Corticotropin-releasing factor (CRF) plays a critical role in the mediation of physiological and behavioral responses to stressors. In the present study, we investigated the role played by the CRF system within the medial amygdala (MeA) in the modulation of anxiety and fear-related responses. Male Wistar rats were bilaterally administered into the MeA with CRF (125 and 250 ng/0.2 µl, experiment 1) or with the CRF1 antagonist antalarmin (25 ng/0.2 µl, experiment 2) and 10 min later tested in the elevated T-maze (ETM) for inhibitory avoidance and escape measurements. In clinical terms, these responses have been respectively related to generalized anxiety and panic disorder. To further verify if the anxiogenic effects of CRF were mediated by CRF1 activation, we also investigated the effects of the combined treatment with CRF (250 ng/0.2 µl) and antalarmin (25 ng/0.2 µl) (experiment 3). All animals were tested in an open field, immediately after the ETM, for locomotor activity assessment. Results showed that CRF, in the two doses administered, facilitated ETM avoidance, an anxiogenic response. Antalarmin significantly decreased avoidance latencies, an anxiolytic effect, and was able to counteract the anxiogenic effects of CRF. None of the compounds administered altered escape responses or locomotor activity measurements. These results suggest that CRF in the MeA exerts anxiogenic effects by activating type 1 receptors, which might be of relevance to the physiopathology of generalized anxiety disorder.

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et al., 1996). It has also been previously shown that the MeA presents at medium to high levels both CRF-type I and 2 receptors (Bittencourt and Sawchenko, 2000). Actually, within the amygdaloid complex, CRFR1 mRNA expression was found greater in prenatally stressed females, when compared to non-stressed controls, only in the MeA (Brunton et al., 2011). Furthermore, it has been recently shown that injections of the CRF1 agonist corticosterone into the medial prefrontal cortex decreases Fos protein immunoreactivity in some amygdala nuclei, including the MeA, while at the same time, attenuating predator-induced defensive behaviors (Pentkowski et al., 2013).

Nevertheless, apart from some indirect evidence implicating the MeA CRF system with stress/anxiety, to our knowledge no previous study has investigated the effects of the direct infusion of CRF or CRF-related compounds into the MeA in the modulation of stress/anxiety-related responses. The present study addresses this question by investigating the effects of intra-MeA CRF and antalarmin — a CRF1 antagonist (Takahashi, 2001) — in an animal model of anxiety, the elevated T-maze (ETM). The model allows the measurement of an anxiety (inhibitory avoidance) and a fear-related response (one-way escape). The pharmacological validation of the ETM has shown that compounds representative of three classes of anxiolytics — namely the agonist of benzodiazepine receptors diazepam, the serotonin 1A agonist buspirone, and the nonselective serotonin type 2 antagonist ritalinser — selectively impair inhibitory avoidance while leaving one-way escape unchanged (Graeff and Zangrossi, 2002; Graeff et al., 1993; Viana et al., 1994). These results are compatible with the view that inhibitory avoidance relates to generalized anxiety. In contrast the escape task is impaired by chronic, but not acute administration of imipramine (Teixeira et al., 2000), clomipramine and fluoxetine (Polronieri et al., 2003), drugs that are used to treat panic. As a result, ETM escape has been used as an animal model of panic disorder.

The use of the ETM, thus, allows the investigation of the role played by MeA CRF receptors in two different subtypes of anxiety-related disorders. This is of relevance since it has been previously shown that CRF receptor activation within specific amygdala nuclei may evoke anxiety reactions linked to distinct anxiety disorders found in clinical settings (Lee et al., 2008; Rannie et al., 2004).

To further verify if the anxiogenic effects of CRF were in fact mediated by CRF1 activation, in the present study we also investigated the effects of the combined treatment with CRF and antalarmin. All animals were also tested in an open field, immediately after the ETM, for locomotor activity assessment.

Materials and methods

Subjects

Eighty-seven male Wistar rats (CEDEME, Universidade Federal de Sào Paulo, Campus Santos, Brazil), weighing 280–320 g at the beginning of the experiment, were housed in groups of 5–6 per cage. After surgery, animals were housed in pairs in Plexiglas-walled cages until testing. Room temperature was controlled (22 ± 1 °C) and a light-dark cycle was maintained on a 12-h on-off cycle (0700–1900 h lights on). Food and water were available all throughout the experiments. The study was approved by the Ethical Committee for Animal Research of the Federal University of Sào Paulo (number 0064/12) and was performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior, which are based on the conditions stated by the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, 1996).

