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RESEARCH PAPER

Glucocorticoid receptors participate in the opiate withdrawal-induced stimulation of rats NTS noradrenergic activity and in the somatic signs of morphine withdrawal

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BACKGROUND AND PURPOSE

Recent evidence suggests that glucocorticoid receptor (GR) is a major molecular substrate of addictive properties of drugs of abuse. Hence, we performed a series of experiments to further characterize the role of GR signalling in opiate withdrawal-induced physical signs of dependence, enhanced noradrenaline (NA) turnover in the hypothalamic paraventricular nucleus (PVN) and tyrosine hydroxylase (TH) phosphorylation (activation) as well as GR expression in the nucleus of the solitary tract noradrenergic cell group (NTS-A_2).

EXPERIMENTAL APPROACH

The role of GR signalling was assessed by i.p. pretreatment of the selective GR antagonist, mifepristone. Rats were implanted with two morphine (or placebo) pellets. Six days later, rats were pretreated with mifepristone or vehicle 30 min before naloxone and physical signs of abstinence, NA turnover, TH activation, GR expression and the hypothalamus–pituitary–adrenocortical axis activity were measured using HPLC, immunoblotting and RIA.

KEY RESULTS

Mifepristone alleviated the somatic signs of naloxone-induced opiate withdrawal. Mifepristone attenuated the increase in the NA metabolite, 3-methoxy-4-hydroxyphenylethylen glycol (MHPG), in the PVN, and the enhanced NA turnover observed in morphine-withdrawn rats. Mifepristone antagonized the TH phosphorylation at Ser^{31} and the expression of c-Fos expression induced by morphine withdrawal. Finally, naloxone-precipitated morphine withdrawal induced up-regulation of GR in the NTS.

CONCLUSIONS AND IMPLICATIONS

These results suggest that the physical signs of opiate withdrawal, TH activation and stimulation of noradrenergic pathways innervating the PVN are modulated by GR signalling. Overall, the present data suggest that drugs targeting the GR may ameliorate stress and aversive effects associated with opiate withdrawal.

Abbreviations

GR, glucocorticoid receptor; HPA, hypothalamus–pituitary–adrenocortical; PVN, hypothalamic paraventricular nucleus; NTS-A_2, nucleus of the solitary tract-A_2 noradrenergic cell group

Introduction

Addiction is a complex disorder because many factors contribute to the development and maintenance of this neurological disorder. One factor is stress (Sinha, 2008; Ambroggi *et al.*, 2009; Koob and Volkow, 2010). Glucocorticoids are the final step in the activation of the hypothalamic–pituitary–adrenocortical (HPA) axis and have profound influences on brain function and behaviour. Stress-induced glucocorticoid secretion by the adrenal gland activates the glucocorticoid receptor (GR), triggering changes in genome expression. This response not only facilitates adaptation to acute environmental challenges but also may lead to behavioural pathologies during chronic stress conditions, such as addiction and depression. Glucocorticoids exert their effects in the brain via two types of intracellular corticosteroid receptor (de Kloet *et al.*, 1998), the mineral corticoid receptors (MR) and the GR, which are transcription factors belonging to the nuclear receptor family. Most structures of the brain express GR, which are more widely distributed in the brain and have lower affinity for glucocorticoids than MR, and they are activated by high corticosterone levels such as those after stress (Joëls and de Kloet, 1994).

Evidence has been obtained demonstrating that the GR is a major molecular substrate of the addictive properties of drugs of abuse (Marinelli *et al.*, 1998; Deroche-Gamonet *et al.*, 2003; Shalev *et al.*, 2003). Glucocorticoids have been hypothesized to facilitate behavioural effects of cocaine by acting on mesolimbic dopaminergic neurons (Piazza and Le Moal, 1996;Ambroggi *et al.*, 2009). Blockade of corticosterone secretion reduces the locomotor activity induced by infusions of morphine and cocaine in the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Deroche *et al.*, 1995; Marinelli *et al.*, 1998).

The noradrenergic system and the HPA axis comprise two major adaptation mechanisms to stress. Acute withdrawal from all major drugs with abuse potential, such as opiates, activates the HPA axis in rats, which results in neuronal activation of corticotropin-releasing factor (CRF) transcription in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) and increases in ACTH and corticosterone secretion (Núñez *et al.*, 2007a; 2009; Cleck and Blendy, 2008). Enhanced responsiveness of the HPA axis and aversive symptoms of morphine withdrawal have been associated with activation of noradrenergic neurons in the nucleus of the solitary tract (NTS-A_2) that projects to the hypothalamic PVN (Delfs *et al.*, 2000; Laorden *et al.*, 2002b; Navarro-Zaragoza *et al.*, 2010; 2011). On the other hand, glucocorticoids exert their feedback effects not only by inhibiting the secretion of CRF in the PVN but also by modulating neuronal inputs to the PVN in the central stress pathways (Herman *et al.*, 2003). An important target of glucocorticoids action is the NTS-A_2 noradrenergic cell group. Therefore, we have suggested that one of the neuronal mechanisms that underlie morphine withdrawal-induced activation of glucocorticoids secretion may be dependent on activation of the noradrenergic pathways innervating the PVN.

Noradrenaline (NA) is a key player in stress-induced relapse of drug seeking behaviour for several drugs of abuse (for review, see Smith and Aston-Jones, 2008). Furthermore, NA has been implicated in addiction and in particular in the adverse effects of opiate withdrawal (Maldonado, 1997; Aston-Jones and Kalivas, 2008; Núñez *et al.*, 2009; Navarro-Zaragoza *et al.*, 2010; 2011). Opiate withdrawal results in marked activity of the central noradrenergic neurons, and it has been proposed that noradrenergic afferent neurons to the extended amygdala and the PVN are critically involved in the adverse effects of opiate withdrawal. To emphasize the importance of GR within the NTS-A_2 noradrenergic pathway innervating the PVN, we showed that surgical suppression of glucocorticoids prevents induction of tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine synthesis) mRNA expression and activation in the NTS-A_2 as well as enzyme activity in the PVN (Núñez *et al.*, 2009).

