Rapid Activation of Glucocorticoid Receptors in the Prefrontal Cortex Mediates the Expression of Contextual Conditioned Fear in Rats

Fernando M.C.V. Reis1,3, Rafael C. Almada3,2, Manoela V. Fogaça3,2, and Marcus L. Brandão1,3

1Departamento de Psicologia, FFCLRP, 2Departamento de Farmacologia, FMRP, Universidade de São Paulo, Ribeirão Preto, SP, Brazil and 3Instituto de Neurociências e Comportamento (INeC), Ribeirão Preto, SP, Brazil

Address correspondence to Fernando M.C.V. Reis, Departamento de Psicologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, SP, Brasil. Av. Bandeirantes, 3900, Monte Alegre, Ribeirão Preto, SP 14040-901, Brazil. Email: fereis@usp.br

Abstract

The aim of the present study was to investigate the role of glucocorticoids in medial prefrontal cortex (mPFC) activity and the expression of contextual conditioned fear (freezing). Rats were pretreated with vehicle or metyrapone, a corticosterone synthesis blocker, and exposed to a context previously paired with footshocks. Freezing and Fos-protein expression in different mPFC regions were assessed. Exposure to the aversive context led to increased freezing and Fos expression in the prelimbic (PrL), anterior cingulate areas 1 and 2 (Cg1/Cg2). Pretreatment with metyrapone decreased freezing and Fos expression in these areas. Administration of spironolactone, an MR antagonist, in the PrL before the test decreased freezing. Pretreatment with RU38486, a glucocorticoid receptor (GR) antagonist, reduced this effect of spironolactone, suggesting that the effects of this MR antagonist may be attributable to a redirection of endogenous corticosterone actions to GRs. Consistent with this result, the decrease in freezing that was induced by intra-PrL injections of corticosterone was attenuated by pretreatment with RU38486 but not spironolactone. These findings indicate that corticosterone release during aversive conditioning influences mPFC activity and the retrieval of conditioned fear memory indicating the importance of balance between MR:GR-mediated effects in this brain region in this process.

Key words: corticosterone, mineralocorticoid receptors, nongenomic effects, prelimbic cortex, spironolactone

Introduction

Contextual fear conditioning in rats is a form of associative learning that has been used to elucidate mechanisms that are involved in aversive memory processes. Freezing is a species-specific response to fear that occurs following the presentation of an aversive conditioned stimulus. The expression of this response is associated with an increase in the plasma concentrations of corticosterone, a steroid hormone that is produced by the cortex of the adrenal glands in response to hypothalamic–pituitary–adrenal (HPA) axis activation (Rodrigues et al. 2009). This hormone can act via 2 types of steroid receptors, mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), which are expressed in both neurons and glial cells (Bohn et al. 1991). These intracellular receptors belong to the nuclear receptor family and exert their effects as transcription factors in the nucleus of the cell to change the mRNA and protein synthesis of target genes (McEwen et al. 1979).

MRs and GRs have different affinities for endogenous corticosteroid hormones. Thus, variations in their concentrations in the brain result in changes in the balance of activity between these receptors (Reul and de Kloet 1985). The activation of relatively low-affinity GRs is initiated when higher-affinity MRs are already...
substantially occupied by normally circulating corticosteroids (Wang et al. 2012). Corticosterone coordinates a vast variety of actions, depending on the site, time, context, stress history, and cognitive and genetic inputs (de Kloet 2013). The distinct expression of MRs and GRs and time course of their occupation have far reaching consequences with regard to the extent of receptor occupancy in response to homeostatic challenge, which is reflected by their different modulating processes (de Kloet 2013).

Despite many results in the literature that show that corticosteroids can exert their effects through gene transcription, these hormones may also exert rapid and nongenomic effects on synaptic transmission in several brain regions such as the hypothalamus (Di et al. 2003; Evenson et al. 2010), hippocampus (Karst et al. 2005), basolateral amygdala (Karst et al. 2010) insular cortex (Roozendaal et al. 2010), and medial prefrontal cortex (mPFC; Barsegyan et al. 2010). Some of these rapid effects of corticosterone in the brain appear to require the presence of MRs or GRs that are located on the cell membrane, whereas other effects are mediated by yet unknown receptors (Groeneweg et al. 2011).

Both MRs and GRs are highly expressed and have distinct distributions in the brain, including the mPFC (McEwen et al. 1986; Diroio et al. 1993), a brain region that is critically involved in multiple aspects of emotional and cognitive processes. Specifically, the mPFC has been considered an important area that connects brain regions that are involved in the modulation of defensive behaviors that are evoked by the presence of threatening stimuli (Sotres-Bayon and Quirk 2010). Together with several limbic structures, an increase in neuronal activity in the mPFC has been associated with contextual fear-conditioning responses (Burgos-Robles et al. 2009). Considering the fairly unique set of afferent projections to the dorsal and ventral divisions of the mPFC, including the anterior cingulate cortex (Cg), which receives predominantly sensorimotor (nonlimbic) cortical and thalamic inputs, and prelimbic cortex (PrL) and infralimbic cortex (IL), which receive primarily limbic, cortical, thalamic, and hippocampal inputs (McDonald 1998; Hoover and Vertes 2007), the mPFC subfields have been suggested to have discernible influences on emotional and cognitive processes. Moreover, considering the sensitivity of the mPFC to corticosterone (McEwen et al. 1986; Diroio et al. 1993) and rapid effects induced by glucocorticoids, corticosterone can influence emotional and cognitive functions within the mPFC (Barsegyan et al. 2010).

