Effects of corticotropin-releasing factor receptor-1 (CRF1R) antagonists on the brain stress system responses to morphine withdrawal

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Abbreviations: CRF (corticotrophin-releasing factor); CRF receptor-1 subtype (CRF1R); HPA (hypothalamus-pituitary-adrenocortical); NA (noradrenaline); PVN (hypothalamic paraventricular nucleus); TH (tyrosine hydroxylase); NTS-A₂ (nucleus tractus solitarius).

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Abstract

The role of stress in drug addiction is well established. The negative affective states of withdrawal most probably involve recruitment of brain stress neurocircuitry (e.g. induction of hypothalamo-pituitary-adrenocortical –HPA- axis, noradrenergic and corticotrophin-releasing factor activity). The present study investigated the role of CRF receptor-1 subtype (CRF1R) on the response of brain stress system to morphine withdrawal. The effects of naloxone-precipitated morphine withdrawal on noradrenaline (NA) turnover in the paraventricular nucleus (PVN), HPA axis activity, signs of withdrawal and c-Fos expression were measured in rats pretreated with vehicle, CP-154,526 or antalarmin (selective CRF1R antagonists). Tyrosine hydroxylase (TH)-positive neurons expressing CRF1R were seen at the level of nucleus tractus solitarius (NTS)-A2 cell group both in control and in morphine-withdrawn rats. CP-154,526 and antalarmin attenuated the increase in body weight loss and irritability that were seen during naloxone-induced morphine withdrawal. Pretreatment with CRF1R antagonists resulted in no significant modification of the increased NA turnover at PVN, plasma corticosterone levels or c-Fos expression that were seen during naloxone-induced morphine withdrawal. However, blockade of CRF1R significantly reduced morphine withdrawal-induced increases in plasma ACTH levels. These results suggest that CRF1R subtype may be involved in the behavioral and somatic signs as well as in ACTH release (partially) during morphine withdrawal. However, CRF1R activation may not contribute to the functional interaction between NA and CRF systems in mediating morphine withdrawal-activation of brain stress neurocircuitry.
Corticotropin-releasing factor (CRF) was identified as a hypothalamic releasing factor (Vale et al., 1981) that stimulates the release of ACTH from pituitary, which releases glucocorticoids from adrenal glands. In addition, CRF is widely distributed throughout the brain and plays a major role in coordinating the behavioural and autonomic responses to stress (Owens and Nemeroff, 1991). Alterations in CRF system have been implicated in psychiatric illnesses that are precipitated by stress, such as depression and anxiety (Zorrilla and Koob, 2004). In addition, CRF is reported to contribute to the anxiogenesis and aversive symptoms of withdrawal from exposure to several drugs of abuse, including opiates (Koob, 2008). Two G protein-coupled receptors have been identified that bind CRF with high affinity: CRF receptor 1 (CRF1R) and CRF receptor 2 (CRF2R). CRF1R, involved in anxiety-related behaviour (Bale and Vale, 2004), expresses throughout the entire central nervous system, whereas CRF2R displays more restricted expression that CRF1R (Van Pett et al., 2000). Many lines of evidence indicate that central CRF system is involved in expression of morphine withdrawal signs. Thus, CRF1R antagonists have been shown to attenuate several behavioural signs of morphine withdrawal (Iredale et al., 2000; Lu et al., 2000).

The noradrenergic system and the hypothalamo-pituitary-adrenocortical (HPA) axis comprise two major adaptation mechanisms to stress. Like stressors, morphine withdrawal activates HPA axis in rats, which results in neuronal activation of stress-related neurosecretory neurons in the parvocellular division of the hypothalamic paraventricular nucleus (PVN), an increase in CRF transcription and boost of ACTH and corticosterone secretion (Cleck and
Blendy, 2008; Laorden et al., 2002b; Núñez et al., 2007a). Enhanced responsiveness of HPA axis after morphine withdrawal has been associated with activation of noradrenergic neurons in the nucleus of solitary tract (NTS-A2) that project to the hypothalamic PVN (Laorden et al., 2000b; Laorden et al., 2002b). Therefore, we have suggested that one of the neuronal mechanisms that underlie morphine withdrawal-induced activation of the HPA axis may be dependent on activation of noradrenergic pathways innervating the PVN (Núñez et al., 2007b). However, the possible involvement of CRF1R in the interaction between morphine withdrawal and the noradrenergic system innervating the PVN has not been well documented.

There is strong neurochemical and electrophysiological evidence suggesting an interaction between CRF and catecholaminergic systems. We have previously shown that there is an increase in hnRNA CRF expression which parallels an enhancement in phosphorylation in NTS (activation) and enzymatic activity of tyrosine hydroxylase (TH) in the PVN during morphine withdrawal (Núñez et al., 2007b; Núñez et al., 2007a). CRF-positive terminals have been detected in contact with TH immunoreactive neurons within the locus ceruleus (LC) (Sauvage and Steckler, 2001) and CRF has been demonstrated to increase the activity of noradrenergic neurons projecting to the prefrontal cortex (Lavicky and Dunn, 1993). In addition, it has been demonstrated the presence of CRF1R in the catecholaminergic neurons of the LC, ventral tegmental area (VTA) and NTS (Van Pett et al., 2000; Sauvage and Steckler, 2001). However, direct evidence for the effects of CRF antagonists on morphine withdrawal-stimulated stress axis and brain stress system has been not well documented.
Given the prominent role of NTS in morphine withdrawal responsiveness at PVN level, here we examined (i): whether rat catecholaminergic neurons within the NTS-A2 co-express CRF1R, and the effect of morphine withdrawal on CRF1R expression; (ii): the role of CRF1R in mediating somatic and behavioural states produced during withdrawal from morphine dependence; (iii) the response of noradrenergic pathways innervating the PVN and the activation of the HPA axis induced by morphine withdrawal in morphine-dependent rats pretreated with two selective CRF1R antagonists; and (iv) the effects of CRF1R blockade on activation of NTS, PVN and central amygdala (CeA) during morphine withdrawal, as reflected by c-Fos expression.
Materials and Methods