Because opiate withdrawal and stress involve common neural areas and cell signalling cascades, withdrawal-related changes in processes underlying stress may contribute to addiction. We therefore assessed (i) the role of GR in mediating somatic and behavioural states produced during precipitated morphine withdrawal, (ii) 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 the selective GR antagonist mifepristone, and (iii) the effect of morphine withdrawal on GR expression within the NTS-A_2.

Methods

Materials

Pellets of morphine base (Alcaliber Laboratories., Madrid, Spain) or lactose (control) were prepared in the Department of Pharmacy and Pharmaceutics Technology (School of Pharmacy, Granada, Spain). Naloxone HCl and mifepristone were purchased from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO). Protease inhibitors were purchased from Boehringer Mannheim (Mannheim, Germany). Phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany). HPLC reagents were purchased from Sigma Chemical Co. Naloxone and mifepristone were prepared fresh each day by reconstitution in sterile saline (0.9% NaCl; Laboratorios ERN, Barcelona, Spain) or Milli-Q water with a drop of Tween 80 10% (Sigma-Aldrich), respectively.

Animals

Male Sprague–Dawley rats (220–240 g; Harlan, Barcelona, Spain; n = 32 at the beginning of the experiment) were housed in pairs in cages (length, 45 cm; width, 24 cm; height, 20 cm) on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light–dark cycle (lights on: 08:00 h–20:00 h) for 7 days before the beginning of the experiments. The animals used for molecular analyses were the same animals that were used to observe the somatic expression of naloxone-precipitated morphine withdrawal. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). All surgical and experimental procedures were performed in accordance with the European Communities

Table 1
Distribution of animals used for each experiment

	pla-veh-nx *n* =	pla-mife-nx *n* =	mor-veh-nx *n* =	mor-mife-nx *n* =	Total *n* =
Somatic signs of opiate withdrawal	6	7	7	6	32
c-Fos-IR	6	6	5	5	28
TH pSer^{31}-IR	6	5	5	5	26
TH pSer^{40}-IR	6	7	6	5	29
GR-IR	5		5		10
Corticosterone levels	5	6	7	6	30
MHPG, NA and NA Turnover	5	5	6	5	26

The number of animals at the beginning of the experiment was n = 32. Pla, placebo; veh, vehicle; nx, naloxone; mife, mifepristone; mor, morphine.

Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local Committees for animal research (REGA ES300305440012).

Drug treatment and experimental procedure

At the beginning of the experiment, rats were implanted s.c. with two 75 mg morphine pellets under light ether anaesthesia. Control rats received placebo pellets containing the excipient without morphine. This procedure has been shown to produce consistent plasma morphine concentrations beginning a few hours after the implantation of the pellets and a full withdrawal syndrome after acute injection of opiate 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). Six days after the implantation of morphine or placebo pellets, precipitated withdrawal was induced by s.c. injection of naloxone (1 mg·kg^{-1}; in a volume of 1 mL·kg^{-1} body weight). The four experimental conditions (Table 1) investigated for opiate withdrawal-induced physical signs of dependence, corticosterone release, NA and 3-methoxy-4-hydroxyphenylethylen glycol (MHPG), TH phosphorylated at Ser^{31} and at Ser^{40}, c-Fos and GR determination were (i) placebo-vehicle-naloxone, (ii) placebo-mifepristone(50 mg·kg^{-1})-naloxone, (iii) morphine-vehicle-naloxone and (iv) morphine–mifepristone-naloxone.

Measurement of the withdrawal syndrome

Experiments were carried out in a quiet room. The observer was unaware of the drug combination used. Rats were individually placed into transparent plastic cages 15 min before the naloxone injection and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 30 min after the naloxone injection. Subsequently, previously identified behavioural characteristics of the rat opiate abstinence syndrome (Lu *et al.*, 2000) were evaluated, including wet-dog shakes, jumping, paw tremor, teeth chattering, mastication, ptosis, piloerection, sniffing, writhing, tremor, salivation, rinorrhoea, chromodacryorrhoea and diarrhoea. The number of wet-dog shakes, jumping, sniffing and paw tremor was counted as the number of events occurring during the total test time period (graded signs). Teeth chattering, body tremor, mastication, ptosis, piloerection and diarrhoea were scored 1 for appearance or 0 for non-appearance within each 5 min time. To obtain a comprehensive index of the severity of somatic opiate withdrawal including all the signs examined, a global withdrawal score was calculated for each animal by giving each individual sign a relative weight as previously reported (Maldonado *et al.*, 1996): jumping, ×0.8; wet-dog shakes, ×1; paw tremor, ×0.35; sniffing, ×0.5; writhing, ×0.5; ptosis, ×1.5; teeth chattering, ×1.5; body tremor, ×1.5; mastication, ×1.5; salivation, ×1.5; rinorrhoea, ×1.5; chromodacryorrhoea, ×1.5; diarrhoea, ×1.5; and piloerection, ×1.5. Body weight loss was determined as the difference between the weight determined immediately before naloxone injection and that determined 30 min later. The weight gain of the rats was checked during treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower calorie intake (Houshyar *et al.*, 2004; Núñez *et al.*, 2009). In order to investigate the effect of GR blockade on the physical symptoms of morphine withdrawal, rats were injected with the selective GR antagonist, mifepristone (25 or 50 mg·kg^{-1} i.p.; Saal *et al.*, 2003) or vehicle (control; i.p.) 30 min before the administration of naloxone. On the basis of our initial experiments, the 50 mg·kg^{-1} dose was chosen for our experiments. Thirty minutes after naloxone injection, rats were decapitated (between 11:00 and 12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed and stored immediately at –80°C until use for Western blot analysis of TH pSer^{31}, TH pSer^{40}, c-Fos and GR. A second set of animals from each treatment group was used for NA and MHPG determination. One set of each treatment group was randomly assigned for plasma corticosterone determination. In order to determine the effect of inhibiting GR on the morphine withdrawal-induced activation of the axis, morphine-dependent and control rats were treated with vehicle (a drop of Tween 80 10% added to Milli-Q water) or mifepristone 30 min before the administration of naloxone. The dose of mifepristone was selected on the basis of published reports (Lowery *et al.*, 2010). Mifepristone displays high binding affinity for the glucocorticoid type I receptor