Some evidence has shown that glucocorticoids that act in the mPFC can enhance memory consolidation and impair working memory, effects that depend on membrane steroid receptor activation (Barsegyan et al. 2010). Highlighting the participation of GRs that are located in the mPFC on contextual fear-conditioning responses, a negative relationship was reported between the time spent freezing and expression of GRs in the PFC in high-anxiety rats that were exposed to a conditioned aversive context (Wislowska-Stanek et al. 2013). However, despite this evidence, still unclear is the way in which glucocorticoids can influence mPFC activity and the expression of conditioned fear (Rodrigues et al. 2009). To investigate this issue, metyrapone, a corticosterone synthesis inhibitor, spironolactone (Sigma) and RU38486 (Tocris), MR and GR antagonists, respectively, and corticosterone (Sigma). Metyrapone was dissolved in 0.9% saline that contained 5% Tween 80. Spironolactone, RU38486, and corticosterone were dissolved in 100% DMSO and further diluted in saline (0.9%) so that the final concentration of DMSO was 0.1%. For each respective control group, 0.9% saline containing 5% Tween 80 and 0.9% saline containing 0.1% DMSO were used.

Materials and Methods

Animals

Two hundred and forty-three male Wistar rats weighting 270–300 grams, from the central animal facility of the University of São Paulo, Campus of Ribeirão Preto, Brazil, were used. Rats were housed in groups of 4 per cage (40 × 33 × 17 cm) for at least 72 h under a 12/12 h light/dark cycle (lights on at 07:00 h) at 23 ± 1°C and given free access to food and water. The age of the animals at the time of behavioral testing was ~8 weeks. The experiments reported in this article were performed in accordance with the recommendations of the Brazilian Society of Neuroscience and Behavior and complied with the United States National Institutes of Health Guide for Care and Use of Laboratory Animals. The procedures were approved by the Committee for Animal Care and Use, University of São Paulo (no. 11.1.1300.53.1).

Drugs

The drugs used were metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanone, Sigma, Sigma–Aldrich), a corticosterone synthesis inhibitor, spironolactone (Sigma) and RU38486 (Tocris), MR and GR antagonists, respectively, and corticosterone (Sigma). Metyrapone was dissolved in 0.9% saline to a concentration of 5% Tween 80. Spironolactone, RU38486, and corticosterone were dissolved in 100% DMSO and further diluted in saline (0.9%) so that the final concentration of DMSO was 0.1%. For each respective control group, 0.9% saline containing 5% Tween 80 and 0.9% saline containing 0.1% DMSO were used.

Experimental Procedures

Surgery

Five days before the experimental sessions, rats were anesthetized with intraperitoneal ketamine/xylazine at a dose of 100/7.5 mg/kg (i.p.), in a volume of 1/0.3 mL/kg, respectively, and fixed in a stereotaxic apparatus (David Kopf Instruments). The skull was horizontal between bregma and lambda. After scalp anesthesia with 2% lidocaine, the skull was surgically exposed and stainless steel guide cannulae (10 mm, length; 0.6 mm, outer diameter; 0.4 mm, inner diameter) were bilaterally implanted into the PrL using bregma as the reference point for coordinates: angle of 22°; anterior/posterior, +3.3 mm; medial/lateral, ±1.9 mm; dorsal/ventral, 2.8 mm (Paxinos and Watson 2007). The cannulae were fixed to the skull by means of acrylic resin and 1 stainless steel screws. At the end of the surgery, each guide cannula was sealed with a stainless steel wire to prevent obstruction and rats received an intramuscular injection of penicillin-G benzathine (Pentabiotic, 600 000 IU in a 0.2 mL volume; Fort Dodge) and a subcutaneous injection of the anti-inflammatory analgesic Banamine (flunixin meglumine, 2.5 mg/kg in 0.2 mL, Shering-Plough). After surgery, rats were placed again in their home cages in groups of 4.

Microinjection Procedures

Intracerebral infusions were delivered via an infusion pump (Harvard Apparatus) at a rate of 0.3 µL/min. A thin dental needle (0.3 mm, outer diameter) was introduced through each guide
cannula attached by polyethylene tubing to a 5-µL Hamilton syringe (Reno). The injection needle extended 1 mm below the ventral tip of the implanted guide cannula. The injection needles were left in place for 1 min after the end of the infusion period to allow for diffusion.

