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CRF receptor type 1 (but not type 2) located within the amygdala plays a role in the modulation of anxiety in mice exposed to the elevated plus maze

Ana Cláudia Cipriano [a,b], Karina Santos Gomes [a], Ricardo Luiz Nunes-de-Souza [a,b,*]

[a] School of Pharmaceutical Sciences, Univ. Estadual Paulista — UNESP, 14800-903, Araraquara, SP, Brazil
[b] Joint Graduate Program in Physiological Sciences, UFSCar/UNESP, São Carlos, SP 13565-905, Brazil

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ABSTRACT

The amygdala (Amy) is an important center that processes threatening stimuli. Among the neurotransmitters implicated in the control of emotional states, the corticotrophin releasing factor (CRF) is an important modulator, acting at CRF_1 and CRF_2 receptors. Few studies have investigated the role of CRF and its receptors in the Amy on anxiety in mice. Here, we investigated the effects of intra-Amy (aimed at the basolateral nucleus) injections of CRF (37.5 and 75 pmol/0.1 μl), urocortin 3 (UCn_3, a selective CRF_2 agonist; 4, 8, 16 or 24 pmol/0.1 μl), CP376395 (a selective CRF_1 antagonist; 0.375, 0.75 or 1.5 nmol/0.1 μl), antisauvagine-30 (ASV-30, a selective CRF_2 antagonist; 1 or 3 nmol/0.1 μl) on the behavior of mice exposed to the elevated plus maze (EPM). Both spatiotemporal (e.g., percentage of open-arm entries and percentage of open-arm time; %OE and %OT) and complementary [e.g., frequency of protected and unprotected stretched attend postures (pSAP and uSAP) and head dips (pHD and uHD); frequency and time spent on open arm end exploration (OAEE)] measures were recorded during a 5-min test in the EPM. While intra-Amy injections of CRF decreased %OE, %OT and OAEE, suggesting an anxiogenic-like action, UCn_3 (all doses) did not change any behavior. In contrast, injections of CP376395 (0.75 nmol) produced an anxiolytic-like effect, by increasing %OT and OAEE and decreasing pSAP and pHD. Neither spatiotemporal nor complementary measures were changed by intra-Amy ASV-30. These results suggest that CRF plays a marked anxiogenic role at CRF_1 receptors in the amygdala of mice exposed to the EPM.

Introduction

The amygdala (Amy) is a crucial structure of the central nervous system that processes aversive stimuli, integrating information coming from sensory areas, autonomic and memory systems and projecting dense and reciprocally to cortical and subcortical regions (LeDoux, 2000; Phelps and LeDoux, 2005; Sah et al., 2003; Zald, 2003). For instance, the basolateral nucleus of the Amy (BLA) projects to cortical regions, thalamus, hypothalamic nuclei, hippocampus, basal forebrain and brainstem (for a extensive review, see Knapska et al., 2007). It has been proposed two neural pathways involved in the modulation of defensive reactions within the amygdaloid complex of rats. The first, more related to fear responses, would consist of the central (CeA), medial (MeA) and BLA nuclei. Activation of these nuclei would originate fast responses to specific threats. The second pathway, which would be more related to the processing of emotional states of anxiety, would consist of projections from BLA to bed nucleus of the stria terminalis (BNST), and its activation would originate sustained responses to potential threats (Davis, 2006).

There are several neurotransmitter systems [e.g., serotonergic, gama-aminobutyric acid (GABAergic), glutamatergic, noradrenergic, acetylcholinergic, and corticotrophin release factor (CRFergic) systems] within the Amy that play a role in the modulation of emotional responses (Shekhar et al., 2005). Besides its well-known role in the control of the neuroendrocrine system regulating the functioning of the hypothalamo-pituitary-adrenal axis (Whitnall, 1993), this 41 amino acid peptide is also found within other brain areas [e.g., periaqueductal gray matter (PAG), hippocampus, BNST, prefrontal cortex (PFC)] where it modulates emotional states via CRF_1 and CRF_2 receptors (Blank et al., 2003; De Souza et al., 1985; Keck et al., 2001; Miguel and Nunes-de-Souza, 2011; Miguel et al., 2014; Ohata and Shibasaki, 2011; Sahuque et al., 2006).

CRF_1 receptor mRNA can be found in various parts of the amygdaloid complex (medial nucleus, cortical nucleus, nucleus of the lateral olfactory tract, anterior amygdaloid area, lateral nucleus, basolateral nucleus, basomedial nucleus and intercalated nuclei). Few isolated positively labeled cells for CRF1 receptor mRNA are present on the central nucleus (Kuhne et al., 2012; Justice et al., 2008; Van Pett et al., 2000; Chen et al., 2000). On the other hand, CRF_2 receptor mRNA displays a more

* Corresponding author at: Laboratório de Neuropsicofarmacologia, FCFAr-UNESP, Rodovia Araraquara Jaú, Km 01 - s/n, Campos Ville - Araraquara/SP, 14.800-903, Brasil.
E-mail addresses: anac_cipri@yahoo.com.br (A.C. Cipriano), karinasg@gmail.com (K.S. Gomes), souzarn@fcfar.unesp.br (R.L. Nunes-de-Souza).

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confined expression, particularly in the cortical, medial and basomedial nucleus (although also present in the lateral and basolateral nucleus — Chalmers et al., 1995; Van Pett et al., 2000). Besides CRF, the CRF-related peptides urocortins (UCn 1, 2 and 3) also bind to CRF_1 and CRF_2 receptors in mammalian. Briefly, while CRF and UCn_1 display similar high affinity to CRF_1, both urocortin 2 and 3 show higher affinity for CRF_2 than for CRF_1 (Hillhouse and Grammatopoulos, 2006).

