

Effects of Corticotropin-Releasing Factor Receptor-1 Antagonists on the Brain Stress System Responses to Morphine Withdrawal

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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 activity, and corticotropin-releasing factor (CRF) 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-154526 [*N*-butyl-*N*-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)pyrrolo[3,2-*e*]pyrimidin-4-amine], or antalarmin (selective CRF1R antagonists). Tyrosine hydroxylase-positive neurons expressing CRF1R were seen at the level of the nucleus tractus

solitarius-A₂ cell group in both control and morphine-withdrawn rats. CP-154526 and antalarmin attenuated the increases 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 was seen during naloxone-induced morphine withdrawal. However, blockade of CRF1R significantly reduced morphine withdrawal-induced increases in plasma adrenocorticotropin levels. These results suggest that the CRF1R subtype may be involved in the behavioral and somatic signs and in adrenocorticotropin 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 adrenocorticotropin from the pituitary, which releases glucocorticoids from adrenal glands. In addition, CRF is widely distributed throughout the brain and plays a major role in coordinating behavioral and autonomic responses to stress (Owens and Nemeroff, 1991). Alterations in the 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, which is involved in anxiety-related behavior (Bale and Vale, 2004), expresses throughout the entire central nervous system, whereas CRF2R displays more restricted expression than CRF1R (Van Pett et al., 2000). Many lines of evidence indicate that the central CRF system is involved in the expression of morphine withdrawal signs. Thus, CRF1R antagonists have been shown to attenuate several behavioral signs of morphine withdrawal (Iredale et al., 2000; Lu et al., 2000).

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ABBREVIATIONS: CRF, corticotropin-releasing factor; CRFR, CRF receptor; CRF1R, CRF receptor-1 subtype; HPA, hypothalamus-pituitary-adrenocortical; NA, noradrenaline; PVN, paraventricular nucleus; TH, tyrosine hydroxylase; NTS, nucleus tractus solitarius; CeA, central amygdala; AP, area postrema; DAB, diaminobenzidine; MHPG, 3-methoxy-4-hydroxyphenylethylen glycol; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; CP-154526, *N*-butyl-*N*-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)pyrrolo[3,2-*e*]pyrimidin-4-amine; AVP, arginine-vasopressin; CRA1000, [2-[*N*-(2-methylthio-4-isopropylphenyl)-*N*-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidine]; R121919, 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N,N*-dipropyl-pyrazolo[2,3-*a*]pyrimidin-7-amine.

The noradrenergic system and the hypothalamo-pituitary-adrenocortical (HPA) axis comprise two major adaptation mechanisms to stress. Like stressors, morphine withdrawal activates the 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 adrenocorticotropin and corticosterone secretion (Laorden et al., 2002b; Núñez et al., 2007a; Cleck and Blendy, 2008). Enhanced responsiveness of HPA axis after morphine withdrawal has been associated with activation of noradrenergic neurons in the nucleus tractus solitarius (NTS)-A₂ that project to the hypothalamic PVN (Laorden et al., 2000b, 2002b). Therefore, we have suggested that one of the neuronal mechanisms that underlie morphine withdrawal-induced activation of the HPA axis may depend 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 heterogeneous nuclear RNA CRF expression that parallels an enhancement in phosphorylation in NTS (activation) and enzymatic activity of tyrosine hydroxylase (TH) in the PVN during morphine withdrawal (Núñez et al., 2007a,b). CRF-positive terminals have been detected in contact with TH-immunoreactive neurons within the locus ceruleus (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, the presence of CRF1R has been demonstrated in the catecholaminergic neurons of the locus ceruleus, ventral tegmental area, 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 the brain stress system has been not well documented.

Given the prominent role of NTS in morphine withdrawal responsiveness at the PVN level, here we examined 1) whether rat catecholaminergic neurons within the NTS-A₂ coexpress CRF1R, and the effect of morphine withdrawal on CRF1R expression; 2) the role of CRF1R in mediating somatic and behavioral states produced during withdrawal from morphine dependence; 3) 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 4) 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 No-

vember 24, 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 subcutaneous implantation of two 75-mg morphine pellets (provided by the Ministerio de Sanidad, Madrid, España) under light ether anesthesia. 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 short-term treatment with opioid antagonists (Frenois et al., 2002). Dependence on morphine was 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 injection of naloxone (1 mg/kg s.c. in a volume of 1 ml/kg body weight) or saline (controls; 1 ml/kg s.c.), and then the rats were observed for behavioral 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 long-term morphine treatment induces a decrease in body weight gain caused by lower caloric intake (Núñez et al., 2009).

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, CeA, and PVN (magno- and parvocellular parts), and plasma corticosterone and adrenocorticotropin concentrations were determined in morphine-dependent and control rats treated with the selective CRF1R antagonists CP-154526 [N-butyl-N-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)pyrrolo[3,2-e]pyrimidin-4-amine] (20 or 30 mg/kg i.p.) or antalarmin (20 mg/kg i.p.) 30 min before the administration of naloxone or saline. Doses of CRF1R antagonists were selected based on existing literature and the antiwithdrawal effects of the antagonists that were observed in previous reports. CP-154526 was dissolved in 10% Tween 80 and given in a volume of 1 ml/kg body weight. Antalarmin was dissolved in Tween 80.

Sixty minutes after saline or naloxone injection, rats were sacrificed (between 11:00 AM and 12:00 noon to avoid circadian variations in the 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 colocalization and c-Fos immunohistochemistry. One set of each treatment group was randomly assigned for plasma adrenocorticotropin and corticosterone determination.

Radioimmunoassay. Plasma levels of corticosterone and adrenocorticotropin were measured by commercially available kits for rats (¹²⁵I-corticosterone and ¹²⁵I-adrenocorticotropin radioimmunoassay; MP Biomedicals, Solon, OH). The sensitivity of the assay was 7.7 ng/ml for corticosterone and 5.7 pg/ml for adrenocorticotropin.