Fear Conditioning

The animals were subjected to fear conditioning paradigm using context as conditioned stimulus. During the training session, the rats were individually placed in an experimental chamber (48 × 26 × 25 cm) illuminated by a 15-W red light. The side walls were made of acrylic and gray roof and front door of transparent Plexiglas. The floor consisted of a grid with 36 stainless steel rods (3 mm in diameter), spaced 1.5 cm apart. A stimulator linked to an interface controlled by a computer delivered footshocks (Insight Instruments). After 5 min of habituation, the rats received 10 electric 0.6-mA footshocks (duration of 1 s each), varying the interval between shocks (range 30–120 s), as previously reported (Albrechet-Souza et al. 2013). The rats were removed from the conditioning chamber 3 min after the presentation of the last shock and put back into their home cage. The chamber was cleaned with 20% ethanol, before and after use. The test session to assess fear conditioning expression was conducted 24 h after training and consisted of an exposure of 8 min to an environment in which footshocks were previously administered. The behavioral response used in this study was the time spent freezing during the test session. Freezing was operationally defined as the complete absence of movement of the vibrissa and body, except those required for respiration, for at least 6 s (Bouton and Bolles 1980; Fanselow 1980). All scoring procedure was made by a single experimenter who manually timed freezing response during 8 min in a continuous manner, 1 rat at a time. All experiments were monitored in real time by a video camera mounted 12 cm above the door and located on the top side of the box so that the presence of the experimenter did not interfere with the experimental condition.

Control groups were added in this study to assess possible generalization of fear conditioning and drug effects per se. To this end, during testing, a separate group of rats was exposed to an experimental chamber different from the one used during the training session. This different context consisted of a chamber (31 × 21 × 21 cm) illuminated by a 15-W white lamp. The side and back walls were made of steel, and the ceiling and front door were made of transparent Plexiglas. The grid was covered with thick plastic. The chamber was cleaned with 20% alcohol and scented with 0.5% acetic acid after each session. Both chambers were enclosed in wooden sound-attenuating boxes.

Experiment 1: Effects of Metyrapone on Freezing and Fos-Protein Expression in the mPFC

Twenty-four hours after the training session, rats that did not undergo stereotaxic surgery were divided in 2 groups: those who would be exposed to the same context (training chamber) and those who would be exposed to a different context. Both groups received either an intraperitoneal injection of vehicle or metyrapone at a dose of 30 mg/kg in a volume of 1 mL/kg and 20 min after freezing behavior was registered over 8 min. This dose of metyrapone has been shown to significantly reduce plasma corticosterone concentrations within 20 min (Mikics et al. 2004), an effect that lasts for at least 20 min (de Oliveira et al. 2012) without altering locomotor activity (Mikics et al. 2005). Two hours after the beginning of the test session, they were deeply anaesthetized with urethane (3 g/kg i.p.; Sigma–Aldrich) and intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) (Albrechet-Souza et al. 2013).

Immunohistochemical Analysis

After removal from the skull, the brains were immersed overnight in paraformaldehyde and stored for 72 h in 30% sucrose in 0.1 M PBS for cryoprotection. The brains were quickly frozen in isopentane (−40°C) and sliced in a cryostat (−19°C). Sections (40 µm) were collected in antifreeze solution, and Fos-protein immunoreactivity was detected using standard techniques, as previously described (Albrechet-Souza et al. 2013). Briefly, the sections were first treated with 1% hydrogen peroxide in 0.1 M PBS for 10 min and incubated with primary c-fos antibody (1:4000, rabbit polyclonal; Santa Cruz Biotechnology) in 0.1 M PBS enriched with 0.2% Triton-X and 0.1% bovine serum albumin overnight at 23 ± 1°C. The sections were then incubated in biotinylated goat antirabbit secondary antibody (1:400; Vector Laboratories) and avidin–biotin complex (1:200; Vector Laboratories) for 1 h each. Fos immunoreactivity was revealed by the addition of the chromogen 3,3′ diaminobenzidine (DAB; 0.02%; Sigma–Aldrich) in 0.1 M PBS, to which 0.04% hydrogen peroxide was added before use. After ~10 min, the tissue sections were rinsed with buffer solution, mounted on gelatine-coated slides and dehydrated. Fos-positive cells were visualized and counted under a 10× objective using a bright-field microscope (Olympus BX-50; Olympus Corporation) equipped with a video camera module (Leica DFC320; Leica Camera AG). The average number of labeled cells “per 0.1-mm² structure was obtained. The analyzed mPFC areas comprised the prelimbic (PrL) and infralimbic (IL) cortices (anterior/posterior coordinates from bregma: 4.20–3.00 mm), cingulate cortex, area 1 (Cg1) and 2 (Cg2) (2.16–1.68 mm) according to the Paxinos and Watson Atlas (Paxinos and Watson 2007).

Experiment 2: Effects of the MR and GR Antagonists Injected into the PrL on the Expression of Conditioned Fear Response

Twenty-four hours after the training session, independent groups that underwent stereotaxic surgery received microinjections of vehicle or different doses of spironolactone or RU38468 (0.5, 2.5, or 5 ng/0.25 µL “per” hemisphere) 10 min prior to the test session. The doses and time of the injection were based on previous studies (Roozendaal and McGaugh 1997; Barsegyan et al. 2010).