In the Amy, CRF is released when rats are exposed to acute or chronic stressors (Merali et al., 1998; Merlo-Pich et al., 1992, 1995). Moreover, activation of the amygdala CRF receptors has been postulated as a possible neurochemical substrate responsible for the changes (e.g., long-term synaptic plasticity and increased excitability of BLA neurons) that occur in behavioral disorders induced by stress (Shekhar et al., 2005).

While there is a large body of evidence emphasizing the role of CRF_1 receptor subtype in the modulation of fear and anxiety (e.g., Miguel and Nunes-de-Souza, 2011; Miguel et al., 2014; Smith et al., 1998; Spina et al., 2000; Timpl et al., 1998), contrasting results regarding the role of CRF_2 receptor in the modulation of anxiety have been reported, with findings showing anxiolytic- and anxiogenic-like effects or even lack of effects (e.g., Bale et al., 2000; Kishimoto et al., 2000; Takahashi et al., 2001). Although in most studies CRF receptor agonists and antagonists have been injected systemically or intracerebroventricularly, rendering it difficult to identify where in the central nervous system CRF modulates anxiety and fear, some brain areas are potential candidates to play such function. For instance, the midbrain PAG, medial hypothalamus, amygdala and the medial PFC (mPFC) have been suggested to be part of the brain defensive system (for a review, see Gray and McNaughton, 2000; McNaughton and Corr, 2004). In this context, intra-PAG injection of CRF or CRF agonists produced anxiogenic-like effects in rats (Borelli and Brandão, 2008) and mice (Miguel and Nunes-de-Souza, 2011) exposed to the elevated plus-maze (EPM), a widely used animal test of anxiety (review, see Carobrez and Bertoglio, 2005). In mice, intra-PAG injection of CRF_1 antagonist selectively and completely blocked the anxiogenic effects of CRF (Miguel and Nunes-de-Souza, 2011). Interestingly, neither non-selective CRF antagonists nor selective CRF_1 antagonist per sechanged anxiety indices, respectively, in rats (Martins et al., 2000) and mice (Miguel and Nunes-de-Souza, 2011) when injected into the PAG. Recently, Pentkowski et al. (2013) observed that intra-mPFC injection of cortagine, a highly selective CRF_1 agonist (Tezval et al., 2004), attenuated (instead of enhancing) defensive response in mice exposed to a predator. Moreover, these authors found that intra-mPFC cortagine also reduced Fos protein staining in specific amygdalar nuclei, suggesting that the anxiolytic-like effects following mPFC CRF_1 activation are likely via amygdala inhibition. However, few studies have investigated the effects of the neuropeptide CRF or CRF_1 and CRF_2 receptor agonists/antagonists injected directly into the Amy on anxiety-related behaviors in rats (e.g., Abiri et al., 2014; Heinrichs et al., 1992; Iemolo et al., 2013; Isogawa et al., 2013; Roozendaal et al., 2008). Moreover, as far as we know, there are no studies in the recent literature that investigated the role of CRF or selective CRF_1 and CRF_2 agonists or antagonists injected within the Amy of mice exposed exclusively to an animal model of anxiety. In this study we investigated, for the first time, the effects of intra-amygdala (aimed at the BLA nucleus) injections of CRF, UCn_3 (a CRF_2 agonist), CRF_1 (CP376395) and CRF_2 (antisauvagine-30) antagonists on behavior of mice exposed to the elevated plus-maze.

Material and methods

Animals

Subjects were male adult Swiss mice (Univ. Estadual Paulista - UNESP, SP, Brazil), weighing 25–35 g at testing. They were housed in groups of 10 per cage (41 cm × 34 cm × 16 cm) and maintained under a 12-h light cycle (lights on 07:00 a.m.) in a temperature-controlled environment (23 ± 2 °C). Food and water were freely available except during the test periods. All mice were experimentally naive.

Drugs

Drugs used were: corticotropin-releasing factor (CRF: 37.5 and 75 pmol; Sigma-Aldrich, Brazil); CP 376395 [*N*-(1-ethylpropyl)-3.6-dimethyl-2-(2,4,6-trimethylphenoxy)-4-pyridinamine hydrochloride], a selective CRF_1 antagonist (0.375, 0.75 and 1.5 nmol; Tocris Cookson Inc., Ballwin, USA); antisauvagine-30 (ASV-30), a selective CRF_2 antagonist (1 and 3 nmol; Tocris Cookson Inc., Ballwin, USA); urocortin 3 (UCn_3), a selective CRF_2 agonist (4, 8, 16 and 24 pmol; Sigma-Aldrich, Brazil). The doses used were based on previous studies (Blacktop et al., 2011; Funk and Koob, 2007; Miguel and Nunes-de-Souza, 2011; Miguel et al., 2012, 2014; Myers and Greenwood-Van Meerveld, 2010; Telegdy and Adamik, 2013; Valdez et al., 2003). All drugs were diluted in physiological saline (0.9% NaCl), which was injected in the control group.

Surgery and microinjection

The surgery was similar to described by Cornélio and Nunes-de-Souza (2007). Stainless steel guide cannulae (7 mm long, 26-gauge; Insight Equipamentos Científicos Ltd., Brazil) were bilaterally implanted into the Amy in mice anesthetized by intraperitoneal injection of ketamine (80 mg/kg) plus xylasin (8 mg/kg). The guide cannulae were fixed to the skull with dental acrylic and jeweler's screws. Stereotaxic coordinates (Paxinos and Franklin, 2001) for the amygdala were, respectively, 1.1 mm posterior to bregma, ±3.1 mm lateral to the midline, and 3.7 mm ventral to the skull surface, aiming at the basolateral nucleus. A dummy cannula (33-gauge stainless steel wire; Fishtex Industry and Commerce of Plastics Ltd.), inserted into each guide cannula immediately after surgery, served to reduce the incidence of occlusion.