Western Blotting. Brainstem tissue corresponding to the NTS-A₂ cell group was dissected between the area postrema (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), equal quantities of total protein (40 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, and Western analysis was performed with goat polyclonal anti-CRF1R antibody (against peptide mapping within an internal region of CRF1R; 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 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 (25 mM glycine and 1%

SDS, pH 2), for 1 h at 37°C. Blots were subsequently reblocked and probed with 1:1000 anti- β -actin (rabbit polyclonal antibody; Cell Signaling Technology Inc., Danvers, MA) 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 by using previously published techniques (Núñez et al., 2007b). Analysis of CRF1R expression in the NTS was made on free-floating sections (30 μ m) at various levels, ranging rostrally from the AP to the pyramidal decussation (plane of sections relative to bregma: -13.68 to -14.60) (Paxinos and Watson, 2007). Analysis of c-Fos immunoreactivity was made at the NTS, CeA, and PVN. Tissue sections were incubated in the following primary antibodies: goat polyclonal anti-CRF1R (1:200; Santa Cruz Biotechnology) for 48 h at 4°C and rabbit polyclonal anti-c-Fos (1:10,000; Santa Cruz Biotechnology). Antigens were visualized by using a mixture of NiSO₄·6H₂O, diaminobenzidine (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 (Millipore Corporation, Waltham, MA) to stop the color reaction. Sections were mounted onto chrome-alum gelatin-coated slides, dehydrated, and coverslipped.

Double-Labeling 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 TH was revealed with DAB in the second position. CRF1R immunohistochemistry was performed as described previously. After CRF1R staining, sections were rinsed twice in phosphate-buffered saline, and then incubated with a rabbit polyclonal anti-TH antibody (diluted 1:6000; Millipore Bioscience Research Reagents, Temecula, CA) overnight at 4°C. 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 phosphate-buffered saline. 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 gelatin, air-dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped with dibutyl phthalate.

Quantification of c-Fos Immunoreactivity. Evidence of c-Fos immunoreactivity was examined under a light microscope. c-Fos immunostaining within sections from the different nuclei was quantified bilaterally for each rat and all treatment groups by an observer blinded to the treatment protocol. Images were captured with a DMLB microscope (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, bed nucleus stria terminalis, CeA, and the dorsal medial parvocellular, ventral medial parvocellular, dorsal parvocellular, and lateral magnocellular 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 \pm S.E.M.

Colocalization of CRF1R Immunoreactivity with TH-Positive Neurons. Sections in the NTS-A₂ were collected from the AP to the obex, according to the map of Paxinos and Watson, 2007. Positive CRF1R immunoreactivity was detected by 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 μ m) was superimposed on a captured image for use as a reference area. The high density of TH-positive fibers at the level of NTS rendered the distinction of individual TH neurons difficult and therefore did not allow a proper quantification of colocalization of TH neurons with CRF1R immunoreactivity.

Estimation of Noradrenaline and its Metabolite 3-Methoxy-4-hydroxyphenylethylen Glycol. NA and its metabolite in the central nervous system, 3-methoxy-4-hydroxyphenylethylen glycol (MHPG), were determined by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Laorden et al., 2000a). Bilateral tissue samples of the PVN were dissected according to the technique of Palkovits (1973), weighed, placed in perchloric acid (0.1 M), homogenized, and centrifuged, and the supernatants were taken for analysis and filtered through 0.22- μ m GV filter (Millipore Corporation). 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 microliters of the first aliquot of each sample was injected into a 5- μ m C18 reversed-phase column (Waters, Milford, MA) through a Rheodyne syringe-loading injector (Waters). Electrochemical detection was 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 were analyzed with chromatography equipment (Empower 2 software; Waters). Because in the rat central nervous system most MHPG is present in a sulfate conjugate form, the method for the determination of total MHPG in the PVN is based on acid-catalyzed hydrolysis of MHPG-sulfate. The aliquots for MHPG analysis were kept in a water bath at 100°C. After centrifugation, the supernatant of the hydrolyzed samples was injected (10 μ l) 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 nanograms per gram of wet weight of tissue. The NA turnover was determined as the NA ratio, which was calculated as follows: NA ratio = MHPG/NA.

Drugs and Reagents. Pellets of morphine base (Alcaliber Labs, Madrid, Spain) or lactose were prepared at the Department of Pharmacy and Pharmaceutics Technology, School of Pharmacy, University of Granada (Granada, Spain). Naloxone HCl was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile 0.9% NaCl (saline). CP-154526, kindly provided by Pfizer (New York, NY), was dissolved in 10% Tween 80 (Sigma-Aldrich). Antalarmin was dissolved in Tween 80 (Sigma-Aldrich). Drugs were prepared fresh every day. Reagents used were: protease inhibitors (Roche Diagnostics, Indianapolis, IN); phosphatase inhibitor cocktail set (Calbiochem, San Diego, CA); goat serum (Sigma-Aldrich); avidin-biotin complex (Vector Laboratories, Burlingame, CA); and nickel sulfate (Sigma-Aldrich). HPLC reagents were purchased from Sigma-Aldrich.

Statistical Analysis. Data are presented as mean \pm S.E.M. Two-way or one-way analysis of variance (ANOVA) was used to analyze the data. Newman Keuls or Dunnett post hoc test was 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 χ^2 test. Differences with *p* < 0.05 were considered significant.

Results

TH Immunoreactivity in the NTS-A₂ and Colocalization of TH and CRF1R. As shown previously (Núñez et al., 2007b, 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 + vehicle + naloxone and morphine + vehicle + naloxone. However, the high density of TH cell bodies and fibers prevented counting TH-positive neurons and, therefore, allowed no evaluation of the percentage of TH-positive neurons coexpressing CRF1R (Fig. 1A, B, and 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 the control group receiving naloxone and with morphine plus saline-treated rats. No changes were observed in CRF1R levels in the NTS in control rats receiving naloxone compared with the control pellet-treated group injected with saline.

