Brain stress system response after morphine-conditioned place preference

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Abstract

This study examined the involvement of the brain stress system in the reinforcing effects of morphine. One group of mice was conditioned to morphine using the conditioned place preference (CPP) paradigm and the other group received morphine in a home-cage (non-conditioned). Adrenocorticotropic hormone and corticosterone levels were measured by radioimmunoassay; phospho (p) CREB expression and the number of corticotropin-releasing factor (CRF) neurons and fibres were measured by immunohistochemistry in different brain areas. We observed that the number of CRF neurons in the paraventricular nucleus (PVN) was increased after morphine-induced CPP, which was paralleled with enhanced CRF-immunoreactivity fibres in the nucleus tractus solitarius (NTS) and ventral tegmental area (VTA) vs. home-cage group injected with morphine. Morphine exposure induced an increase in CREB phosphorylated at Ser133 in the PVN and central amygdale (CeA), whereas mice exhibiting morphine CPP had higher levels of pCREB in the PVN, CeA and bed nucleus of the stria terminalis (BNST). We also found that most of the CRF-positive neurons in the PVN, CeA and BNST co-express pCREB after morphine CPP expression, suggesting that the drug-associated environmental contexts can elicit neuronal activity in the brain stress system. From the present results it is clear that exposure to a drug-associated context remains a potent activator of signalling pathways leading to CRF activation in the brain stress system.

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Introduction

Opiate addiction is a complex relapsing brain disease process that is characterized by the compulsive seeking and taking of opiates, despite adverse consequences and the emergence of a negative emotional state when access to the opiate is denied (Koob et al., 1998). Drug addiction is characterized by a high incidence of relapse among recovered addicts, even after many years of abstinence (See, 2002). Three factors are thought to be responsible for the high relapse rates: (1) the potent positive reinforcing effects of drugs of abuse (O’Brien et al., 1998); (2) environmental stimuli previously associated with drugs; thus, drug-related cues can increase both drug craving and subsequent drug use (O’Brien et al., 1992); (3) psychological stress can produce drug craving and increase the risk of drug relapse (Sinha, 2001).

The conditioned place preference (CPP) paradigm is a model widely used to investigate the mechanisms underlying context-dependent learning associated with drugs of abuse (Bardo et al., 1995). The CPP paradigm is based on Pavlovian conditioning in which, during the training phase, one context is paired with drug injection, whereas another context is paired with vehicle injection. During subsequent drug-free CPP test, animals choose between the drug- and vehicle-paired contexts. Increased preference for the drug context serves as a measure of the rewarding effects of drugs of abuse, such as opiates and cocaine (Van der Kooy, 1987; Tzschentke, 2007).

In general, the field of addiction research has focused primarily on the mesolimbic dopamine (DA) pathway, which originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAC) and prefrontal cortex, because of its critical role in mediating the rewarding actions of virtually all drugs of abuse (Everitt and Wolf, 2002). Recently, importance of the brain stress system in the reinforcing
properties of drugs (Piazza and Le Moal, 1996; Goeders and Guerin, 2000) and drug-cue exposure (Sinha et al., 2003; Waldrop et al., 2010) has also been proposed.