Animals. Male Sprague-Dawley rats (220–240 g; Harlan, Barcelona, Spain) were housed four-to-five per cage (length, 45 cm; width, 24 cm; height, 20 cm) in a room with controlled temperature (22±2°C) and humidity (50±10%), with free access to water and food. Animals were adapted to a standard 12-h light-dark cycle for 7 days before the beginning of the experiments. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the local Committees for animal research (REGA ES300305440012).

Drug treatment and experimental procedure. Rats were made dependent on morphine by subcutaneously (sc) implantation of two 75 mg morphine pellets (provided by the Ministerio de Sanidad, Madrid, España) under light ether anaesthesia. This procedure has been shown to produce consistent plasma morphine concentration beginning a few hours after the implantation of the pellets and a full withdrawal syndrome after acute injection of opioid antagonists (Frenois et al., 2002). Dependence on morphine is achieved 24 h after implantation of pellets and remained constant for 15 days (Gold et al., 1994). Control rats received placebo pellets containing the excipient without morphine. Six days after the implantation of morphine or placebo pellets, precipitated withdrawal was induced by sc injection of naloxone (1 mg/kg; in a volume of 1 ml/kg body weight) or saline (controls; 1 ml/kg) and then were observed for behavioural signs of withdrawal. The incidence of irritability was scored for 10 min at 5 min intervals. In addition, body weight loss was determined as the difference between the weight determined immediately before saline or
naloxone injection and a second determination made 60 min later. These signs are reliable markers of opiate withdrawal in morphine-dependent rats. The weight gain of the rats was checked during treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (Núñez et al., 2009).

In order to determine the effect of inhibiting CRF1R on the morphine withdrawal-induced activation of the axis and brain stress system, NA turnover in the PVN, c-Fos expression in NTS-A₂, central amygdala (CeA) and PVN (magno and parvocellular parts) and plasma corticosterone and ACTH concentrations were determined in morphine dependent and control rats treated with the selective CRF1 receptor antagonists CP-154,526 (20 or 30 mg/kg ip) or antalarmin (20 mg/kg ip) 30 min before the administration of naloxone or saline. Doses of CRF1R antagonists were selected based on existing literature and the anti-withdrawal effects of the antagonists that were observed in previous reports. CP-154,526 was dissolved in 10 % Tween-80 and given in a volume of 1 ml/kg body weight. Antalarmin was dissolved in Tween 80.

Sixty min after saline or naloxone injection, rats were sacrificed (between 11:00 and 12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, fresh-frozen, and stored immediately at -80°C until use for Western-blot analysis and NA turnover. A second set of animals from each treatment group was used for CRF1R/TH co-localization and for c-Fos immunohistochemistry. One set of each treatment group was randomly assigned for plasma ACTH and corticosterone determination.
Radioimmunoassay. Plasma levels of corticosterone and ACTH were measured by commercially available kits for rats (125I-CORT and 125I-hACTH RIA; MP Biomedicals, Orangeburg, NY). The sensitivity of the assay was 7.7 ng/ml for corticosterone and 5.7 pg/ml for ACTH.

Western blotting. Brainstem tissue corresponding to NTS-A2 cell group was dissected between the AP, rostrally, to the pyramidal decussation caudally (plane of sections relative to bregma: -13.68 to -14.60). Samples were placed in homogenization buffer (Núñez et al., 2007b) and equal quantities of total protein (40 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes, and Western analysis was performed with goat polyclonal anti-CRF1R antibody (against peptide mapping within an internal region of CRF1R; 1:200 dilution; sc-12381; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Quantification of immunoreactivity was carried out by densitometry. The integrated optical density of the bands was normalized to the background values. Antibody was stripped from the blots by incubation with stripping buffer (glycine 25mM and SDS 1%, pH 2), for 1 h at 37°C. Blots were subsequently reblocked and probed with 1:1000 anti-beta actin (rabbit polyclonal antibody, Cell Signalling #4967) as loading control. Protein levels were corrected for individual β-actin levels.

Immunohistochemistry. Animals were perfused transcardially and brains were processed for visualization of CRF1R and TH using previously published
techniques (Núñez et al., 2007b). Analysis of CRF1R expression in the NTS was made on free-floating sections (30 μm) at various levels, ranging rostrally from the AP, to the pyramidal decussation (plane of sections relative to bregma: -13.68 to -14.60; (Paxinos, 2007). Analysis of c-Fos immunoreactivity was made at NTS, CeA and PVN. Tissue sections were incubated in the following primary antibodies: goat polyclonal anti-CRF1R (1:200; Santa Cruz), 48 h at 4ºC; rabbit polyclonal anti-c-Fos (sc-52, 1:10000; Santa Cruz). Antigens were visualized by using a mixture of NiSO₄.6H₂O, DAB and H₂O₂ in 0.175 M sodium acetate solution (pH 7.5). When the level of staining was appropriate, tissue sections were transferred into Milli-Q water to stop the colour reaction. Sections were mounted onto chrome-alum gelatin coated slides, dehydrated and coverslipped.