Effects of CRF1R Antagonists on Naloxone-Precipitated Withdrawal Signs in Morphine-Dependent Rats. Student's *t* test showed that rats receiving long-term morphine treatment had significantly lower weight gain (4.62 ± 1.60 g; $t_{97} = 10.17$; $p < 0.001$; $n = 41$) than the placebo control group (24.04 ± 1.42 g; $n = 58$). Figure 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 pellet-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-154526 at doses of 20 mg/kg ($p < 0.001$) and 30 mg/kg ($p < 0.01$) (Fig. 2, B and C) compared with control pellet-treated rats receiving CP-154526 and naloxone. However, a post hoc Dunnett's test showed a significant decrease in body weight loss during morphine withdrawal in rats

treated with CP-154526 at doses of 20 mg/kg ($p < 0.01$) and 30 mg/kg ($p < 0.001$) compared with morphine-withdrawn rats receiving vehicle instead of CP-154526. To ensure that the reduction in body weight loss produced by CP-154526 was not a nonspecific effect, another experiment was performed with the selective CRF1R antagonist antalarmin. As shown in Fig. 2D, antalarmin significantly ($p < 0.001$) reduced body weight loss in rats during morphine withdrawal compared with morphine-withdrawn rats receiving vehicle.

Table 1 shows the effects of pretreatment with CP-154526 (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 the dependent group pretreated with CP-154526 or antalarmin at 5 and 10 min after naloxone administration. Signs of withdrawal were not observed in the control group receiving vehicle and naloxone.

Effects of CRF1R Blockade on Morphine Withdrawal-Induced HPA Axis Activation. We measured plasma corticosterone and adrenocorticotropin 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. 2 E and F, naloxone-precipitated morphine withdrawal evoked a dramatic increase of both corticosterone and adrenocorticotropin secretion.

To evaluate whether a causal link exists between CRF1R activation and HPA axis hyperactivation during morphine withdrawal, we measured plasma corticosterone and adrenocorticotropin concentrations in animals made dependent on morphine and pretreated with CP-154526 (20 or 30 mg/kg

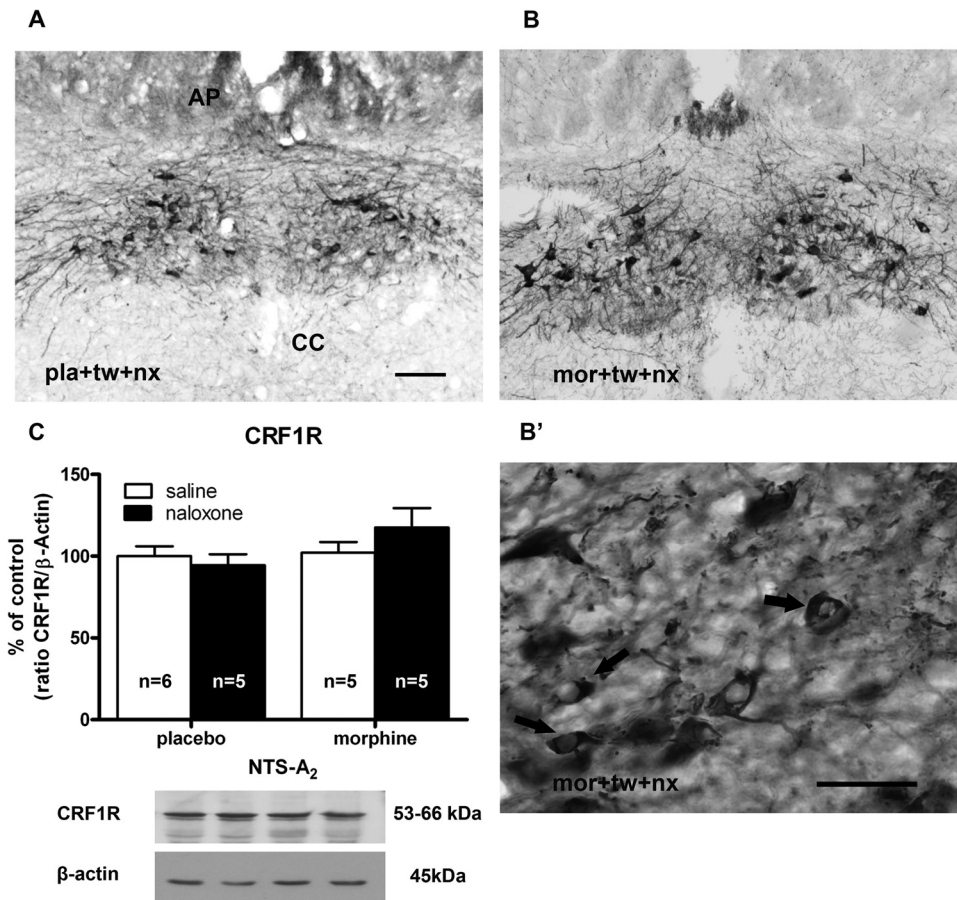


Fig. 1. Colocalization of TH-positive neurons and CRF1R immunoreactivity in the rat NTS-A₂ noradrenergic cell group A, B, and B', photographs represent immunohistochemical detection of CRF1R in TH neurons in the NTS from control pellets (A) or morphine (mor)-pretreated rats (B and B') 60 min after naloxone (nx) injection. Scale bars, 100 μ m (A and B) and 50 μ m (B'). CC, canal central; tw, Tween; pla, placebo. Arrows indicate CRF1R in TH-positive neurons. C, representative immunoblots of CRF1R expression in NTS tissue isolated from control pellet-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 \pm S.E.M. of placebo plus saline control band.

i.p.) 30 min before naloxone administration. A 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 + CP-154526 + naloxone and morphine + CP-154526 + saline. Post hoc Dunnett's tests showed no significant changes in corticosterone levels during morphine withdrawal in rats treated with CP-154526 at doses of 20 and 30 mg/kg compared with morphine-withdrawn rats receiving vehicle instead of CP-154526. In Fig. 2I, a Newman-Keuls post hoc test shows that naloxone-induced morphine withdrawal produced a significant increase in adrenocortico-

tropin levels in animals receiving CP 154526 at a dose of 20 mg/kg ($p < 0.001$) compared with morphine-treated rats receiving CP-154526 and saline and placebo rats treated with the CRF1R antagonist and naloxone. However, Dunnett's test showed that CP-154526 significantly ($p < 0.001$) reduced morphine withdrawal-induced increases in plasma adrenocorticotropin compared with rats receiving vehicle instead of CP-154526, whereas plasma adrenocorticotropin levels in morphine rats treated with saline or placebo rats receiving saline or naloxone were not modified by CP-154526.