Apparatus

The elevated T-maze was made of wood and had 3 arms of equal dimensions (50 × 12 cm). One of the arms was enclosed by 40 cm high walls and was oriented perpendicularly to two opposed open arms. The whole apparatus was elevated 50 cm above the floor. To avoid falls, a 1 cm high Plexiglas rim surrounded the open arms.

The open field was a round arena (60 × 60 cm), with the floor divided into 12 parts, and walls 50 cm high.

Laminosity at the level of the T-maze arms or at the open field center was 60 lx. After the experimental sessions, each experimental apparatus was cleaned with a 10% ethanol solution.

Compounds

Rats/human CRF (r/h CRF #102-282-15 - PBL, The Salk Institute for Biological Studies, USA) was initially dissolved in a solution of 0.04% acetic acid and 0.02 M KPB and diluted 1:1 in sterile saline (0.9%). Control animals were administered with a solution of 0.04%acetic acid and 0.02 M KPB in sterile saline (0.9%) (1:1). The CRF1 antagonist antalarmin (#A8727, Sigma, USA) was dissolved in sterile saline (0.9%) with 2% Tween 80. Control animals were injected with a solution of sterile saline (0.9%) with 2% Tween 80. Compounds and vehicles were administered in a volume of 0.2 μl 10 min prior to the test sessions.

Surgery

Three days after their arrival to the laboratory, rats were anesthetized with an IP injection of ketamine hydrochloride (80 mg/kg; Agibrands, Brazil) and xylazine (10 mg/kg; Agibrands, Brazil) and fixed to a stereotaxic frame (David Kopf, USA). Local anesthesia was also performed (2%lidocaine with a vasoconstrictor; Harvey, Brazil) before the implant of stainless steel guide cannulae into the MeA.

Guide cannulae (0.6 mm outer diameter and 0.4 mm inner diameter) were inserted bilaterally into the brain through a hole drilled in the skull above the MeA, following the coordinates from the atlas of Paxinos and Watson (2008): AP = − 2.5 mm from bregma; ML = ± 3.5 mm and DV = − 6.9 mm from skull. Cannulae were attached to the skull by means of acrylic resin and two stainless steel screws. Stylets with the same length of the guide cannulae were introduced inside them to prevent obstruction.

To prevent infections, at the end of the surgery, all animals were injected (IM) with a 0.2 ml of pentabiotic preparation (Pentabiotico Veterinário Pequeno Porto; Forte Dodge, Brazil). In addition, flunixin meglumine (Schering–Plough, Brazil; 3 mg/kg), a drug with analgesic, antipyretic and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia.

The animals were left undisturbed in their home cages for 6 days after the surgery, except for normal handling for cage cleaning, and monitoring of signs of postoperative pain or behavioral alterations.

Microinjections

For microinjections, needles (0.3 mm outer diameter) were introduced through the guide cannulae until their tip were 1 mm below the cannulae end. Compounds and vehicles were injected over a period of 120 s using 5 μl microsyringes (Hamilton 701-RN, USA) attached to a microinfusion pump (Insight, Brazil). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intra-cerebral needle was used to monitor the microinjection. The intra-cerebral needles were removed 60 s after the end of injection.

Procedure

On the fifth and sixth days after surgery, the experimenter gently handled animals for 5 min. On the sixth day, immediately after handling, rats were exposed to one of the open arms of the T-maze for 30 min. A wood barrier mounted on the border of the central area of the maze and the open arm’s proximal end isolated this arm from the rest of the maze. It has been shown that this pre-exposure to the open arm renders
the escape task more sensitive to the effects of antipanic compounds, because it shortens the latencies of withdrawal from the open arm during the test (Sena et al., 2003).

On the seventh day after surgery, animals were injected (0.2 µl) with CRF (125 or 250 ng; N = 5–7) (experiment 1) or antalarmin (25 ng; N = 6–7) (experiment 2) into the MeA, as described above, and 10 min later tested in the ETM for inhibitory avoidance and escape measurements. The doses of the compounds were chosen on the basis of previously published studies (Forster et al., 2008; Lakkes et al., 2008; Miguel et al., 2012).

