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RESEARCH PAPER

Corticotropin-releasing factor (CRF) receptor-1 is involved in cardiac noradrenergic activity observed during naloxone-precipitated morphine withdrawal

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BACKGROUND AND PURPOSE

The negative affective states of withdrawal involve the recruitment of brain and peripheral stress circuitry [noradrenergic activity, induction of the hypothalamic–pituitary–adrenocortical (HPA) axis and activation of heat shock proteins (Hsps)]. Corticotropin-releasing factor (CRF) pathways are important mediators in the negative symptoms of opioid withdrawal. We performed a series of experiments to characterize the role of the CRF_1 receptor in the response of stress systems to morphine withdrawal and its effect in the heart using genetically engineered mice lacking functional CRF_1 receptors.

EXPERIMENTAL APPROACH

Wild-type and CRF_1 receptor-knockout mice were treated with increasing doses of morphine. Precipitated withdrawal was induced by naloxone. Plasma adrenocorticotropic hormone (ACTH) and corticosterone levels, the expression of myocardial Hsp27, Hsp27 phosphorylated at Ser^{82}, membrane (MB)- COMT, soluble (S)-COMT protein and NA turnover were evaluated by RIA, immunoblotting and HPLC.

KEY RESULTS

During morphine withdrawal we observed an enhancement of NA turnover in parallel with an increase in mean arterial blood pressure (MAP) and heart rate (HR) in wild-type mice. In addition, naloxone-precipitated morphine withdrawal induced an activation of HPA axis and Hsp27. The principal finding of the present study was that plasma ACTH and corticosterone levels, MB-COMT, S-COMT, NA turnover, and Hsp27 expression and activation observed during morphine withdrawal were significantly inhibited in the CRF_1 receptor-knockout mice.

CONCLUSION AND IMPLICATIONS

Our results demonstrate that CRF/CRF_1 receptor activation may contribute to stress-induced cardiovascular dysfunction after naloxone-precipitated morphine withdrawal and suggest that CRF/CRF_1 receptor pathways could contribute to cardiovascular disease associated with opioid addiction.

Abbreviations

ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; Hsp27, Heat shock protein 27; HPA, hypothalamic–pituitary–adrenocortical; MAP, mean arterial BP; NMN, normetanephrine; PVN, paraventricular nucleus; TBST, tris buffer saline tween

Introduction

Morphine belongs to the most commonly used opioids in medical practice due to its strong analgesic effects. However, sustained administration of morphine can lead to the development of opioid addiction and causes complex adaptive changes in opioid receptors and associated signalling systems, leading to alterations in neuronal plasticity in the brain regions projecting to different systems including the cardiovascular system. The hypothalamic–pituitary–adrenocortical (HPA) axis, a system largely controlled by corticotropin-releasing factor (CRF), is an important adaptive mechanism in morphine withdrawal (Koob and Kreek, 2007; Koob and Le Moal, 2008; Nuñez *et al.*, 2010; Ueno *et al.*, 2011). The paraventricular nucleus (PVN) of the hypothalamus projects to autonomic nuclei in the brain stem and spinal cord and is responsible for the activation of the sympathetic nervous system, including cardiovascular regulation (Sawchenko and Swanson, 1982). In addition, the PVN receives afferent projections from several limbic structures that are implicated in behavioural and cardiovascular control, such as the medial amygdala, the prefrontal cortex and the lateral septum (Risold and Swanson, 1997; Ongur *et al.*, 1998). Therefore, adaptive changes induced by drugs of abuse not only affect the brain, but also peripheral tissues and cells expressing opioid receptors, such as the heart (Pugsley, 2002).

In addition to the neurobehavioural consequences of opiate addiction, there is a strong association between drug addiction and cardiac disorders. There are studies in the literature supporting mainly the effect of cocaine abuse (Aquaro *et al.*, 2011; Basso *et al.*, 2011) and less that of heroin abuse. However, heroin and other opiates can cause arrhythmias and non-cardiac pulmonary oedema and may reduce cardiac output (Frishman *et al.*, 2003). It has been postulated that heroin affects the sinus node and the surrounding nerves, ganglia and atria myocardium replacing their tissues with quantities of fatty and/or fibrous tissues. These changes may result in a dysfunction of the aforementioned structures and probably in the origin of different types of arrhythmias, which are partly responsible for the sudden cardiac death observed in heroin addicts (Nerantzis *et al.*, 2011). Despite the clinical relevance of an association between addiction and cardiovascular disorders, little is known about the pathophysiology or mediators underlying this comorbidity. The majority of cardiology studies were oriented on clinical usage of this drug and current cardiovascular research has been limited to the evaluation of factors or pathways believed to contribute to its physiological actions (Jiang *et al.*, 2006; Xu *et al.*, 2011). So, investigation about the mechanisms implicated in the cardiac adaptive changes that occur during morphine withdrawal deserve more attention.

Like stress, morphine withdrawal leads to the activation of two systems: the catecholaminergic system and the HPA axis. Activation of the former, results in enhanced circulating catecholamines levels that can damage the heart (Kasch, 1987). All major drugs of abuse stimulate the HPA axis, during acute withdrawal via the activation of CRF in PVN, with a common response of elevated adrenocorticotropic hormone (ACTH) and corticosterone (Koob and Le Moal, 2008; Ueno *et al.*, 2011), which mediate somatic and negative affective-like components of withdrawal (Contarino and Papaleo, 2005; Harris and Aston-Jones, 2007; Papaleo *et al.*, 2007; Koob, 2008). CRF exerts its actions through activation of two different types of GPCRs: CRF_1 and CRF_2, which are distributed throughout the periphery and the brain (De Souza, 1995; Bale and Vale, 2004). CRF_1 receptor expression has been demonstrated in several key brain areas involved in reward, reinforcement, craving and aversive effects of drugs of abuse (Korosi *et al.*, 2006). Moreover, the decreased brain reward function associated with drug withdrawal is CRF_1 receptor-dependent (Bruijnzeel *et al.*, 2009; Koob, 2010). Although, under normal physiological conditions, the CRF_1 receptor only has a scant presence in the heart (Kishimoto *et al.*, 1995), its levels are up-regulated in cardiac diseases (Yuan *et al.*, 2010). In addition, it has been postulated that CRF_1 receptor activation may contribute to stress-induced cardiovascular dysfunction (Wood *et al.*, 2012).

Despite the extensive research supporting the role of CRF in drug addiction, the specific participation of CRF in drug-induced cardiac alterations remains undetermined. Because stress is a common risk factor for both addiction and cardiovascular disorders, investigating the neuronal pathways or substrates that mediate the stress response may provide clues to the shared pathophysiology that links addiction and cardiovascular disease. Therefore, in this study, we have evaluated the role of CRF/ CRF_1 receptor pathways in the activation of: (i) cardiac sympathetic pathways, (ii) HPA axis and (iii) heat shock protein 27 (Hsp27) that occur after morphine withdrawal, using genetically engineered mice lacking functional CRF_1 receptors.