Effects of CRF1R Antagonists on Naloxone-Induced NA Turnover in the Hypothalamic PVN. A Newman-Keuls post hoc test shows that naloxone injection

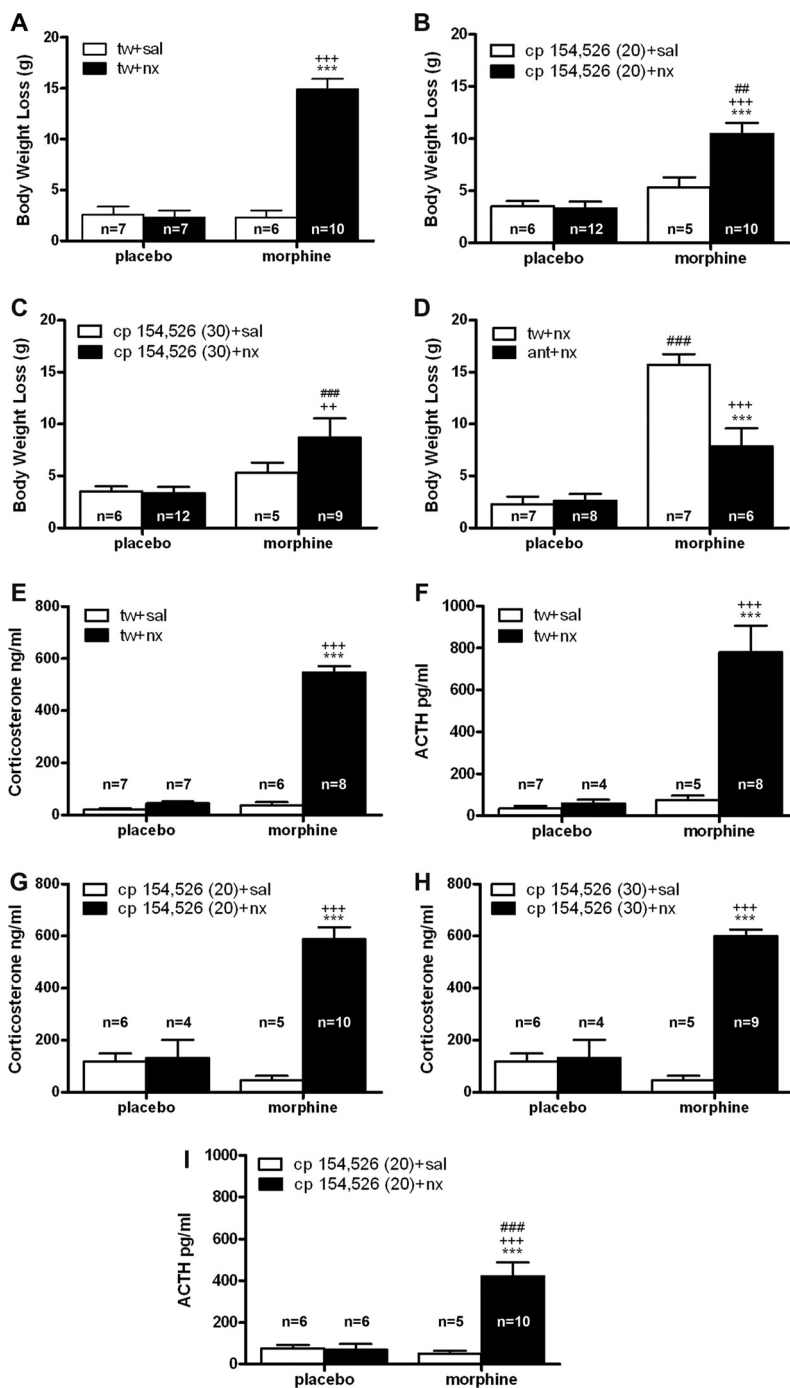


Fig. 2. CP-154526 attenuated the body weight loss (A–D) and the plasma adrenocorticotropin (F and I) but not the corticosterone (E, G, and H) response to naloxone-induced morphine withdrawal. Two-way ANOVA for body weight loss revealed a main effect of morphine pretreatment ($F_{1,26} = 45.81$; $p < 0.0001$), naloxone injection ($F_{1,26} = 45.11$; $p < 0.0001$), and a significant interaction between morphine pretreatment \times short-term treatment ($F_{1,26} = 49.40$; $p < 0.0001$). Two-way ANOVA for body weight loss after CP-154526 at the dose of 20 mg/kg (B) revealed a main effect of morphine pretreatment ($F_{1,31} = 23.52$; $p < 0.0001$), short-term treatment ($F_{1,31} = 7.20$; $p < 0.0116$), and a significant interaction between pretreatment and short-term treatment ($F_{1,31} = 8.20$; $p = 0.0074$). CP-154526 pretreatment at the dose of 30 mg/kg (C) 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 after antalarmin pretreatment showed a main effect of morphine pretreatment ($F_{1,24} = 81.31$; $p < 0.0001$), short-term treatment ($F_{1,24} = 13.31$; $p < 0.0013$), and a significant interaction between pretreatment and short-term treatment ($F_{1,24} = 15.82$; $p = 0.0006$). Two-way ANOVA for corticosterone and adrenocorticotropin revealed a main effect of morphine treatment (corticosterone: $F_{1,20} = 207.51$; $p < 0.0001$; adrenocorticotropin: $F_{1,20} = 18.03$; $p < 0.0004$), naloxone injection (corticosterone: $F_{1,20} = 220.31$; $p < 0.0001$; adrenocorticotropin: $F_{1,20} = 16.47$; $p = 0.0006$), and an interaction between pretreatment and short-term treatment (corticosterone: $F_{1,20} = 182.22$; $p < 0.0001$; adrenocorticotropin: $F_{1,20} = 14.23$; $p = 0.0012$). Two-way ANOVA for corticosterone in animals treated with CP-154526 revealed a main effect of pretreatment (20 mg/kg: $F_{1,21} = 14.70$; $p < 0.0010$; 30 mg/kg: $F_{1,20} = 32.09$; $p < 0.0001$), short-term 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 short-term treatment (20 mg/kg: $F_{1,21} = 28.24$; $p < 0.0001$; 30 mg/kg: $F_{1,20} = 60.46$; $p < 0.0001$). Two-way ANOVA for adrenocorticotropin in animals treated with CP-154526 showed a main effect of pretreatment ($F_{1,23} = 9.15$; $p = 0.0060$), short-term treatment ($F_{1,23} = 11.30$; $p = 0.0027$), and a pretreatment \times short-term treatment interaction ($F_{1,20} = 12.07$; $p = 0.0021$). Data represent the mean \pm S.E.M. of body weight loss and plasma adrenocorticotropin and corticosterone concentration 60 min after saline or naloxone injection to control pellet- or morphine-treated rats receiving vehicle, CP-154526 (cp), or antalarmin (ant) 30 min before saline (sal) or naloxone (nx) administration. ***, $p < 0.001$ versus control pellets + Tween (tw; 10% Tween 80 or 100% Tween 80) or CP-154526 (20 or 30 mg/kg) + naloxone. ++, $p < 0.01$ and +, $p < 0.001$ versus morphine + 10% Tween 80 or 100% Tween 80 + saline; ##, $p < 0.01$ and ###, $p < 0.001$ versus morphine + Tween (10% Tween 80 or 100% Tween 80) + naloxone.

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 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 Fig. 3 B–D, no alteration

TABLE 1

Effects of pretreatment with CP-154526 (20 and 30 mg/kg) and antalarmin on the incidence of irritability in morphine-withdrawn rats. Control pellet- or morphine-treated rats were injected with vehicle, CP-154526 at 20 or 30 mg/kg, or antalarmin (20 mg/kg) 30 min before naloxone 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.

Treatment	Irritability	
	5 min	10 min
Control/vehicle/naloxone	0/12	0/12
Morphine/vehicle/naloxone	6/6 ⁺⁺⁺	6/6 ⁺⁺⁺
Morphine/CP-154526 (20 mg/kg)/naloxone	9/15	0/15 ^{***}
Morphine/CP-154526 (30 mg/kg)/naloxone	5/9	0/9 ^{***}
Morphine/antalarmin/naloxone	7/10	0/10 ^{***}

***, $P < 0.001$ vs. morphine/vehicle/naloxone for 10 min; χ^2 .

+++, $P < 0.001$ vs. placebo/vehicle/naloxone for 5 and 10 min; χ^2 .