(K_i = 0.4 nM), and mifepristone and its active metabolites are known to cross the blood–brain barrier (Peeters *et al.*, 2004). After treatment, the following parameters were determined: NA and MHPG content and NA turnover in the PVN (HPLC); TH phosphorylation (activation) at serine 31 ($pSer^{31}$) and/or serine 40 ($pSer^{40}$) in the NTS-A_2 noradrenergic cell group (Western blot); GR expression in the NTS-A_2 (Western blot); c-Fos expression in the NTS-A_2 (Western blot); and plasma corticosterone concentration (RIA).

Western blotting

Brainstem tissue corresponding to the NTS-A_2 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; Paxinos and Watson, 2007). Samples were placed in homogenization buffer (Núñez *et al.*, 2007b), homogenized and sonicated for 30 s before centrifugation at 6000× *g* for 5 min at 4°C. Samples containing equal quantities of total proteins (60 mg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). Western analysis was performed with the following primary antibodies: 1:500 rabbit polyclonal anti-GR antibody (Santa Cruz Biotechnology, Santa Cruz, CA); 1:500 rabbit polyclonal anti-tyrosine-hydroxylase phosphorylated at Ser^{31} ($pSer^{31}$; Millipore, Temecula, CA); 1:500 rabbit polyclonal anti-tyrosine-hydroxylase $phosphoSer^{40}$ ($pSer^{40}$; Millipore); 1:500 rabbit polyclonal anti-cFos antibody (Santa Cruz Biotechnology) and 1:1000 anti-β-actin (rabbit polyclonal antibody, Cell Signaling Technology Inc., Danvers, MA). We used β-actin as our loading control for all the experiments. Before re-probing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with anti β-actin (1:1000, overnight at room temperature). The ratios of GR/β-actin, $pSer^{31}$-TH/β-actin and $pSer^{40}$-TH/β-actin and c-Fos/β-actin were plotted and analysed. Protein levels were corrected for individual levels.

Estimation of NA and its metabolite MHPG in the PVN

NA and its metabolite in the CNS, MHPG, were determined by HPLC with electrochemical detection as described previously (Navarro-Zaragoza *et al.*, 2010). PVN was punched with needles of 1 mm diameter (Leng *et al.*, 2004) from slices of 500 µm; 60 µL of a solution composed of 1 M $HClO_4$ and 2.7 mM EDTA was added to each tissue sample. The samples were homogenized by slight sonication for about 1 min, centrifuged at 10 000× *g* and 4°C for 20 min and the supernatants taken for analysis and filtered through 0.22 mm GV (Millipore). Then levels of proteins from each sample were measured by spectrophotometry. Tissue samples of the PVN were dissected according to the technique of Palkovits and Brownstein (1988). Fifteen millilitres of each sample was injected into a 5 mm C18 reversed-phase column (Waters, Milford, MA) through a Rheodyne syringe loading injector (Waters). Electrochemical detection was accomplished with an electrochemical detector (Waters 2465). NA and MHPG were quantified by reference to calibration curves run at the beginning and the end of each series of assays. The levels of NA and MHPG in the PVN are expressed as $ng \cdot g^{-1}$ wet weight of tissue. The NA turnover was determined as the NA ratio, which was calculated as: NA ratio = MHPG/NA.

RIA

After the rats had been decapitated, trunk blood was collected into ice-cooled tubes containing 5% EDTA and was then centrifuged (500× *g*; 4°C; 15 min). Plasma was separated, divided into two aliquots and stored at –80°C until assayed for corticosterone. Plasma levels of corticosterone were quantified using specific corticosterone antibody for rats ([^{125}I]-corticosterone (CORT) RIA; MP Biomedicals, Orangeburg, NY). The sensitivity of the assay was 7.7 $ng \cdot mL^{-1}$.

Statistical analysis

Data are presented as mean ± SEM. Somatic signs of withdrawal, body weight loss and hormonal and biochemical parameters were analysed by two-way ANOVA with pretreatment (placebo, morphine) and acute treatment (vehicle, mifepristone) as independent variables. The Newman–Keuls *post hoc* test was used for individual group comparisons. Differences with a $P < 0.05$ were considered significant.

Nomenclature

Drug/molecular target nomenclature conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Results

GR blockade attenuates the somatic expression of naloxone-precipitated morphine withdrawal

Six days after the morphine or placebo pellet implantation, rats received vehicle and were challenged with naloxone (1 $mg \cdot kg^{-1}$ s.c.) and immediately tested for the occurrence of somatic signs of opiate withdrawal. The following somatic signs were significantly expressed in morphine-treated groups when compared with placebo-treated groups: wet-dog shakes ($P < 0.001$), tremor ($P < 0.001$), sniffing ($P < 0.001$), teeth chattering ($P < 0.001$), ptosis ($P < 0.001$), piloerection ($P < 0.001$), rinorrhoea ($P < 0.01$), chromodacryorrhoea ($P < 0.001$) and weight loss ($P < 0.001$). The analysis of the global withdrawal score confirmed these differences between morphine- and placebo-treated rats ($P < 0.001$). The results for two-way ANOVA analysis are shown in Table 2.