Double-labelling immunohistochemistry of CRF1R and TH-positive neurons in the NTS-A₂. For CRF1R and TH double-label immunohistochemistry, sections were processed as follows: CRF1R was revealed with DAB intensified with nickel in the first position, and the TH revealed with DAB in the second position. CRF1R immunohistochemistry was performed as described previously. Following the CRF1R staining, sections were rinsed twice in PBS, and then incubated with a rabbit polyclonal anti-TH antibody (diluted 1:6000; Chemicon, Temecula, CA) overnight at 4ºC. The same immunohistochemistry procedures described above were followed. A biotinylated anti-rabbit IgG (diluted 1:200 for 1 h) was used as a secondary antibody. The TH antibody–peroxidase complex was stained in DAB and H₂O₂ in 0.05 M Tris–HCl buffer. The reaction was stopped in PBS. With this staining
procedure, the CRF1R protein stains blue-black, and the cytoplasmic TH stains yellow-brown. The sections were mounted onto glass slides coated with gelatine, air-dried, dehydrated through graded alcohols, cleared in xylene and coverslipped with dibutylphtalate (DPX).

**Quantification of c-Fos immunoreactivity.** Evidence of c-Fos-immunoreactivity (IR) was examined under a light microscope. C-Fos immunostaining within section from the different nuclei was quantified bilaterally for each rat and for all treatment groups by an observer blinded to the treatment protocol. Images were captured by means of a DMLB microscopy (Leica, Madrid, Spain) connected to a video camera (Leica) and an image analysis computer (Q500MC; Leica). C-Fos positive cell nuclei were counted. The boundaries of the NTS, the BNST, the CeA, and the dorsal medial parvocellular (mpd), ventral medial parvocellular (mpv), dorsal parvocellular (dp) and lateral magnocellular (pm) subdivisions of the PVN were outlined and the number of positive profiles was recorded after thresholding the images to a common level. Total counts of the different brain regions were taken bilaterally at regularly spaced intervals and expressed as mean ± SEM.

**Colocalization of CRF1R immunoreactivity with TH-positive neurons.** Sections in the NTS-A2 were collected from area postrema (AP) to obex, according to the map of (Paxinos and Watson, 2007). Positive CRF1R immunoreactivity was detected using the same conventional light microscopy described above. CRF1R-positive TH cells were identified as cells with brown
cytosolic deposits for TH-positive staining and blue/dark staining for CRF1R. A square field (195 µm) was superimposed upon captured image for using as reference area. The high density of TH-positive fibers at level of NTS rendered the distinction of individual TH neurons difficult and therefore did not allow a proper quantification of co-localization of TH neurons with CRF1R immunoreactivity.

**Estimation of noradrenaline and its metabolite MHPG.** NA and its metabolite in the central nervous system 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) were determined by HPLC with electrochemical detection as in the previous paper (Laorden et al., 2000a). Bilateral tissue samples of the PVN were dissected according to the technique of Palkovits (Palkovits, 1973), weighed, placed in perchloric acid (0.1 M), homogenized and centrifuged and the supernatants taken for analysis and filtered through 0.22-µm GV (Millipore, Bedford, Mass, USA). Two aliquots of the supernatant from the same tissue sample were used, the first for analysis of NA and the second for analysis of MHPG. Ten µl of the first aliquot of each sample is injected into a 5-µm C18 reversed-phase column (Waters, Mildford, MA) through a Rheodyne syringe-loading injector (Waters). Electrochemical detection is accomplished with an electrode set at a potential of +0.65 V versus the reference electrode. The mobile phase consisted of a 90:10 (v/v) mixture of water and methanol and chromatographic data are analyzed with chromatography equipment (Empower 2 software, Waters). Because in the rat central nervous system most of MHPG is present in a sulphate conjugate form, the method for the determination of total MHPG in the PVN is based on the acid-catalyzed hydrolysis of MHPG-sulphate. The aliquots for MHPG analysis are kept in a water bath at 100°C.
centrifugation, the supernatant of the hydrolyzed samples was injected (10 µl) into the HPLC equipment. NA and MHPG were quantified by reference to calibration curves run at the beginning and the end of each series of assays. The content of NA and MHPG in the PVN was expressed as ng per g wet weight of tissue. The NA turnover was determined as the NA ratio, which was calculated as: NA ratio = MHPG/NA.

**Drugs and reagents.** Pellets of morphine base (Alcaliber Labs., Madrid, Spain) or lactose were prepared by the Department of Pharmacy and Pharmaceutics Technology (School of Pharmacy, Granada, Spain); naloxone HCl was purchased from Sigma Chemical Co. (St Louis, MO) and dissolved in sterile 0.9 % NaCl (saline). CP-154,526, kindly provided by Pfizer (Bristol-Myers Squibb Co, Princeton, NY) was dissolved in 10 % Tween 80 (Sigma). Antalarmin was dissolved in Tween 80 (Sigma). Drugs were prepared fresh every day. Reagents: Protease inhibitors (Boehringer Mannhein, Germany); phosphatase inhibitor Cocktail Set (Calbiochem, Germany); goat serum (Sigma); avidin–biotin complex (ABC kits; Vector); nickel sulphate (Sigma). HPLC reagents were purchased from Sigma.