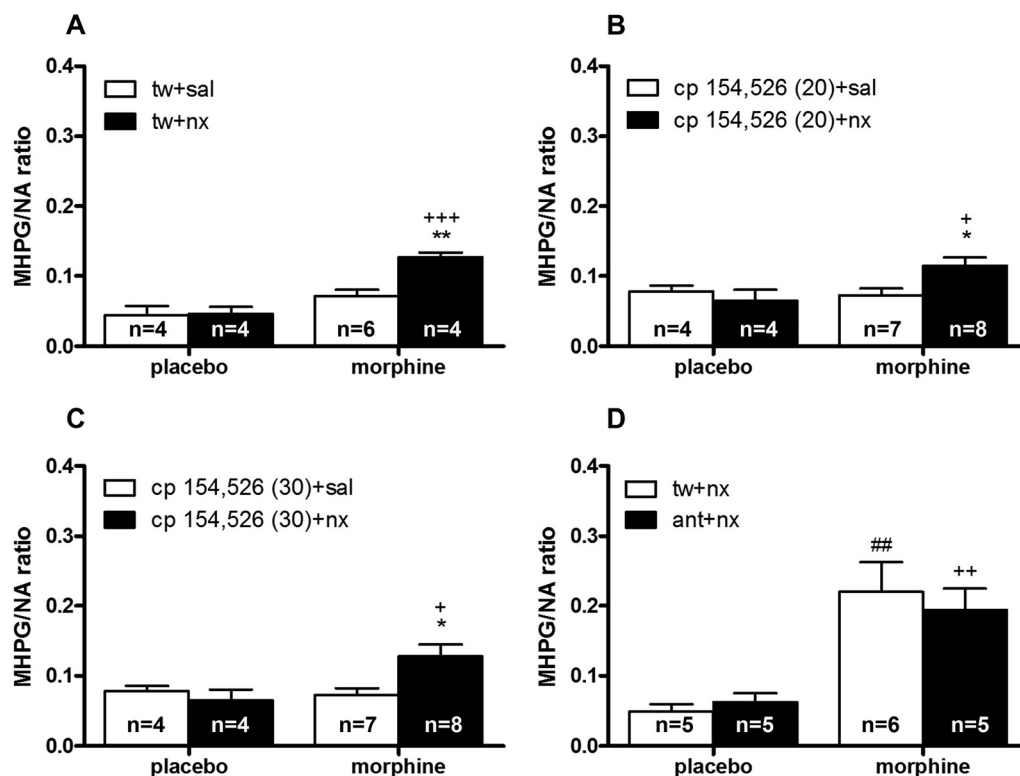


Fig. 3. Effects of CRF1R antagonists on the morphine withdrawal-induced increased NA turnover (as estimated by the MHPG/NA ratio) in control and morphine-dependent rats after administration of saline (sal) or naloxone (nx). Two-way ANOVA revealed a main effect on NA turnover for morphine pretreatment ($F_{1,14} = 28.30$; $p = 0.0001$), naloxone injection ($F_{1,14} = 8.19$; $p = 0.0126$), and a significant interaction between morphine pretreatment \times short-term treatment ($F_{1,14} = 7.21$; $p = 0.0178$). Neither CP-154526 (cp) nor antalarmin (ant) attenuated morphine withdrawal-induced increase in NA turnover. Two-way ANOVA for MHPG/NA ratio in animals treated with CP-154526 (20 or 30 mg/kg) showed a pretreatment \times short-term treatment interaction (20 mg/kg: $F_{1,19} = 4.84$, $p = 0.0403$; 30 mg/kg: $F_{1,19} = 4.77$, $p = 0.0418$). Two-way ANOVA for NA turnover in rats pretreated with antalarmin revealed a main effect of morphine pretreatment ($F_{1,17} = 29.29$; $p < 0.0001$). Data represent the mean \pm S.E.M. of NA turnover 60 min after saline or naloxone injection to control pellet- or morphine-treated rats receiving vehicle, CP-154526, or antalarmin 30 min before saline or naloxone administration. *, $p < 0.05$ and **, $p < 0.001$ versus control pellets + 10% Tween 80 or CP-154526 + naloxone. +, $p < 0.05$ and +++, $p < 0.001$ versus morphine + 10% Tween 80 or CP-154526 (20 or 30 mg/kg) + saline. ##, $p < 0.01$ versus control group + 100% Tween 80 + naloxone. ++, $p < 0.01$ versus control group + antalarmin + naloxone.