In the GR blockade study after naloxone-precipitated morphine withdrawal, comparisons between morphine groups showed that wet-dog shakes ($P < 0.001$), tremor ($P < 0.01$), sniffing ($P < 0.001$), ptosis ($P < 0.001$), teeth-chattering ($P < 0.01$), weight loss ($P < 0.001$), piloerection ($P < 0.001$) and chromodacryorrhoea ($P < 0.001$) were significantly decreased in rats receiving 50 $mg \cdot kg^{-1}$ mifepristone (Figure 1A–I). The analysis of the global withdrawal score confirmed that mifepristone significantly reduced the somatic expression of withdrawal in morphine-treated rats ($P < 0.001$; Figure 1J). The results for two-way ANOVA analysis are shown in Table 2. Thus, the blockade of GR overall decreased the expression of

Table 2

Mifepristone (50 mg·kg^{-1}) attenuates the somatic expression of naloxone-precipitated morphine withdrawal

Signs	Chronic treatment (morphine vs. placebo) $F_{(1, 22)}$	P	Two-way ANOVA Pretreatment (mifepristone vs. vehicle) $F_{(1, 22)}$	P	Interaction $F_{(1, 22)}$	P
Wet-dog shakes	26.26	≤0.0001	56.83	≤0.0001	22.07	≤0.0001
Tremor	4.72	≤0.05	26.17	≤0.0001	4.72	≤0.05
Sniffing	13.83	≤0.01	13.83	≤0.01	13.83	≤0.01
Teeth chattering	4.34	≤0.05	41.28	≤0.0001	4.34	≤0.05
Ptosis	15.79	≤0.0006	227.96	≤0.0001	15.79	≤0.0006
Piloerection	197.74	≤0.0001	236.65	≤0.0001	197.74	≤0.0001
Rinorrhoea	2.00	n.s.	16.76	≤0.0005	2.00	n.s.
Chromodacryorrhoea	7.60	≤0.05	27.53	≤0.0001	7.60	≤0.05
Weight loss	26.47	≤0.0001	19.64	≤0.0002	8.83	≤0.0070
Global score	26.06	≤0.0001	96.82	≤0.0001	26.06	≤0.0001

Two-way ANOVA with chronic treatment (morphine vs. placebo) and pretreatment before naloxone (mifepristone 50 mg·kg^{-1} vs. vehicle) as between-subject factors. When significant interactions in pretreatment or between these two factors were observed, subsequent *post hoc* test was applied.

naloxone-precipitated somatic signs of opiate withdrawal, reducing the global score of morphine-dependent mifepristone-treated rats.

GR blockade decreases naloxone-induced MHPG production and elevation in NA turnover in the hypothalamic PVN

Figure 2 summarizes the changes in NA content, MHPG production and NA turnover (as estimated by the ratio MHPG/NA) after injection of naloxone to control and morphine-dependent rats injected with vehicle or mifepristone. The overall ANOVA on NA content in the PVN for mifepristone revealed no effects of acute mifepristone administration ($F_{(1, 16)} = 0.19$; $P = 0.6698$), morphine pretreatment ($F_{(1, 16)} = 1.22$; $P = 0.2854$) or significant interaction between pretreatment and acute treatment ($F_{(1, 16)} = 3.00$; $P = 0.1023$.

The ANOVA for MHPG production in animals receiving vehicle or mifepristone showed a significant effect of acute treatment ($F_{(1, 16)} = 7.84$; $P = 0.0128$) and a significant interaction between morphine pretreatment and mifepristone administration ($F_{(1, 16)} = 6.85$; $P = 0.0187$). *Post hoc* analysis showed that the MHPG levels increased significantly ($P < 0.05$) in naloxone-precipitated morphine withdrawal group receiving vehicle, as compared with the placebo-treated group receiving vehicle plus naloxone (Figure 2B). *Post hoc* analysis also showed that pretreatment with 50 mg·kg^{-1} mifepristone 30 min before naloxone injection significantly ($P < 0.01$) reduced morphine withdrawal-induced increases in MHPG levels compared with morphine-dependent rats receiving vehicle instead of mifepristone.

Results for the two-way ANOVA for MHPG/NA ratio in the PVN in rats pretreated with mifepristone revealed a significant effect of chronic pretreatment ($F_{(1, 17)} = 5.68$; $P = 0.0291$). As shown in Figure 2C, rats made dependent on morphine and injected with vehicle plus naloxone showed a significantly ($P < 0.01$) higher NA turnover in the PVN than the placebo group also receiving vehicle plus naloxone. Administration of mifepristone 30 min before naloxone to morphine-dependent rats significantly ($P < 0.05$) antagonized that elevation in NA turnover compared with the morphine-pelleted group pretreated with vehicle before naloxone.

Effects of GR blockade on morphine withdrawal-induced c-Fos expression in the NTS-A_2

The ANOVA for c-Fos expression in the NTS in animals receiving vehicle or mifepristone showed a significant effect of chronic pretreatment ($F_{(1, 18)} = 7.88$; $P = 0.0117$). *Post hoc* analysis showed that c-Fos expression increased significantly ($P < 0.05$) in the naloxone-precipitated morphine withdrawal group receiving vehicle, as compared with the placebo-treated group receiving vehicle plus naloxone (Figure 3). *Post hoc* analysis also showed that pretreatment with mifepristone 30 min before naloxone injection significantly ($P < 0.05$) reduced morphine withdrawal-induced c-Fos expression in the NTS compared with morphine-dependent rats receiving vehicle instead of mifepristone.