Experiment 3: Effects of Combined Administration of MR and GR Antagonists Injected into the PrL on the Expression of Conditioned Fear Response

Once dose–response curves of spironolactone and RU38468 were determined, drugs were administered in a combined way into the PrL of independent groups of rats. The first treatment (vehicle or RU38468, 5 ng/0.25 µL) was performed 5 min before the second treatment (vehicle or spironolactone, 2.5 or 5 ng/0.25 µL) whereas this treatment was given 10 min before the test session.

Experiment 4: Effects of Corticosterone Injected into the PrL on the Expression of Conditioned Fear Response

To assess possible rapid effects of corticosterone injections into the PrL, a dose–response curve of corticosterone was performed. Twenty-four hours after the training session, independent groups of rats received microinjections of vehicle or different doses of corticosterone (0.25, 0.5, or 1 ng/0.25 µL “per” hemisphere) 5 min prior to the test session. The doses and time of
injections were based on previous studies (Morrow et al. 1996; Chauveau et al. 2009).

**Experiment 5: Effects of Administration of MR or GR Antagonists Combined with Corticosterone Injected into the PrL on the Expression of Conditioned Fear Response**

In order to evaluate the mechanisms by which corticosterone exerts its rapid effects into the PrL, RU38486 (5 ng/0.25 µL) or spironolactone (0.5 ng/0.25 µL) were locally administered prior to corticosterone infusions (1 ng/0.25 µL) into the PrL. In these experiments, the first treatment was performed 5 min before the second treatment whereas this latter administration was carried out 5 min before the test session.

**Experiment 6: Effects of Spironolactone and Corticosterone into the PrL on Time of Freezing Behavior in Rats Submitted to a Different Context**

To evaluate possible general effects of spironolactone and corticosterone in the PrL on freezing expression, a separated group of rats was conditioned and treated as previously described but submitted to a different context for the assessment of freezing behavior.

**Histology**

Upon conclusion of the experiments, the rats were deeply anesthetized with intraperitoneal urethane (3 g/kg i.p.; Sigma–Aldrich) and perfused transcardially with 0.9% saline followed by buffered 4% formalin. Brains were removed from the skulls, maintained in formalin solution for 2 h and cryoprotected in 30% sucrose for 72 h. Coronal brain slices (50 µm) that contained the PrL cortex were stained with Cresyl Violet (5%, Sigma–Aldrich) to localize the microinjection sites by microscopic examination according to the Paxinos and Watson Atlas (Paxinos and Watson 2007). Rats receiving injections outside the aimed area were not included in the analysis.

**Data Analysis**

The software used for all statistical analysis was STATISTICA version 6.0. Data are expressed as mean ± standard error of the mean (SEM). In the first experiment, behavioral and Fos-positive cells data were assessed by two-way analysis of variance (ANOVA) to evaluate the effects of treatment (metyrapone or vehicle) and condition (same context or different context) factors. In the second and fourth experiment, freezing response was analyzed using one-way ANOVA for same context conditioning. In the case where combined treatments were performed for same context conditioning, freezing response was analyzed using two-way ANOVA considering pretreatment and treatment as factors. The assessment of the effective drug doses on freezing response in a different context was done using one-way ANOVA. Significant comparisons were followed by Newman–Keuls post-hoc test. Values of $P < 0.05$ were considered statistically significant.

**Results**

**Effects of Metyrapone on the Expression of Conditioned Fear Response and Fos Protein in the mPFC**

Figure 1 shows the average freezing time in rats treated with vehicle or metyrapone before exposure to the same or different context where footshocks were previously administered. Two-way ANOVA showed significant effects of treatment, condition, and treatment × condition interaction on freezing time ($F_{1,28} = 13.15; 11.38,$ and 4.76 respectively, $P < 0.05$ in all cases). The post-hoc analysis showed that exposure to the same context increased freezing expression in the vehicle-treated group when compared with all other groups and that metyrapone treatment was associated with decreased fear expression only in the context where footshocks were previously administered ($P < 0.05$, Newman–Keuls).

Fos-positive cells in different areas of the mPFC and representative photomicrographs that illustrate Fos immunoreactivity in the PrL are shown in Figure 2A,B, respectively. A two-way ANOVA showed significant effects of the drug treatment and the interaction drug treatment × condition on the expression of Fos–protein expression in the Cg1 ($F_{1,25} = 10.56$ and 18.74, respectively, $P < 0.05$ in both cases), but not in the condition factor alone ($F_{1,25} = 0.14$, $P > 0.05$). Significant effects were observed on the interaction treatment × condition in the PrL, IL, and Cg2 ($F_{1,25} = 11.05, 6.47$, and 5.99, respectively, $P < 0.05$ in all cases), but no significant effects on treatment ($F_{1,25} = 3.60, 0.26$, and 0.70, $P > 0.05$ in all cases) and condition factors ($F_{1,25} = 0.6.0, 3.77$, and 1.11, respectively, $P > 0.05$ in all cases) when analyzed separately. The post-hoc comparisons showed that rats treated with vehicle and exposed to the same context showed an increased expression of Fos expression in the PrL, Cg1, and Cg2 compared with the vehicle-treated groups and exposed to different context and with metyrapone-treated groups and exposed to the same context ($P < 0.05$, Newman–Keuls). The comparisons also demonstrated that metyrapone-treated group exposed to the different context had a lower expression of Fos in the Cg1 and Cg2 when compared with vehicle-treated group exposed to the same context ($P < 0.05$, Newman–Keuls). The post-hoc analysis found no significant difference between groups in the expression of Fos protein in the IL ($P > 0.05$, Newman–Keuls).