At the end of surgery, mice received an intramuscular injection of penicillin-G benzathine (Pentabiotic, 56.7 mg/kg in a 0.1 ml volume) and a subcutaneous injection of the anti-inflammatory analgesic Banamine (3.5 mg/kg flunixin meglumine in a volume of 0.3 ml).

Four to six days after surgery, various solutions (see item 2.2) were bilaterally injected into the Amy through microinjection units (33-gauge stainless steel cannula; Insight Equipamentos Científicos Ltd., Brazil), which extended 1.0 mm beyond the tip of the guide cannula. Each microinjection unit was attached to a 2-μl Hamilton microsyringe via polyethylene tubing (PE-10). The microinjection procedure consisted of gently restraining the animal, removing the dummy cannula, inserting the injection unit in situ and proceeding with the microinjection, after which the needle was left for a further 30 s. The drug/vehicle volume delivered was 0.1 μl. The successful procedure was verified by monitoring the movement of a small air bubble in the PE-10 tubing.

Elevated plus maze (EPM) and behavioral analysis

The EPM design was very similar to that described by Lister (1987) and comprised two open arms (30 × 5 × 0.25 cm) and two closed arms (30 × 5 × 15 cm) connected via a common central platform (5 × 5 cm). The apparatus was constructed from wood (floor) and transparent glass (clear walls) and was raised to a height of 38.5 cm above floor level.

Ten minutes after each drug or saline (control group) injection into the Amy, each mouse was placed on the central platform of the maze (facing an open arm). The test sessions were 5 min in duration and, between subjects, the maze was thoroughly cleaned with 20% alcohol. All experiments were performed under normal laboratory illumination (50 lx on the EPM floor), during the light phase of the light–dark cycle.

The scorer was unaware of the experimental design and treatment for each animal. Behavioral parameters were scored using an

ethological analysis package developed by Dr. Morato's group at Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo (personal communication), and comprised both conventional spatiotemporal and ethological measures (Rodgers and Johnson, 1995). Conventional measures were the frequencies of closed-arm entries (arm entry = all four paws into an arm), percentage of open-arm entries [(open/total) × 100)], and percentage of open-arm time [e.g., (time open/300) × 100]. Ethological measures comprised frequency and time scores for open arm end exploration (OAEE: entering the open arm 10-cm distal section from the central square) as well as frequency scores for head dipping (HD: exploratory movement of head/shoulders over the side of the maze) and stretched-attend postures (SAP: exploratory posture in which the body stretched forward then retracted to the original position without any forward locomotion). In view of importance of thigmotactic cues to patterns of plus-maze exploration (Treit et al., 1993) head dipping and SAP were further differentiated as a function of whereabouts on the maze they were displayed. Consistent with earlier reports (Rodgers and Johnson, 1995) the closed arms and the central platform were together designated "protected" areas (i.e., offering relative security), while the open arms were designated "unprotected" areas. Data for the HD and SAP measures are reported both as protected and unprotected scores. Interpretation of the results of complementary measures was based on the natural aversion of rodents to open and elevated places (e.g., Carobrez and Bertoglio, 2005; Lister, 1987; Pellow et al., 1985). For instance, it is expected that anxiogenic-like drugs decrease the frequency of SAP and HD displayed on unprotected areas of the EPM, while anxiolytic-like compounds produce the opposite effects on these parameters (e.g., Carobrez and Bertoglio, 2005).

Experimental Procedures

Experiments 1–3: Intra-amygdala injections of CRF, UCn_3, CP 376395 or ASV-30 in mice exposed to the EPM

Animals received intra-amygdala injection of saline (control; $n = 7$) or CRF 37.5 pmol ($n = 6$) or 75 pmol ($n = 9$) in 0.1 μl (Experiment 1); saline (control; $n = 14$) or UCn_3 4 pmol ($n = 10$), 8 pmol ($n = 12$), 16 pmol ($n = 14$) or 24 pmol ($n = 6$) in 0.1 μl; (Experiment 2); saline (control; $n = 7$), CP 0.375 nmol ($n = 8$), 0.75 nmol ($n = 9$) or 1.5 nmol ($n = 5$) or ASV-30 1 nmol ($n = 6$) or 3 nmol ($n = 5$) in 0.1 μl (Experiment 3) and 10 min later were individually placed in the EPM to record the behavioral parameters described on section *Elevated plus maze (EPM) and behavioral analysis.*

Histological analysis

At the end of testing, all animals received bilateral injection of 0.1 μl of 1% Evans blue into the amygdala by the same microinjection procedure as for the drugs. Animals were then sacrificed by anesthetic overdose, their brains removed and injection sites checked histologically by reference to the atlas of Paxinos and Franklin (2001). Data from animals with injection sites outside the amygdala were excluded from the study.

Statistical analysis

All results were initially submitted to Levene's test for homogeneity of variance. Where Levene's test yielded significance, results were log, square root or cube root-transformed and confirmed for homogeneity of variance before being submitted to one- way analyses of variance (ANOVA) followed, where significant, by Duncan test. Eta-squared ($\eta 2$) effect size for ANOVA was calculated according to the method described by Cohen (1973). In all cases, a P value ≤0.05 was considered significant.

Ethics

The experiments carried out in this study comply with the norms of the Brazilian Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (DBCA) and approved by the local Research Ethics Committee (CEUA/FCF/CAr: protocol number 19/2011).

Results

Histology confirmed that the majority of the microinfusion sites was in the basolateral (lateral + basal nuclei) nucleus (Experiment 1: 82%; Experiment 2: 77%; Experiment 3: 74%) (see Fig. 1). The other sites hit comprised the central amygdala (Experiment 1: 18%; Experiment 2: 23%; Experiment 3: 26%). Behavioral results are summarized in Figs. 2–4.