Effects of GR blockade on morphine withdrawal-induced TH phosphorylation at Ser^{31} and Ser^{40} in the NTS

Additional experiments were performed in the NTS to determine whether naloxone-induced morphine withdrawal would activate phosphorylation of TH at Ser^{31} (pSer^{31}) and/or Ser^{40} (pSer^{40}). Next, we evaluated the effects of GR blockade on phosphorylation of TH after naloxone-induced morphine withdrawal. Two-way ANOVA for pSer^{31}-TH revealed a main effect of chronic pretreatment ($F_{(1, 17)} = 5.43$; $P = 0.0324$). As

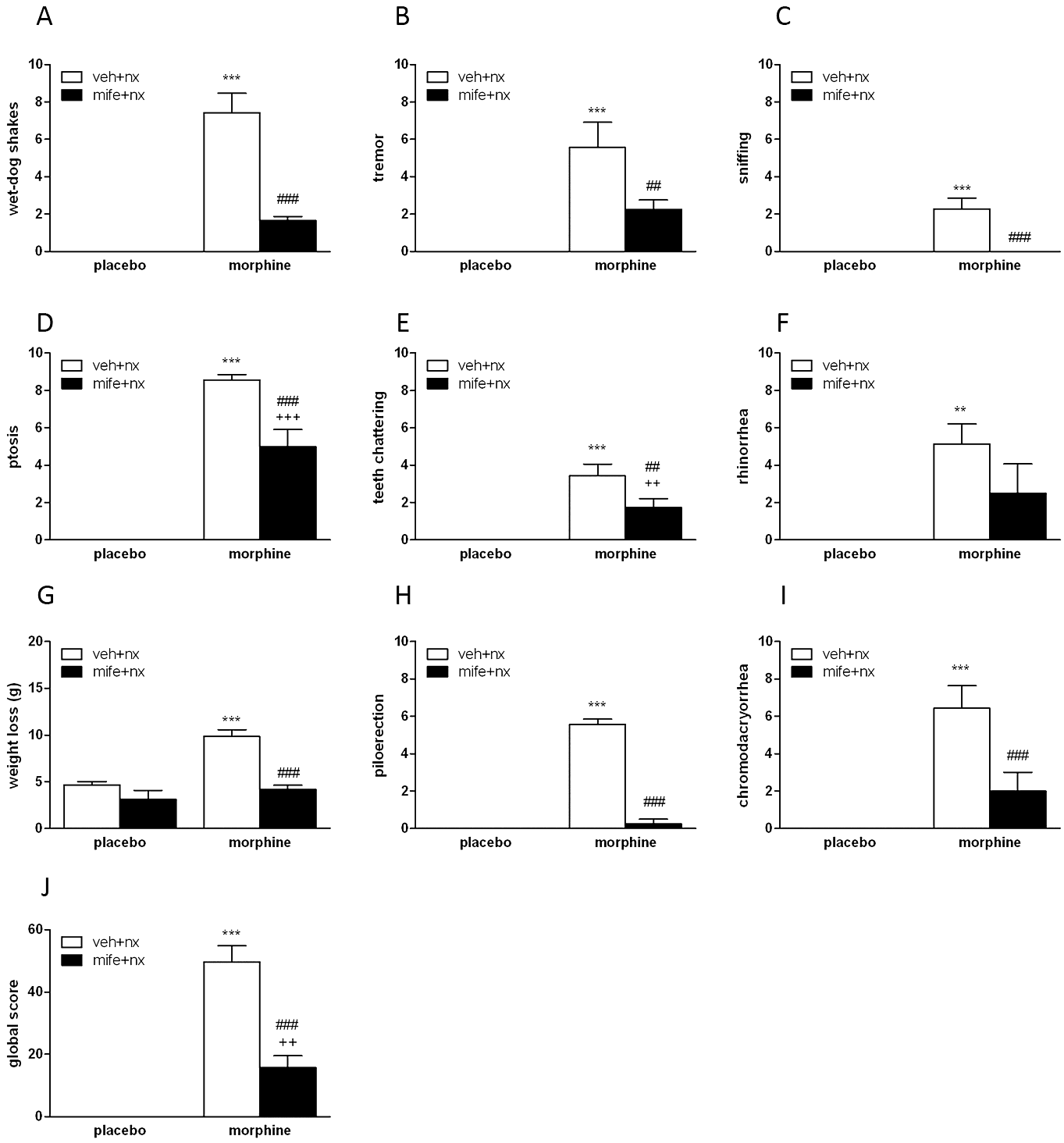

Figure 1

Attenuation of the severity of somatic signs of naloxone-precipitated morphine withdrawal by mifepristone. Counted (A: wet-dog shakes; G: body weight loss) and assessed (B: tremor; C: sniffing; D: ptosis; E: teeth chattering; F: rinorrhoea; H: piloerection; I: chromodacryorrhoea). Somatic signs of withdrawal were observed for 30 min immediately after naloxone injection (1 mg·kg^{-1} s.c.). A global withdrawal score (J) was calculated for each animal as described in the Methods. Data are expressed as mean ± SEM. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus morphine + vehicle (veh) + naloxone (nx); $^{**}P < 0.01$, $^{***}P < 0.001$, versus placebo + veh + nx; $^{++}P < 0.01$, $^{+++}P < 0.001$, versus placebo + mifepristone (mife) + naloxone (nx).