Corticotropin-releasing factor (CRF) participates in two separate (but connected) stress systems. In the hypothalamo–pituitary–adrenal (HPA) axis stress system, CRF neurons from the parvocellular region of the hypothalamic paraventricular nucleus (PVN) regulate the release of pituitary adrenocorticotropic hormone (ACTH) and adrenal glucocorticoids. In the extrahypothalamic stress system, CRF neurons from the central nucleus of amygdale (CeA) and bed nucleus of the stria terminalis (BNST) project to mesolimbic structures (Rodaros et al., 2007) and to the brainstem catecholaminergic cell group (Koob, 1999). Recent studies suggest that the release of CRF from the PVN and/or extrahypothalamic areas, and subsequent activation of CRF₁ receptors, could mediate stressor-induced potentiation of mesolimbic DA activation, thus increasing drug reward (Wanat et al., 2008; Kreibich et al., 2009; Brielmaier et al., 2012). Activation of CRF neurons in the PVN has been associated with increased activity of the nucleus tractus solitarius (NTS) noradrenergic cell group (Delfs et al., 2000; Navarro-Zaragoza et al., 2011), which projects to the PVN, BNST, VTA and NAc (Weinshenker and Schroeder, 2007). In addition, the activation of CRF₁ receptor leads to activation of the transcription factor cAMP response element binding protein (CREB; Kasagi et al., 2002). However, it remains unclear whether drug-related cues alter the brain CRF systems activity in opioid addicts. Although there is some evidence that drug-related cues increase craving and physiological arousal, there has been little examination about the role of brain stress systems during such exposure. Therefore, the objective of our study was to investigate the implication of the brain stress system pathways on morphine reward and the possible activation of CRF neurons by the environment. For these purposes, we examined: (1) plasma ACTH and corticosterone levels; (2) CRF neurons in the PVN, CeA and BNST and their projections to NTS, NAc (shell) and VTA; (3) CREB phosphorylation and CRF activation in the PVN, CeA and BNST after morphine-induced CPP; (4) the effects of morphine in mice without being subjected to CPP, on the different parameters described above.

Method

Animals

Adult male Swiss mice (25–30 g) were maintained under controlled temperature (23±2°C) and 12-h light/dark cycle (lights on 08:00 hours), with free access to food and water. Animals were handled daily during the first week after arrival to minimize stress and conditioned and tested during the light phase of the cycle. All studies were performed in compliance with the Royal Decree 223/1998 of 14 March (BOE 8 18) and the Ministerial Order of 13 October 1989 (BOE 18) as well as with the European Council Directive of 24 November 1986 (86/609/EEC) and the local Animal Ethics Committee.

Conditioned place preference paradigm

The rewarding effects of morphine were examined by using the CPP paradigm. The place conditioning apparatus is based on that used by Valverde et al. (1996), with some modifications. It consisted of two rectangular polycarbonate compartments (length, 20 cm; width, 18 cm; height, 25 cm) spaced 4 cm from each other, both accessible from a rectangular polyvinyl chloride exterior area (length, 20 cm; width, 7 cm; height, 25 cm). In order to distinguish the three compartments, visual and sensory texture cues were used. One compartment was grey striped wall with black smooth floor whereas the other compartment was black spotted wall with grey rough floor. The neutral area providing access to the compartments had transparent wall and floor. The guillotine doors, which were made with the colour corresponding to the respective wall colour, were inserted during the conditioning sessions and removed during the pre-conditioning and post-conditioning tests. The CPP schedule consisted of three phases: pre-conditioning test, conditioning phase; post-conditioning test. On day 0 (pre-conditioning test), each mouse was allowed to explore freely the CPP apparatus for 18 min and time spent in each of the two compartments was recorded for each mouse by a computer program (CPP Win 2.0, Panlab, Spain). Animals that spent <7 min in either the white or black compartment were considered not to be neutral in preference for either side and were excluded from further study. In the conditioning phase, animals were randomly assigned to receive saline (control group) or morphine (6 mg/kg i.p.). Mice received morphine on days 1, 3 and 5 and saline on days 2, 4 and 6. Control animals received saline every day. Immediately after saline or morphine injections, the animals were placed in the compartment assigned and then doors matching the walls of the compartment allowed confinement of the mice for 20 min. Twenty-four hours after the end of conditioning, the post-conditioning test (day 7) allowed free exploration of the CPP apparatus for
18 min and was conducted exactly as the pre-conditioning test. A score was calculated for each mouse as the difference between post-conditioning and pre-conditioning time spent in the drug-paired compartment.