in NA turnover in PVN was found after naloxone-precipitated morphine withdrawal in rats pretreated with CP-154526 (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 of the brain stress system areas that have been studied: PVN, NTS-A₂, and CeA (Figs. 4 and 5). Naloxone-precipitated morphine withdrawal resulted in a robust induction of c-Fos immunoreactivity throughout all of the areas 60 min after naloxone injection. As shown in Fig. 4, naloxone-precipitated morphine withdrawal results in a dramatic increase in c-Fos staining in all four functionally distinct subdivisions of the PVN. Significant labeling 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 dorsal parvocellular subdivisions and the magnocellular division.

Pretreatment with CP-154526 (20 mg/kg i.p.) did not significantly modify the increase in c-Fos expression after naloxone-precipitated morphine withdrawal that was seen in NTS, CeA, and PVN (Figs. 4 and 5). In addition, c-Fos expression in placebo rats was significantly elevated over control levels at PVN in animals receiving CP-154526 (Fig. 4B).

Discussion

The principal findings of the present study are as follows: 1) CRF1R is expressed in the NTS-A₂ cell group; 2) CRF1R antagonists significantly decreased body weight loss and irritability during morphine withdrawal; 3) pretreatment with CRF1R antagonists did not block either the increased NA turnover or corticosterone release, whereas plasma adrenocorticotropin levels were significantly attenuated by CP-154526; and 4) the activation of the brain stress system (which includes, among other nuclei, the NTS-A₂, the CeA, and the PVN) after naloxone injection to morphine-dependent rats was not attenuated by CRF1R antagonist pretreatment.

Evidence for CRF1R mRNA within the 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 anxiolytic-like action

A

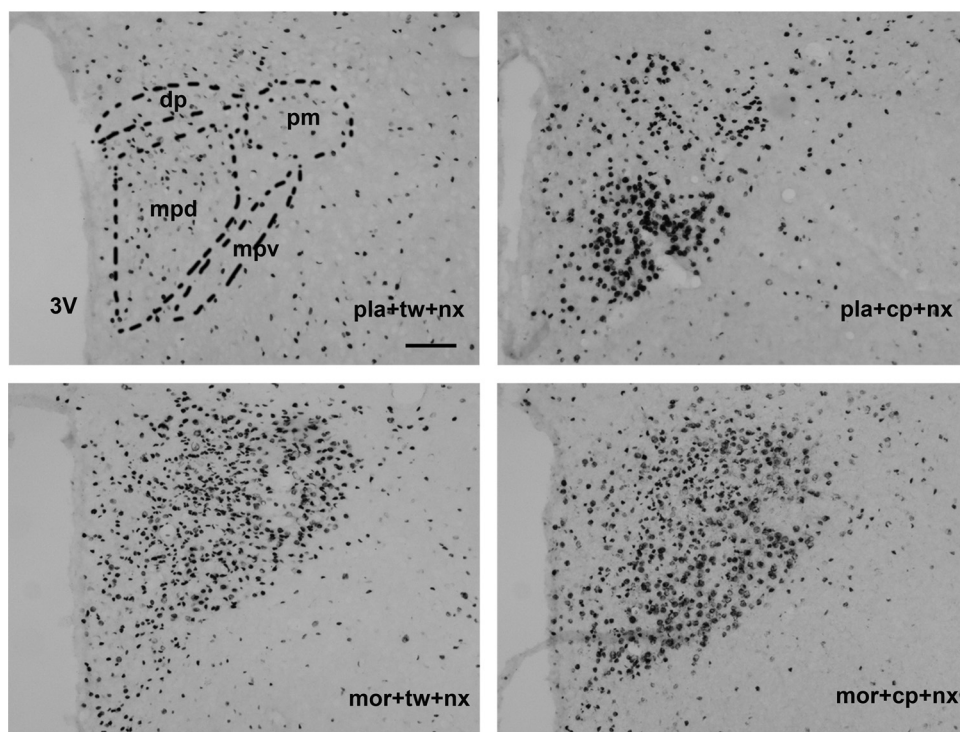
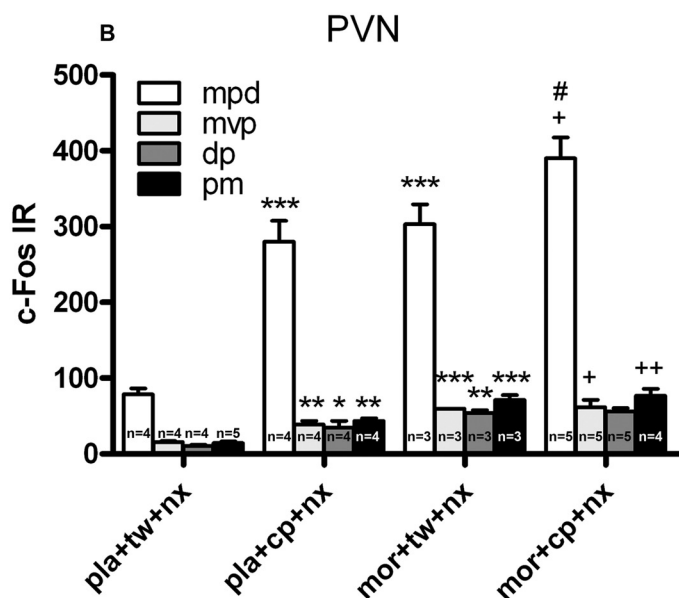