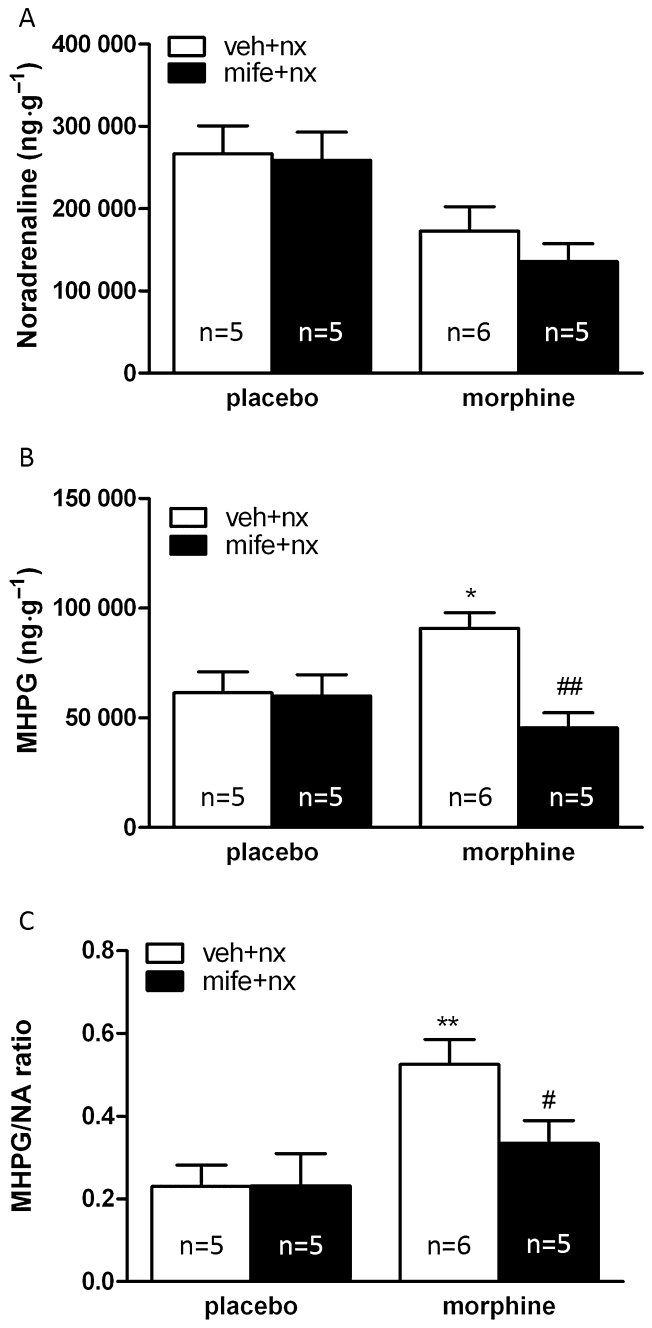

Figure 2

Effects of GR blockade on NA (A) and MHPG (B) levels at the PVN and on the morphine withdrawal-induced increased NA turnover (as estimated by the MHPG/NA ratio; C) in control and in morphine-dependent rats after administration of naloxone. Mifepristone (50 mg·kg^{-1}) attenuated morphine withdrawal-induced increase in MHPG levels and NA turnover. Data represent the mean ± SEM 30 min after naloxone injection to control pellets- or morphine-treated rats receiving vehicle or mifepristone thirty min before naloxone administration. *P < 0.05, **P < 0.01, versus control pellets (placebo) + vehicle (veh) + naloxone (nx); $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, versus morphine + veh + nx.

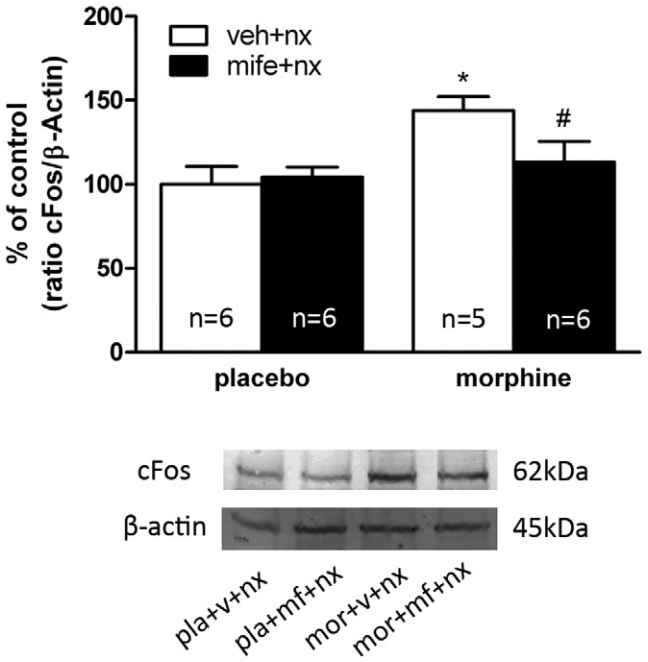

Figure 3

Effects of mifepristone on neuronal activation in the NTS in response to morphine withdrawal. Representative immunoblots of c-Fos in NTS tissues isolated from placebo or morphine-dependent rats 30 min after administration of naloxone in absence or presence of mifepristone (50 mg·kg^{-1}) 30 min before naloxone administration. β-Actin was used as a loading control. Data represent the optical density of immunoreactive bands expressed as a percentage (%) of the mean ± SEM of placebo control band. *P < 0.05, versus the corresponding control group receiving placebo + vehicle (veh) + naloxone (nx); $^{\#}P$ < 0.05, versus morphine + veh + nx.

shown in Figure 4A, rats dependent on morphine and given vehicle 30 min before naloxone (1 mg·kg^{-1} s.c.) injection showed a significant (P < 0.05) enhancement in pSer^{31}-TH levels in the NTS compared with the placebo-pretreated group also receiving vehicle plus naloxone. Administration of mifepristone to morphine-dependent rats significantly (P < 0.05) prevented the enhancement in pSer^{31}-TH levels that was seen 30 min after naloxone injection.