Experiment 1: Effects of intra-amygdala injections of saline or CRF on behavior of mice exposed to the EPM

Fig. 2 illustrates the effects of intra-amygdala injections of saline or CRF (35.5 or 75 pmol) on (A) anxiety indices and (B) frequency of closed arm entries recorded over a 5-min period in the EPM. One-way ANOVA followed by Duncan's test revealed that both doses of CRF decreased the percentage of open-arm entries ($F2,19 = 5.32$; $\eta_2 = 0.36$; $p < 0.05$) and percentage of open-arm time ($F2,19 = 6.19$; $\eta_2 = 0.39$; $p < 0.05$). Furthermore, analysis revealed that the CRF 75 pmol increased the closed-arm entries ($F2,19 = 3.81$; $\eta_2 l = 0.29$; $p < 0.05$).

Regarding the effects of CRF on ethological measures, one-way ANOVA followed by Duncan's test revealed that this neuropeptide decreased the frequency ($F2,19 = 8.78$; $\eta_2 = 0.48$;$p < 0.05$) and time ($F2,19 = 6.78$; $\eta_2 = 0.42$;$p < 0.05$) of open arm end exploration (Fig. 2D). One-way also revealed that CRF tended to change pSAP ($F2,19 = 2.68$; $\eta_2 = 0.22$;$p = 0.09$) and post-hoc analysis revealed that CRF (75 pmol) increased this measure ($p = 0.05$). Intra-amygdala injections of CRF did not significantly change any other behavior of mice exposed to the EPM ($F2,19 \leq 0.40$; $\eta_2 \leq 0.16$; $p \geq 0.17$).

Experiment 2: Lack of effects of UCn_3 injections into the Amy on behavior of mice exposed to the EPM

Fig. 3 shows the lack of effects of intra-Amy infusions of saline or UCn_3 (4, 8, 16 or 24 pmol) on anxiety indices and locomotor activity as well as on complementary measures in mice exposed to the EPM. UCn_3 did not change any behavior in mice in the EPM ($F4,51 \leq 2.39$; $\eta_2 \leq 0.16$; $p > 0.05$).

Experiment 3: Effects of intra-Amy injections of CP 376395 or ASV-30 on behavior of mice exposed to the EPM

Fig. 4 shows the effects of intra-Amy infusions of saline, CP376395 (0.375, 0.75 or 1.5 nmol) or ASV-30 (1 or 3 nmol) on (A) percentage of open arm entries and percentage of open arm time (anxiety indices) and (B) frequency of closed arm entries (locomotor activity) in mice exposed to the EPM. One-way ANOVA followed by Duncan's test revealed that intra-Amy CP376395 increased the percentage of open-arm time ($F5,34 = 3.15$; $\eta_2 = 0.32$; $p = 0.02$), without changing percentage of open-arm entries ($F5,34 = 1.31$; $\eta_2 = 0.16$; $p = 0.28$). This antianxiety-like effect of CP376395 was statistically confirmed with the dose of 0.75 nmol. At the dose of 1.5 nmol, CP376396 tended to increase %OT ($p = 0.087$). Importantly, intra-Amy injections of ASV-30 did not modify the anxiety indices in mice exposed to the EPM. In addition, neither CP 376395 nor ASV-30 changed closed arm entries ($F5,34 = 1.66$; $\eta_2 = 0.20$; $p > 0.05$).

