidation of extinction) the first extinction session. The treatment was repeated for each animal on the following 2 days (extinction sessions 2 and 3). On the fourth day, rats were returned to the chamber without drug treatment and the percentage of time spent freezing was recorded.

2.8.4. Experiment 4: vmPFC-immunoreactivity in the groups systemically treated with propranolol, isoproterenol or saline prior to the extinction sessions

Animals in the first group were systemically administered propranolol ($N = 6$; 10 mg/kg), isoproterenol ($N = 6$; 2.5 mg/kg) or saline ($N = 6$) 30 min prior to the first extinction session. A second group of animals received systemic injection of propranolol ($N = 8$; 10 mg/kg); isoproterenol ($N = 6$; 2.5 mg/kg) or saline ($N = 6$) 30 min before extinction sessions 1–3. Rats from an additional saline control group ($N = 6$; Home) were kept in their home cage and were not submitted to the extinction sessions. Ninety minutes after extinction session 1 for the first group or 4 for the second group the animals were deeply anesthetized with chloral hydrate (150 mg/kg) and perfused transcardially with a solution of 4.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were removed and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4 °C.

The brains were then frozen and four series of 40 μ m section were cut with a cryostat (Leica, CM 1850) in the frontal plane and collected from the prefrontal cortex. A complete series of sections was processed for immunohistochemistry with anti-Fos serum raised in rabbit (Ab-5, lot#4191–1–2; Calbiochem) at a dilution of 1:20,000. The primary antiserum was localized using a variation of the avidin–biotin complex system (ABC). In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector laboratories) and then placed in the mixed avidin–biotin–horseradish peroxidase complex solution (ABC Elite kit; Vector Laboratories) for the same period of time. The peroxidase complex was visualized by a 10 min exposure to a chromogen solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride with 0.3% nickel-ammonium sulfate (DAB-Ni) in 0.05 M Tris buffer (pH 7.6) followed by incubation for 5 min in a chromogen solution with glucose oxidase (10%) and β -D-glucose (10%) to produce a blue–black product. The reaction was stopped by extensive washing in potassium–PBS, pH 7.4. Sections were mounted on gelatin-coated slides and then dehydrated and coverslipped with DPX.

Counts of the number of Fos-immunoreactive neurons as a function of experimental status were generated for the vmPFC (infralimbic cortex and ventral border of the prelimbic cortex) with the 10 \times objective of an Olympus microscope (BX-41) equipped with a digital camera (3.3 Mpixel QCOLOR3C, Qimaging™) to capture the images (software Qcapture Pro 5.1, Qimaging™). To be considered positive for Fos-like immunoreactivity, the nucleus of the neurons had to be of appropriate size (ranging approximately from 8 to 15 μ m) and shape (oval or round), show the characteristic blue–black staining of oxidized DAB-Ni, and be distinct from the

background at magnification of 10 \times . For each animal, Fos-positive cells were plotted and counted in five distinct rostrocaudal levels of the vmPFC (120 μ m apart) using a specialized computer program (Image J 1.42 g; National Institute of Health, USA).

2.8.5. Experiment 5: effects of intra-vmPFC microinjection of atenolol, isoproterenol or PBS on the acquisition or consolidation of contextual conditioned fear extinction

Rats were contextually fear conditioned as described for Experiment 2. Subjects were then randomly divided for bilateral intra-vmPFC microinjections into the following groups: atenolol (10 or 40 nmol); isoproterenol (10 or 40 nmol); atenolol (40 nmol) followed by isoproterenol (40 nmol; 10 min apart), and PBS. Subjects were microinjected 10 min before (acquisition of extinction) or immediately after (consolidation of extinction) extinction session 1. After 24 h, all animals were placed into the conditioning chamber without drug treatment (extinction session 2) and the percentage of time spent freezing was recorded. A histological analysis was performed to evaluate the microinjection sites as described above.

2.9. Data analysis

A student's *t*-test for independent samples was used to compare changes in heart rate between the propranolol and saline groups after an isoproterenol-induced increase. A one-way ANOVA followed by Duncan's post hoc test was used to compare the effects of three consecutive days of propranolol, isoproterenol or saline injections on the heart rate values during the fourth day and to evaluate the extinction index parameter. A two-way ANOVA followed by Duncan's post hoc test was used to compare the number of vmPFC-Fos-positive cells between the first and the fourth extinction sessions in the group that was kept in the home cage and the groups treated with propranolol, isoproterenol or saline for three consecutive days. A repeated-measures ANOVA followed by Duncan's post hoc test was used to compare the effects of single or repeated systemic treatments or intra-vmPFC administration of beta-adrenergic drugs on the mean percentage of freezing time during the extinction sessions. The level of statistical significance adopted was $p < .05$. All statistical analyses were performed using the Statistica[®] software package (version 8.0, Statsoft[®], Tulsa, EUA).

3. Results

3.1. Experiment 1: effects of single or repeated systemic administrations of propranolol or isoproterenol on heart rate

As illustrated in Fig. 1A, a single injection of propranolol (10 mg/kg IP) given 30 min in advance, was able to block ($p < .05$) the increased heart rate elicited by increasing doses of isoproterenol (0.1, 0.3 and 1.0 nmol/kg), with no changes in basal heart rate and mean arterial pressure (Fig. 1A, inset). These findings suggest that the doses and injection interval regimen were consistent with behavioral studies. On the other hand, repeated treatments with propranolol (20 mg/kg IP) during three consecutive days was not able to prevent the tachycardia induced by different doses of isoproterenol on the fourth day, thus we ruled out the presence of residual propranolol during the last extinction session (Fig. 1B).