**Perfusion and tissue processing**

One hour after the post-conditioning test, mice were deeply anaesthetized with an overdose of pentobarbital (10 mg/kg i.p.) and perfused transcardially with 30 ml PBS, pH 7.4, followed by 50 ml fixative containing 4% paraformaldehyde in PBS (pH 7.4). After removing, brains were post-fixed in sucrose (20% in paraformaldehyde) and stored at 4 °C overnight. Series of 30 μm frontal sections were cut on a freezing microtome, collected in cryoprotectant and stored at −20 °C until processing.

**Home-cage administration**

Mice in the home-cage were injected following the same schedule of drug administration as in CPP groups, but without any behaviour components of CPP training and testing. Mice received morphine (6 mg/kg i.p.) on days 1, 3 and 5 and saline on days 2, 4 and 6. Control animals received saline every day. One day after the last injection, mice were perfused and the tissues processed on the same schedule as in CPP groups.

**Immunohistochemistry detection of CRF and pCREB**

After blocking with 0.3% normal goat serum (Vector Laboratories, USA), tissue sections were incubated for 60 h at 4 °C with the following antibodies: rabbit anti-CRF (1:500, a gift from Wylie W Vale, The Salk Institute, USA); anti-phospho (p) CREB (1:750, Millipore, USA). This was followed by application of a biotinylated anti-rabbit IgG (Vector) and then with avidin–biotin complex. Visualization of the antigen–antibody reaction sites was performed using 3,3′-diaminobenzidine (DAB, Sigma, USA) for CRF or DAB intensified with nickel in the first position; CRF revealed with DAB in the second position. pCREB immunohistochemistry was performed as described previously (dilution of primary antibody: 1:750) and pCREB antibody–peroxidase complex was visualized using a mixture of NiSO₄·6H₂O (33.2 mg/ml), DAB (0.033%) and 0.014% H₂O₂ in 0.175 M sodium acetate solution, pH 7.5. Sections were then incubated with the anti-CRF antibody (diluted 1:500) for 60 h at 4 °C. A biotinylated anti-rabbit IgG (diluted 1:200 for 1 h) was used as secondary antibody. The CRF antibody–peroxidase complex was stained in 0.033% DAB and 0.014% H₂O₂ in 0.05 M Tris–HCl buffer.

**Quantification of CRF and pCREB immnoreactivity**

CRF and pCREB immunostaining within section of the PVN, CeA and BNST were quantified bilaterally for each mouse and for all treatment groups by an observer blinded to the treatment protocol. The density of pCREB-like immunoreactivity was determined using a computer assisted image analysis system (Qwin; Leica, Spain). This system consists of a light microscope (DM4000; Leica) connected to a video camera (DFC290, Leica) and the image analysis computer. A square field (195 μm side) was superimposed upon the captured image (×20 magnification) to use as reference area.

**Quantification of pCREB-positive/CRF-positive neurons**

pCREB-positive CRF neurons were identified as cells with brown cytosolic deposits for CRF-positive staining and blue/dark nuclear staining for pCREB. A square field (195 μm) was superimposed upon captured image to use as reference area. Leica-Qwin V3 software was used to determine the CRF-positive neurons and pCREB immunoreactivity. The number of double-labelled neurons was counted bilaterally in four to five sections from each animal in the PVN, CeA and BNST.

**Quantification of CRF fibres**

The CRF fibres in the NTS, NAc shell and VTA were analysed by measuring the optical density of the CRF immunoreactivity with a computer analyser (ScionImage; Barcia et al., 2004).