Methods

Subjects

All surgical and experimental procedures were performed in accordance with the European Communities Council Directive 24 November 1986 (86/609EEC) and the local Animal Ethics Committee. Adult male B6, 129 CRH^{tklee} mice (25–30 g at the beginning of the experiments) that were wild-type ($CRF_1R+/+$) and recessive homozygous or knockout ($CRF_1R-/-$) were housed six per cage. The total number of

mice used was 90. Mice were 4–8 months old and derived from mating CRF_1R+/– breeders (Jackson Laboratory, Jackson, CA, USA). Wild-type and CRF_1R–/– offspring from CRF_1R+/– breeders were identified by PCR analysis of tail DNA. The mice colony room was maintained under a 12 h light/dark cycle (lights on from 0800 until 2000 h) at 22 ± 2°C. Food and water were available *ad libitum*. They were handled daily during the week preceding the experiment start to minimize stress. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Drug treatment and experimental procedure

For 4 consecutive days, wild-type and CRF_1R–/–mice were treated every 12 h (at 0800 and 2000 h) with saline or increasing doses of morphine (10–60 mg·kg^{-1}, i.p) according to the following protocol: day 1, 10 mg·kg^{-1}; day 2, 30 mg·kg^{-1}; day 3, 50 mg·kg^{-1}; day 4, 60 mg·kg^{-1}. On day 4 and 1 h after last morphine injection, naloxone or saline was administered s.c. at a dose of 1 mg·kg^{-1} to the mice treated chronically with saline or morphine.

The animals undergoing morphine withdrawal displayed characteristic withdrawal symptoms. Loss of body weight was calculated as the difference between the body weight determined immediately before saline or naloxone injection and a second determination made 60 min later. After that, the animals were killed by decapitation, the chest was opened with a midsternal incision and the left ventricle dissected and stored at –80°C to determined NA turnover, COMT and total Hsp expression and phosphorylation at Ser^{82}.

Haemodynamic variables

Another group of animals were treated with the same morphine protocol and anaesthetized with thiopental sodium (40 mg·kg^{-1}, i.p.), 30 min after the last dose of morphine, intubated and placed on a heated table to maintain body temperature at 37°C. The tail reflex was used to assess the depth of anaesthesia. A polyethylene cannula (PE-50) was placed in the right femoral artery for measuring haemodynamic variables. Catheters were connected to pressure transducers (L969-A07 Abbott Ireland, Sligo, Rep. of Ireland) and BP and HR were monitored on a PowerLab8/30 (ADInstruments, Pty Ltd., Oxford, UK) and analysed with LabChart software (ADInstruments, Pty Ltd.). Naloxone (1 mg·kg^{-1}) was injected s.c. after a 30 min stabilization period and its effect on mean arterial BP (MAP) and HR was evaluated in mice treated with saline or morphine.

Determination of NA and its metabolite normetanephrine (NMN) in the left ventricle

NA and NMN, extraneuronal NA metabolite generated by COMT, were determined by HPLC with electrochemical detection. Each tissue was weighed, placed in a dry-cooled propylene vial and homogenized. The homogenates were centrifuged (8000 x *g*, 4°C), the supernatant layer was removed into a 1 mL syringe and filtered through a 0.45 µm filter (Millipore, Bedford, MA, USA) and centrifuged (6000 x *g*, 4°C) again through Ultra free MC 0.2 filter (Millipore). From each sample, 10 µL was injected into a 5 µm C_{18} reverse phase column (Waters, Milford, MA, USA) through a Rheodyne syringe-loading injector 200 µL loop. The mobile phase consisted of a 95% (v/v) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), L-octyl-sodium sulfonate (3.75 mM), di-n-butylamine (1 mM) and EDTA (0.135 mM), adjusted to pH 4.3. Chromatographic data were analysed with Millenium 2010 Chromatography Manager Equipment (Millipore). NA and NMN were simultaneously detected at an elution time of 4.25 and 7.32 min respectively. NA and NMN were quantified by reference to calibration curves run at the beginning and at the end of each series of assays. The content of NA and NMN in the left ventricle was expressed as ng g^{-1} tissue weight.

Western blot analysis

Western blot analysis was performed for total Hsp27, Hsp27 phosphorylated at Ser^{82} and COMT protein determination. Samples were placed in homogenization buffer [PBS, 2 % SDS plus protease inhibitors (Roche, Mannheim, Germany) and phosphatase inhibitors Cocktail Set (Calbiochem, Darmstadt, Germany)], homogenized and centrifuged at 6000 x *g* at 4°C. Equal amounts of protein (50 µg per lane) from each sample were loaded on a 10% SDS-PAGE, electrophoresed and transferred onto a PVDF membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Lab, Hercules, CA, USA). Non-specific binding of antibodies was prevented by incubating membranes with 1% BSA in tris buffered saline with Tween (TBST: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20). Blots were incubated overnight with the following primary antibodies: polyclonal anti-total Hsp27 antibody (1:500; sc-1048, Santa Cruz Biotecnology, Santa Cruz, CA, USA); polyclonal anti-phospho Ser^{82} Hsp27 (1:400 dilution; ab39399, Abcam Ltd., Cambridge, UK) and monoclonal anti-COMT (1:5000; AB5873, Chemicon International, Temecula, MA, USA) in TBST with BSA. After extensive washings with TBST, the membranes were incubated for 1 h, at room temperature, with peroxidase-labelled secondary antibodies (anti-goat sc-2350 for total Hsp27; anti-rabbit sc-2004 for Hsp27 phosphorylated at Ser^{82}; anti-mouse sc-2005 for COMT, Santa Cruz) at 1:5000 dilution. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent Western blot detection system (ECL Plus, GE Healthcare, Middlesex, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2), for 1 h at 37°C. We used anti-β actin (45 kDa, Cell Signaling Technology, Inc., Beverly, MA, USA) as our loading control for all the experiments. The ratio of total Hsp27/β-actin, phospho-Hsp27/β-actin, phospho-Hsp27/total-Hsp27 and COMT/β-actin was plotted and analysed. Quantification of immunoreactivity bands corresponding to total Hsp27 (27 kDa), Hsp27 phoshorylated at Ser^{82} (27 kDa), soluble-COMT (S-COMT) and membrane-COMT (MB-COMT) (25 and 30 kDa respectively) was carried out by densitometry (AlphaImager, Nucliber, Madrid, Spain). Experimental and control samples were included in the same blots and relative variations between bands were calculated in the same image.

RIA

Plasma ACTH and corticosterone concentrations were measured 1 and 24 h after saline or naloxone injection by commercially available kits for rats ([^{125}I]-ACTH and [^{125}I]-corticosterone RIA; MP Biomedicals, Solon, OH, USA). The sensitivity of the assay was 5.7 pg·mL^{-1} for ACTH and 7.7 ng·mL^{-1} for corticosterone.

Drugs and chemicals

Morphine HCl (Alcaliber, Madrid, Spain); SDS-PAGE and PVDF membrane were obtained from Bio-Rad Laboratory (Teknovas, Bilbao, Spain). NA bitartrate, NMN (used as an HPLC standard), naloxone HCl and Western blot reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA). Morphine HCL and naloxone HCl were dissolved in sterile 0.9 % NaCl (saline) and administered in volumes of 0.1 mL 100 g^{-1} body weight.

Data analysis

Data are expressed as mean ± SEM. Two-way ANOVA with genotype (wild-type and CRF_1R–/– mice) and chronic treatment (saline or morphine) as independent variables was used to examine body weight gain, basal MAP and basal HR. Three-way ANOVA with genotype, chronic and acute treatment (saline or naloxone) as independent factors was used to examine body weight loss, NA and NMN content, NA turnover, MB-COMT, S-COMT, total Hsp expression and Hsp phosphorylation at Ser^{82}. Newman–Keuls *post hoc* test was used to indentify individual mean differences. Haemodynamic variables (Δ MAP and Δ HR), ACTH and corticosterone levels were analysed by repeated measures ANOVA and Bonferroni's multiple comparison *post hoc* test. Differences with a *P*-value < 0.05 were considered significant.