3.2. Experiment 2: effects of a single systemic administration of propranolol, isoproterenol or saline on the acquisition of contextual conditioned fear extinction

The first set of experiments was conducted to investigate a possible effect of a single injection of the beta-adrenergic agonist

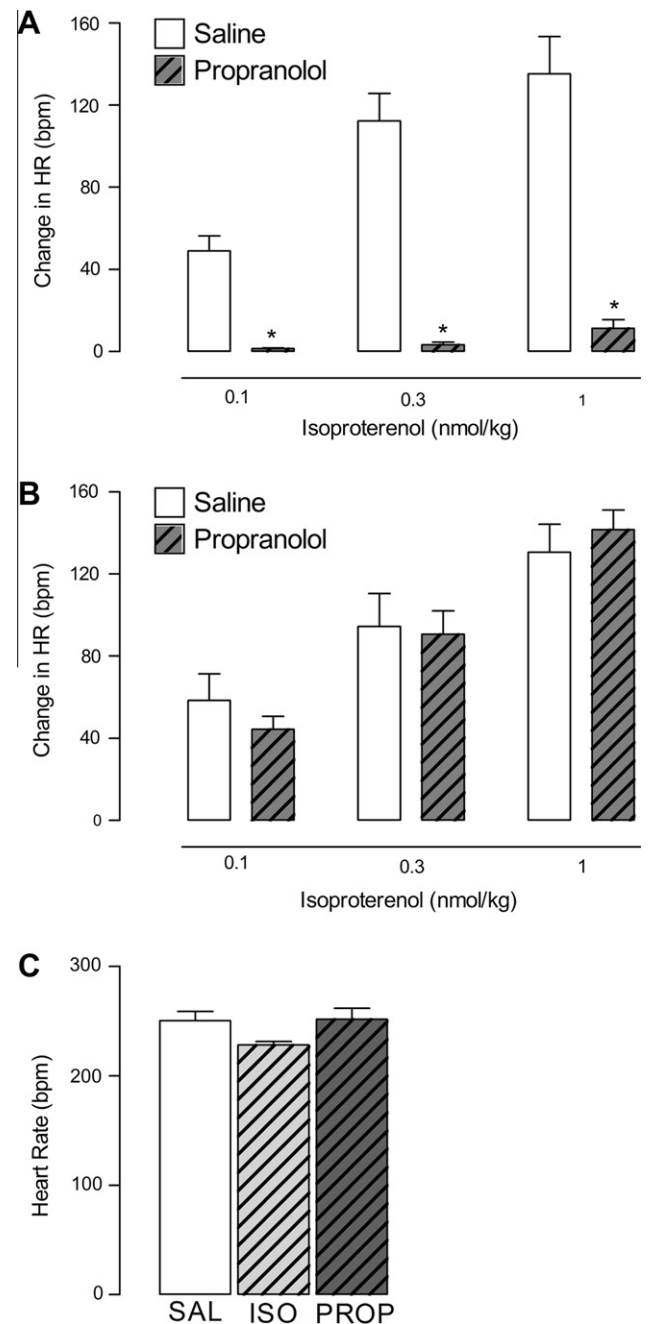


Fig. 1. Effects of a single (A; 10 mg/kg, IP, 30 min before) or a repeated (B; 20 mg/kg, IP, three consecutive days) systemic administration of propranolol on the basal values of mean arterial pressure (MAP) and heart rate (HR; inset) and on the efficacy to block beta-adrenoceptors after the administration of isoproterenol (0.025, 0.075, 0.25 μ g/kg, IV, 30 min after) in anesthetized rats submitted to a cardiovascular assay. Data are expressed as the mean \pm SEM. Student's *t*-test. ($N = 4-5$ per group). Legend = * $p < .05$ compared to respective group before treatment.

(isoproterenol) or beta-adrenergic antagonist (propranolol) on the acquisition of contextual conditioned fear extinction. A repeated-measures ANOVA revealed a significant effect for the extinction sessions [$F_{(3,54)} = 18.51$; $p < .0001$] among the distinct days of extinction only, indicating the development of contextual conditioned fear extinction. No significant difference was observed for the main treatment factor [$F_{(2,18)} = 0.78$; $p = 0.47$] or for the interaction between the extinction session and treatment [$F_{(6,54)} = 0.77$; $p = 0.59$]. Subsequent analysis using Duncan's test showed a significant ($p < .05$) decrease in the percentage of time

spent freezing from the first to the third or fourth extinction session for the saline or treated groups, respectively (Fig. 2A). One-way ANOVA followed by Duncan's post hoc test did not show significant differences [$F_{(2,18)} = 0.5$; $p = 0.6$] in the extinction index parameter between the different groups (Fig. 2B).