The ANOVA for pSer^{40}-TH showed a major effect of morphine pretreatment ($F_{(1,\ 20)}$ = 13.15; P = 0.0017). Figure 4B depicts that there was a significant (P < 0.01) increase in pSer^{40}-TH levels in the NTS during naloxone-induced morphine withdrawal in rats receiving vehicle, compared with the corresponding placebo control group receiving vehicle plus naloxone (1 mg·kg^{-1} s.c.). Administration of mifepristone to morphine-dependent rats did not prevent the enhancement in pSer^{40}-TH levels that was seen 30 min after naloxone injection.

Influence of morphine withdrawal on GR immunoreactivity in the NTS

Figure 5 shows that there was a significant (t_{11} = 3.281; P < 0.01) increase in GR levels in the NTS during morphine

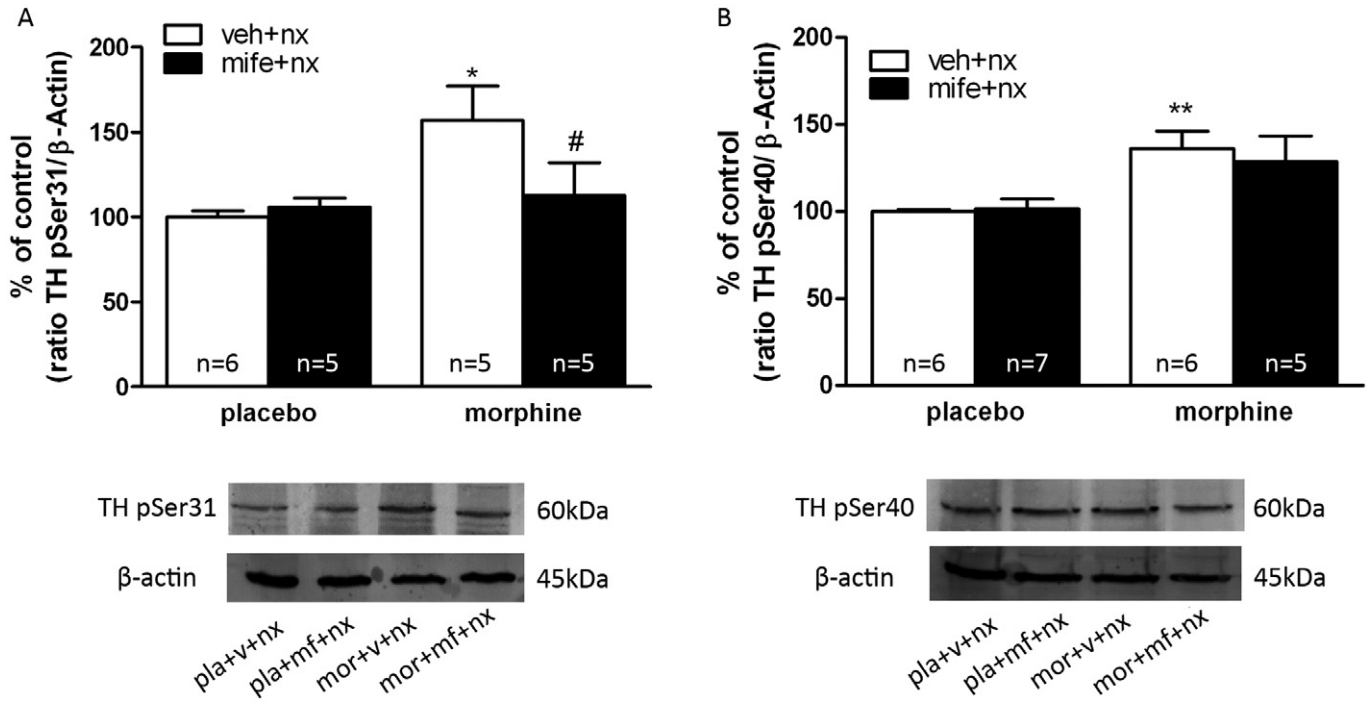

Figure 4

Effects of GR blockade on morphine withdrawal-induced TH phosphorylation at Ser^{31} and Ser^{40} in the NTS-A2 noradrenergic cell group. Representative immunoblots of TH phosphorylated at Ser^{31} (A) and Ser^{40} (B) in NTS tissues isolated from placebo or morphine-dependent rats 30 min after administration of naloxone in the absence or presence of mifepristone (50 mg·kg^{-1}) 30 min before naloxone administration. β-Actin was used as a loading control. Data represent the optical density of immunoreactive bands expressed as a percentage (%) of the mean ± SEM of placebo control band. In morphine-dependent rats, *post hoc* comparison test revealed a significant increase in TH phosphorylation at Ser^{31} during morphine withdrawal, which was attenuated by mifepristone. By contrast, the increase in TH phosphorylated at Ser^{40} after naloxone-precipitated morphine withdrawal did not was attenuated in rats pretreated with mifepristone $^{*}P < 0.05$, $^{**}P < 0.01$, versus control pellets (placebo) + vehicle (veh) + naloxone (nx); $^{\#}P < 0.05$, versus morphine-treated rats + veh + nx.

withdrawal compared with the control placebo-treated group also receiving naloxone.

HPA axis response to morphine withdrawal in mifepristone-treated rats

We measured plasma corticosterone concentrations (as a marker of HPA axis activation) in blood samples obtained from morphine-dependent or control rats 30 min after injection of naloxone. Two-way ANOVA for corticosterone revealed a major effect of chronic morphine treatment ($F_{(1, 20)} = 198.89$; $P < 0.0001$). Newman–Keuls *post hoc* test showed that naloxone-precipitated morphine withdrawal in animals receiving vehicle evoked a marked increase ($P < 0.001$) in corticosterone secretion (Figure 6) compared with placebo-treated rats receiving vehicle plus naloxone.