**Statistical analysis.** Data are presented as mean±SEM. Two-way or one-way analysis of variance (ANOVA) was use to analyze the data. Newman Keuls or Dunnett post hoc test were used, when appropriate, to identify individual mean differences. To compare two groups, Student’s t-test was used. Irritability was quantified as the number of animals exhibiting the sign/total number of animals observed, and data obtained were analyzed nonparametrically with the $\chi^2$ test. Differences with a $p < 0.05$ were considered significant.
Results

TH immunoreactivity in the NTS-A2 and co-localization of TH and CRF1R.

As previously shown (Núñez et al., 2007b; Núñez et al., 2009), high densities of TH positive cell bodies and fibers were seen at the level of NTS, which corresponds to the acknowledged distribution of TH mRNA (Rusnak et al., 2001). TH neurons were very strongly and ubiquitously stained for CRF1R. In addition, a high number of TH-positive cells expressing CRF1R were found in the two treatment groups tested: control pellets plus vehicle plus naloxone and morphine plus vehicle plus naloxone. However, the high density of TH cell bodies and fibers prevented counting of TH-positive neurons and, therefore, did not allow any evaluation of the percentage of TH-positive neurons co-expressing CRF1R (Fig. 1A, B, B').

Influence of morphine withdrawal on CRF1R immunoreactivity in NTS as determined by Western blot. Figure 1C shows that there was no significant modification of CRF1R immunoreactivity levels during morphine withdrawal compared with control group receiving naloxone and also compared with morphine plus saline-treated rats. No changes were observed in CRF1R levels in the NTS in control rats receiving naloxone compared with control pellets-treated group injected with saline.

Effects of CRF1R antagonists on naloxone-precipitated withdrawal signs in morphine-dependent rats. Student t-test showed that chronic morphine-treated rats had significantly lower weight gain (4.62±1.60 g; t97=10.17; p <0.001; n=41) than placebo control group (24.0400±1.42 g; n=58). Fig. 2A shows that naloxone injection to morphine-dependent rats pretreated with
vehicle produced a significant \((p < 0.001)\) increase in body weight loss (Fig. 2A) compared with the control pellets-treated group also receiving naloxone and with morphine-treated rats given saline. However, administration of naloxone to the control rats resulted in no significant changes in body weight loss 1 h after drug injection, compared with control rats receiving saline.

A significant increase in body weight loss was seen in morphine-withdrawn rats pretreated with the CRF1R antagonist CP-154,526 at the doses of 20 \((p < 0.001)\) and 30 mg/kg \((p < 0.01)\) (Fig. 2B,C) compared with control pellets-treated rats receiving CP-154,526 plus naloxone. However, post hoc Dunnett’s test showed a significant decrease in body weight loss during morphine withdrawal in rats treated with CP-154,526 at the doses of 20 \((p < 0.01)\) and 30 mg/kg \((p < 0.001)\) compared with morphine withdrawn rats receiving vehicle instead of CP-154,526. To ensure that the reduction in body weight loss produced by CP-154,526 was not a non-specific effect, another experiment was performed with the selective CRF1R antagonist antalarmin. As shown in figure 2D, antalarmin significantly \((p<0.001)\) reduced the body weight loss during morphine withdrawal compared with morphine withdrawn rats receiving vehicle.

Table 1 shows the effects of pretreatment with CP-154,526 (20 and 30 mg/kg) and antalarmin on the incidence of naloxone-precipitated irritability in morphine-dependent rats. A lower frequency or total suppression of irritability was noted in dependent group pretreated with CP-154,526 or antalarmin at 5 and 10 min after naloxone administration. Signs of withdrawal were not observed in the control group receiving vehicle plus naloxone.
Effects of CRF1R blockade on morphine withdrawal-induced HPA axis activation. We measured plasma corticosterone and ACTH concentrations (as HPA axis activation markers) in blood samples obtained from morphine dependent or control rats 60 min after injection of saline or naloxone. As shown in Fig. 2E,F, naloxone-precipitated morphine withdrawal evoked a dramatic increase of both corticosterone and ACTH secretion.

To evaluate if a causal link exists between CRF1R activation and HPA axis hyper activation during morphine withdrawal, we measured plasma corticosterone and ACTH concentrations in animals made dependent on morphine and pretreated with CP-154,526 (20 or 30 mg/kg i.p.) 30 min before naloxone administration. Newman-Keuls post hoc test showed that naloxone-precipitated morphine withdrawal evoked a dramatic increase of corticosterone in animals treated with the CRF1R antagonist at 20 mg/kg (Fig. 2G; $p < 0.001$) or 30 mg/kg (Fig. 2H; $p < 0.001$) versus placebo plus CP-154,526 plus naloxone and morphine plus CP-154,526 plus saline. Post hoc Dunnett’s tests showed no significant changes in corticosterone levels during morphine withdrawal in rats treated with CP-154,526 at the doses of 20 and 30 mg/kg compared with morphine withdrawn rats receiving vehicle instead of CP-154,526. In Fig. 2I, Newman-Keuls post hoc test shows that naloxone-induced morphine withdrawal produced a significant increase in ACTH levels in animals receiving CP 154,526 at the dose of 20 mg/kg ($p < 0.001$) compared with morphine-treated rats receiving CP-154,526 plus saline and placebo rats treated with the CRF1R antagonist and naloxone. However, Dunnett’s test showed that CP-154,526 significantly ($p<0.001$) reduced morphine withdrawal-induced increases in plasma ACTH compared with rats receiving vehicle instead of CP-154,526,
whereas plasma ACTH levels in morphine rats treated with saline or in placebo rats receiving saline or naloxone were not modified by CP-154,526.