In order to investigate whether antalarmin was able to block the effects of CRF (experiment 3), animals received intra-MeA microinjection of antalarmin (25 ng) or vehicle (KPBS + sterile saline), ten minutes before the microinjection of CRF (250 ng) or vehicle (sterile saline + Tween 80). Thus, the following groups (N = 5–8) were formed: vehicle (sterile saline + Tween 80)/vehicle (KPBS + sterile saline), antalarmin/vehicle (KPBS + sterile saline), vehicle (sterile saline + Tween 80)/CRF, and antalarmin/CRF. Ten minutes after the last microinjection, the animals were submitted to the behavioral tests as described below. The dose of 250 ng of CRF was chosen for this experiment since it altered both avoidance 1 and 2 latencies in experiment 1.

For inhibitory avoidance measurement, each animal was placed at the distal end of the enclosed arm of the elevated T-maze facing the intersection of the arms. The time taken by the rat to leave this arm with the four paws, for the first time, was recorded (baseline latency) and used as a measure of locomotor activity (Graeff and Zangrossi, 2002; Graeff et al., 1993; Viana et al., 1994). After baseline latency measurements, the time taken by the rat to leave the enclosed arm was again recorded in two subsequent trials (avoidance 1 and 2) at 30 s inter-trial intervals, during which animals were placed in a Plexiglas cage to which they had been previously habituated. Since being in the open arms seems to be an aversive experience, in these subsequent trials, the animals usually take a longer time to withdraw from the enclosed arm towards the open space, thus showing avoidance learning. Following avoidance measurements (30 s), rats were placed at the end of one of the open arms and the latency to leave this arm with the four paws was recorded for 3 consecutive times (escape 1, 2 and 3), with 30 s inter-trial intervals. The latencies were always evaluated in the same previously experienced open arm. A cutoff time of 300 s was established for the avoidance and escape latencies. Immediately after being tested in the ETM, each animal was placed for 5 min in the center of the open field and the total number of lines crossed and number of rearings were recorded.

Histology

After the experiments, animals were sacrificed under deep anesthesia with urethane. Their brains were perfused through the heart with saline solution followed by 10% formalin solution, before being removed and fixed in 10% formalin. Frozen sections of 55 µm were cut using a microtome in order to localize the site of injections, according to the Paxinos and Watson's atlas (2008). Only data from rats with injection sites into the MeA were included in the statistical analysis.

Statistical analysis

Data were tested for homogeneity. In case of heterogeneous results, data were submitted to a logarithmic transform. For experiments 1 and 2, two-way analysis of variance (ANOVA) with repeated measures was used to analyze avoidance and escape data from the ETM, with treatment as the independent factor and trials (baseline, avoidance 1 and 2, or escape 1 to 3) as the dependent factors. A three-factor design was used to analyze ETM results from experiment 3, with the two treatments (treatment 1: vehicle or antalarmin; treatment 2: vehicle or CRF) as the independent factors and trials as the dependent factor. Significant effects of the independent factors or of the interaction between the independent and dependent factors were analyzed by one-way ANOVA followed by the Tukey post-hoc test (experiments 1 and 3) or by the Student’s t test (experiment 2). Locomotor activity in the open field was analyzed by one or two-way ANOVA followed by the Tukey post-hoc test (experiments 1 and 3, respectively) or by Student's t test (experiment 2). A value of P ≤ 0.05 was considered significant. Effect size estimates were calculated using eta squared (η² = sum of squares for whatever effect is of interest/total sum of squares for all effects, interactions and errors) for ANOVA results and Cohen’s d (d = mean of group 1 – mean of group 2/square root ([standard deviation of group 1 + standard deviation of group 2] / 2)) for pair-wise comparisons.

Results

Fig. 1 illustrates the site of injections in both hemispheres. Only animals with microinjection sites in the MeA were included in the statistical analysis. Thirty animals were discarded due to cannulae misplacements.

Experiment 1 — Effects of CRF

Fig. 2 (panel A) shows the effects of CRF (125 and 250 ng/0.2 µl) on inhibitory avoidance measurements. Repeated measures ANOVA revealed a significant effect of trials (F(2,32) = 9.36; P = 0.001; η² = 0.342) and treatment (F(2,16) = 5.48; P = 0.015; η² = 0.407), but no trials by treatment interaction (F(4,32) = 0.13; P = 0.416; η² = 0.073). The Tukey post-hoc test showed that in avoidance 1 animals treated with 250 ng of CRF (mean difference: = − 0.81; standard error: 0.32; P = 0.050; d = − 2.200), and in avoidance 2 animals treated with 125 ng (mean difference: = 0.94; standard error: 0.25; P = 0.004; d = − 3.445) and with 250 ng (mean difference: = − 0.84; standard error: 0.23; P = 0.006; d = − 2.132), took a significantly longer time to leave the enclosed arm when compared to the control group.