3.3. Experiment 3: effects of repeated systemic administrations of propranolol, isoproterenol or saline on the acquisition or consolidation of contextual conditioned fear extinction

A repeated-measures ANOVA (treatment vs. extinction session) revealed a significant effect for the main factors, treatment [$F_{(4,41)} = 3.83$, $p < .01$], extinction session [$F_{(3,123)} = 16.61$, $p < .0001$], and their interaction [$F_{(12,123)} = 2.25$, $p < .05$], for the percentage of time spent freezing in subjects that received repeated infusions of saline, propranolol or isoproterenol during the acquisition of contextual conditioned fear extinction. Duncan's post hoc test revealed a significant ($p < .05$) decrease in the percentage of time spent freezing from the first to the third or fourth extinction sessions in the saline group (Fig. 3A). This reduction in freezing behavior indicates the development of contextual fear extinction. Rats administered with both doses of isoproterenol (1.0 and 2.5 mg/kg) or 5 mg/kg propranolol showed a significant reduction in freezing behavior during the fourth extinction session when compared to the first extinction day (Fig. 3A). In contrast, the percentage of time spent freezing in subjects treated with propranolol (10 mg/kg) was not found altered during the extinction sessions. In addition, the group of rats treated with propranolol (10 mg/kg) was significantly different from saline group on the fourth extinction

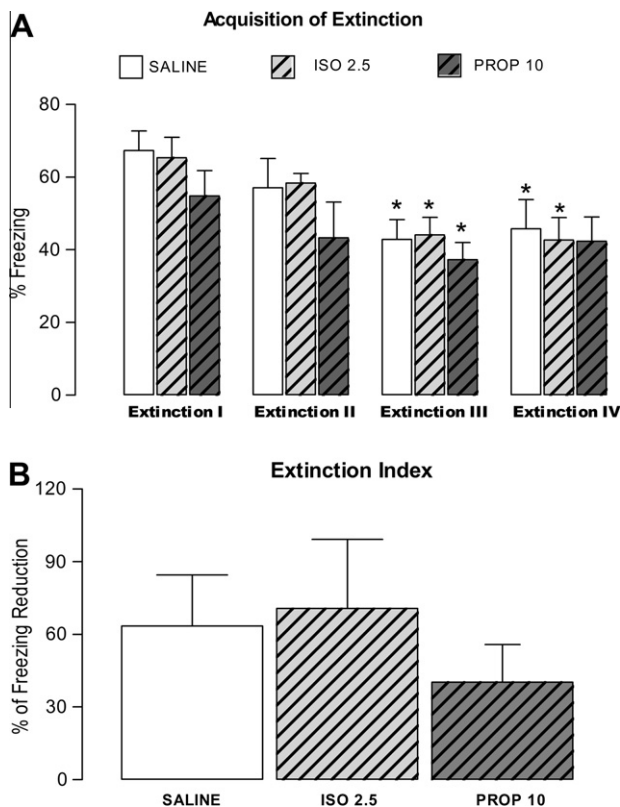


Fig. 2. (A) Effects of a single injection of propranolol (10 mg/kg, IP) 30 min before extinction session 1 on the percentage of freezing behavior in the extinction sessions. (B) Extinction index parameter represented by the reduction of percentage of freezing from the first to the last extinction session. Data are expressed as the mean \pm SEM. A repeated-measures ANOVA repeated measures followed by Duncan's post hoc test. ($N = 7$ per group). Legend: * $p < .05$ compared to respective group in extinction session 1. Legend: ISO = isoproterenol; PROP = propranolol.

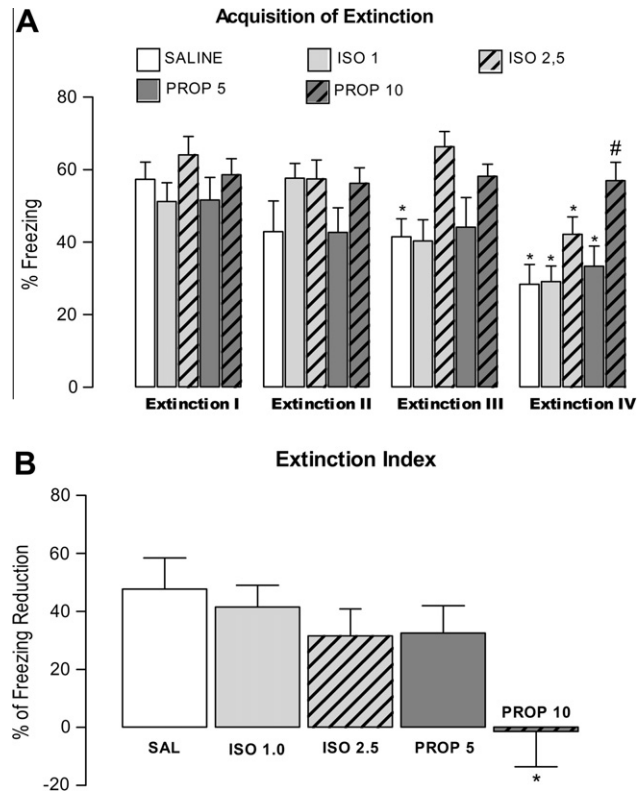


Fig. 3. (A) Effects of repeated injections of propranolol (5 or 10 mg/kg, IP), isoproterenol (1.0 or 2.5 mg/kg, IP) or saline during the acquisition of extinction (30 min before extinction session 1–3) on the percentage of freezing behavior in the extinction sessions. (B) Extinction index parameter represented by the reduction in the percentage of freezing from the first to the last extinction session. Data are expressed as the mean \pm SEM. Repeated-measures ANOVA followed by Duncan's post hoc test. ($N = 9–10$ per group). Legend: * $p < .05$ compared to respective group in extinction session 1; # $p < .05$ compared to saline control group on the same day. ISO = isoproterenol; PROP = propranolol.

session, in which no drug injection was administered, suggesting that this dose of propranolol was able to impair the acquisition of contextual conditioned fear extinction (Fig. 3A). In fact, an ANOVA followed by Duncan's post hoc test revealed a significant reduction [$F_{(4,41)} = 3.64$; $p < .05$] in the extinction index for this group when compared to the saline control group (Fig. 3B).