Fig. 4. CRF1R antagonists did not modify the neuronal activation of the hypothalamic PVN in response to morphine withdrawal. A, photomicrographs showing c-Fos immunoreactive cell nuclei in the PVN 60 min after naloxone (nx) injection to control pellet (pla)- or morphine (mor)-implanted rats receiving vehicle (10% Tween 80; tw) or CP-154526 (cp) 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 after naloxone-precipitated morphine withdrawal in both vehicle- and CP-154526-pretreated animal. B, the mean \pm S.E.M. values for c-Fos-positive nuclei at different PVN subdivisions. dp, dorsal parvocellular; mdp, ventral aspect of the medial parvocellular; pm, posterior magnocellular subdivision. 3V, third ventricle. Scale bar, 100 μ m. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus the corresponding group receiving control pellets + 10% Tween 80 + naloxone. #, $p < 0.05$ versus morphine + 10% Tween 80 + naloxone. +, $p < 0.05$ versus control pellets + CP-154526 + naloxone.



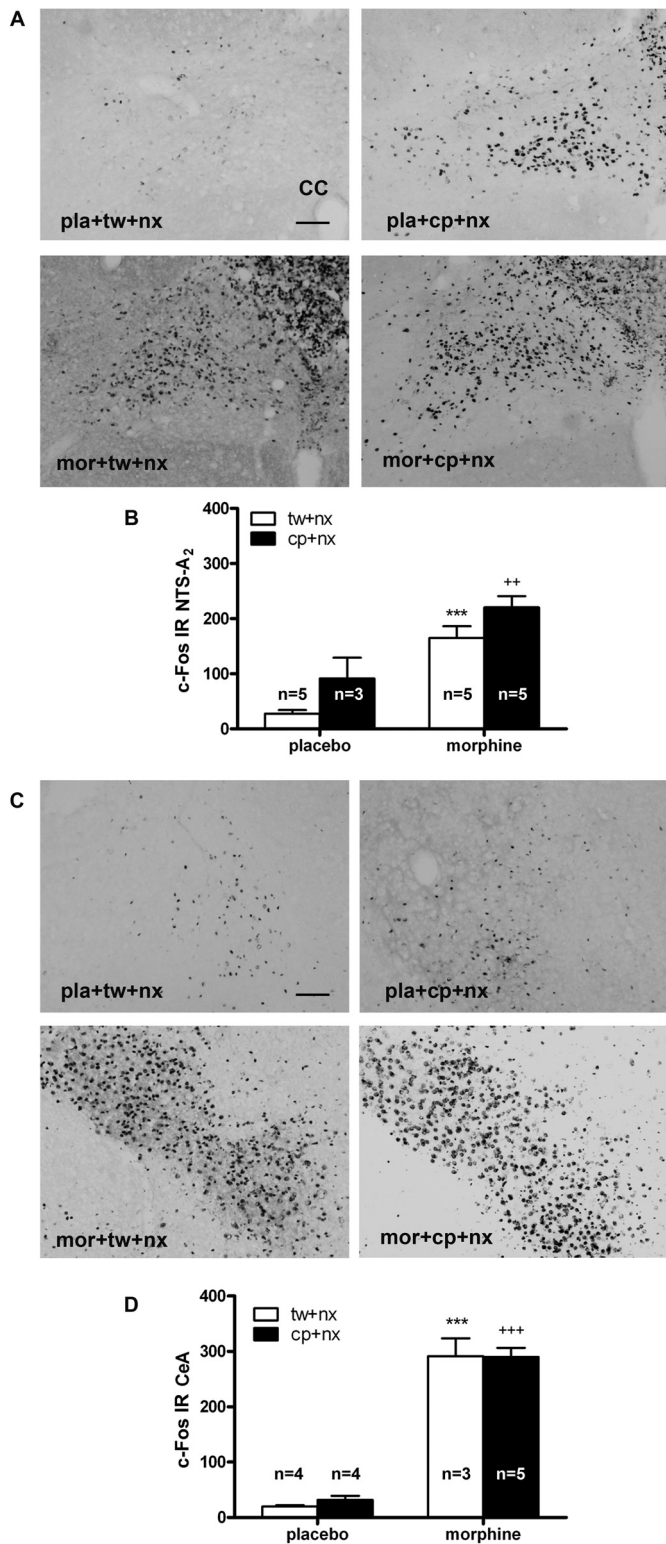


Fig. 5. Effects of CRF1R blockade on neuronal activation at selected brain sites in response to morphine withdrawal. A and C, representative photomicrographs show c-Fos immunoreactive cell nuclei 60 min after naloxone injection to control pellet (pla)- or morphine (mor)-implanted rats. Animals received vehicle (10% Tween 80, tw) or CP-154526 (cp; 20 mg/kg i.p.) 30 min before saline or naloxone (nx) administration. Specific induction of c-Fos staining was found in the NTS (A) and CeA (C). B and D, mean \pm S.E.M. 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 after naloxone-precipitated morphine withdrawal in both