**Effects of CRF1R antagonists on naloxone-induced NA turnover in the hypothalamic PVN.** Newman-Keuls post hoc test shows that naloxone injection to morphine-dependent rats pretreated with vehicle produced a significant increase in NA turnover (Fig. 3A) compared with the placebo-pelleted group also receiving naloxone ($p<0.001$) and with morphine-treated rats given saline ($p<0.01$). However, administration of naloxone to placebo-pelleted rats resulted in no significant changes in NA turnover 1 h after drug injection, compared with control rats receiving saline. As shown in Figure 3B,C,D no alteration in NA turnover in PVN were found following naloxone-precipitated morphine withdrawal in rats pretreated with CP-154,526 (20 or 30 mg/kg) or antalarmin.

**Effects of CRF1R blockade on morphine withdrawal-induced c-Fos induction in the brain stress system.** Placebo implanted, vehicle and naloxone-injected rats display weak, scattered c-Fos immunoreactivity in all the brain stress system areas that have been studied: PVN, NTS-$A_2$, and CeA (Figs. 4, 5). Naloxone precipitated morphine withdrawal resulted in a robust induction of c-Fos-ir throughout all the areas 60 min following naloxone injection. As shown in Fig. 4A,B naloxone precipitated morphine withdrawal results in a dramatic increase in c-Fos staining in all four, functionally distinct subdivisions of the PVN. Significant labelling was found in the medial parvocellular subdivision. In addition, inducible c-Fos reaction was detected in
the autonomic-related projection neurons in the ventral aspect of the medial and in the dorsal parvocellular subdivisions as well as in the magnocellular division.

Pretreatment with CP-154,526 (20 mg/kg i.p.) did not significantly modify the increase in c-Fos expression following naloxone-precipitated morphine withdrawal that was seen in NTS, CeA and PVN (Figs. 4, 5). In addition, c-Fos expression in placebo rats was significantly elevated over control levels at PVN in animals receiving CP-154,526 (Fig. 4B).
Discussion

The principal findings of the present study are: (i): CRF1R is expressed in the NTS-A2 cell group; (ii): CRF1R antagonists significantly decreased body weight loss and irritability during morphine withdrawal; (iii): pretreatment with CRF1R antagonists did not block either the increased NA turnover or corticosterone release, whereas plasma ACTH levels were significantly attenuated by CP-154,526; and (iv): brain stress system (which includes, among other nuclei, the NTS-A2, the CeA and the PVN) activation after naloxone injection to morphine-dependent rats was not attenuated by CRF1R antagonists pretreatment.

Evidence for CRF1R mRNA within NTS has been demonstrated in the rat (Van Pett et al., 2000). The present study extended these findings by demonstrating immunochemical evidence for CRF1R protein expression in rat NTS-A2 catecholaminergic neurons innervating the PVN. Several studies have shown that increased CRF release contributes to the anxiety and aversive states produced by drug withdrawal (Zorrilla and Koob, 2004; Gallagher et al., 2008), and recruitment of the CRF system has been hypothesized to be involved in drug dependence (Koob, 2008). Accordingly, antagonism of CRF neurotransmission attenuated the anxiety-like and aversive effects of drug withdrawal (Stinus et al., 2005). In addition, previous findings have shown anxiolitic-like action of CRF1R antagonists (Contarino and Papaleo, 2005). Consistent with these studies, we observed that both CP-156,526 and antalarmin suppressed the anxiety-like behaviour during morphine withdrawal.
Normally, when naloxone was injected to morphine-dependent rats, animals were very irritable and showed high motor activity. Instead, rats receiving CRF1R antagonists showed neither motor activity nor irritability along 30 min of observation. In addition, pretreatment of rats with CP-154,526 or antalarmin significantly attenuated weight loss (another somatic sign of opiate withdrawal) in morphine-withdrawn rats. These results indicate that activation of CRF1R subtype contributes to the somatic signs of opiate withdrawal and suggest that reduced body weight loss and irritability induced by pretreatment with CP-154,526 is not related to nonspecific effects of this drug. These findings are consistent with different reports showing the potent anxiolitic-like activity of CRF1R antagonists (Lu et al., 2000b; Koob, 2008).