Nomenclature

Drug/molecular target nomenclature conforms to BJP's *Concise Guide to PHARMACOLOGY* (Alexander *et al.*, 2013).

Results

The weight of the animals was checked as it is known that chronic morphine treatment induces a decrease in body weight gain due to a lower caloric intake. The weight of the mice was recorded on the days of morphine or saline injection and on the day of killing. Two-way ANOVA for body weight gain revealed a main effect of morphine treatment ($F_{1,44}$ = 194.57, P < 0.0001), but there was neither significant genotype effects ($F_{1,44}$ = 0.70, P = 0.4060) nor significant interaction between genotype and morphine treatment ($F_{1,44}$ = 0.00, P = 0.9983). Our results showed that wild-type and CRF_1 receptor knockout mice receiving morphine treatment had a significantly (P < 0.001) lower body weight than animals receiving saline injection (Figure 1A). The present results are in agreement with previous studies (Papaleo *et al.*, 2007), which demonstrated that loss of CRF_1 receptors did not affect body weight reduction induced by escalating morphine doses.

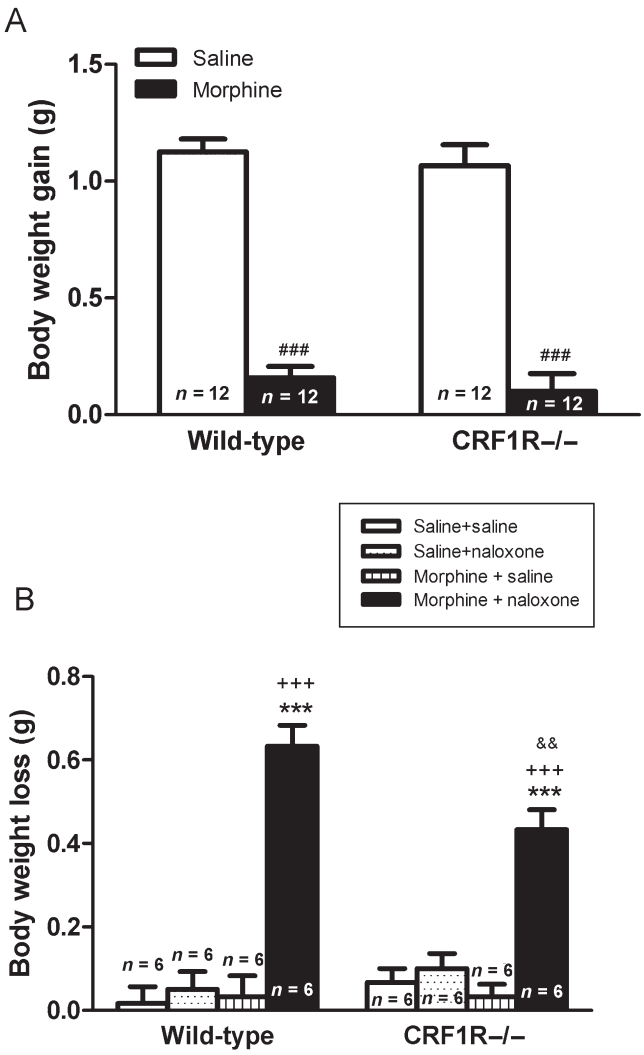

Figure 1

(A) Effect of saline or morphine injection on body weight gain in wild-type or CRF_1 receptor knockout (CRF/–) mice. The animals received increasing doses of morphine (10–60 mg·kg^{-1}, i.p.) or saline every 12 h for four days. (B) Effect of naloxone (1 mg·kg^{-1}, s.c.) injection on body weight loss in wild-type and CRF_1 receptor knockout mice treated with morphine or saline. Data are the mean ± SEM. ###P < 0.001 versus saline; ***P < 0.001 versus morphine + saline; +++P < 0.001 versus saline + naloxone; &&P < 0.01 versus wild-type mice.

Body weight loss after naloxone-precipitated morphine withdrawal

Because body weight loss induced by naloxone-precipitated withdrawal is an objective and accurate measurable sign of opioid withdrawal, we evaluated the effect of loss of CRF_1 receptors on this parameter. Three-way ANOVA revealed significant main effects on body weight loss for chronic morphine treatment ($F_{1,24}$ = 28.063, P = 0.0005), withdrawal (naloxone injection; $F_{1,24}$ = 115.148, P = 0.0005) and genotype ($F_{1,24}$ = 5.332, P = 0.026). In addition, there was interaction between the factors of genotype × withdrawal ($F_{1,24}$ = 11.531, P = 0.002) and chronic morphine treatment × withdrawal ($F_{1,24}$ = 40.756,