The effects of repeated injections of propranolol, isoproterenol or saline in the consolidation of contextual conditioned fear extinction were evaluated by administering these drugs immediately after extinction sessions 1–3. A repeated-measures ANOVA did not find any significant effect of treatment. However, a significant effect was revealed for the extinction session factor [$F_{(3,12)} = 12.49$, $p < .0001$] and for the interaction between treatment and extinction session [$F_{(12,129)} = 2.30$, $p < .05$]. Further analysis using Duncan's test revealed a significant decrease ($p < .05$) in the percentage of time spent freezing from the first to the fourth extinction session in saline-treated subjects, ensuring that four sessions of extinction were enough to develop contextual conditioned fear extinction (Fig. 4A). This reduction in the freezing behavior was observed on the second extinction session for the group treated with 5 mg/kg isoproterenol or on the third extinction session for the group injected with 2.5 mg/kg isoproterenol. On the other hand, as shown in Fig. 4A, animals treated with both doses of propranolol (10 or 20 mg/kg) did not show differences in the percentage of time spent freezing during the extinction sessions, suggesting that the administration of beta-blocker immediately after each extinction session was able to impair the extinction of contextual conditioned fear. In fact, an ANOVA followed by Duncan's post

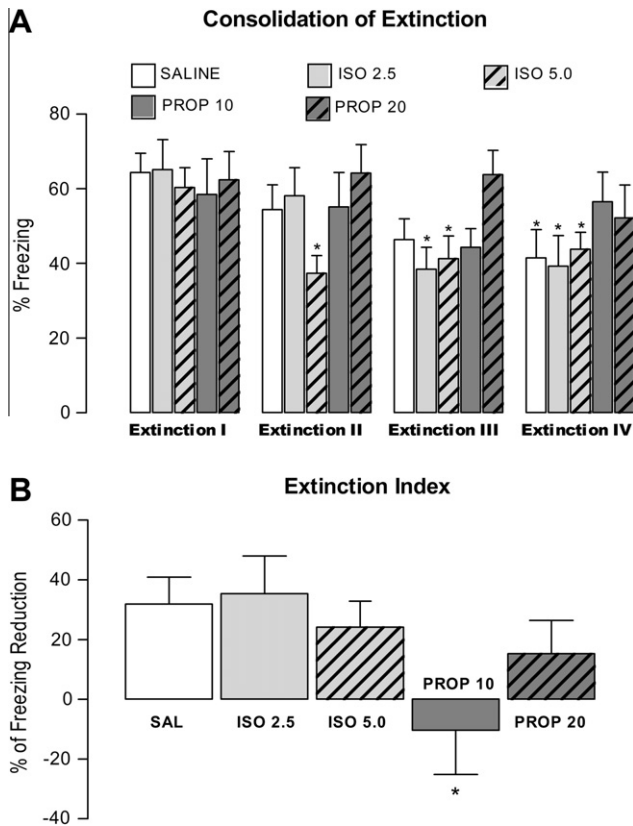


Fig. 4. (A) Effects of repeated injections of propranolol (10 or 20 mg/kg, IP), isoproterenol (2.5 or 5 mg/kg, IP) or saline during the consolidation of extinction (immediately after extinction sessions 1–3) on the percentage of freezing behavior in the extinction sessions. (B) Extinction index parameter represented by the reduction in the percentage of freezing from the first to the last extinction session. Data are expressed as mean \pm SEM. A repeated-measures ANOVA followed by Duncan's post hoc test. ($N = 8$ –12 per group). Legend: * $p < .05$ compared to the respective group in extinction session 1; ISO = isoproterenol; PROP = propranolol.

hoc test revealed a significant reduction [$F_{(4,43)} = 2.51$; $p < .05$] in the extinction index in the group treated with propranolol 10 mg/kg when compared to saline control group (Fig. 4B).

3.4. Experiment 4: vmPFC-immunoreactivity in the groups systemically treated with propranolol, isoproterenol or saline prior to the extinction sessions

Fig. 5A shows photomicrographs illustrating the Fos-positive-vmPFC cells from the different groups during the first and the fourth extinction session. A two-way ANOVA revealed a significant difference ($p < .05$) in the number of Fos-positive-vmPFC cells for the treatment [$F_{(3,42)} = 52.49$, $p < .0001$], extinction session [$F_{(1,42)} = 7.00$, $p < .05$] and the interaction between these factors [$F_{(3,42)} = 11.03$, $p < .0001$]. During the first extinction session, Duncan's post hoc test demonstrated a significant ($p < .05$) increase in the number of Fos-positive cells from the saline group compared with the home cage-control group. In addition, animals treated with either isoproterenol or propranolol showed a reduction in the number of Fos-immunoreactive cells in the vmPFC when compared to the saline group. Post hoc analysis also revealed a significant ($p < .05$) increase in the vmPFC-Fos-immunoreactivity when the subjects were repeatedly treated with isoproterenol or propranolol for 3 days and prior to the drug-free fourth day of extinction compared to the saline group. A significant ($p < .05$) reduction in vmPFC-immunoreactivity from the first to the fourth extinction sessions was observed in the saline group only (Fig. 5B).