NA has been implicated in addiction, in particular in opiate withdrawal (Maldonado, 1997; Smith and Aston-Jones, 2008). Clinical findings and studies in animals have shown that systemic administration of adrenoceptor antagonists reduces the somatic signs of opiate withdrawal (Laorden et al., 2000b; Aston-Jones and Harris, 2004). The present findings demonstrate that administration of naloxone to morphine dependent rats significantly elevated NA turnover in the PVN, which projects from NTS-A2. Previous reports have shown that activation of noradrenergic terminals innervating the PVN modulates the HPA axis activity in response to morphine withdrawal. Thus, naloxone-induced morphine withdrawal increases NA turnover and c-Fos expression in the PVN concomitantly with an increase in the activity of NTS-A2 TH positive neurons (as reflected by c-Fos expression) (Laorden et al., 2002b; Laorden et al., 2002a; Smith and Aston-Jones, 2008). These effects were dependent on adrenoceptor activation, which indicates that the hyperactivity of the HPA axis
during morphine withdrawal is mediated via stimulatory noradrenergic pathways (Laorden et al., 2000b). Additionally, we reported that morphine withdrawal is associated with an increase in TH enzymatic activity in the PVN as well as TH phosphorylation (activation) at Ser31 and TH mRNA expression in NTS-A2 noradrenergic cell group (Núñez et al., 2009; Núñez et al., 2007b), which occurred in parallel to increased hnRNA CRF and AVP expression in the PVN (Núñez et al., 2007a). There are evidences supporting that CRF neurons in the PVN innervate noradrenergic brain stem nuclei (Gray and Magnuson, 1987) and it has been proposed the existence of a NA-CRF loop in which NA would stimulate the release of CRF in CeA, the PVN and the bed nucleus stria terminalis (BNST). CRF from these nuclei would induce the release of NA by the brain stem noradrenergic areas (Koob, 1999). The results of the present study demonstrated that pretreatment with the selective CRF1R antagonists CP-154,526 or antalarmin, did not block the increased noradrenergic activity in the PVN during morphine withdrawal. These findings suggest that activation of CRF1R subtype is not responsible for the elevation of NA neurotransmission innervating the PVN. Our results are inconsistent with findings of previous studies in mice (Funada et al., 2001), showing that the CRF1R antagonist CRA1000 attenuated the noradrenergic activity in the cerebral cortex. The conflicting findings may be the result of species differences, although the different brain areas studied - NTS versus cerebral cortex – may have caused the different results.

Present findings show that elevated plasma glucocorticoid and ACTH levels in morphine withdrawn rats occur in parallel with enhanced NA turnover in the PVN, which provide evidence that HPA axis was activated as a
consequence of morphine withdrawal. Our results show that pretreatment with CP-154,526 did not block the corticosterone release that is produced as a consequence of morphine withdrawal. However, ACTH concentrations were found to be decreased in animals pretreated with CP-154,526 (20 mg/kg i.p.). The relation between HPA activity and drugs withdrawal-induced behaviour alteration and changes in the brain stress system has not been elucidated and contradictory results have been shown. For example, morphine-induced conditioned place preference (CPP) was decreased by antalarmin, while this antagonist did not antagonized the increase in plasma corticosterone levels (Grakalic et al., 2006), which suggests that the changes in place preference conditioning induced by CRF1R blockade are not related to changes in HPA activity. In addition, CRF1R has been shown to play a major role in mediating the effects of CRF on behavioural responses to lorazepam withdrawal but not some of the neuroendocrine effects. Thus, according to present results, the CRF1R antagonist R121919 attenuated the behavioural and autonomic signs of lorazepam withdrawal, as well as the ACTH secretion, whereas corticosterone levels, were not altered (Skelton et al., 2007). Furthermore, in clinical studies CRFR1 antagonists produced anxiolitic-like effects in depressed patients in absence of effects on CRF-stimulated HPA axis activity (Zobel et al., 2000). An additional potential explanation for present findings is that, although CRF is thought to be the major secretagogue in stimulating ACTH secretion, AVP, catecholamine and other factors also play a role (Tilders et al., 1985). In addition, there is increasing evidence that AVP and CRF production and release from the parvocellular PVN neurons are under independent regulation. Thus, it is possible that AVP may play an important role in mediating pituitary-adrenal
response to drug withdrawal and stress (Deak et al., 1999). Taken into account the above findings, it is possible that pituitary-adrenal response is not related with the behavioural signs of precipitated opiate withdrawal in rats.

It is well known that CRF receptors, in particular CRF1R subtype, are widely distributed in the brain, as well in the anterior pituitary gland. Mice and rats seem to show comparable distribution, while density of immunoreactivity differs in a structure-dependent manner (Van Pett et al., 2000). This differential distribution of CRF1R and CRF2R implicates that these receptors exert different functions that are controlled by different mechanisms. According to the distribution of CRF receptors, the results of the present study would suggest that the effects of CRF1R antagonists were at anterior pituitary level (so attenuating ACTH release), but not at NTS, where robust hybridization signal for CRF2R mRNA has also been found (Van Pett et al., 2000). Taken together, these findings would suggest that CRF2R subtype might mediate the activation of NA system innervating the PVN during morphine withdrawal.

According to previous data from our laboratory (Laorden et al., 2002a; Núñez et al., 2007a; Laorden et al., 2002c), c-Fos expression was induced in several nuclei of the brain stress system (NTS, CeA, and PVN) 60 min after naloxone-induced morphine withdrawal. Activation of HPA axis during naloxone-precipitated morphine withdrawal involves transcriptional up-regulation of CRF and AVP expression in the PVN (Núñez et al., 2007a). We also have previously shown that increased c-Fos expression in the PVN during morphine withdrawal was abolished by adrenergic receptor antagonists, which indicates that neuronal activation of PVN is depending on increased NA release (Laorden et al., 2002b). In the present study, pretreatment with CP-154,526 did
not modify the increase in c-Fos expression that was seen in NTS, CeA and PVN. This suggests that neurons in those nuclei are not stimulated by CRF1R activation. Since all these structures also express CRF2R (Van Pett et al., 2000), it is possible that morphine withdrawal may activate neurons in the brain stress system via CRF2R. In addition, high c-Fos expression was found in the PVN from placebo-pelleted rats receiving CP-154,526. A number of cellular and molecular mechanisms may be involved. One might include a compensatory agonist activation of the CRF2R subtype as a result of the blockade of the CRF1R.