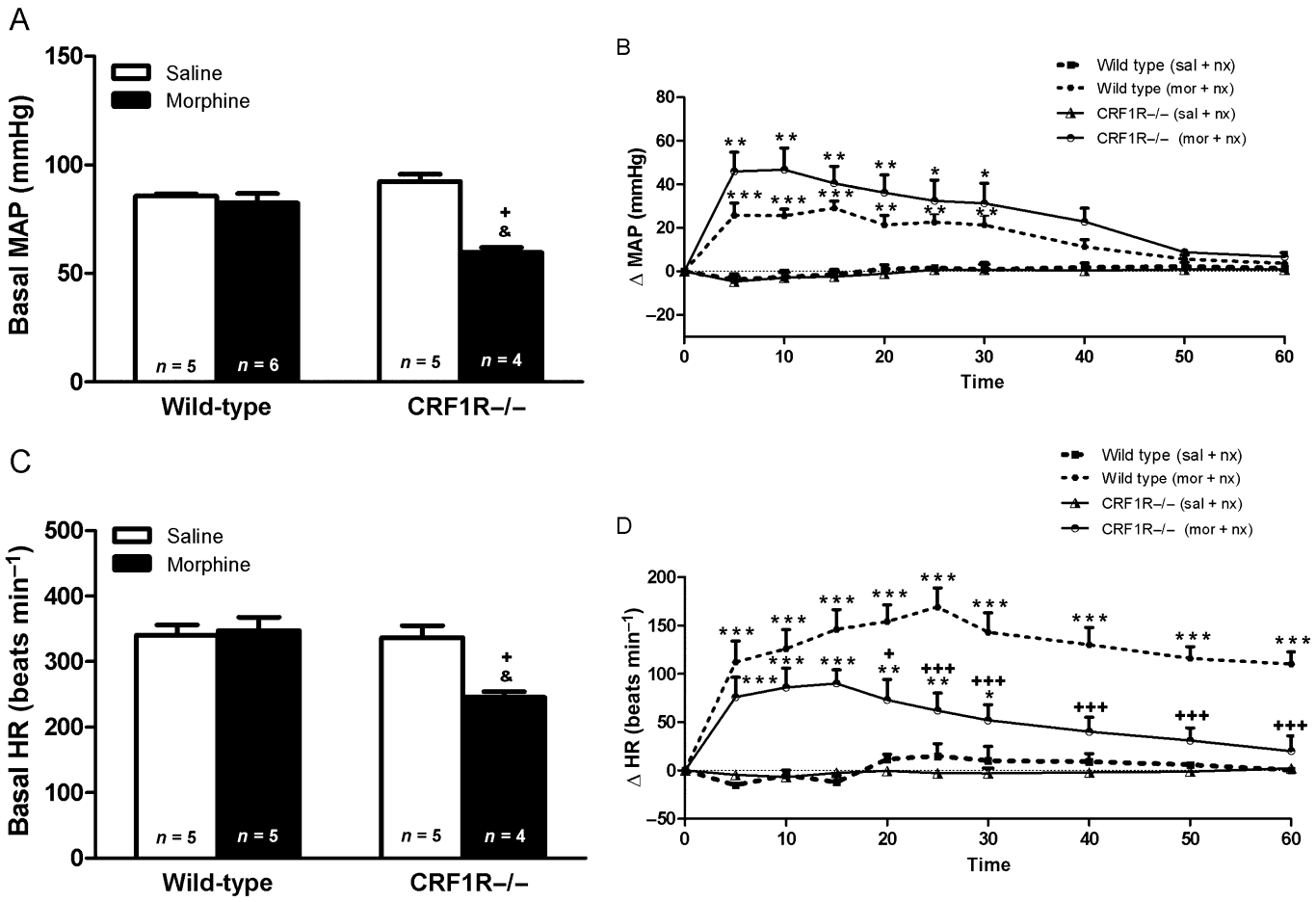

Figure 2

Baseline MAP (mmHg) (A) and HR (beats·min^{-1}) (C) in wild-type and CRF$_1$ receptor knockout mice treated with saline or morphine (A, C). Effects of naloxone (1 mg·kg^{-1} s.c.) on changes in MAP and HR rate in wild-type and CRF$_1$ receptor-deficient mice (B, D). Naloxone was injected at time 0. Data are the mean ± SEM (n = 4–5). *P < 0.05, **P < 0.01, ***P < 0.001 versus saline + naloxone; + P < 0.05, +++P < 0.001 versus wild-type mice; &P < 0.05 versus saline.

P = 0.0005). Naloxone injection to wild-type morphine-treated mice produced a significant (P < 0.001) increase in body weight loss. A significant (P < 0.001) increase in body weight loss after naloxone injection to morphine-treated mice was also observed in CRF$_1$ receptor knockout mice. However, the weight loss in morphine-withdrawn CRF$_1$R–/– animals was significantly (P < 0.01) less than that observed in wild-type animals after naloxone-precipitated morphine withdrawal (Figure 1B).

Haemodynamic changes after naloxone-induced morphine withdrawal

We analysed the effects of morphine withdrawal on MAP and HR in wild-type and CRF$_1$R–/– mice to determine the involvement of CRF$_1$ receptors in the haemodynamic changes observed after morphine withdrawal. Two-way ANOVA for basal MAP and HR revealed significant main effect of chronic morphine treatment (MAP: $F_{1,16}$ = 29.69, P < 0.0001; HR: $F_{1,15}$ = 6.21, P = 0.0249), genotype (MAP: $F_{1,16}$ = 5.82, P = 0.02.820; HR: $F_{1,15}$ = 9.68, P = 0.0071), and significant interaction between the factors genotype × chronic morphine treatment (MAP: $F_{(1,16)}$ = 20.19, P = 0.0004; HR: $F_{1,15}$ = 8.34, P = 0.0113). *Post hoc* test showed that the basal MAP and HR were significantly (P < 0.05) decreased in CRF$_1$R–/– mice treated chronically with morphine versus saline (control) group or morphine-treated wild-type mice (P < 0.05). However, there were not changes in the wild-type mice after chronic morphine administration versus saline group (Figure 2A, C).

Repeated measures ANOVA of MAP and HR revealed significant main effect of treatment (MAP: $F_{1,14}$ = 52.81, P < 0.0001; HR: $F_{1,16}$ = 26.222, P < 0.0001). Naloxone administration to morphine-treated CRF$_1$ receptor knockout or wild-type mice induced an immediate and significant increase (P < 0.05, P < 0.01, P < 0.001) in MAP (5–30 min) versus mice chronically treated with saline and injected with naloxone (Figure 2B). Five minutes after naloxone administration to wild-type mice treated with morphine, there was also a significant (P < 0.001) enhancement of HR. The time of peak effect was 25 min post-naloxone injection, although HR remained (P < 0.001) increased for 60 min after naloxone (Figure 2D). CRF$_1$ receptor knockout mice also showed an increase in HR after

naloxone administration to morphine-treated mice (5–30 min). However, the enhancement of HR observed in this group was significantly ($P < 0.05$, $P < 0.001$) less than that observed in wild-type mice (Figure 2D).

NA turnover in the left ventricle

Additional experiments were performed to evaluate the response of noradrenergic pathways innervating the heart in morphine withdrawal wild-type and CRF$_1$ receptor deficient mice. Three-way ANOVA for NA in the left ventricle showed significant main effects of genotype ($F_{1,20} = 4.216$, $P = 0.050$), genotype × naloxone injection (withdrawal; $F_{1,20} = 5.300$, $P = 0.028$) and genotype × chronic morphine treatment × withdrawal interaction ($F_{1,20} = 5.272$, $P = 0.028$). Three-way ANOVA examining effects of genotype, chronic morphine and acute naloxone on NMN concentration and NMN/NA ratio showed significant main effects of chronic drug treatment (NMN: $F_{1,20} = 13.691$, $P = 0.001$; NMN/NA ratio: $F_{1,20} = 19.069$, $P = 0.0005$), genotype (NMN: $F_{1,20} = 6.404$, $P = 0.01$; NMN/NA ratio: $F_{1,20} = 19.516$, $P = 0.0005$), acute treatment (NMN/NA ratio: $F_{1,20} = 13.100$, $P = 0.001$), genotype × chronic treatment (NMN/NA ratio: $F_{1,20} = 10.719$, $P = 0.003$), genotype × acute treatment (NMN/NA ratio: $F_{1,20} = 16.700$, $P = 0.0005$), chronic treatment × acute treatment (NMN/NA ratio: $F_{1,20} = 8.063$, $P = 0.008$) and genotype × chronic treatment × acute treatment interaction (NMN: $F_{1,20} = 5.825$, $P = 0.022$; NMN/NA ratio: $F_{1,20} = 20.450$, $P = 0.0005$). *Post hoc* tests revealed that there was an increase ($P < 0.01$, $P < 0.001$, $P < 0.05$) of NMN content and NMN/NA ratio (as index of NA turnover) concomitantly with a decrease in NA content in wild-type mice 24 h after naloxone-precipitated morphine withdrawal (Figure 3A–C). However, CRF$_1$R–/– mice showed a significant increase in NA content concomitantly with a decrease ($P < 0.01$, $P < 0.001$) in NMN content and NA turnover after naloxone induced withdrawal compared to that in wild-type mice.

Three-way ANOVA for MB-COMT expression revealed a significant main effect of genotype ($F_{1,24} = 7.048$, $P = 0.011$), naloxone injection ($F_{1,24} = 7.985$, $P = 0.007$) and there was interaction between the factors of chronic morphine treatment × acute naloxone injection (withdrawal; $F_{1,24} = 7.647$, $P = 0.009$), genotype × chronic treatment ($F_{1,24} = 5.271$, $P = 0.027$) and genotype × chronic treatment × acute withdrawal ($F_{1,24} = 7.647$, $P = 0.009$) interactions. Three-way ANOVA for S-COMT expression showed a main effect of morphine chronic treatment ($F_{1,24} = 10.295$, $P = 0.03$). Overall there was an increase in MB-COMT ($P < 0.001$) and S-COMT ($P < 0.01$) in wild-type mice after naloxone-precipitated morphine withdrawal whereas CRF$_1$ receptor knockout mice showed a significant decrease in MB-COMT ($P < 0.001$) and S-COMT ($P < 0.01$) compared to wild-type mice (Figure 4A,B).