Fig. 2 (panel B) shows the effects of CRF intra-MeA on ETM escape measurements. Repeated measures ANOVA revealed a significant effect of trials (F(2,32) = 3.87; P = 0.030; η² = 0.162), but not of treatment (F(2,16) = 0.96; P = 0.410; η² = 0.017) or trials by treatment interaction (F(4,32) = 1.98; P = 0.120; η² = 0.166).

One-way ANOVA revealed no significant differences in the open field between animals treated with CRF or vehicle. Neither the number of crossings (F(2,16) = 0.19; P = 0.832; η² = 0.023), nor the number of rearings (F(2,16) = 0.58; P = 0.572; η² = 0.067) were altered by CRF treatment (see Table 1).

Experiment 2 — Effects of antalarmin

Fig. 3 (upper panel) shows the effects of antalarmin (25 ng/0.2 µl) intra-MeA on inhibitory avoidance measurements. Repeated measures ANOVA revealed a significant effect of trials (F(2,22) = 16.44; P = 0.001; η² = 0.596), treatment (F(1,11) = 9.10; P = 0.012; η² = 0.453), but not of trials by treatment interaction (F(2,22) = 0.14; P = 0.874; η² = 0.005). Student’s t test showed that avoidance 1 (t(19) = − 2.37; P = 0.042; d = 1.634) and 2 (t(8.91) = − 5.70; P ≥ 0.001; d = 1.934) latencies of animals treated with antalarmin were significantly smaller than the ones presented by control animals.

Fig. 3 (lower panel) shows the effects of antalarmin in intra-MeA escape measurements. Repeated measures ANOVA did not show a significant effect of trials (F(1,11) = 0.01; P = 0.936; η² = 0.001), trials (F(2,22) = 0.77; P = 0.477; η² = 0.058) or trials by treatment interaction (F(2,22) = 1.50; P = 0.245; η² = 0.113).

As indicated in Table 1, unpaired Student’s t test showed that neither the number of crossings (t(11) = − 0.61; P = 0.557; d = − 0.375) nor the number of rearings (t(11) = − 1.13; P = 0.282; d = − 0.680) measured in the open field were affected by treatment with antalarmin.
Experiment 3—Effects of the combined treatment with CRF and antalarmin

Fig. 4 (upper panel) shows the effects of the intra-MeA combined treatment with antalarmin and CRF on inhibitory avoidance measurements. Three-way ANOVA showed a significant effect of trials (F(2,42) = 57.66; \( P < 0.001 \); \( \eta^2 = 0.607 \)), trials by antalarmin (F(2,42) = 7.37; \( P = 0.002 \); \( \eta^2 = 0.079 \)), trials by CRF (F(2,42) = 4.34; \( P = 0.014 \); \( \eta^2 = 0.050 \)), trials by antalarmin by CRF (F(2,42) = 4.05; \( P = 0.025 \); \( \eta^2 = 0.043 \)), antalarmin (F(1,21) = 23.97; \( P < 0.001 \); \( \eta^2 = 0.264 \)), CRF (F(1,20) = 29.02; \( P < 0.001 \); \( \eta^2 = 0.453 \)) and antalarmin by CRF interaction (F(1,21) = 4.62; \( P = 0.043 \); \( \eta^2 = 0.051 \)). The Tukey post-hoc test showed that the groups treated with vehicle/CRF (mean difference: 0.60; standard deviation: 0.20; \( P = 0.035 \); \( d = 1.327 \)) and antalarmin/vehicle (mean difference: 1.19; standard deviation: 0.20; \( P < 0.001 \); \( d = 1.905 \)) were significantly different from the control group in avoidance 1, and that the group treated with antalarmin/vehicle was significantly different from the vehicle/vehicle (mean difference: −0.99; standard deviation:
Fig. 2. Effect (mean ± SEM) of the intra-MeA injection of CRF (125 or 250 ng/0.2 µl) or vehicle on inhibitory avoidance (panel A) and escape (panel B) latencies of rats in the elevated T-maze. N = 5 (control), 6 (CRF 125 ng) and 8 (CRF 250 ng). *P < 0.05, compared with the control group in the same trial (ANOVA, followed by the Tukey test). Avoidance data were analyzed after log transform. Raw data are displayed for better comprehension of the testing procedure.