**Radioimmunoassay**

Sixty minutes after CPP and 24 h after morphine or saline injection (home-cage groups), mice were revealed with DAB intensified with nickel in the first position; CRF revealed with DAB in the second position. pCREB immunohistochemistry was performed as described previously (dilution of primary antibody: 1:750) and pCREB antibody–peroxidase complex was visualized using a mixture of NiSO₄·6H₂O (33.2 mg/ml), DAB (0.033%) and 0.014% H₂O₂ in 0.175 M sodium acetate solution, pH 7.5. Sections were then incubated with the anti-CRF antibody (diluted 1:500) for 60 h at 4 °C. A biotinylated anti-rabbit IgG (diluted 1:200 for 1 h) was used as secondary antibody. The CRF antibody–peroxidase complex was stained in 0.033% DAB and 0.014% H₂O₂ in 0.05 M Tris–HCl buffer.
known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (Berhow et al., 1995). For this purpose the weight of animals was recorded on the days of morphine or saline injection and on the day of killing after the testing phase (day 7). CPP mice conditioned by morphine and home-cage groups injected with morphine showed a significantly ($t_{28}=10.37$ and $t_{19}=5.016$, $p<0.001$ respectively) lower body weight gain (0.32±0.12 g, $n=10$ and 0.200±0.06 g, $n=15$, respectively) than animals receiving saline injection (1.12±0.10 g, $n=11$ and 1.26±0.08 g, $n=15$, respectively), indicating that morphine was correctly administered.

**Effects of morphine on CPP test**

It is known that a certain number of conditioning sessions are necessary for the conditioning of morphine reward in mice that are opiate naive before place conditioning. In this study, 6 d conditioning led to a significant place preference to the drug as compared to saline controls. Student’s $t$ test showed that score was significantly ($t_{17}=7.141$, $p<0.001$) stronger in mice conditioned by morphine (161.8±16.09 s) than in saline-paired group (17.46±11.55 s), indicating motivational effects of morphine cues.

**HPA axis activity after morphine CPP expression**

We measured plasma ACTH (Fig. 1a) and corticosterone (Fig. 1b) concentrations (as markers of HPA axis activity) in blood samples obtained from CPP and home-cage mice. Two-way ANOVA for ACTH plasma levels revealed a main effect of morphine treatment ($F_{1,12}=17.75$, $p=0.0012$). However, ANOVA failed to show significant main effects of CPP ($F_{1,12}=2.22$, $p=0.1618$) or significant morphine treatment×CPP interaction ($F_{1,12}=0.67$, $p=0.4276$). Post hoc test revealed an increase ($p<0.05$) of plasma ACTH levels after morphine-induced CPP (173.3±24.11 pg/ml) compared with the saline-paired group (74.71±18.66 pg/ml). We examined whether these changes in the HPA activation were specific to morphine-induced CPP or could be due only to morphine treatment. For that, animals were treated in their home-cages with the same volumes of saline. Reagents used were: goat serum (Sigma Aldrich, USA); nickel sulphate (Sigma Aldrich).

**Results**

Mice weight gain was checked during the treatment to ensure the efficacy of morphine treatment because it is...
p = 0.4980) or CPP (F_{1,16} = 0.42, p = 0.5244). ANOVA also failed to show significant morphine treatment × CPP interaction (F_{1,16} = 0.13, p = 0.7185).

**CRF-immunoreactivity in the PVN, CeA and BNST after morphine CPP expression**

The possible changes in the number of CRF-positive neurons in the PVN, CeA and BNST were studied by immunohistochemistry (Fig. 2a–c). At the PVN level, two-way ANOVA for the number of CRF neurons showed that there was a main effect of morphine treatment (F_{1,13} = 3.13, p = 0.1003), CPP paradigm (F_{1,13} = 8.98, p = 0.0103) and a morphine treatment × CPP interaction (F_{1,13} = 4.90, p = 0.0454). As shown in Fig. 2(e), Newman–Keuls post hoc test showed that there was an increase (p < 0.05) in the number of CRF positive neurons in the morphine-paired group (25.38 ± 2.03) compared with the home-cage group injected with morphine (17.90 ± 0.95). There were no changes in the home-cage groups injected with morphine vs. saline-treated animals (18.75 ± 1.54). In addition, mice conditioned to morphine and mice conditioned to saline (20.25 ± 1.93) showed similar number of CRF neurons.