Expression of Hsp27 and phospho-Hsp27 in chronic morphine treatment and withdrawal

We examined Hsp27 expression and phospho-Hsp27 at Ser^{82}, which is highly expressed in the heart (Kato *et al.*, 1992), to determine the magnitude and severity of cellular stress during chronic morphine treatment and withdrawal. Three-way ANOVA for Hsp27 expression revealed a main effect of genotype ($F_{1,22} = 8.382$, $P = 0.006$), chronic morphine treatment ($F_{1,22} = 32.904$, $P = 0.0005$) and genotype × chronic

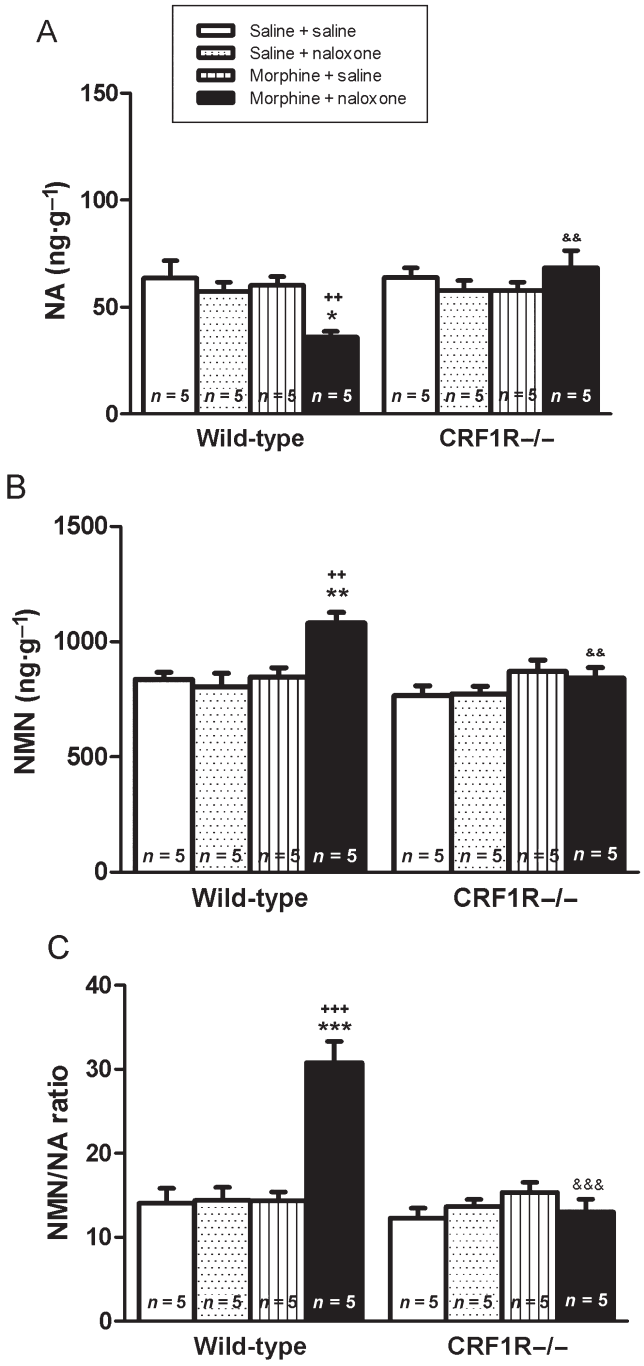

Figure 3

NA (A), NMN (B) content and NMN/NA ratio (C) in left ventricle after naloxone or saline administration to placebo (saline) or morphine-treated wild-type and CRF$_1$ receptor knockout mice. Data are the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus morphine+saline; ++$P < 0.01$, +++$P < 0.001$ versus saline + naloxone; &&$P < 0.01$, &&&$P < 0.001$ versus wild-type mice.

treatment ($F_{1,22} = 4.352$, $P = 0.044$). *Post hoc* revealed that in wild-type mice chronic treatment with morphine and its withdrawal induced an increase ($P < 0.05$, $P < 0.01$) in Hsp27 expression in the left ventricle compared with the corre-

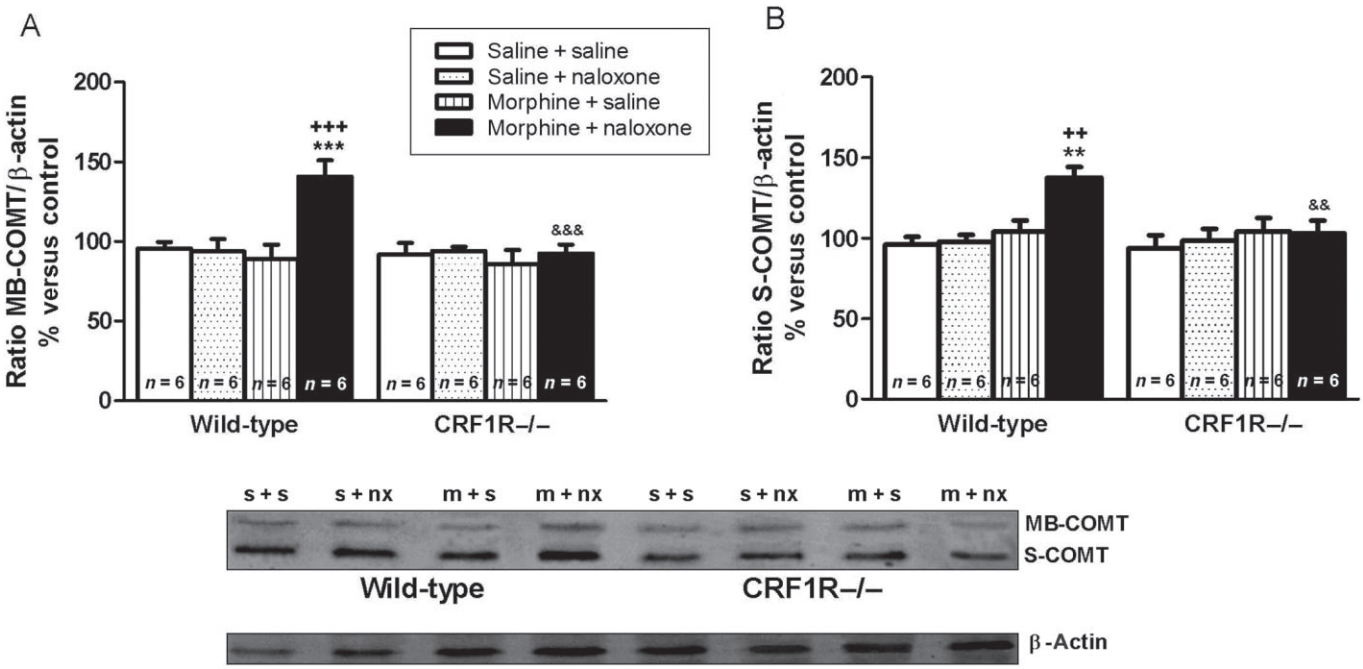

Figure 4

Western-blotting analysis of membrane-COMT (MB-COMT) (A) and soluble-COMT (S-COMT) (B) in left ventricle after saline or naloxone (nx) administration to placebo (saline, s) or morphine (m)-treated wild-type and CRF_1 receptor knockout mice. The immunoreactivity corresponding to MB-COMT or S-COMT is expressed as a percentage of that in the control group defined as 100%. Data are the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, versus morphine+saline; $^{++}P < 0.01$, $^{+++}P < 0.001$ versus saline+naloxone; $\&\&P < 0.01$, $\&\&\&P < 0.001$ versus wild-type mice.

sponding control groups (Figure 5A). These increases were significantly ($P < 0.05$) attenuated in CRF_1 receptor knockout mice (Figure 5A).