In conclusion, the present study indicated that systemically bioavailable and selective CRF1R antagonists attenuated the irritability and weight loss signs of opiate withdrawal as well as ACTH hypersecretion. However, these antagonists had no detectable impact on NA turnover and c-Fos expression during naloxone-induced morphine withdrawal. These data suggest that CRF1R activation may not contribute to the increased brain stress system response in morphine-withdrawn rats. Future research is needed to determine the regions in which CRF1R signalling modulates ACTH release associated with opiate withdrawal and if the CRF2R subtype plays a role.
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References


Footnotes

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**Figure Legends**

**Fig. 1.** Co-localization of TH-positive neurons and CRF1R immunoreactivity in rat NTS-A₂ noradrenergic cell group A, B, B’. Photographs represent immunohistochemical detection of CRF1R in TH neurons in the NTS from control pellets (A) or morphine pretreated rats B, B’) 60 min after naloxone injection. Scale bar, 100 μm in A and B; 50 μm in B”. AP: area postrema; CC: canal central. Arrows show CRF1R in TH-positive neurons. C, representative immunoblots of CRF1R expression in NTS tissue isolated from control pellets-treated or morphine-dependent rats 60 min after administration of saline or naloxone. β-actin was used as loading control. Data represent the optical density of immunoreactive bands expressed as the percentage (%) of the mean ± SEM of placebo plus saline control band.

**Fig. 2.** CP-154,526 attenuated the body weight loss (A-D) and the plasma ACTH (F, I) but not the corticosterone (E, G, H) response to naloxone-induced morphine withdrawal. Two-way ANOVA for body weight loss revealed a main effects of morphine pre-treatment (F₁,₂₆=45.81; p <0.0001), naloxone injection (F₁,₂₆=45.11; p <0.0001) and a significant interaction between morphine pre-treatment X acute treatment (F₁,₂₆=49.40; p <0.0001). Two-way ANOVA for body weight loss following CP-154,526 at the dose of 20 mg/kg (Fig. 2B) revealed a main effect of morphine pretreatment (F₁,₃₁=23.52; p <0.0001), acute treatment (F₁,₃₁=7.20; p <0.0116), and a significant interaction between pretreatment and acute treatment (F₁,₃₁=8.20; p =0.0074). CP-154,526 pretreatment at the dose of 30 mg/kg (Fig. 2C) revealed a main effect of
morphine pretreatment ($F_{1,29}=8.36; p =0.0072$) on body weight loss. Two-way ANOVA for body weight loss following antalarmin pretreatment showed a main effect of morphine pretreatment ($F_{1,24}=81.31; p <0.0001$), acute treatment ($F_{1,24}=13.31; p <0.0013$), and a significant interaction between pretreatment and acute treatment ($F_{1,24}=15.82; p =0.0006$). Two-way ANOVA for corticosterone and ACTH revealed a main effect of morphine treatment (corticosterone: $F_{(1, 20)}=207.51; p < 0.0001$; ACTH: $F_{(1, 20)}=18.03; p <=0.0004$), naloxone injection (corticosterone: $F_{(1, 20)}=220.31; p < 0.0001$; ACTH: $F_{(1, 20)}=16.47; p =0.0006$) and an interaction between pre-treatment and acute treatment (corticosterone: $F_{(1, 20)}=182.22; p < 0.0001$; ACTH: $F_{(1, 20)}=14.23; p = 0.0012$). Two-way ANOVA for corticosterone in animals treated with CP-154,526 revealed a main effect of pretreatment (20 mg/kg: $F_{(1, 21)}=14.70; p < 0.0010$; 30 mg/kg: $F_{(1, 20)}=32.09; p < 0.0001$), acute treatment (20 mg/kg: $F_{(1, 21)}=31.31; p < 0.0001$; 30 mg/kg: $F_{(1, 20)}=66.86; p < 0.0001$) and an interaction between pretreatment and acute treatment (20mg/kg: $F_{(1, 21)}=28.24; p < 0.0001$; 30 mg/kg: $F_{(1, 20)}=60.46; p < 0.0001$). Two-way ANOVA for ACTH in animals treated with CP-154,526 showed a main effect of pretreatment ($F_{(1, 23)}=9.15; p = 0.0060$), acute treatment ($F_{(1, 23)}=11.30; p = 0.0027$) and a pretreatment X acute treatment interaction ($F_{(1, 20)}=12.07; p = 0.0021$). Data represent the mean ± SEM of body weight loss and plasma ACTH and corticosterone concentration 60 min after saline or naloxone injection to control pellets- or morphine-treated rats receiving vehicle, CP-154,526 (CP) or antalarmin (Ant) 30 min before saline or naloxone administration. **p< 0.001 vs control pellets+Tween (10% or 80) or CP-154,526 (20 or 30 mg/kg)+naloxone; ††p < 0.01, †††p < 0.001 vs
morphine+Tween 10%+saline; \#\#p < 0.01; \###p < 0.001 vs morphine+Tween (10% or 80)+naloxone.