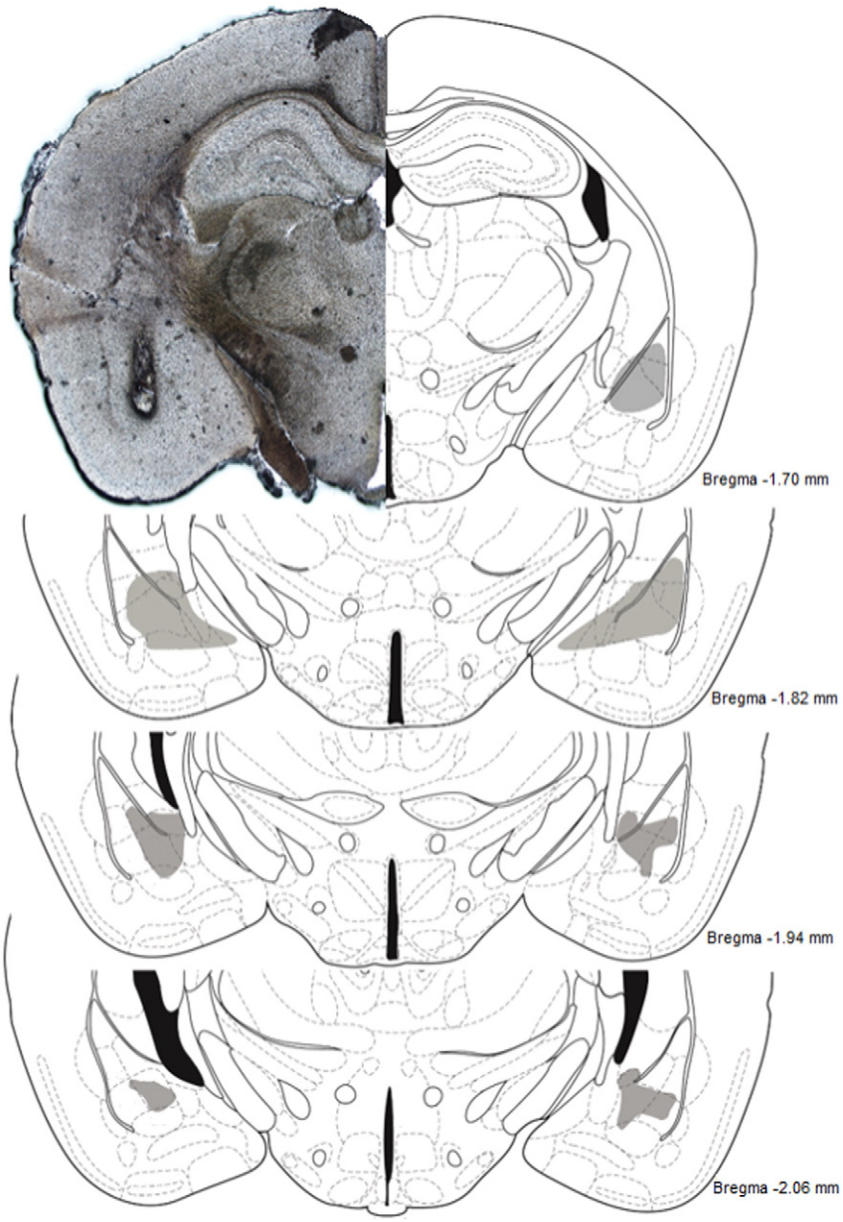

Fig. 1. A photomicrograph from a representative subject showing the injection site into the amygdala (top left) and a sketch (top right and down) of microinfusion sites within the amygdala of the mouse. Gray area is corresponding to whole area where various microinjections were placed at different slices (distance from bregma in mm) described in the Paxinos and Franklin (2001).

The effects of intra-Amy infusions of CP376395 or ASV-30 on ethological measures in mice exposed to the EPM are shown in Fig. 4C and D. One-way ANOVA followed by Duncan's test revealed that CP 376395 (0.75 nmol) reduced the protected SAP ($F_{5,34} = 3.25$; $\eta_2 = 0.32$; $p < 0.05$) and protected HD ($F_{5,34} = 5.08$; $\eta_2 = 0.43$; $p < 0.05$) as well as increased the time of open arm end exploration ($F_{5,34} = 3.39$, $\eta_2 = 0.33$; $p < 0.05$). Duncan's test did not show any significant effect of intra-Amy ASV-30 on ethological measures.

Discussion

This study demonstrated that intra-Amy injections of CRF (a preferential CRF_1 receptor agonist) enhanced mouse aversion to the open arms of the EPM. Moreover, the selective blockade of CRF_1 receptors (with intra-Amy injection of CP 376395) produced opposite effects, i.e. anxiolysis. In contrast, neither activation nor blockade of CRF_2 receptors located in this forebrain structure changed anxiety-like behavior in mice exposed to the EPM.

Intra-Amy infusions of CRF (37.5 and 75 pmol) decreased both percentage of open arm entries and percentage of open arm time (Fig. 2), two main parameters used as anxiety indices in rodents exposed to the EPM (e.g., Carobrez and Bertoglio, 2005; Pellow et al., 1985; Rodgers and Johnson, 1995). Moreover, the dose of 75 pmol increased the closed arm entries, a widely used measure of general locomotor activity (e.g., Rodgers and Johnson, 1995), indicating that the decrease in open arm exploration was not due to an impairment in general locomotion produced by this neuropeptide. Actually, the increase in closed arm entries produced by CRF seems to corroborate its anxiogenic-like effect in mice exposed to the EPM. The anxiogenic-like profile observed with intra-amygdala injections of CRF was also observed through the complementary (ethological) measures, i.e., besides decreasing the spatio-temporal measures (%OE and %OT), CRF also increased pSAP (Fig. 2C) and reduced open arm end exploration (Fig. 2D), indicating that mice avoided the open arms. It would be expected a reduction of uSAP and