We also studied the phosphorylation of Hsp27 at Ser^{82} in the left ventricle. Three-way ANOVA results for Hsp27 phosphorylated in Ser^{82} revealed a significant effect of genotype ($F_{1,20}$ = 8.240,P = 0.007), and significant interaction between genotype and chronic morphine treatment ($F_{1,20}$ = 7.215, P = 0.011) and genotype × acute treatment ($F_{1,20}$ = 7.250, P = 0.011). As shown in Figure 5B, after saline injection to morphine-treated wild-type mice, levels of phospho-Ser^{82}-Hsp27 were not changed. However, wild-type mice chronically treated with morphine that received naloxone showed significant ($P < 0.01$, $P < 0.05$) increases in Hsp27 phosphorylated at Ser^{82}. In contrast, CRF_1 receptor knockout mice showed a significant ($P < 0.01$) decrease in phospho-Hsp27 after naloxone-induced morphine withdrawal versus wild-type mice (Figure 5B). Three-way ANOVA results for phospho-Hsp27/Hsp27 ratio revealed a significant effect of acute naloxone (withdrawal; $F_{1,20}$ = 6.062, P = 0.019) and an interaction between genotype and acute withdrawal ($F_{1,20}$ = 5.166, P = 0.030) and chronic morphine treatment × acute treatment ($F_{1,20}$ = 13.655, P = 0.001). *Post hoc* test showed that morphine-treated wild-type mice receiving naloxone presented a significant ($P < 0.001$, $P < 0.05$) increase in this ratio versus the group treated with morphine injected with saline and the saline group receiving naloxone. However, the administration of naloxone to morphine-treated CRF_1 receptor knockout mice did not increase the ratio phospho-Hsp27/Hsp27, this ratio being significantly ($P < 0.05$) lower than that observed in wild-type mice (Figure 5C).

Influence of morphine withdrawal on HPA axis activation

To evaluate whether a causal link exists between CRF_1 receptor activation and the HPA axis during naloxone-precipitated morphine withdrawal, we measured plasma ACTH and corticosterone concentrations in wild-type and CRF_1R–/– mice. Repeated measures ANOVA of ACTH plasma levels revealed a significant main effect of genotype ($F_{1,33}$ = 37.805, P = 0.0001), chronic morphine treatment ($F_{1,33}$ = 105.815, P = 0.0001), acute naloxone ($F_{1,33}$ = 116.433, P = 0.0001), genotype × chronic treatment ($F_{1,33}$ = 54.513, P = 0.0001), genotype × acute treatment ($F_{1,33}$ = 40.099, P = 0.0001), acute (naloxone) × chronic treatment ($F_{1,33}$ = 85.082, P = 0.0001) and genotype × acute treatment × chronic treatment interaction ($F_{1,33}$ = 52.243, P = 0.0001). As shown in Figure 6A similar results were obtained 1 or 24 h after naloxone administration to chronic morphine-treated wild-type mice. Thus, naloxone induced a dramatic increase ($P < 0.001$) in ACTH plasma levels in morphine-treated mice. Morphine-treated CRF_1R–/– mice injected with naloxone showed a significant ($P < 0.001$) decrease in plasma ACTH levels versus wild-type mice. With respect to corticosterone a repeated measures ANOVA revealed a significant main effect of acute naloxone injection ($F_{1,33}$ = 11.656, P = 0.002) and as well as significant chronic morphine treatment × acute naloxone injection interaction ($F_{1,33}$ = 8.745, P = 0.006). Corticosterone plasma levels were also

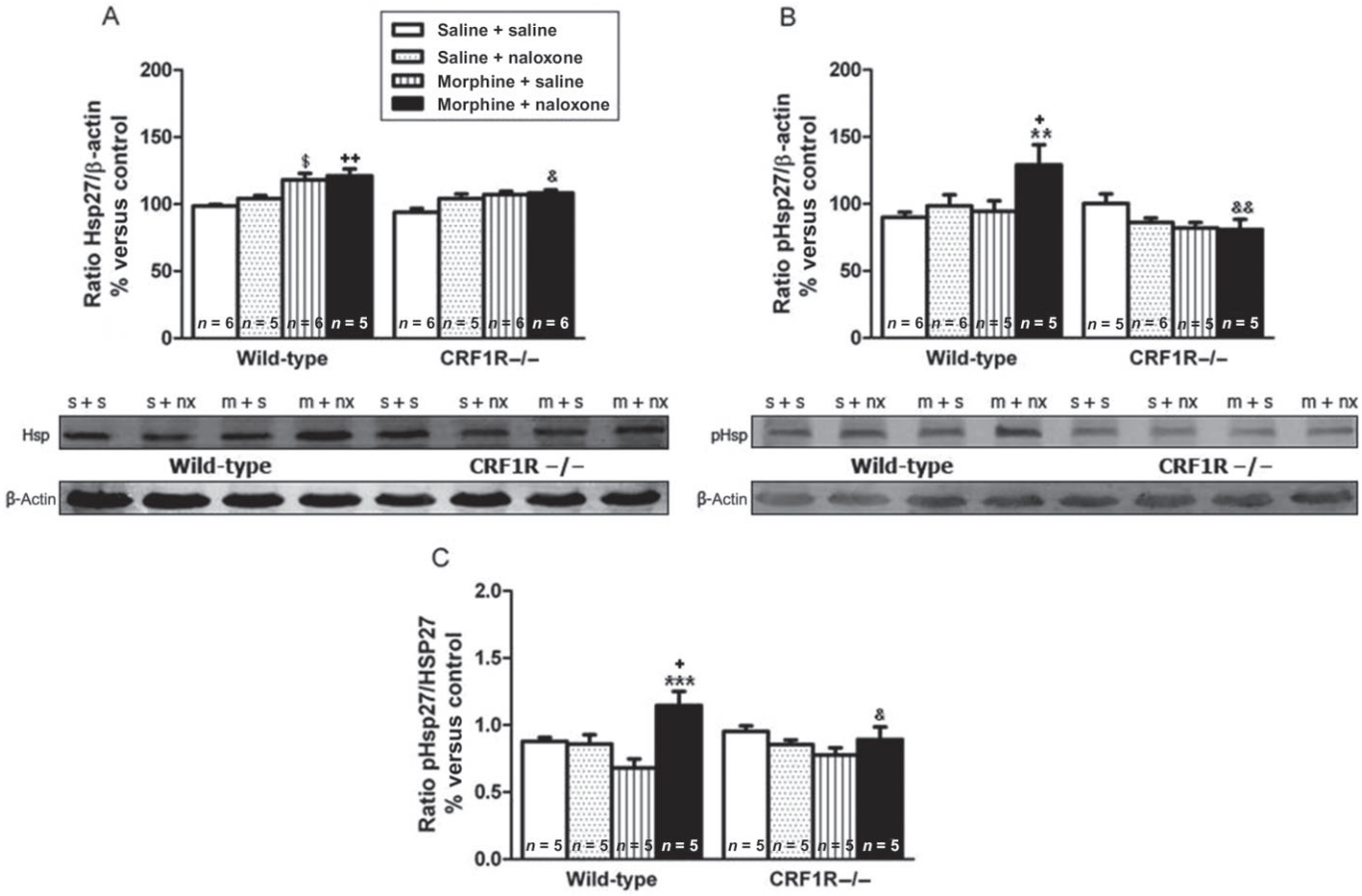

Figure 5

Western-blotting analysis of Hsp27 (A) phospho (p)-Ser^{82} (B) and phospho(p)-Hsp27/Hsp27 ratio in left ventricle after saline or naloxone (nx) administration to placebo (saline, s) or morphine (m)- treated wild-type and CRF_1 receptor knockout mice. The immunoreactivity corresponding to Hsp27 and pHsp27 is expressed as a percentage of that in the control group defined as 100%. Data are the mean ± SEM $^{**}P < 0.01$, $^{***}P < 0.001$ versus morphine + saline; + $P < 0.05$, ++$P < 0.01$ versus saline + naloxone; \$$P < 0.05$ versus saline+saline; &$P < 0.05$, && $P < 0.01$ versus wild-type mice.

significantly increased 1 h after naloxone administration to the wild-type morphine-treated group. CRF_1 receptor knockout mice treated with morphine showed a significant ($P < 0.05$) decrease in corticosterone plasma levels 1 h after naloxone administration versus morphine-treated wild-type mice. However, 24 h after naloxone administration, no changes in plasma corticosterone levels were observed. (Figure 6B).