0.15; P < 0.001; d = 3.347), the vehicle/CRF (mean difference: -1.28; standard deviation: 0.16; P < 0.001; d = 6.507), and the antalarmin/CRF group (mean difference: -1.12; standard deviation: 0.16; P < 0.001; d = 4.102) in avoidance 2.

Fig. 3 (upper panel) shows the effects of the combined treatment with antalarmin and CRF on escape measurements. Three-way ANOVA showed a significant effect of trials (F(2,42) = 3.94; P = 0.027; η² = 0.147). No effect of trials by antalarmin (F(2,42) = 0.19; P = 0.826; η² = 0.007), trials by CRF (F(2,42) = 0.37; P = 0.691; η² = 0.014), trials by antalarmin by CRF (F(2,42) = 1.20; P = 0.310; η² = 0.045), antalarmin (F(1,21) = 2.40; P = 0.137; η² = 0.098), CRF (F(1,21) = 0.11; P = 0.749; η² = 0.004), and antalarmin by CRF (F(1,21) = 1.05; P = 0.316; η² = 0.043) was found.

Table 1 shows the effects of the combined treatment with CRF and antalarmin intra-MeA locomotor activity measured in an open

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Crossings</th>
<th>Rearings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>63.20 ± 4.36</td>
<td>12.40 ± 0.68</td>
</tr>
<tr>
<td>CRF 125 ng</td>
<td>69.50 ± 8.92</td>
<td>13.83 ± 1.64</td>
</tr>
<tr>
<td>CRF 250 ng</td>
<td>72.00 ± 11.68</td>
<td>16.50 ± 3.51</td>
</tr>
<tr>
<td>Vehicle</td>
<td>60.86 ± 10.38</td>
<td>15.00 ± 2.09</td>
</tr>
<tr>
<td>Antalarmin</td>
<td>66.17 ± 7.71</td>
<td>17.83 ± 1.14</td>
</tr>
<tr>
<td>Vehicle/vehicle</td>
<td>71.00 ± 4.68</td>
<td>16.00 ± 1.15</td>
</tr>
<tr>
<td>Vehicle/CRF</td>
<td>74.40 ± 6.84</td>
<td>16.00 ± 2.51</td>
</tr>
<tr>
<td>Antalarmin/vehicle</td>
<td>74.00 ± 3.22</td>
<td>15.12 ± 1.34</td>
</tr>
<tr>
<td>Antalarmin/CRF</td>
<td>72.40 ± 2.33</td>
<td>12.60 ± 0.93</td>
</tr>
</tbody>
</table>

Fig. 4. Effect (mean ± SEM) of the combined intra-MeA treatment with CRF (250 ng/0.2 µl) and antalarmin (25 ng/0.2 µl) on inhibitory avoidance (panel B) and escape (panel B) latencies of rats in the elevated T-maze. N = 7 (vehicle/vehicle), 5 (antalarmin/vehicle), 8 (vehicle/CRF), 5 (antalarmin/CRF). *P < 0.05, compared with the vehicle/vehicle group; η² = 0.05, compared with all the other groups in the same trial (ANOVA, followed by the Tukey test). Avoidance data were analyzed after log transform. Raw data are displayed for better comprehension of the testing procedure.
field. Two-way ANOVA did not show any significant differences, neither with respect to the number of crossings nor with respect to the number of rearings (antalarmin: crossings F(1,21) = 0.01; P = 0.922; \( \eta^2 = 0.0001 \), rearings F(1,21) = 1.91; P = 0.181; \( \eta^2 = 0.080 \); CRF; crossings F(1,21) = 0.03; P = 0.860; \( \eta^2 = 0.001 \), rearings F(1,21) = 0.67; P = 0.423; \( \eta^2 = 0.028 \)); antalarmin by CRF; crossings F(1,21) = 0.25; P = 0.630; \( \eta^2 = 0.012 \), rearings F(1,21) = 0.69; P = 0.423; \( \eta^2 = 0.028 \).