3.5. Experiment 5: effects of intra-vmPFC microinjections of atenolol, isoproterenol or PBS on the acquisition or consolidation of contextual conditioned fear extinction

A repeated-measures ANOVA comparing the effects of intra-vmPFC microinjection of PBS, atenolol, isoproterenol, or atenolol plus isoproterenol 10 min prior to extinction session 1 showed a significant effect of treatment [$F_{(5,40)} = 4.1$, $p < .005$], extinction session [$F_{(1,40)} = 8.25$, $p < .01$] and the interaction treatment and extinction session [$F_{(5,40)} = 2.76$, $p < .05$]. Post hoc comparisons on data collected from extinction session 1 revealed a significant ($p < .05$) decrease in the percentage of freezing in the group microinjected with isoproterenol 40 nmol compared to the PBS group (Fig. 6A). This reduction in freezing behavior was not observed in rats that had been microinjected with the beta1-blocker atenolol (40 nmol) 10 min before the isoproterenol treatment, suggesting that the behavioral effects observed in the isoproterenol group were mediated by the beta1-adrenergic receptor. As depicted in Fig. 6A, a decrease in the percentage of time spent freezing was detected in extinction session 2 for the PBS control group, showing that two 10-min extinction sessions 24 h apart were sufficient for subjects to develop contextual conditioned fear extinction. However, this reduction in freezing behavior observed in the PBS control group from the first to the second extinction session was not seen in rats microinjected with 40 nmol atenolol 10 min before extinction session 1, suggesting a participation of vmPFC-beta1-adrenergic receptors in the extinction of contextual conditioned fear. In fact, an ANOVA followed by Duncan's post hoc test revealed a significant reduction [$F_{(5,40)} = 2.65$; $p < .05$] in the extinction index for this group when compared to the PBS control group (Fig. 6B).

An ANOVA performed on data obtained from the group receiving intra-vmPFC microinjections immediately after the first extinction session showed a significant effect of the extinction session [$F_{(1,36)} = 105.58$; $p < .0001$] only. Duncan's post hoc test revealed a decrease in the percentage of freezing time from the first to the second extinction sessions in all groups regardless of the treatment (Fig. 7A). ANOVA followed by Duncan's post hoc test did not reveal any significant differences in the extinction index parameter between the different groups (Fig. 7B).

4. Discussion

The results obtained in this study confirm the influence of beta-adrenergic receptors in the acquisition and consolidation of extinction in rats submitted to the contextual conditioned fear extinction paradigm.

A single systemic administration of beta-adrenergic compounds was not able to modify the rate of contextual conditioned fear extinction. These findings are in agreement with previous studies that show a lack of effects following a single injection of the beta-blocker propranolol on contextual conditioned fear extinction in mice (Cain, Blouin, & Barad, 2004) or on auditory conditioned fear extinction in rats (Rodriguez-Romaguera et al., 2009). In addition, the pharmacological stimulation or inhibition of beta-adrenergic receptors did not interfere with the percentage of freezing behavior during the first 3 or 5 min of the extinction session 1 (data not shown), suggesting an absence of effects on the contextual fear expression. Contradicting results regarding the involvement of beta-adrenoceptors in the expression of conditioned fear have been described by different authors (Cain et al., 2004; Davis, Redmond, & Baraban, 1979; Do Monte, Canteras, Fernandes, Assreuy, and Carobrez, 2008; Rodriguez-Romaguera et al., 2009). These differences have been attributed to variability in the exper-

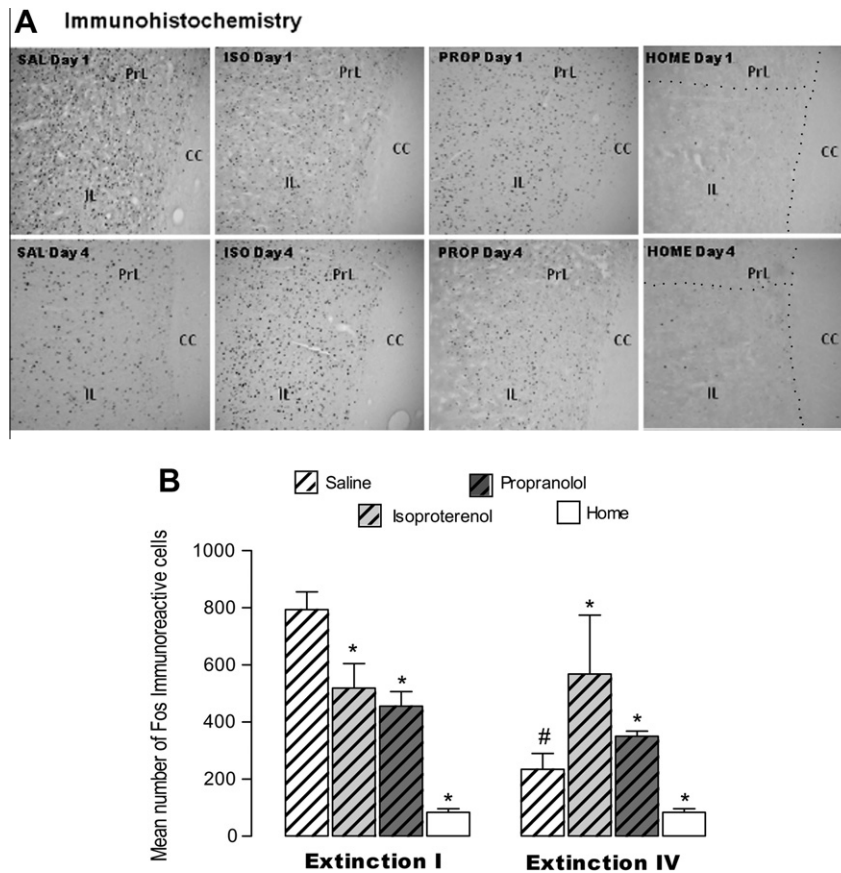


Fig. 5. Effects of systemic beta-adrenergic manipulation on the vmPFC Fos-immunoreactivity during contextual conditioned fear extinction. (A) Photomicrographs of vmPFC transverse sections showing the Fos-positive stained cells from rats treated with saline, isoproterenol or propranolol, 30 min before each extinction session and during the first and fourth extinction sessions. (B) Histograms showing the mean number of mPFC immunoreactive cells in the different groups. Data are expressed as the mean \pm SEM for 6–8 rats per group. Legend: * $p < .05$ compared to the saline control group in the same extinction session; # $p < .05$ compared to the same group in the first extinction session.

imental parameters, such as training intensity or differences in the conditioned stimulus used.