Discussion

The role of integrating stress signals producing a neuroendocrine output to HPA axis is played by neurons secreting CRF. Beyond CRF function as primary activators of the HPA system, CRF and its analogues also have effects on the heart and vasculature by increasing the secretion of NA and adrenaline from the sympathetic nervous system and adrenal medulla, respectively, and play a role within the heart as local mediators (Yang *et al.*, 2010). In this regard, the present data demonstrated that naloxone-induced morphine withdrawal increases plasma ACTH and corticosterone concentrations in parallel with an enhancement in MB-COMT and S-COMT expression, which could be responsible for the enhancement of NMN content and NA turnover, which are implicated in the increase in MAP and HR observed in wild-type mice; the severity of cellular stress during chronic morphine treatment and withdrawal was also evidenced by the expression and activation of Hsp27. The principal finding of the present study is that plasma ACTH and corticosterone levels, MB-COMT, S-COMT, NMN content, NA turnover, and Hsp expression and activation (phosphorylation) were significantly attenuated in CRF_1 receptor knockout mice.

In the current study, withdrawal was precipitated by naloxone administration to morphine-treated wild-type or CRF_1R–/– mice. Although the spontaneous withdrawal is more similar to the human condition (Cobuzzi and Riley, 2011), in the current assessment, we used antagonist-precipitated withdrawal because the effects of spontaneous withdrawal are less pronounced and are more difficult to detect and because acute withdrawal occurs more reliably when it is precipitated by an antagonist. Our data indicate a weight loss after naloxone-precipitated withdrawal in wild-

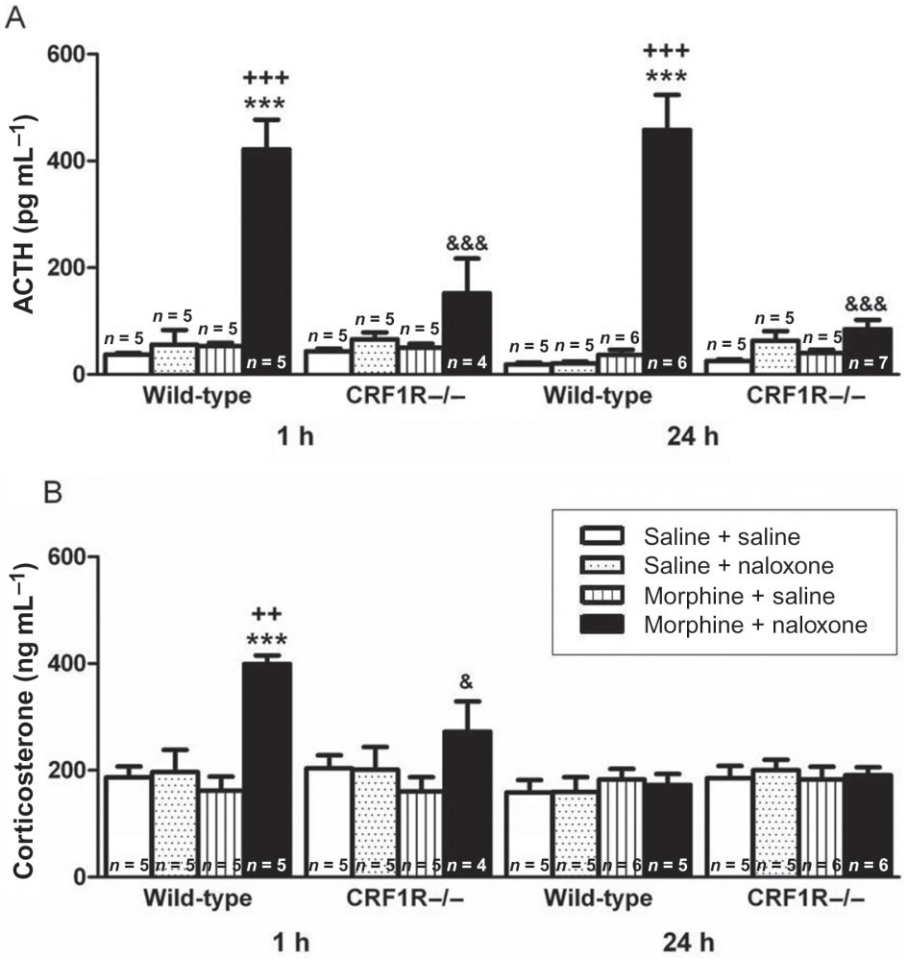

Figure 6

Plasma ACTH (A) and corticosterone (B) concentrations 1 and 24 h after saline or naloxone (nx) injection to placebo (saline) or morphine-treated wild-type and CRF_1 receptor knockout mice. Data are the mean ± SEM. ***$P < 0.001$, versus morphine + saline; ++$P < 0.01$, +++$P < 0.001$ versus saline + naloxone; &$P < 0.05$, &&&$P < 0.001$ versus wild-type mice.

type mice; the weight loss in morphine-withdrawn CRF_1 receptor knockout animals was significantly attenuated. In general, when naloxone was injected to morphine-treated wild-type mice, the animals showed higher motor activity and diarrhoea than CRF_1 receptor knockout mice. These findings are consistent with previous reports from our laboratory (Navarro-Zaragoza *et al.*, 2010; Garcia-Carmona *et al.*, 2011) and other laboratories (Iredale *et al.*, 2000; Lu *et al.*, 2000). However, our findings contrast with those from the studies ofPapaleo *et al.* (2007), where it was shown that there is an increase in some signs of opiate withdrawal in CRF_1R–/– mice, but in these latter studies spontaneous morphine withdrawal was employed whereas we used opioid-antagonist precipitated morphine withdrawal. Major behavioural and molecular differences between naloxone-precipitated withdrawal and spontaneous withdrawal procedures make it difficult to compare the present findings with those obtained previously.