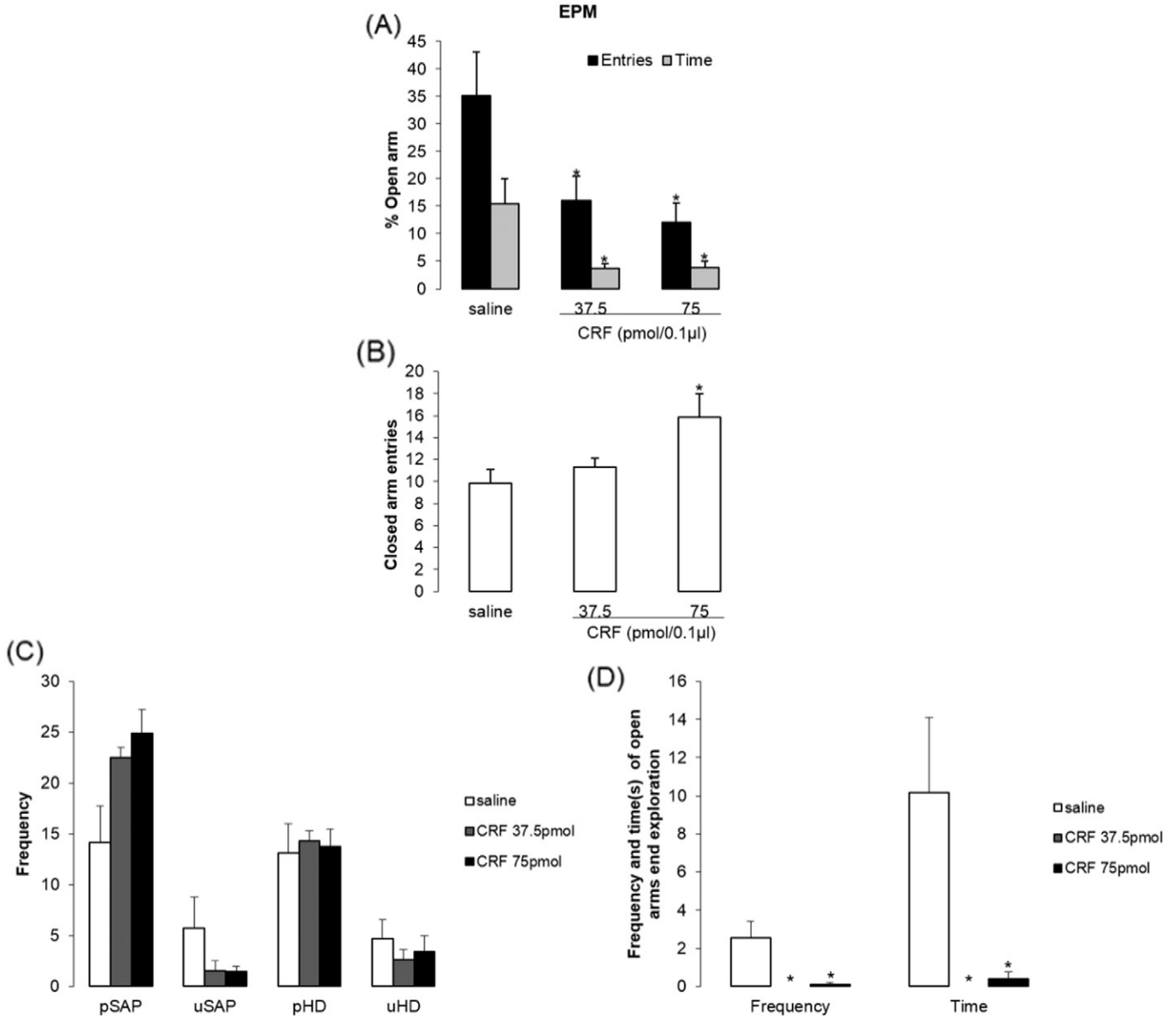

Fig. 2. Effects of intra-Amy injections of CRF (37.5 or 75 pmol) on anxiety indices (A: %OE and %OT), general activity (B: frequency of closed-arm entries) and complementary (ethological) measures [C (frequency of pSAP, uSAP, pHD and uHD) and D (frequency and time (in *sec*) of OAEE)] in mice exposed to the EPM. Bars represent means (±SEM). $N = 6–9$. $^{*}p \leq 0.05$ compared to saline group.

uHD as well as an increase in pHD following intra-Amy injection of CRF, confirming its anxiogenic-like profile (e.g., Espejo, 1997). We do not have a clear explanation for these unexpected results. Although CRF binds preferentially to CRF_1 receptors (Ki = 3.3 nmol), this neuropeptide can also activate CRF_2 receptors (Ki = 42 nmol — Reul and Holsboer, 2002). Given that the role of CRF_2 receptors on anxiety is still poorly understood, we cannot discard an eventual modulatory effect of CRF_2 receptors on anxiogenic-like effects of CRF. However, it must be emphasized that the main conventional measures of anxiety (%OE and %OT) were markedly changed by both doses of CRF in the present study.

The lack of studies showing consistent effects of CRF_2 agonists and antagonists on anxiety led us to investigate the effects of UCn_3 injected into the amygdala on anxiety-related behaviors assessed in the EPM. UCn_3 displays high affinity for CRF_2 receptor (Hillhouse and Grammatopoulos, 2006). It has been shown that intracerebroventricular (icv) injection of UCn_3 is innocuous upon the behaviors exhibited for mice in EPM (Bruchas et al., 2009; Venihaki et al., 2004). However, some studies showed an anxiolytic-like effect in the EPM when UCn_3 was injected icv in mice (Telegdy and Adamik, 2013) and rats (Valdez et al., 2003). Here, intra-Amy injections of UCn_3 did not change any conventional and complementary measure in mice exposed to the EPM, suggesting that CRF_2 receptors located within the mouse amygdala do not play a pivotal role on anxiety modulation. Corroborating these results, intra-Amy injection of ASV-30, a selective CRF_2 receptor antagonist (Ruhmann et al., 1998), did not modify any anxiety-like behavior of mice in the EPM. The lack of effects observed with CRF_2 agonist and antagonist injected into the amygdala on anxiety of mice exposed to the EPM suggests that this CRF receptor subtype does not play a crucial role to acute injection of CRF_2 ligands.

Although our study does not show a role of the amygdala CRF_2 receptors in the modulation of anxiety-related behaviors observed in the EPM, other studies involve the participation of these receptors in drug withdrawal-induced anxiety. In this sense, several evidences points to a role of amygdala CRF_2 receptors of rodents in the modulation of anxiety related to the withdrawal syndrome and the preference for