However, when propranolol was repeatedly administered before or immediately after the extinction session or when isoproterenol was injected immediately after the extinction session, rats exhibited changes in the pattern of contextual fear extinction. While propranolol impaired the acquisition and consolidation of contextual fear extinction, isoproterenol facilitated the consolidation of the extinction processes. These results are in agreement with previous studies (Ouyang & Thomas, 2005) in which dopamine-beta-hydroxylase knockout mice and wild-type mice injected with a beta-blocker both exhibited impairment in the acquisition and consolidation of contextual conditioned fear extinction.

Beta-adrenoceptor blockade has been associated with a disruption in the acquisition (Ebrahimi, Rashidy-Pour, Vafaei, & Akhavan, 2010; Grillon, Cordova, Morgan, Charney, & Davis, 2004; King & Williams, 2009) and consolidation (Adamec, Muir, Grimes, & Pearcey, 2007; Cahill et al., 2000) of aversive memories. In this study, beta-adrenoceptors were also implicated in the contextual fear extinction processes. In fact, these results are unsurprising given the substantial amount of evidence indicating that the extinction mechanism involves new learning that masks or inhibits the original conditioned fear (see Bouton (2004) for review).

Surprisingly, the percentage of freezing in the group of subjects treated with 2.5 mg/kg isoproterenol prior to the extinction sessions was not significantly different from the saline control group during the third extinction session (Fig. 3A). This group failed to

show a decrease in the percentage of freezing behavior from the third to the first day of extinction, similar to the saline group. This effect could be attributed to the well-known anxiogenic effect of isoproterenol (Pohl et al., 1985, 1988). In fact, it is possible to exclude impairment of the fear extinction mechanisms because freezing behavior during the drug-free session performed 24 h later was reduced and the extinction index parameter in this group was similar to the saline control group.

Similarly, rats treated with propranolol (20 mg/kg) during the consolidation of extinction showed non-significant increase in the percentage of freezing behavior during the third extinction session (Fig. 4A). In this case, however, the increased defensive response persisted to the last drug-free extinction session, suggesting impairment in the consolidation of the fear extinction processes.

Because systemic beta-adrenergic drugs are known to interfere with cardiovascular function, we examined a possible carryover drug effect on the baseline heart rate levels 24 h after three consecutive days of isoproterenol or propranolol treatment. The basal heart rate did not change during the drug-free state, suggesting that the behavioral differences observed on the last day of extinction were due to the cognitive effects of the drugs during the previous sessions and not a residual drug effect.

An investigation on the ventral portion of the mPFC as a potential neural system for the noradrenergic modulation of fear extinction was also performed. This region has been widely implicated as a key structure in the mechanisms of extinction for aversive memories (for a review see Quirk and Mueller (2008) and Sotres-Bayon,

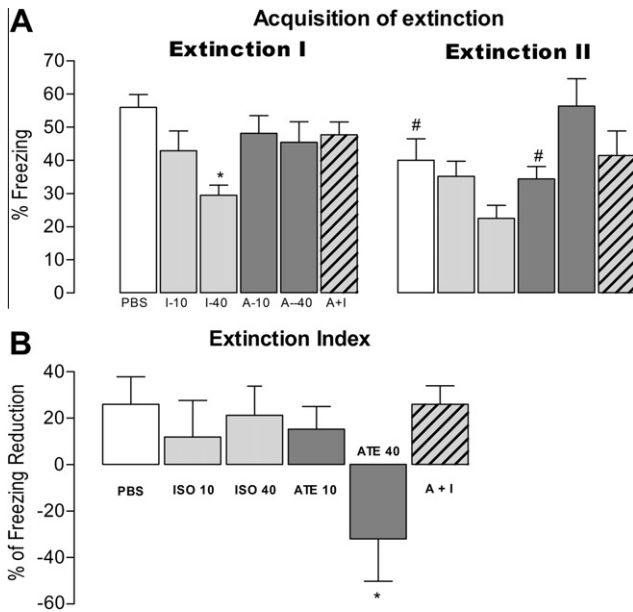


Fig. 6. (A) Effects of intra-vmPFC microinjection of atenolol (10 or 40 nmol per side), isoproterenol (10 or 40 nmol per side), atenolol plus isoproterenol (both 40 nmol per side, 10 min apart) or PBS during the acquisition of extinction (10 min before extinction session 1) on the percentage of freezing behavior in the extinction sessions. (B) Extinction index parameter represented by the reduction in the percentage of freezing from the first to the last extinction session. Data are expressed as the mean \pm SEM. A repeated-measures ANOVA followed by Duncan's post hoc test. ($N = 7-9$ per group). Legend: * $p < .05$ compared to respective group in extinction session 1; # $p < .05$ compared to saline control group on the same day. I = isoproterenol; A = atenolol, A + I = atenolol plus isoproterenol.