In the CeA, two-way ANOVA for total CRF-positive neurons revealed that there was a main effect of morphine treatment (F_{1,12} = 19.84, p = 0.0008). However, there were not significant effects for CPP (F_{1,12} = 0.84, p = 0.3763). ANOVA also failed to show significant morphine treatment × CPP interaction (F_{1,12} = 4.48, p = 0.059). Post hoc test (Fig. 2) showed a significant (p < 0.01) enhancement of the number of CRF-positive neurons in the morphine CPP group (49.24 ± 3.99) vs. saline-paired group (25.10 ± 4.40). There were no changes in the home-cage groups (Fig. 2f–j).

In the BNST, two-way ANOVA for the number of CRF-positive neurons revealed that there was no effect of morphine treatment (F_{1,12} = 3.69, p = 0.0787), CPP (F_{1,12} = 0.52, p = 0.4867) or a morphine treatment × CPP interaction (F_{1,12} = 0.86, p = 0.3727). In addition the number of CRF neurons did not differ significantly between home-cage mice (saline: 37.13 ± 4.79; morphine: 42.50 ± 6.20) and mice subjected to CPP (saline: 36.00 ± 4.54; morphine: 51.38 ± 5.86; Fig. 2k–o).

**CRF fibres in the NTS, NAc shell and VTA after morphine CPP expression**

We also examined whether the changes in the number of CRF neurons (PVN and CeA) observed after morphine CPP expression were accompanied by alterations in the CRF fibres in the terminals (NTS, NAc shell and VTA). In NTS (Fig. 3a–c), two-way ANOVA showed a main effect of morphine treatment (F_{1,12} = 13.61, p = 0.0027). ANOVA failed to show significant CPP effects (F_{1,13} = 4.04, p = 0.0656) or significant morphine treatment × CPP interaction (F_{1,13} = 3.45, p = 0.0859). As shown in Fig. 3e post hoc analysis showed that mice conditioned to morphine (15.20 ± 0.46) showed a significant increase of CRF-immunoreactive fibres compared with saline-paired group (10.67 ± 1.05; p < 0.01) or with morphine home-cage treated mice (12.04 ± 0.74; p < 0.05). There were not differences between the groups injected with saline or morphine in their home-cages (saline: 10.55 ± 0.83; morphine: 31.86 ± 0.76).

At the NAc shell level (Fig. 3f–j), two-way ANOVA for the CRF-immunoreactive fibres revealed no significant effect of morphine treatment (F_{1,13} = 0.02, p = 0.8942), CPP (F_{1,13} = 0.92, p = 0.3562) or a morphine treatment × CPP interaction (F_{1,13} = 0.23, p = 0.6416). Post hoc analysis did not show significant differences in the CRF fibres in the groups subjected to CPP test (saline: 34.27 ± 1.01; morphine: 33.27) or the groups treated in their home-cages (saline: 30.06 ± 3.83; morphine: 31.86 ± 0.76).

Two-way ANOVA for CRF-immunoreactive fibres in the VTA (Fig. 3k–o) revealed a main effect of morphine treatment (F_{1,14} = 4.62, p = 0.0496) and CPP (F_{1,14} = 5.21, p = 0.0386). ANOVA failed to show significant interaction (F_{1,14} = 2.36, p = 0.1469). Post hoc test showed a significant (p < 0.05) increase in the CRF-immunoreactive fibres in the morphine CPP group (286.3 ± 26.87) compared with its control (206.2 ± 14.14) and with morphine-treated home-cage mice (203.3 ± 20.56).