In the present study we evaluated ACTH and plasma corticosterone levels in saline and morphine-treated mice after saline or naloxone administration. Our results showed that plasma corticosterone levels obtained in control mice are higher than those described in previous studies in rats (reviewed by Joels *et al.*, 2012). These discrepancies could be due to species differences. According to previous reports (Koob and Kreek, 2007; Koob, 2008), 1 h after naloxone administration to morphine-dependent mice, plasma ACTH and corticosterone levels were increased. In addition, our findings demonstrated that 24 h after naloxone-precipitated withdrawal plasma ACTH levels remain increased while plasma corticosterone returned to basal levels. Although the presence of pituitary ACTH is clearly essential for adrenocortical function, ACTH-independent mechanisms seem to have an important role in modulating the highly sensitive adrenal stress system to adapt its response appropriately to physiological needs. Numerous studies have been published indicating that a large number of neuropeptides, neurotransmitters, growth factors and even bacterial ligands are capable of modulating the release of adrenal glucocorticoids independently of pituitary ACTH (Bornstein *et al.*, 2008). Adrenocortical cells express a great variety of receptors for these factors, thus enabling potential direct actions on corticoids release. Lesions of upstream stress regulatory pathways in the brain

lead to a dissociation between ACTH and corticosterone, suggesting that CNS pathways are capable of regulating HPA axis function at both the pituitary and adrenal level. (Choi *et al.*, 2007). Our results also showed that ACTH and corticosterone release, which are produced as a consequence of morphine withdrawal, was attenuated in CRF_1 receptor knockout mice. In accord with these results, plasma ACTH levels were found to be decreased in morphine-withdrawn animals treated with CRF_1 receptor antagonists (Navarro-Zaragoza *et al.*, 2010). In addition, a role for the HPA axis and brain extraphypothalamic CRF/CRF_1 receptor circuitry in somatic, molecular and endocrine alterations induced by opioid withdrawal has been reported (Papaleo *et al.*, 2007). The CRF_1 receptor is also required for normal development and function of chromaffin cells in the adrenal medulla and deletion of this gene is associated with a significant impairment of adrenaline biosynthesis (Yoshida-Hiroi *et al.*, 2002). Furthermore, CRF_1 receptor antagonists attenuated the increase of plasma ACTH levels in heart failure rats (Kang *et al.*, 2011). Altogether, these results suggest a role for the CRF/CRF_1 receptor pathway in the regulation of HPA axis activity.

Animals and clinical studies suggest that chronic exposure to drugs of abuse is associated with elevated cardiac sympathetic activity, which is one of the major factors contributing to the morbidity and mortality of addict patients. Previous studies have demonstrated that chronic μ-opioid receptor stimulation decreases muscle sympathetic nerve activity (Kienbaum *et al.*, 2001; 2002), NA plasma concentration (Kienbaum *et al.*, 2001), MAP and HR (Almela *et al.*, 2011; Shanazari *et al.*, 2011). In the present study, we clearly show that chronic treatment with morphine decrease MAP and HR in CRF–/–, but not in wild-type mice. It is possible that the decrease in BP and HR observed after chronic morphine treatment in CRF_1 receptor-deficient mice could be due to a significant impairment of adrenaline biosynthesis associated with the deletion of the CRF_1 receptor (Yoshida-Hiroi *et al.*, 2002). However, μ-opioid receptor blockade by naloxone in patients with chronic opioid abuse or in morphine-dependent rats, unmasks these effects, resulting in markedly increased muscle sympathetic nerve activity, plasma NA concentrations (Peart and Gross, 2006), NA turnover, total tyrosine hydroxylase expression and an enhancement in MAP and HR (Almela *et al.*, 2008; 2011), two objective and accurate measurable signs of opioid withdrawal in humans. CRF has interesting effects on BP in rodents. Intracerebroventricular administration of CRF produces a marked hypertension in rats (Morimoto *et al.*, 1993). When it is given i.v., it produces a hypotensive effect (Corder *et al.*, 1992). However, it is unclear which type of CRF receptor was responsible for the CRF-induced autonomic response. After naloxone-precipitated morphine withdrawal, we observed an increase in HR and MAP, similar to that seen after central CRF delivery (Arlt *et al.*, 2003). Interestingly, mice lacking CRF_1 receptors exhibit an increase in MAP after naloxone administration to morphine-treated mice similar to that observed in wild-type mice, while HR was decreased in CRF_1 receptor-deficient mice. These results demonstrate that the CRF_1 receptor is not involved in the increases in MAP and suggest that another receptor, such as the CRF_2 receptor, could be involved. In this regard, the effects of urocortin on BP were inhibited by a selective CRF_2 receptor antagonist, antisauvagine-30, suggesting a role for CRF_2 receptors in BP regulation (Mackay *et al.*, 2003).

However, COMT activity has repeatedly been suggested to be associated with increased vulnerability to the development of drugs abuse (Li *et al.*, 2004; Beuten *et al.*, 2005; Ersche *et al.*, 2011) and reward processing (Tunbridge *et al.*, 2013), but there are few data about its role during morphine withdrawal (Martínez-Laoden *et al.*, 2012). The present results demonstrated that naloxone-precipitated morphine withdrawal caused a decrease in myocardial NA levels and increases in myocardial NMN (extraneuronal NA metabolite generated by COMT), in parallel with an increased expression of MB-COMT and S-COMT, suggesting that both isoforms of COMT are implicated in the degradation of NA. This increased cardiac sympathetic activity observed in wild-type mice after naloxone-precipitated morphine withdrawal could be responsible for the increase in MAP and HR observed in our study. It is known that CRF_1 receptor activation in selected brain regions contributes to stress-induced sympathetic activation (Nijsen *et al.*, 2000; Chu *et al.*, 2004). The present results showed an increase in NMN concentration, NMN/NA ratio, HR, MB-COMT and S-COMT in wild-type mice after naloxone-precipitated withdrawal. However, these changes were abolished in CRF_1R–/– mice. Taken together these results suggest that CRF_1 receptors may be responsible for the observed cardiac sympathetic hyperactivity observed during naloxone-precipitated morphine withdrawal.

As the repeated exposure to morphine and its withdrawal induces profound and severe stress reactions that are also evidenced by over expression of Hsp27 (Almela *et al.*, 2011; Drastichova *et al.*, 2012; Martínez-Laoden *et al.*, 2012), we evaluated Hsp27 expression and phosphorylation in the left ventricle in wild-type and CRF_1 receptor knockout mice. In accord with previous data (Almela *et al.*, 2011; Drastichova *et al.*, 2012; Martínez-Laoden *et al.*, 2012), the present investigation showed that both chronic morphine treatment and its withdrawal are associated with an increase of Hsp27 expression. However, chronic morphine treatment did not modify Hsp27 phosphorylation, whereas naloxone administration to morphine-treated rats induced an enhancement of Hsp27 phosphorylation at Ser^{82}. While Hsp27 can block actin polymerization, the phosphorylation of Hsp27 is related to re-organization of the actin-based cytoskeletal structures (Bitar, 2002). It has been suggested that this re-organization of the actin cytoskeleton induced by phosphorylation of Hsp27 could lead to cytoprotection due to stabilization of actin filaments (Robinson *et al.*, 2010). Altogether, these results support the idea that morphine dependence and its withdrawal induces profound cellular stress that could damage the myocardium (Dettmeyer *et al.*, 2009). The enhancement of Hsp expression and phosphorylation seen in wild-type morphine-dependent mice was abolished in CRF_1 receptor knockout mice. These results suggest that CRF pathways contribute to the cardiac stress response observed during morphine withdrawal, by activating CRF_1 receptors.

In conclusion, our results demonstrate an increased activity of HPA axis and sympathetic pathways in cardiac tissues from morphine-withdrawn wild-type mice. In spite of all the insults inflicted to the cardiomyocytes, they were able to increase Hsp27 expression and phosphorylation, which could be protecting the heart. The cardiac changes observed after

naloxone-precipitated morphine withdrawal were inhibited in CRF_1 receptor knockout mice, suggesting a role for the CRF/CRF_1 receptor pathway in thee effects. Therefore, the present results indicate that CRF activity is a contributing factor involved in the cardiovascular alterations seen in drug addicts and offer insight into potential therapy to treat this condition.

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Conflict of interest

The authors have no conflict of interest.

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