**Effects of Spironolactone and RU38486 Bilaterally Injected into the PrL on the Expression of Conditioned Fear Response**

As shown in Figure 3A, one-way ANOVA followed by the post-hoc Newman–Keuls tests showed that only the intermediate dose of
Figure 2. Effects of aversive context and metyrapone on the expression of Fos protein in the mPFC. Average number of Fos-positive cells in the PrL, IL, Cg1, and Cg2 subregions of rats treated with vehicle or metyrapone and exposed to a different or to the same context previously paired with footshock (A). Representative photomicrographs illustrating the Fos-protein immunoreactivity in the PrL of rats treated with vehicle or metyrapone and exposed to the same context (B). * Different from the vehicle group exposed to a different context, # different from the vehicle group exposed to the same context ($P < 0.05$). $n = 7–8$ “per” group. Scale bar equal to 100 µm. DC, different context; SC, same context; PrL, prelimbic cortex; IL, infralimbic cortex; Cg1, anterior cingulate cortex area 1; and Cg2, anterior cingulate cortex area 2.

Figure 3. Effects of MR and GR antagonists microinjected in the PrL in the expression of contextual fear conditioning. Average of freezing responses in rats treated with vehicle ($n = 10$) or spironolactone at doses of 0.5 ng ($n = 7$), 2.5 ng ($n = 10$), or 5 ng ($n = 10$) and exposed the same context previously paired with footshocks (A). Average of freezing responses in rats that received bilateral microinjections of vehicle ($n = 12$) or RU38486 at doses of 0.5 ng ($n = 9$), 2.5 ng ($n = 10$), or 5 ng ($n = 10$); exposed to the same context (B). Photomicrograph representative of microinjection sites in the PrL and diagrams representing the microinjection sites in cross-sections of the Paxinos and Watson atlas (Paxinos and Watson 2007) (C). Scale bar equal to 1.0 mm. PrL, prelimbic cortex. The number of points in the figure is less than the total number of animals because of overlapping injection sites. * Different from vehicle group ($P < 0.05$). Spiron, spironolactone.
spironolactone (2.5 ng) decreased freezing time response when administered bilaterally in the PrL before the test ($F_{3,33} = 9.99, P < 0.05$). The bilateral administration of RU38486 in the PrL before the test session did not change the freezing expression in any of the doses tested, as shown in Figure 3B ($F_{3,37} = 0.46, P > 0.05$).

A representative photomicrograph of microinjection into the PrL and the sites of injections located on diagrams of cross-sections from the Paxinos and Watson Atlas (Paxinos and Watson 2007) are shown in Figure 3C.

Effects of Combined Administration of RU38486 and Spironolactone BilaterallyInjected into the PrL on the Expression of Conditioned Fear Response

The combined effects of RU38486 and spironolactone injections are illustrated in Figure 4. Two-way ANOVA analysis revealed significant effects of treatment and interaction among pretreatment × treatment ($F_{2,35} = 8.17$ and $3.22$, respectively, $P < 0.05$ in both cases). There was no significant effect of pretreatment factor ($F_{1,35} = 1.14, P > 0.05$). The post-hoc analysis showed that the combined injection of vehicle and spironolactone (2.5 ng) decreased the freezing time response compared with all other treatments, an effect that was blocked by prior microinjection of RU38486 (5 ng). The same analysis revealed no significant differences between the other groups ($P > 0.05$, Newman–Keuls).

Effects of Corticosterone Bilaterally Injected into the PrL on the Expression of Conditioned Fear Response

As shown in Figure 5A, the one-way ANOVA followed by the post-hoc Newman–Keuls tests showed that the higher dose of corticosterone tested (1 ng) decreased freezing response when injected into the PrL before the test ($F_{3,19} = 6.28, P < 0.05$).

Effects of Combined Administration of Corticosterone and its Antagonists Injected into the PrL on the Expression of Conditioned Fear Response

As shown in Figure 5B, the two-way ANOVA analysis revealed significant effects of the pretreatment ($F_{2,43} = 4.60$, treatment ($F_{1,43} = 10.15$), and interaction between pretreatment × treatment ($F_{2,43} = 4.38, P < 0.05$ in all cases). The post-hoc analysis showed that the groups treated with corticosterone (1 ng) showed decreased time of freezing response (Newman–Keuls, $P < 0.05$), an effect that was blocked by prior microinjection of RU38486 but not by spironolactone. The analysis revealed no significant differences between the groups treated with vehicle + corticosterone (1 ng) and spironolactone (0.5 ng) + corticosterone (1 ng).

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**Figure 4.** Effect of prior administration of RU38486 on the effects promoted by spironolactone microinjected in the PrL in the expression of contextual fear conditioning. Average freezing responses in rats treated with vehicle (n = 5) or corticosterone tested (1 ng) decreased freezing response when injected into the PrL before the test ($F_{3,19} = 6.28, P < 0.05$).