Discussion

The results from the present study showed that intra-MeA injection of rat/human CRF in the two doses administered, facilitated ETM avoidance, an anxiogenic effect (experiment 1). Intra-MeA administration of the CRF\(_{\text{R1}}\) antagonist antalarmin significantly decreased avoidance latencies (experiment 2), an anxiolytic effect. To confirm the anxiogenic action of CRF and to verify if antalarmin was able to counteract this effect, experiment 3 was conducted. The results of this last experiment confirmed the anxiogenic profile of intra-MeA CRF. Antalarmin administration was able to prevent the drug’s anxiogenic effect. Furthermore, our results also showed that none of the compounds administered altered escape responses or locomotor activity measurements.

The anxiogenic effects of intra-MeA administration of CRF agree with data obtained from other amygdala nuclei. It was previously shown that CRF administration into the basolateral amygdala exerts anxiogenic-like effects (Sadiky et al., 1999), as measured by the social interaction test. Using this same anxiety test, it was also demonstrated that the administration of urocortin 1, which has a high affinity for both CRF\(_{\text{R1}}\) and 2, into the basolateral amygdala induced anxiety-like behavior and c-Fos expression in brainstem serotonergic neurons (Spiga et al., 2006). Furthermore, nonanxiety-inducing doses of urocortin 1 infused locally into the basolateral amygdala of rats for a period of 5 days induced anxiety-like responses in the social interaction test and elevated plus-maze, which were accompanied by hyperexcitability of the basolateral network apparently related to NMDA receptor and calcium-calmodulin-dependent protein kinase II activation (Rannnie et al., 2004). These animals also showed a physiological sensitivity to intravenous sodium lactate infusions, which is present in patients with panic or post-traumatic stress disorders, but not social or generalized anxiety disorders. Thus, according to the authors (Rannnie et al., 2004), these observations implicate basolateral amygdala CRF receptors in the pathophysiology of panic disorder.

Anxiogenic effects of intra-amygdala CRF administration were also found in the central amygdaloid nucleus (Wiersma et al., 1997) and in the bed nucleus of the stria terminalis (BST) (Lee et al., 2008), which is part of the so-called extended amygdala. Interestingly, in this last region, repeated intra-BSTN injections of a sub-anxiogenic dose of urocortin 1 elicited persistent anxiety-like responses in the social interaction test, but not in the elevated plus-maze. Prior local injection of astressin, a potent CRF\(_{\text{R1}}\) antagonist, into the BSTN blocked this effect. Additionally, unlike what was observed by the same research group for the basolateral amygdala (Rannnie et al., 2004), animals showed no cardiovascular changes following lactate infusion. These observations led to the conclusion that the CRF system of the BSTN is involved with the pathophysiologic of specific subtypes of anxiety disorders, which are not lactate sensitive.

While intra-MeA CRF was anxiogenic, facilitating ETM avoidance, the CRF\(_{\text{R1}}\) antagonist antalarmin decreased inhibitory avoidance latencies, an anxiolytic-like effect (experiment 2). Again, these results corroborate data obtained from other amygdala nuclei. Thus, it has been previously shown that the injection of a CRF\(_{\text{R1}}\) antagonist or of CRF\(_{\text{R1}}\) antisense nucleotides into the central amygdala decreases social defeat behavior and contextual freezing (Heimrichs et al., 1992; Liebsch et al., 1995) in ethanol-dependent (Rassnick et al., 1993) or opiate-dependent (Heimrichs et al., 1995) rats and in rats previously exposed to foot-shock (Pitts et al., 2009; Swiergiel et al., 1993). In a similar way, the administration of 30 ng of DMP96, a selective CRF\(_{\text{R1}}\) antagonist, into the basolateral amygdala was able to reduce contextual freezing responses (Hubbard et al., 2007).

Antalarmin also counteracted the anxiogenic effect induced by CRF administration in avoidance 1 (experiment 3), suggesting that this effect was in fact mediated by CRF\(_{\text{R1}}\) activation. It is important to point out, however, that in this same experiment CRF did not show an anxiogenic action in avoidance 2. This most probably happened because of a ceiling effect, since in this trial both the vehicle/vehicle and the vehicle/CRF groups had latencies very close to the cutoff time imposed by the test (300 s). On the other hand, avoidance 2 latencies of the antalarmin/vehicle group were different from the ones shown by the other 3 groups. This last observation raises two possibilities: 1) the antilarmin-treated group did not show an ideal avoidance learning (this is made clear when we compare avoidance 2 latencies in experiments 2 and 3); and, 2) the antalarmin/CRF group was different from the antalarmin/vehicle group due to the activation of type 2 receptors by CRF. Regarding this second possibility, it has been previously suggested that CRF\(_{\text{R1}}\) and CRF\(_{\text{R2}}\) play complementary roles: while CRF\(_{\text{R1}}\) are involved with the hormonal and behavioral adaptation to stress, the CRF\(_{\text{R2}}\) system is important for the reestablishment of energy homeostasis in response to metabolic alterations and stressors (Chen et al., 2012). Nevertheless, the role played by MeA CRF\(_{\text{R2}}\) receptors still warrants investigation.