**Effects of morphine CPP paradigm on pCREB levels in the brain stress system**

Within the hypothalamic PVN (Fig. 4a–c), two-way ANOVA for pCREB revealed a main effect of morphine treatment (F_{1,12} = 47.02, p < 0.0001). However, ANOVA showed no significant main effects of CPP (F_{1,12} = 0.78, p = 0.3937) or significant morphine treatment × CPP interaction (F_{1,12} = 0.24, p = 0.6365). Newman–Keuls post hoc test showed that there was a significant enhancement in pCREB levels in the groups treated with morphine (home-cage: 446.5 ± 18.95, p < 0.01; CPP: 434.8 ± 11.49, p < 0.001) compared with saline-treated mice (home-cage: 259.3 ± 53.22; CPP: 219.0 ± 11.43; Fig. 4d).

At the CeA level (Fig. 4f–j), two-way ANOVA revealed a main effect of morphine treatment (F_{1,12} = 33.37, p < 0.0001) but there was neither significant CPP (F_{1,12} = 0.27, p = 0.6133) nor significant morphine treatment × CPP interaction (F_{1,12} = 4.48, p = 0.0559).

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Newman–Keuls post hoc test (Fig. 4f) showed high levels of pCREB immunoreactivity in the groups treated with morphine (home-cage: 493.5±20.82, p<0.01; CPP: 597.0±55.40, p<0.001) compared with saline injected animals (home-cage: 349.8±43.54; CPP: 287.0±27.78).

In the oval nucleus of the BNST (Fig. 4k–o), two-way ANOVA showed a main effect of morphine treatment (F[1,13]=11.68, p=0.0046) and CPP (F[1,13]=4.76, p=0.0046), but failed to show significant morphine treatment×CPP interaction (F[1,13]=2.21, p=0.1608). As shown in Fig. 5m, Newman–Keuls post hoc test revealed that...
Fig. 3. Immunoreactivity of corticotropin-releasing factor (CRF) fibres in conditioned (CPP) and home-cage mice. Photographs represent immunohistochemical detection of CRF fibres in the nucleus tractus solitarius (NTS; a–d), nucleus accumbens (NAc; f–i) and ventral tegmental area (VTA; k–n). Scale bar 100 μM. Each column represents the optical density of CRF immunostaining in the NTS (e), NAc (j) and VTA (o). Data are expressed as mean±S.E.M. * p<0.05, ** p<0.01 vs. saline (Sal); + p<0.05 vs. morphine (Mor)-treated group in the home-cage (two-way analysis of variance followed by the Newman–Keuls post hoc test).
there was an increase in pCREB immunoreactivity in morphine-injected mice subjected to CPP (449.2±39.13) compared with saline-injected mice (274.5±20.97, p<0.01) and with the group of mice treated with morphine in their home-cages (318.5±36.29, p<0.05). There were not significant differences between

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**Fig. 4.** cAMP response element binding protein (CREB) phosphorylation in conditioned (CPP) and home-cage mice. Photographs represent immunohistochemical detection of phospho (p)CREB in the paraventricular nucleus (PVN; b–e), central nucleus of amygdale (CeA; f–j) and bed nucleus of the stria terminalis (BNST; k–o). Scale bar 100 μM. Quantitative analysis of pCREB immunohistochemistry in the PVN (a), CeA (f) and BNST (k). Data are expressed as mean±S.E.M. ** p<0.01, *** p<0.001 vs. saline (Sal); + p<0.05 vs. morphine (Mor)-treated group in the home-cage (two-way analysis of variance followed by the Newman–Keuls post hoc test).
saline (249.8±38.45) and morphine-injected animals in their home-cages.