**Figure 5.** Effect of corticosterone and prior administration of spironolactone or RU38486 microinjected in the PrL in the expression of contextual fear conditioning. Average freezing responses in rats treated with vehicle (n = 5) or corticosterone at doses of 0.25 ng (n = 5), 0.5 ng (n = 7), or 1 ng (n = 6) and exposed to the same context previously paired with footshocks (A). * Different from vehicle group ($P < 0.05$). Average freezing responses in rats treated with the combination of V + V, (n = 7); V + Cort (1 ng), (n = 7); Spiron (0.5 ng) + V, (n = 7); Spiron (0.5 ng) + Cort (1 ng), (n = 6), RU38468 (5 ng) + V, (n = 7); and RU38468 (5 ng) + Cort (1 ng), (n = 7) and exposed to the same context previously paired with footshocks (B). * Different from V + V, Spiron (0.5 ng) + V, and V + RU38468 (5 ng) groups. # Different from the V + Cort (1 ng) and Spiron (0.5 ng) + Cort (1 ng) groups ($P < 0.05$). V, vehicle; Spiron, spironolactone; Cort, corticosterone.
Table 1 Effects of spironolactone and corticosterone on the expression of freezing behavior in rats submitted to a different context (n = 6–7 “per” group)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Freezing (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh + Veh</td>
<td>146.50 ± 30.07</td>
</tr>
<tr>
<td>Veh + Spiron (2.5 ng)</td>
<td>188.57 ± 37.38</td>
</tr>
<tr>
<td>Veh + Cort (1 ng)</td>
<td>163.29 ± 45.57</td>
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Effects of Corticosterone and Spironolactone into the PrL on Time of Freezing Behavior in Rats Submitted to a Different Context

One-way ANOVA revealed that injections of spironolactone (2.5 ng) or corticosterone (1 ng) into the PrL did not influence freezing response of rats that were subjected to a context different from the conditioning context compared with the vehicle-treated group (Table 1 [$F_{2,17} = 0.28, P > 0.05$]).

Discussion

The present results suggest that glucocorticoid release during contextual fear-conditioning responses influences mPFC activity and the expression of conditioned fear. Metyrapone, a glucocorticoid synthesis inhibitor, attenuated this behavioral response accompanied by a decrease in Fos-protein expression in the mPFC. Moreover, spironolactone, an MR antagonist, decreased the freezing response when it was injected in the PrL, an effect that was prevented by pretreatment with the GR antagonist RU38486. Endogenous corticosterone can activate both MRs and GRs, and the effects that were observed after MR antagonist administration could be attributable to changes in the balance of MR and GR signaling and a redirection of corticosterone actions to GRs. Supporting the hypothesis that the antiaversive effect of corticosterone is mediated by an increase in GR activation, direct injections of corticosterone into the PrL decreased the freezing response, which was prevented by pretreatment with a GR antagonist but not MR antagonist.

The inhibition of corticosterone synthesis prior to exposure to the environment where footshocks were previously administered reduced the freezing response, associated with a decrease in Fos expression in the PrL, Cg1, and Cg2, suggesting an important role of hormone release in the expression of conditioned fear responses that are organized in the mPFC. A previous study demonstrated that the freezing response was attenuated by removing the adrenal glands in rats 1 h before the test, an effect that was reversed by systemic corticosterone administration (Bohus et al. 1987). Moreover, metyrapone administration before the test session in nonadrenalectomized rats decreased the duration of conditioned freezing (Roozenaall et al. 1996; de Oliveira et al. 2012). Furthermore, a clinical study reported that metyrapone administration effectively reduced both plasma cortisol concentrations and the strength of an emotional memory in a long-lasting manner, without affecting the retrieval of neutral responses (Marin et al. 2011).

The most obvious explanation for the effects of metyrapone is that the activation of GRs and MRs receptors is influenced by decreases in corticosteroid levels (Roozenaall et al. 1996). However, some alternative explanations should be considered. Metyrapone administration may result in increases in progesterone and deoxycorticosterone concentrations (Kruegers et al. 2000). These hormones can potentiate responses that are mediated by γ-aminobutyric acid-A (GABA_A) receptors and block Ca^{2+}-dependent channels (French-Mullen and Spence 1991; Reddy and Rogawski 2002). Such effects may underlie an anxiolytic action of the drug.

Interestingly, some studies have demonstrated that increases in plasma corticosteroid concentrations can also reduce conditioned freezing responses. Atsak et al. (2012) showed that systemic corticosterone administration before the test session decreased the expression of contextual conditioned fear in rats. In another study, mice were systemically treated with corticosterone before the test session and exhibited a reduction of the expression of conditioned fear (Cai et al. 2006). Consistent with these results, some human studies indicated that the exogenous administration of cortisone, which is rapidly absorbed and converted to cortisol, may impair the declarative memory retrieval of emotional valence information (de Quervain et al. 2007). Based on the results that indicated a reduction of the expression of conditioned fear after opposing manipulations of plasma corticosterone concentrations, an inverted U-shaped function may be observed between the circulating levels of corticosterone and magnitude of contextual conditioned fear expression (Joels and de Kloet 1994).