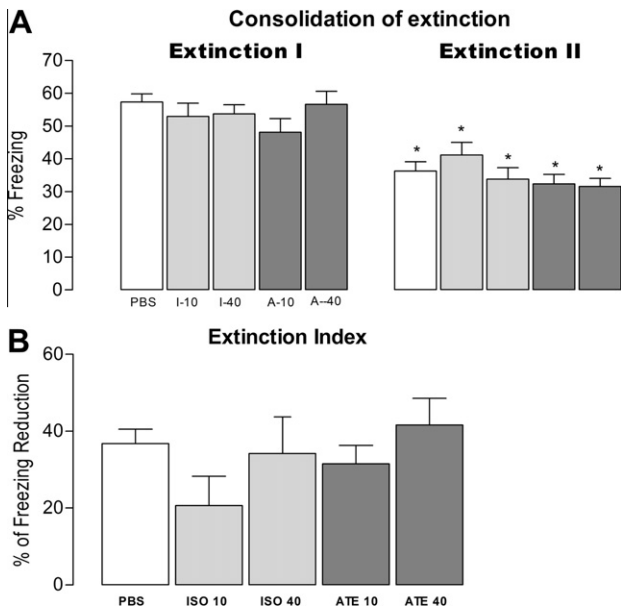


Fig. 7. (A) Effects of intra-vmPFC microinjection of atenolol (10 or 40 nmol per side), isoproterenol (10 or 40 nmol per side) or PBS during the consolidation of extinction (immediately after extinction session 1) on the percentage of freezing behavior in the extinction sessions. (B) Extinction index parameter represented by the reduction in the percentage of freezing from the first to the last extinction session. Data are expressed as the mean \pm SEM. A repeated-measures ANOVA followed by Duncan's post hoc test. ($N = 7-9$ per group). Legend: * $p < .05$ compared to the respective group in extinction session 1. I = isoproterenol; A = atenolol.

& McEchron, 2005; Laurent & Westbrook, 2009). While the vmPFC has been described as a critical region for fear extinction processing (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Herry & Garcia, 2002; Quirk, Garcia, & González Lima, 2006), the dmPFC seems to drive the expression of conditioned fear (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009; Corcoran & Quirk, 2007). In agreement with the opposing influences of the mPFC subdivisions, Vidal-Gonzalez and coworkers (2006) demonstrated that while the electrical microstimulation of the dmPFC elicited an increase in the expression of conditioned fear, the microstimulation of the vmPFC lead to a reduction in defensive responses. Based on neuro-anatomical findings (Brinley-Reed, Mascagni, & McDonald, 1995; Cassell & Wright, 1986), it has been suggested that these functional differences may be associated with a bi-directional mechanism for fear modulation in which the dorsal and ventral parts of the mPFC would be exciting and inhibiting the amygdala activity, respectively.

The mPFC receives a dense plexus of noradrenergic fibers from *Locus Coeruleus*, the main noradrenergic nucleus of the central nervous system (Aston-Jones, Foote, and Segal, 1985; Robbins, 1984). In addition, the mPFC contains a high concentration of beta-1-adrenergic receptors (Rainbow et al., 1984) and shows prominent immunoreactivity for dopamine beta-hydroxylase, the enzyme that synthesizes noradrenaline from dopamine (Oropeza, Mackie, & Van Bockstaele, 2007).

Our immunohistochemical results revealed an increase in Fos expression in the vmPFC after the first extinction session in the saline control group when compared to the home cage-control group. An increase in Fos-protein activity in this structure has been associated with the extinction of conditioned fear (Herry & Mons, 2004; Santini, Ge, Ren, Peña de Ortiz, & Quirk, 2004; Knaspka & Maren, 2009; Kim, Jo, Kim, Kim, & Choi, 2010).

In fact, this increase in Fos-immunoreactivity in the vmPFC of the saline group following the first extinction session was significantly reduced after the fourth day of extinction, suggesting that the high vmPFC activity seems to be associated with the initial stages of extinction processes. Subjects treated with propranolol showed a reduced number of Fos-positive vmPFC cells after the first extinction session compared to the saline control group. In contrast to the saline group, the number of immunoreactive vmPFC cells in the propranolol group was not decreased between the first and last extinction sessions. The extinction mechanism has been correlated with an increased activity in the vmPFC, and thus the reduced immunoreactivity in the vmPFC during the first extinction session and the absence of differences in the level of Fos-protein expression during the first and the last extinction day in propranolol-treated rats may be associated with an impairment of contextual fear extinction.

Similar to the propranolol-treated group, rats treated with isoproterenol also showed a decrease in the Fos-protein expression in the vmPFC after the first extinction session compared to the saline group. However, the isoproterenol-treated group exhibited an extinction index similar to the saline group, suggesting that this decreased immunoreactivity observed after first extinction session may be associated with the previously reported anxiogenic properties of this compound, which could temporarily counteract the extinction mechanisms. In fact, an increase in vmPFC activity was observed after the last extinction session when isoproterenol-treated rats were exposed to the context without the presence of drug.

Although in our experimental settings we cannot verify the specificity for the increased vmPFC Fos expression in relation to the extinction process, recently Kim and coworkers (2010) reported that a reduced Fos-immunoreactivity in the vmPFC underlies the extinction deficits observed after an immediate extinction protocol. In this study, they showed that the extinction impairment was alleviated when rats received immediate extinc-

Cain and LeDoux (2006). Distinct roles for the dorsal (dmPFC) and ventral subdivisions of the mPFC in the different phases of aversive memory processing have been demonstrated (Gilmartin

tion paired with electrical stimulation of the vmPFC. In addition, Knapska and Maren (2009) demonstrated that Fos expression in the vmPFC is selectively related to the extinction processes, because the simple presentation of the conditioned cue did not increase Fos expression in this region.