**Induction of pCREB in CRF-positive neurons**

To explore the specificity of morphine CPP-induction of CREB phosphorylation observed in the brain stress system, sections of PVN, CeA and BNST were immunohistochemically double-labelled for pCREB and CRF. Two-way ANOVA revealed a main effect of morphine treatment (PVN: $F_{1,13}=14.35$, $p=0.0023$; CeA: $F_{1,12}=16.95$, $p=0.0014$; BNST: $F_{1,12}=24.14$, $p=0.0004$), with non-significant effect of CPP or morphine treatment×CPP interaction. Post hoc test (Fig. 5a, f, k)
showed a significant increase in the number of CRF neurons expressing pCREB in the morphine-CPP group (PVN: 18.94±1.12, p<0.01; CeA: 27.90±1.69, p<0.05; BNST: 32.58±2.05, p<0.01) vs. its control (PVN: 9.93±0.69; CeA: 17.55±2.76; BNST: 20.00±1.95). There were not statistical differences between morphine (PVN: 15.75±1.82; CeA: 21.85±2.52; BNST: 233.56±2.93) and saline groups (PVN: 9.93±0.69; CeA: 17.55±2.76; BNST: 20.00±1.95) injected in their home-cages. No significant effects were observed between the group of mice conditioned to morphine and the morphine-injected mice in their home-cages (PVN: 15.75±1.82; CeA: 21.85±2.52; BNST: 33.56±2.93; Fig. 5a–d).

Discussion

CPP is an important behavioural assay that is widely used to assess the reward value of drugs and natural reinforcers. Present data indicate that morphine produced a significant CPP for the drug-associated place. These findings support previous studies (Tzschentke, 1998; Zarrindast et al., 2007; Ma et al., 2009; Moaddab et al., 2009; González-Cuello et al., 2010) and indicate that morphine induced rewarding effects, which, through a mechanism of associative learning, become connected to the environment in which these effects occurred (Tzschentke and Schmidt, 1995).

Opioid-induced place preference depends on activation of the mesolimbic dopaminergic system (Manzanedo et al., 2001). The mesolimbic and mesocortical DA system comprise projections from the VTA to the NAc and prefrontal cortex, respectively. The NAc is well-known for its important role in reward and motivation (Di Chiara and North, 1992). As part of the extended amygdala, NAc contains CRF receptors, as well as CRF fibres (Merchenthaler et al., 1982; Merchenthaler, 1984; Lim et al., 2007). In this regard, the importance of CRF pathways in the reinforcing properties of drugs and drug-cue exposure is clearly increasing (Piazza and Le Moal, 1996; Goeders and Guerin, 2000; Sinha et al., 2003; Waldrop et al., 2010).

In the present study we have investigated the involvement of brain stress-related nuclei (PVN, CeA, BNST) and their projections to NAc and VTA (mesolimbic areas), as well as to the NTS (the main noradrenergic system innervating the stress neurocircuity) in the rewarding effects of morphine. The HPA axis, the primary endocrine stress pathway, and the extrahypothalamic stress system (which includes the extended amygdala and the NTS) are dysregulated not only by chronic administration of drugs with dependence potential (Koob and Le Moal, 2008) but also by reward-related stimuli (Sinha et al., 2006; Fox et al., 2007). Present findings showed an enhancement of plasma ACTH levels after morphine-induced CPP, whereas plasma ACTH showed a non-significant tendency to increase in home-cage treated animals. However, changes in plasma corticosterone levels were not observed after morphine-induced CPP. Although ACTH is clearly essential for adrenocortical function, a number of ACTH-independent mechanisms have been proposed to have an important role in modulating the highly sensitive adrenal stress system in order to adapt its response to physiological needs. A potential explanation of present findings is that, although CRF is the major secretagogue in stimulating ACTH secretion, arginine vasopressin also plays a role (Tilders et al., 1985). In addition, numerous studies indicate that a large number of neuropeptides, neurotransmitters, growth factors and even bacterial ligands are capable of modulating adrenal glucocorticoid release independently of pituitary ACTH (for review, see Bornstein et al., 2008). Lesions of upstream stress regulatory pathways in the brain lead to a dissociation between ACTH and corticosterone. For example, lesions of the anterior BNST are able to attenuate corticosterone secretion without changes in circulating ACTH (Choi et al., 2007). In this regard, previous studies from our laboratory have demonstrated that the selective CRF1 antagonist CP-154256 blocked ACTH but not corticosterone release after morphine withdrawal (Navarro-Zaragoza et al., 2010; Almela et al., 2012).