Evidence suggest the involvement of the mPFC in regulating stress responses, and this structure appears to be involved in the modulation of endocrine and behavioral responses (Diorio et al. 1993; Mobbs et al. 2007; Radley et al. 2009; Sotres-Bayon and Quirk 2010; Jones et al. 2011). With regard to behavioral data, the PrL and cingulate regions appear to play roles in the expression of defensive behavior, including fear conditioning (Sotres-Bayon and Quirk 2010; Albrechet-Souza et al. 2013). In fact, the importance of mPFC activity in rats that are subjected to contextual fear conditioning has been extensively reported. Pharmacological inactivation by tetrodotoxin (Sierra-Mercado et al. 2006) or cobalt chloride (Lisboa et al. 2010) that was restricted to the PrL reduced the expression of fear that was associated with an explicit or contextual conditioned stimulus. Furthermore, in vivo electrophysiological studies in rats showed that neurons in PrL responded with an increase in firing rate during exposure to aversive conditioned stimuli (Baeg et al. 2001). The intense activity of the PrL that was induced by a conditioned stimulus also impaired the process of aversive memory extinction (Burgos-Robles et al. 2009; Maroun 2013).

The present study found no significant differences between groups in Fos-protein expression in the IL. Previous studies that reported differential functions between subregions of the mPFC suggested that the IL is more involved in extinction than in the expression of aversive memory (Sierra-Mercado et al. 2006; Maroun 2013). The differential participation of the IL and PrL in memory processes has been attributed to the specificity of projection pathways from each cortical region (Gabbott et al. 2005; Hoover and Vertes 2007), although little is known about the complex neuronal network functioning of these areas or how these networks interact during stressful situations (van Aerde et al. 2008). Considering the amygdaloid nuclei, although IL projects directly to inhibitory intercalated cell masses and the lateral division of the central nucleus of the amygdala to regulate the process of extinction, the main projection target of the PrL is the basolateral nucleus of the amygdala (McDonald 1998; Sotres-Bayon and Quirk 2010), suggesting different roles of each region in limbic-cognitive function.

Corticosteroid hormones that are secreted by the adrenal glands rapidly cross the blood–brain barrier and at high concentrations can bind to MRs and GRs (Reul and de Kloet 1985) that are located in both the rat and human mPFC (Meaney and Aitken...
In a classic study that investigated the binding of corticosteroids to brain tissue, Di Dio et al. (1993) showed that the rat mPFC is sensitive to both selective MR and GR agonists. Thus, considering previous findings and the present results, the PrL was chosen to evaluate the ways in which MR and GR activity in the mPFC influences the expression of contextual conditioned fear. To address this issue, we investigated whether microinjections of an MR or GR antagonist in the PrL before the test affects the conditioned fear response. The intermediate dose of spironolactone (2.5 ng) decreased the expression of conditioned fear whereas administration of the other doses and RU3846 did not affect this response.

The present results confirm the effects that were observed after acute systemic or intracerebroventricular (i.c.v.) administration of spironolactone before the test session which reduced conditioned freezing in mice, whereas RU3846 had no effect (Korte et al. 1995; Zhou et al. 2011). Some studies have also suggested that MR antagonism may influence the expression of anxiety-like behavior, in which chronic treatment with an MR antagonist eplerenone decreased open-arm exploration in the elevated plus maze test in rats (Hlavacova et al. 2010).

In the present study, the higher dose of spironolactone (5 ng) lost its effect on the expression of the freezing response. Similarly, the behavioral effects of i.c.v. spironolactone administration on fear conditioning were lost when a higher dose was administered (Korte et al. 1995). To further understand this phenomenon, a previous study characterized the pharmacological MR and GR binding profiles, showing that RU3846 does not have significant affinity for MRs, whereas spironolactone has low affinity for GRs without intrinsic activity (Rupprecht et al. 1993). Thus, it is possible that the higher dose of spironolactone did not cause behavioral effects because of its residual action on GRs.

Given the aforementioned evidence, the present study investigated whether the effects that are observed after MR antagonist administration into the PrL could be attributable to the facilitation of endogenous corticosterone actions on GRs. The results showed that the decrease in conditioned fear that was induced by the microinjection of spironolactone was inhibited by pretreatment with an ineffective dose of RU3846, suggesting that the impairment in memory retrieval that was observed with MR antagonism in the PrL was at least partially caused by changes in the balance between MRs and GRs toward an increase in GR-mediated corticosterone action. Supporting this hypothesis, the present results showed that corticosterone treatment decreased the freezing response, an effect that was abolished by pretreatment with RU3846 but not by spironolactone at the dose of 0.5 ng, which cannot completely exclude the possible role of MRs in this process. Additionally, the present results showed that neither spironolactone nor corticosterone treatment altered the expression of freezing behavior in a context that was different from the one where the rats were conditioned, suggesting that the increase in GR activity in the PrL before the test session led to a selective decrease in the expression of conditioned fear.