Although CRF and antalarmin altered ETM avoidance, escape responses, which in clinical terms have been related to panic disorder, were not changed by the administration of either one of the compounds. These results corroborate previous observations performed by our research group (Diniz et al., 2013). In this particular study, chronic treatment with corticosterone facilitated ETM avoidance responses, at the same time increasing Fos-immunoreactivity, in the MeA paraventricular nucleus of the hypothalamus and in the lateral septal nucleus. Furthermore, in two previously performed studies (Forestiero et al., 2006; Kemble et al., 1984) the relationship between MeA and escape was not demonstrated. In one of these studies, MeA lesions in both wild R. norvegicus and R. rattus reduced defensiveness to nonpainful stimuli, but had no effect on escape/light behavior (Kemble et al., 1984). In another, administration into the MeA of NO synthase inhibitors or of AMPA/kainate and NMDA receptors antagonists caused anxiolytic effects in different animal models used for the screening of generalized anxiety-modulating drugs (Forestiero et al., 2006), i.e. the light–dark transition test and the fear-potentiated startle.

On the other hand, the absence of involvement of the MeA with ETM escape responses contrasts with a previous study performed by our research group (Herdade et al., 2006). In this particular study, it was shown that intra-MeA injection of the GABA\(_{\alpha}\) agonist muscimol significantly impaired ETM escape, not altering, however, ETM avoidance.

It is important to mention that in humans, investigations of CRF dysregulation, quantified in cerebral spinal fluid collected through lumbar puncture, showed no significant differences among controls, generalized anxiety and panic disorder patients (for a review see Risbrough and Stein, 2006). Nevertheless, lumbar puncture is a stressful technique, which can increase CRF release in all subjects. Thus, it is possible that the technique masked relevant differences between anxiety patients and controls.

HPA axis abnormalities have also been used as an indirect marker of CRF dysregulation. In this respect, clinical evidence indicates that although increases in HPA function accompany generalized anxiety, panic disorder patients show significantly lower ACTH and cortisol responses to CRF administration (Roy-Byrne et al., 1986). Also, different panicogenic stimuli (for instance, CO\(_2\) or sodium lactate) can trigger a panic attack without significantly increasing cortisol release in panic disorder patients (Terleph et al., 2006; Van Duijn et al., 2007). Thus, it has been proposed (Coplan et al., 1995; Graeff et al., 2005; Klein, 1993) that apart from the differences in symptomatology and pharmacological response, generalized anxiety and panic disorder also differently affect...
stress hormones. Taking into account these last observations, it is possible to conceive that the CRF-system of the MeA is preferentially involved with the neurobiology of generalized anxiety disorder, as presently suggested. The lack of an anatomical control (i.e., animals with microinjection sites outside the MeA), however, limits this assumption. Furthermore, how CRF receptors of other amygdala nuclei modulate anxiety and panic-related responses needs to be better investigated through the use of more selective animal models. In this regard, it is also important to point out that previous observations (Lee et al., 2008; Rainnie et al., 2004) performed with the administration of CRF or CRF-related compounds into the amygdala complex used animal models of anxiety (i.e., the social interaction and the elevated plus-maze) that do not specifically model generalized anxiety or panic disorder (Graeff and Zangrossi, 2002). In conclusion, the results reported herein provide, for the first time, direct evidence for the involvement of CRFR1 receptors of the MeA in the modulation of a defensive response that has been associated with generalized anxiety disorder and might be of relevance to the better understanding of the neural mechanisms underlying this pathological condition.

Abbreviations

CRF corticotropin-releasing factor
CRF1 type 1 receptor
CRF2 type 2 receptor
MeA medial amygdala
ETM elevated T-maze
IP intraperitoneal
IM intramuscular
KPBS potassium phosphate-buffered saline
ANOVA analysis of variance

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