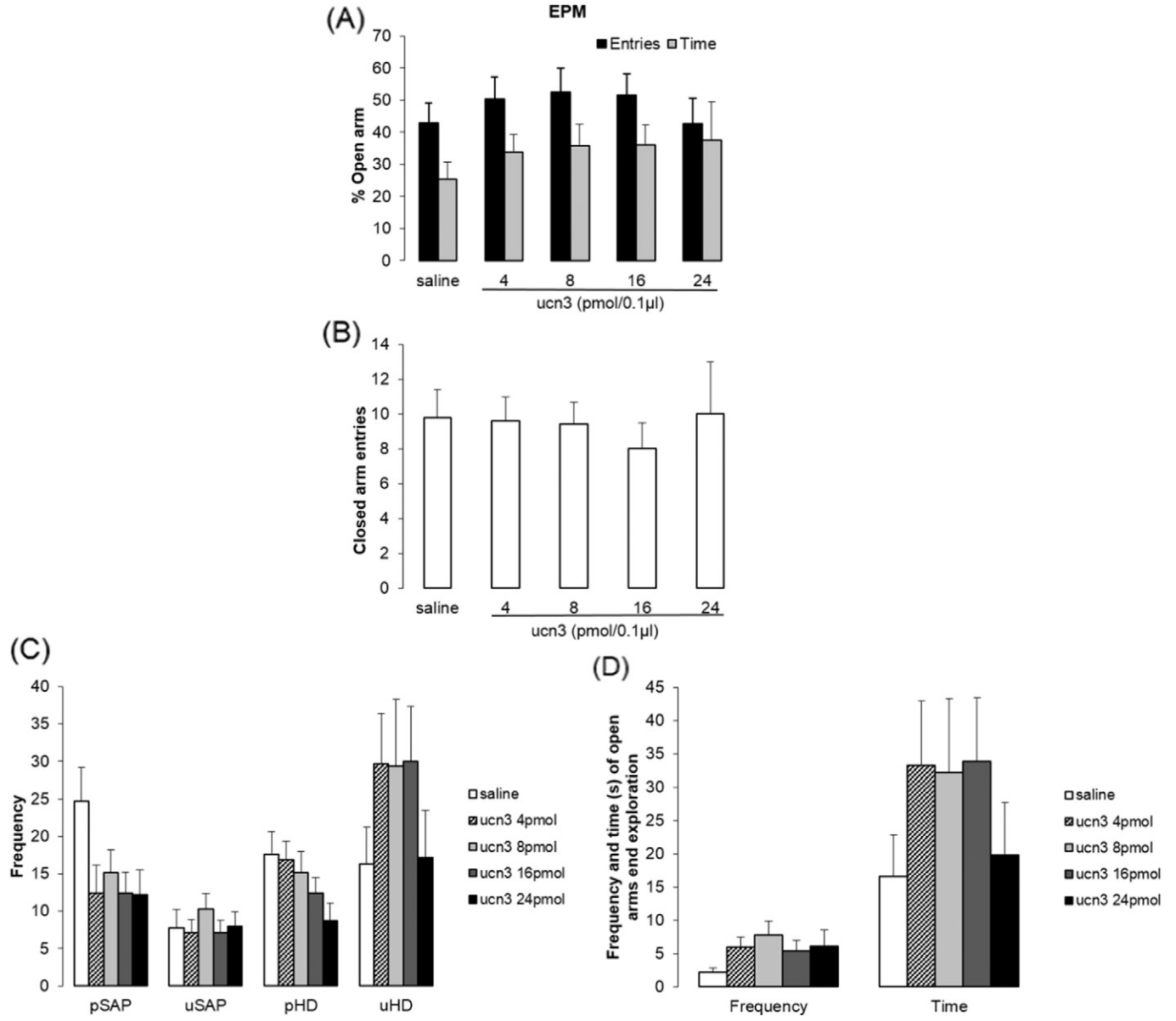

Fig. 3. Lack of effects of intra-Amy injections of UCn_3 (4, 8, 16 or 24 pmol) on anxiety indices (A: %OE and %OT), general activity (B: frequency of closed-arm entries) and complementary (ethological) measures [C (frequency of pSAP, uSAP, pHD and uHD) and D (frequency and time (in *sec*) of OAEE)] in mice exposed to the EPM. Bars represent means (± SEM). $N = 6–14$.

consumption. For example, Morisot et al. (2015) revealed that GABA biosynthesis within the amygdala of mice after opiate withdrawal requires functional CRF_2 receptors. Orozco-Cabal et al. (2008), using an electrophysiological approach, showed a synergism between D1-like dopamine receptors and CRF_2 receptors in BLA-to-medial prefrontal cortex synaptic transmission in rats subjected to repeated cocaine treatment. Sommer et al. (2008) found a decreased CRF_2 mRNA levels in the BLA of rats following ethanol withdrawal. Moreover, decreased CRF_2 receptor density was detected in the amygdala of alcohol-preferring compared to non-preferring rats. Given that the former are more anxyous than the latter, the CRF_2 receptors may provide an interesting target for the treatment of alcoholism related to anxiety (Yong et al., 2014).

Perhaps the most relevant result obtained in the current study was the intrinsic antianxiety effect provoked by the blockade of the CRF_1 receptors within the amygdala. Intra-Amy injections of CP376395 per se were able to change both spatiotemporal and ethological measures of anxiety in mice exposed to the EPM. This highly selective CRF_1 antagonist (Chen et al., 2008) attenuated the %OT, the frequencies of pSAP and pHD as well as the open arm end exploration when injected alone into the amygdala. Interestingly, intra-Amy CP376395 did not change closed arm entries, indicating that its anxiolytic-like profile does not depend on any effect on locomotion. This selective anxiolytic-like profile induced by the blockade of CRF_1 in the mouse amygdala suggests that CRF plays a marked role in the modulation of defensive behavior.

The selective anxiolytic-like effects and the lack of effects on anxiety behavior during maze exposure observed with intra-Amy injection of CRF_1 antagonist and CRF_2 agonist/antagonist, respectively, strongly suggest that the neuropeptide CRF most likely exerts a marked anxiogenic-like action at CRF_1 receptors within this limbic forebrain area in the mouse. Previous reports have demonstrated that intra-Amy CRF_1 antagonists attenuate defensive reactions (Bakshi et al., 2002; Ji et al., 2007; Risbrough et al., 2003; Robison et al., 2004); however, in those studies the animals were exposed to a stressful situation (e.g., footshock, pain, acoustic startle, social defeat) prior to being exposed to an anxiety test. Thus, as far as we know, the results shown here suggest, for the first time, that the CRF_1 (but not CRF_2) receptors located in the amygdala play a marked role in the modulation of anxiety in non previously stressed mice exposed to the EPM.