The PFC has been implicated in the inhibition of inappropriate responses and consequently adaptive processes. It is plausible that the balance between GRs and MRs in this brain region is essential for behavioral adaptation. Repeated exposure to stressful situations has been reported to decrease GR expression and increase MR expression in the mPFC (Chiba et al. 2012; Zhang et al. 2012), suggesting that an imbalance between MR and GR activity could underlie some aspects of emotional disorders that are influenced by stress. Corticosterone is well known to act primarily on MRs because it has higher affinity and intrinsic activity for these receptors (Reul and de Kloet 1985). In the PrL region, when corticosterone acts secondarily on GRs, it produces opposite effects. The extent to which these opposing actions of glucocorticoids on MRs and GRs contribute to the overall effect, as seen during stressful situations, remains to be determined.

A review by de Kloet (2013) discussed the functional profile of this binary brain corticosteroid receptor system, proposing that modulating processes that are coordinated by corticosterone in response to homeostatic challenge operate via MRs and GRs in 2 complementary modes. He suggested that MRs mediate appraisal processes, such as risk assessment and the onset of stress reactions, whereas GR-mediated actions are related to controllability of the situation, adaptation, and turning off the stress reaction. Because the test situation in the present study was aversive, a rapid rise in circulating corticosterone levels in vehicle-treated animals was expected (Roozendaal et al. 1992), which begin to fully occupy MRs and increasingly occupy GRs (Yau and Seckl 2012). As mentioned previously, some of the effects of metyrapone in decreasing freezing responses are mediated by decreases in endogenous corticosterone concentrations and might influence not only GR—but also MR-mediated effects (Roozendaal et al. 1996). Considering the present findings and previous studies that reported decreases in the expression of freezing responses that were induced by corticosterone administration (Atsak et al. 2012), the behavioral responses that are induced by this hormone may be mediated by a rapid increase in both MR and GR activity. Therefore, although our results showed that an increase in GR-mediated corticosterone actions is crucial for this process in the mPFC, both corticosterone and metyrapone may induce the same behavioral outcome by influencing the balance between MR and GR activity and the retrieval of conditioned fear memory in an inverted U-shaped function (Joels and de Kloet 1994).

Regarding the lack of an effect of GR antagonism per se, although we observed an effect of MR blockade that depended on GRs, the opposite is not necessarily true (i.e., the facilitation of memory retrieval). The balance between MR and GR signaling in homeostatic control reflects different and complementary roles of GR and MR activation (de Kloet 2013). These receptors have different signaling pathways and mediate distinct rapid effects on neurotransmission (Tasker et al. 2006).

Glucocorticoids can indirectly influence neuronal activity by modulating neurotransmitter systems. Glucocorticoids were reported to exert their actions by increasing endocannabinoid (eCB) mobilization (Di et al. 2003; Wang et al. 2012). This mechanism is mediated by GR activation, also in the membrane, which rapidly stimulates the production of eCBs in the brain, which in turn can bind to cannabinoid 1 receptors (CB1) that are located presynaptically and inhibit neurotransmitter release (Popoli et al. 2012). Previous studies that confirmed this interaction between glucocorticoids and the eCB system showed that the effects of systemic corticosterone administration in rats decreased contextual conditioned fear by increasing eCB levels in the hippocampus (Atsak et al. 2012) and acute stress increased eCB production in the mPFC through GR activation (Hill et al. 2011). The activation of CB1 receptors by eCBs, such as 2-arachidonoylglycerol and anandamide, can modulate synaptic transmission by regulating GABA and glutamate release (Kano et al. 2009; Popoli et al. 2012; Wang et al. 2012). With regard to glutamate, acute stress was recently shown to rapidly increase glutamate release in the mPFC, and systemic GR antagonist administration prevented this response (Musazzi et al. 2010). Furthermore, the in vitro application of corticosterone in this region rapidly induced the mobilization of synaptic vesicles, priming
the terminals to enhance glutamate release in response to acute stress (Treccani et al. 2014).

In the present study, we cannot exclude the possibility that increases in GR activation in the PrL as a result of corticosterone challenge may influence HPA axis activation and consequently behavioral responses. The mPFC is a target structure for the effects of glucocorticoids in the negative feedback mechanism of the HPA axis in responses to acute psychological stressors (Herman et al. 2005; Radley et al. 2009; Jones et al. 2011). Despite the short time interval between corticosterone administration in the PrL and the fear conditioning test, GR activation in the PrL is suggested to contribute to the recovery and termination of stress-induced HPA axis activation (Hall et al. 2011).

In summary, the present study showed that the inhibition of corticosterone synthesis during the expression of contextual conditioned fear decreased PrL activity and the freezing response. The results also showed that MR antagonism in this region decreased conditioned fear, an effect that depended on GR activation by endogenous glucocorticoids. Corticosterone administration in the PrL also reduced freezing behavior, an effect that is attributable to changes in the MR:GR balance toward an increase in GR-mediated corticosterone action. The results suggest an important role of rapid nongenomic effects of glucocorticoids in the mPFC in the cognitive control of emotional learning experiences.

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References