**Fig. 3.** Effects of CRF1R antagonists on the morphine withdrawal-induced increased NA turnover (as estimated by the MHPG/NA ratio) in control and in morphine-dependent rats after administration of saline or naloxone. Two-way ANOVA revealed a main effects on NA turnover for morphine pre-treatment ($F_{1,14}=28.30; \ p = 0.0001$), naloxone injection ($F_{1,14}=8.19; \ p = 0.0126$) and a significant interaction between morphine pre-treatment X acute treatment ($F_{1,14}=7.21; \ p = 0.0178$). Neither CP-154,526 (CP) nor antalarmin (ant) attenuated morphine withdrawal-induced increase in NA turnover. Two-way ANOVA for MHPG/NA ratio in animals treated with CP-154,526 (20 or 30 mg kg) showed a pretreatment X acute treatment interaction (20: $F_{1, 19}= 4.84; \ p = 0.0403$; 30: $F_{1, 19}= 4.77; \ p = 0.0418$). Two-way ANOVA for NA turnover in rats pretreated with antalarmin revealed a main effect of morphine pre-treatment ($F_{1,17}=29.29; \ p < 0.0001$). Data represent the mean ± SEM of NA turnover 60 min after saline or naloxone injection to control pellets- or morphine-treated rats receiving vehicle, CP-154,526 or antalarmin 30 min before saline or naloxone administration. *p < 0.05; **p < 0.001 vs control pellets+Tween (10% or 80) or CP-154,526+naloxone; *p < 0.05; **p < 0.001 vs morphine+tween or CP-154,526 (20 or 30 mg/kg)+saline; \#\#p < 0.01 vs control group+TWEEN+naloxone; \&\&p < 0.01 vs control group+antalarmin+naloxone;
**Fig. 4.** CRF1R antagonists did not modify the neuronal activation of the hypothalamic paraventricular nucleus (PVN) in response to morphine withdrawal. A: photomicrographs showing c-Fos immunoreactive cell nuclei in the PVN, 60 min after naloxone injection to control pellets- or morphine-implanted rats receiving vehicle (Tween 10%) or CP-154,526 30 min before saline or naloxone administration. Note the absence of c-Fos immunoreactivity cell nuclei in the control group receiving vehicle and induction of c-Fos staining in all subdivisions of the PVN following naloxone-precipitated morphine withdrawal in both vehicle-and CP-154,526-pretreated animal. Panel B shows the mean ± SEM values for c-Fos positive nuclei at different PVN subdivisions. dp, dorsal parvocellular; mdp, dorsal aspect of the medial parvocellular; mpd, ventral aspect of the medial parvocellular; pm, posterior magnocellular subdivision. 3V, third ventricle. Scale bar: 100 μm. *p < 0.05, **p < 0.01, ***p < 0.001 vs the corresponding group receiving control pellets+Tween+naloxone; #p < 0.05 vs morphine+Tween+naloxone; +p < 0.05 vs control pellets+CP-154,526+naloxone.

**Fig. 5.** Effects of CRF1R blockade on neuronal activation at selected brain sites in response to morphine withdrawal. Representative photomicrographs showing c-Fos immunoreactive cell nuclei 60 min after naloxone injection to control pellets- or morphine-implanted rats. Animals received vehicle (Tween 20) or CP-154,526 (20 mg/kg i.p.) 30 min before saline or naloxone administration. Specific induction of c-Fos staining was found in the nucleus of the solitary tract (NTS; A) and central nucleus of amygdala (CeA; C). B, D: mean ± SEM values of c-Fos positive cell nuclei at NTS (B) and CeA (D). Note the absence of c-Fos
immunoreactivity cell nuclei in the control group receiving vehicle and induction of c-Fos staining in all subdivisions of the PVN following naloxone-precipitated morphine withdrawal in both vehicle-and CP-154,526-pretreated animal. CC: canal central. Scale bar: 100 μm. ***p < 0.001 vs control pellets+Tween+naloxone; ++p < 0.01; +++p < 0.001 vs control pellets+CP-154,426+naloxone.
Table 1. Effects of pretreatment with CP-154,526 (20 and 30 mg/kg) and antalarmin on the incidence of irritability in morphine-withdrawn rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Irritability 5 min</th>
<th>Irritability 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont/veh/nx</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>Mor/veh/nx</td>
<td>6/6***</td>
<td>6/6***</td>
</tr>
<tr>
<td>Mor/CP 20/nx</td>
<td>9/15</td>
<td>0/15***</td>
</tr>
<tr>
<td>Mor/CP 30/nx</td>
<td>5/9</td>
<td>0/9***</td>
</tr>
<tr>
<td>Mor/Ant/nx</td>
<td>7/10</td>
<td>0/10***</td>
</tr>
</tbody>
</table>

Control pellets (Cont)- or morphine (Mor)-treated rats were injected with vehicle (veh), CP-154,526 20 or 30 mg/kg (CP 20, CP 30) or antalarmin 20 mg/kg (Ant) 30 min before naloxone (nx) administration. The withdrawal symptom of irritability was observed after naloxone injection and scored at 5 min intervals. This symptom is shown as the number of animals exhibiting the sign to the total number of animals observed. Animals were observed for 30 min with similar results. ***p < 0.001 vs mor/veh/nx 10 min; +++p < 0.001 vs pla/veh/nx 5 and 10 min; χ²
Figure 1