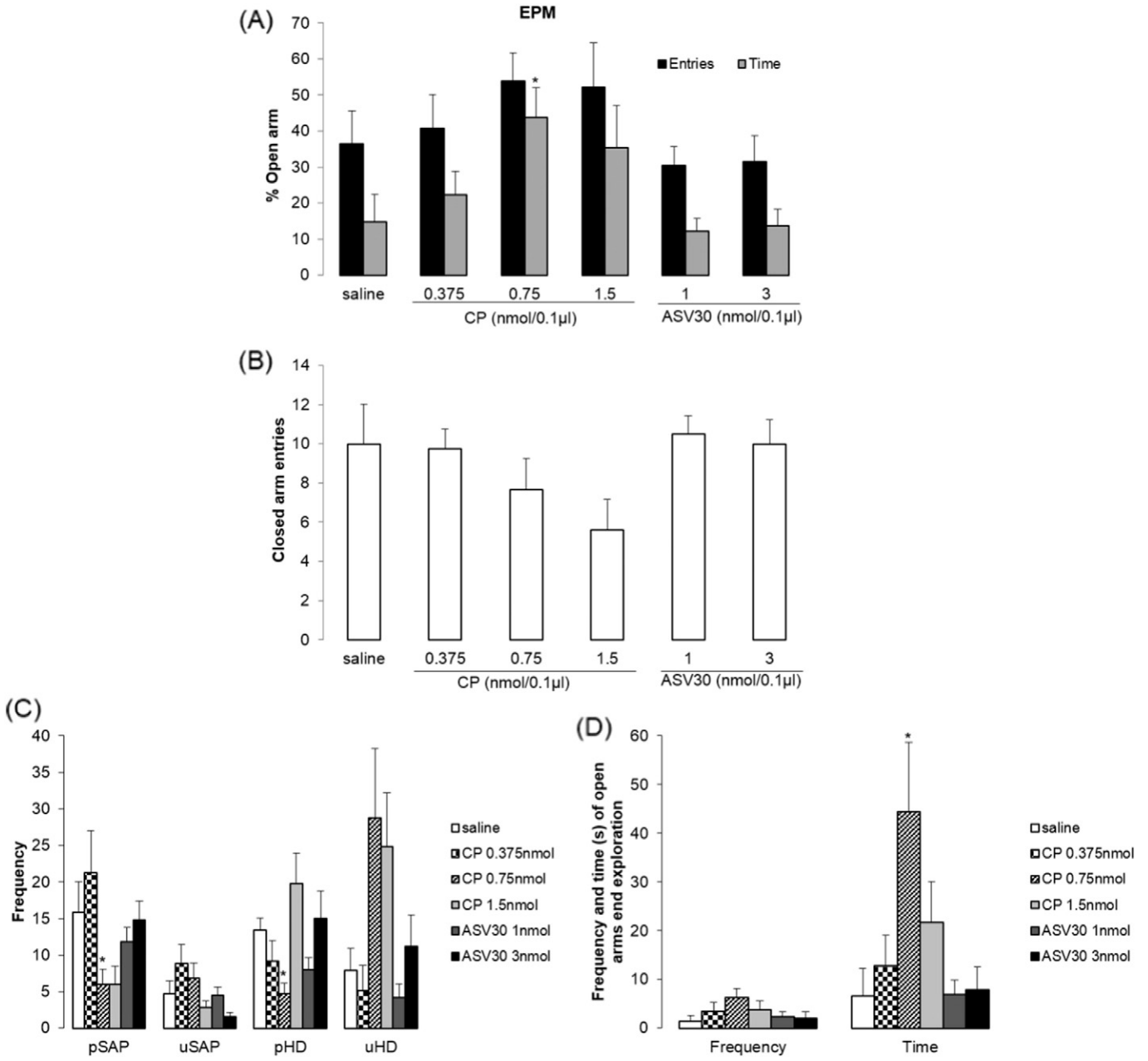

Fig. 4. Effects of intra-Amy injections of CP 376395 (0.375, 0.75 or 1.5 nmol) or antisauvagine-30 (ASV-30: 1 or 3 nmol) on anxiety indices (A: %OE and %OT), general activity (B: frequency of closed-arm entries) and complementary (ethological) measures [C (frequency of pSAP, uSAP, pHD and uHD) and D (frequency and time (in *sec*) of OAEE)] in mice exposed to the EPM. Bars represent means (± SEM). $N = 5–9$. $^{*}p \leq 0.05$ compared to saline group.

The intrinsic effects of CRF receptor antagonists on anxiety have not been observed when they were injected into other brain areas like the midbrain periaqueductal gray (Martins et al., 2000; Miguel and Nunes-de-Souza, 2011) or bed nucleus of the stria terminalis (Faria et al., 2016). However, when injected into the medial prefrontal cortex (mPFC), CP 376395 alone also attenuated anxiety in mice exposed to the EPM (Miguel et al., 2014). Thus, our results add important suggestive elements showing that the role of CRF_1 receptors in the modulation of anxiety seems to depend on the limbic structure where they are located. In other words, while the blockade of CRF_1 receptors in the PAG and BNST fails to alter anxiety-like behavior in mice exposed to the EPM, a clear anxiolytic-like profile has been observed when CRF_1 receptor antagonists are injected into the amygdala (BLA) and mPFC. Importantly, given that previous studies have shown that CRF system manipulations on CeA have not affected anxiety behavior (for a review, see Gafford and Ressler, 2015), the present results can be attributed by the majority of the infusions within the BLA. Moreover, Lee and Davis (1997) demonstrated that the anxiogenic-like effect of intracerebroventricular infusions of CRF was not affected by previous CeA lesions. These pharmacological data are in line with studies demonstrating that the BLA contains a high density of CRF1 receptors, while the CeA has many CRF expressing neurons but only a very low/irrelevant expression of CRF receptors (Kuhne et al., 2012; Justice et al., 2008; Van Pett et al., 2000; Chen et al., 2000). Recent studies using a combination of genetic, pharmacology, immunohistochemistry, in situ hybridization and electrophysiology have dissected the network of CRF1 positive receptor neurons within the amygdala. More specifically, although the BLA contains both spiny glutamatergic neurons and aspiny GABAergic interneurons, CRF activates CAMKII positive glutamatergic neurons via CRF1 receptors (Kuhne et al., 2012; Refojo et al., 2011; Rostkowski et al., 2013).

In conclusion, we suggest that CRF_1 located within the amygdala plays an important role in the modulation of anxiety in mice exposed to the EPM. Although previous results have shown inconsistent effects on anxiety with pharmacological manipulations of CRF_2 receptors, the results of our study are suggestive that acute injections of selective CRF_2 receptor agonist and antagonist into the amygdala do not modulate the defensive responses exhibited by mice in the EPM.

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