To test whether vmPFC-beta-adrenoceptors are directly involved in fear extinction mechanisms, rats were microinjected in the vmPFC with beta-adrenergic compounds before or immediately after the extinction session. In this part of the study, the selective beta-blocker atenolol was used because, unlike propranolol, it is devoid of serotonergic receptor effects (Costain & Green, 1978) and is more selective for beta-1-adrenoceptors (Smith & Teitler, 1999). While rats systemically injected with saline required three to four extinction sessions to show a reduction in freezing time, the PBS control group previously submitted to stereotaxic surgery required only two 10-min extinction sessions to exhibit a significant decrease in freezing behavior.

The results show that the microinjection of the beta-agonist isoproterenol (40 nmol) 10 min prior to the first extinction session was able to reduce the percentage of freezing behavior within the same extinction session. As the first minutes of the first extinction session are considered to be a retrieval memory phase, we sub-divided the statistical analysis of the percentage of freezing behavior into the first 2, 3 or 5 min of the session to evaluate a possible interference of isoproterenol on the retrieval of contextual fear memory. No significant difference in freezing behavior was observed for the first 2, 3 or 5 min of the first extinction session in the isoproterenol (40 nmol) group when compared to control group (data not shown). Therefore, the reduction in freezing behavior produced by isoproterenol during the first extinction session is not due to impairment in the retrieval of the contextual fear conditioning, but rather due to the facilitation of fear extinction mechanisms. Indeed, the attenuation of the percentage of freezing observed during the first extinction session was maintained when the same rats were exposed to the second extinction session 24 h later without drug administration.

Previous studies have shown an increased mPFC noradrenergic transmission in rats exposed to fearful situations (Feenstra, Vogel, Botterblom, Joosten, & de Bruin, 2001; Hugues & Garcia, 2007). In the present study, we found that beta-adrenergic activation in the vmPFC can positively modulate contextual fear extinction. Taking these results together, it is possible to hypothesize that the increased vmPFC noradrenergic activity in aversive situations could be implicated in the learning extinction processes as an intrinsic defensive mechanism to avoid the development of long-lasting aversive memories. In accordance to this idea, Mueller and coworkers (2008) have shown that beta-adrenergic blockade in the vmPFC impairs the acquisition of extinction in an auditory conditioned fear paradigm.

Although the treatment with atenolol (40 nmol) did not alter freezing during the first extinction session, it was able to inhibit the effects of isoproterenol during the extinction sessions, suggesting that the facilitating effects of isoproterenol on contextual fear extinction was mediated by beta-1-adrenoceptor activation. In addition, rats microinjected with atenolol (40 nmol) showed a significant impairment in fear extinction during the second extinction session compared to PBS control group.

Manipulation of the vmPFC-beta-adrenergic receptors immediately after the first extinction session did not interfere with the consolidation of contextual fear extinction. These results corroborated those obtained by Mueller and coworkers (2008), who did not find consolidation effects for auditory fear conditioning in rats microinjected with propranolol into the vmPFC. Taken together, our results suggest that beta-1-adrenergic receptors in the vmPFC are strongly involved in the mechanisms of fear extinction acquisition, but not during the first minutes of the

consolidation phase for extinction in the contextual conditioned fear paradigm.

Several experimental and clinical studies in humans have suggested a potential therapeutic effect of the beta-blocker propranolol to alleviate the symptoms associated with post-traumatic stress disorder (PTSD; Henry, Fishman, & Youngner, 2007; Vaiva et al., 2003; van Stegeren, Everaerd, Cahill, McGaugh, & Gooren, 1998). The fact that repeated, but not single, systemic administration of the beta-blocker impairs the mechanisms of contextual fear extinction in rats suggests that the paradigm used in this study might not be appropriate as a PTSD-model. It has been widely suggested that, after being retrieved, memory processes can follow two distinct and opposite biochemical processes: reconsolidation or extinction. While reconsolidation relates to the original memory formation, the extinction mechanism is a new learning process that can overcome the original stimulus association (see Tronson and Taylor (2007) for more details).

Recent studies have shown that the duration of the retrieval session may be a key determinant of memory processes. Brief retrieval session lead to a reconsolidation, while longer reminders result in memory extinction (Pedreira & Maldonado, 2003; Suzuki et al., 2004). In this case, aside from potential species differences in neurotransmission, opposite results involving the extinction processes between humans and rodents may be associated with the total time of exposure during the extinction session.

In fact, a decrease in defensive behavior has been observed in rodents (Przybylski, Roulet, & Sara, 1999), and more recently in humans (Kindt, Soeter, & Vervilet, 2009), when the experimental protocol involves a beta-adrenergic blockade immediately after a short controlled retrieval session. In addition, the reduction in PTSD symptoms in patients treated with propranolol could also be attributed to the well-known anxiolytic properties of this compound (Neumeister, Daher, & Charney, 2005; Stern et al., 2008). Further studies are needed to clarify if the retrieval session duration can interfere with the progression (to reconsolidate or to extinguish) of an emotional memory and to determine a temporal therapeutic window in which beta-adrenergic receptors could be used in anxiety disorders, such as the PTSD.

Acknowledgments

This work was supported by CAPES, PRONEX, FAPESP and CNPq from which F.H.M.M., G.C.K., E.P. and R.S. receive doctoral fellowships and J.A. and A.P.C. a research fellowship.

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