of CRF1R antagonists (Contarino and Papaleo, 2005). Consistent with these studies, we observed that both CP-154526 and antalarmin suppressed the anxiety-like behavior during morphine withdrawal. In general, 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 during 30 min of observation. In addition, pretreatment of rats with CP-154526 or antalarmin significantly attenuated weight loss (another somatic sign of opiate withdrawal) in morphine-withdrawn rats. These results indicate that activation of the CRF1R subtype contributes to the somatic signs of opiate withdrawal and suggest that reduced body weight loss and irritability induced by pretreatment with CP-154526 are not related to nonspecific effects of this drug. These findings are consistent with different reports showing the potent anxiolytic-like activity of CRF1R antagonists (Lu et al., 2000; 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 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., 2002a,b; Smith and Aston-Jones, 2008). These effects depended 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). In addition, we reported that morphine withdrawal is associated with an increase in TH enzymatic activity in the PVN and TH phosphorylation (activation) at Ser31 and TH mRNA expression in the NTS-A₂ noradrenergic cell group (Núñez et al., 2007b, 2009), which occurred in parallel to increased heterogeneous nuclear RNA CRF and arginine-vasopressin (AVP) expression in the PVN (Núñez et al., 2007a). There is evidence supporting the idea that CRF neurons in the PVN innervate noradrenergic brain stem nuclei (Gray and Magnuson, 1987), and the existence of a NA-CRF loop has been proposed in which NA would stimulate the release of CRF in CeA, the PVN, and the bed nucleus stria terminalis. CRF from these nuclei would induce the release of NA by the brain stem noradrenergic areas (Koob, 1999). The results of the present study demonstrate that pretreatment with the selective CRF1R antagonists CP-154526 or antalarmin did not block increased noradrenergic activity in the PVN during morphine withdrawal. These findings suggest that the activation of the CRF1R subtype is not re-

vehicle- and CP-154526-pretreated animals. CC, canal central. Scale bar, 100 μ m. ***, $p < 0.001$ versus control pellets + 10% Tween 80 + naloxone. ++, $p < 0.01$ and +++, $p < 0.001$ versus control pellets + CP-154526 + naloxone.

sponsible 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 [2-[N-(2-methylthio-4-isopropylphenyl)-N-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidine], 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.

The present findings show that elevated plasma glucocorticoid and adrenocorticotropin levels in morphine-withdrawn rats occur in parallel with enhanced NA turnover in the PVN, which provides evidence that the HPA axis was activated as a consequence of morphine withdrawal. Our results show that pretreatment with CP-154526 did not block the corticosterone release that is produced as a consequence of morphine withdrawal. However, adrenocorticotropin concentrations were found to be decreased in animals pretreated with CP-154526 (20 mg/kg i.p.). The relationship between HPA activity and drug withdrawal-induced behavior 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 was decreased by antalarmin, whereas this antagonist did not antagonize 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 behavioral responses to lorazepam withdrawal but not some of the neuroendocrine effects. Thus, according to present results, the CRF1R antagonist R121919 (3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-N,N-dipropyl-pyrazolo[2,3-*a*]pyrimidin-7-amine) attenuated the behavioral and autonomic signs of lorazepam withdrawal and adrenocorticotropin secretion, whereas corticosterone levels were not altered (Skelton et al., 2007). Furthermore, in clinical studies CRF1R antagonists produced anxiolytic-like effects in depressed patients in the absence of effects on CRF-stimulated HPA axis activity (Zobel et al., 2000). An additional potential explanation for the present findings is that, although CRF is thought to be the major secretagogue in stimulating adrenocorticotropin 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 the pituitary-adrenal response to drug withdrawal and stress (Deak et al., 1999). Taking into account the above findings, it is possible that the pituitary-adrenal response is not related to the behavioral signs of precipitated opiate withdrawal in rats.

It is well known that CRFRs, in particular the CRF1R subtype, are widely distributed in the brain and anterior pituitary gland. Mice and rats seem to show comparable distribution, whereas density of immunoreactivity differs in a structure-dependent manner (Van Pett et al., 2000). This differential distribution of CRF1R and CRF2R indicates that these receptors exert different functions that are controlled

by different mechanisms. According to the distribution of CRFRs, the results of the present study would suggest that the effects of CRF1R antagonists were at the anterior pituitary level (attenuating adrenocorticotropin release), but not at NTS, where a robust hybridization signal for CRF2R mRNA has also been found (Van Pett et al., 2000). Taken together, these findings suggest that the CRF2R subtype might mediate the activation of the NA system innervating the PVN during morphine withdrawal.

According to previous data from our laboratory (Laorden et al., 2002a,b; Núñez et al., 2007a), c-Fos expression was induced in several nuclei of the brain stress system (NTS, CeA, and PVN) 60 min after naloxone-induced morphine withdrawal. The activation of the 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 depends on increased NA release (Laorden et al., 2002b). In the present study, pretreatment with CP-154526 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. Because all of 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-154526. 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 indicates that systemically bioavailable and selective CRF1R antagonists attenuated the irritability and weight-loss signs of opiate withdrawal and adrenocorticotropin 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. Further research is needed to determine the regions in which CRF1R signaling modulates adrenocorticotropin release associated with opiate withdrawal and whether the CRF2R subtype plays a role.

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