Taken together, these results suggest that neurochemical and/or neurohormonal stimulatory factors other than ACTH might be responsible for adrenal hyperactivity associated with drug-cue exposure (Fatselas et al., 2011). It is known that repetitive exposure to drugs of abuse leads to stable regulations that involve adaptations in neuronal circuits and gene expression (Kalivas and Volkow, 2005). CREB has been implicated as transcriptional regulator of many genes, including CRF, and has been proposed as a marker for neuronal plasticity (Nestler, 2004). Present results showed that morphine exposure induced CREB phosphorylation in the PVN and CeA, without any changes in the BNST. These findings indicate that the PVN and CeA may be more susceptible to alterations by morphine exposure than BNST, suggesting substantial differences in CREB activation in the different brain stress-related areas. However, present data indicate that morphine-induced CPP produced CREB phosphorylation not only in the PVN and CeA but also in the BNST, suggesting that the stimulus-related reward can induce changes in CREB expression.
Although many studies have implicated CRF signals in the anxiogenic-like and aversive motivational effects of drug withdrawal, its role in mediating the rewarding effects of opiates remains equivocal. Recent results showed that most of the CRF-positive neurons in the PVN, CeA and BNST co-express pCREB after morphine-induced CPP, which suggests that the drug-associated environment contexts can elicit neuronal activity in the brain stress system. Since the animals treated with morphine in their home-cage did not show any changes in the number of CRF-positive/CREB-positive neurons, this effect could be dependant exclusively on exposure to the environment. It is clear from the present results that exposure to a drug-associated context remains a potent activator of signalling pathways leading to CRF activation in the brain stress system.

There are anatomical and functional evidence indicating connections between CRF and the mesolimbic dopaminergic pathway. CRF-like immunoreactivity has been detected in projections from the PVN and stress extrahypothalamic areas (such as CeA and BNST) to the VTA and NAc (Rodaros et al., 2007; Chen et al., 2012), which have been proposed to regulate release of DA. In addition, it is well known that noradrenergic fibres from the NTS project to the NAc and brain stress areas, regulating the release of DA and CRF, respectively. Conversely, CRF from these nuclei induces the release of noradrenaline (NA) by the brainstem noradrenergic areas (Koob, 1999; Stinus et al., 2005). Taken together, these results underline the complexity of CRF neuronal networks and the CRF neurons×catecholaminergic system interactions, suggesting the existence of a CRF–NA/DA loop. However, the possible involvement of the NTS in the rewarding and motivational effects of morphine is not well documented. Present results showed an increase in the optical density of the CRF fibres in the NTS and VTA from the morphine-induced CPP group. Recent research indicates the necessity of noradrenergic function for the establishment of morphine CPP (Ventura et al., 2005; Olson et al., 2006; Zarrindast et al., 2007). Additionally, previous results from our laboratory demonstrated enhanced 3-methoxy-4-hydroxyphenylglycol levels and NA turnover in the NAc after morphine-induced CPP, suggesting a role for NA in the rewarding properties of opiates (González-Cuello et al., 2010). However, how noradrenergic neurotransmission from the NTS regulates opioid reward remains to be explored. Our data suggest that NA release from the NTS, which projects to the VTA and NAc, may regulate dopaminergic neurons activity and thereby facilitate reward.

In conclusion, we proposed here a role of the brain stress system in morphine-paired cues and suggest that the NTS could be a key mediator for the CRF and dopaminergic reward pathways. These results provide further support to the idea that environment could be of fundamental importance in the treatment of drug addiction.

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**Statement of Interest**

None.

**References**

Chen Y-W, Rada PV, Bützler BP, Leibowitz SF, Hoebel BG (2012) Corticotropin-releasing factor in the nucleus accumbens shell induces swim depression, anxiety, and
anhedonia along with changes in local dopamine/acetylcholine balance. Neurosci 206:155–166.