To evaluate if a causal link exists between GR activation and HPA axis hyperactivation during morphine withdrawal, we measured plasma corticosterone concentrations in animals made dependent on morphine and pretreated with mifepristone 30 min before naloxone administration. As shown in Figure 6, levels of corticosterone in rats pretreated with mifepristone were not significantly different from those measured in morphine-dependent rats receiving vehicle instead of mifepristone.

Discussion

The current results indicate that brain GR signalling modulates the somatic expression of opiate withdrawal. Furthermore, the present results are consistent with a role for GR in the stimulating effects of morphine withdrawal on noradrenergic neurons innervating the stress-related hypothalamic PVN. Administration of mifepristone, a selective GR antagonist, affected several aspect of noradrenergic activity, including TH phosphorylation at Ser^{31}, NA turnover in the PVN and neuronal activity (as estimated by c-Fos expression) of NTS.

All major drugs of abuse stimulate the HPA axis during acute withdrawal from the drug via activation of CRF in the hypothalamic PVN, with a common response of elevated ACTH and corticosterone (Koob and Kreek, 2007; Núñez *et al.*, 2007a; Koob, 2008). However, the relationship between HPA axis activity and drug withdrawal-induced alterations in behaviour has not been elucidated. GR are widely distributed within the CNS (Ahima and Harlan, 1990) and regulate a broad range of neuronal systems. These systems include those that mediate motivated behaviour and serve as targets for abuse drugs (Mantsch *et al.*, 2008). Over the past few years, pharmacological evidence has accumulated showing that glucocorticoids and GR facilitate several responses to different

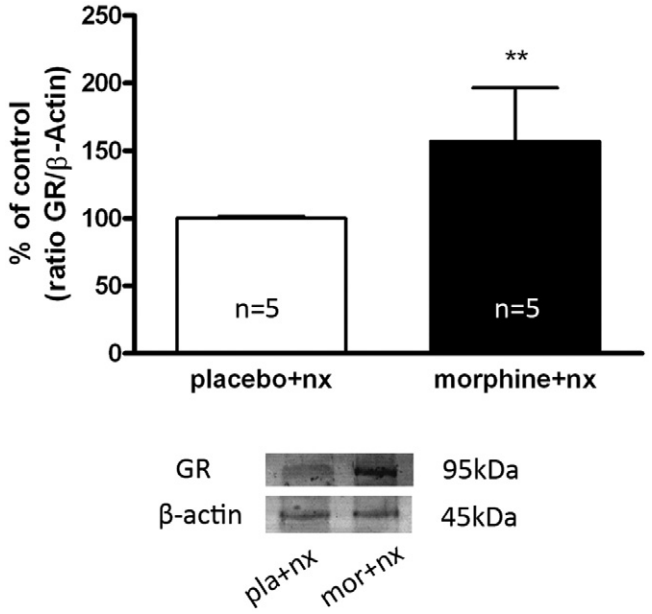

Figure 5

Representative immunoblots of GR expression in NTS tissue isolated from control pellets-treated (placebo) or morphine-dependent rats 30 min after administration of naloxone. β-Actin was used as a loading control. Data represent the optical density of immunoreactive bands expressed as the percentage (%) of the mean ± SEM of placebo control band. $^{**}P < 0.01$, versus control group.

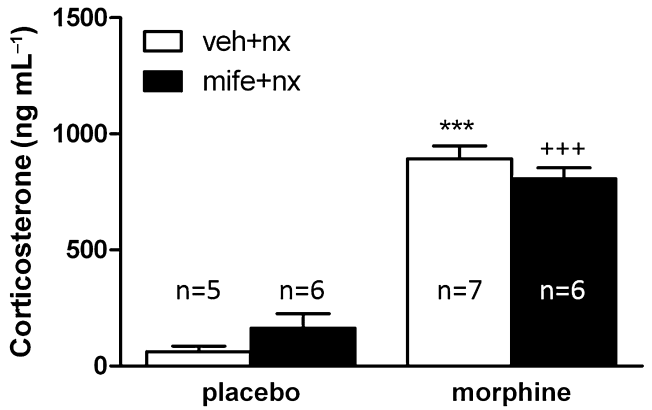

Figure 6

GR blockade did not modify the plasma corticosterone response to naloxone-induced morphine withdrawal. Data represent the mean ± SEM of plasma corticosterone concentration 30 min after naloxone injection to control pellets- or morphine-treated rats receiving vehicle or mifepristone (50 mg.kg^{-1}) 30 min before naloxone administration. $^{***}P < 0.001$, versus control pellets + vehicle (veh) + naloxone; $^{+++}P < 0.001$, versus placebo + mifepristone (mife) + naloxone.

drugs of abuse, including the locomotor response to cocaine and cocaine self-administration (Deroche-Gamonet *et al.*, 2003; Ambroggi *et al.*, 2009; Fiancette *et al.*, 2010), ethanol intake and memory deficit during alcohol withdrawal (Koenig and Olive, 2004; Jacquot *et al.*, 2008). The present results demonstrate an important role for the GR pathways in the somatic expression of morphine withdrawal. Thus, morphine-dependent rats infused with the GR antagonist mifepristone before naloxone injection displayed lower levels of major somatic reactions to the stressful condition of naloxone-induced morphine withdrawal than rats infused with vehicle instead mifepristone. In addition, morphine-dependent rats infused with mifepristone also showed a lower global morphine withdrawal score than morphine-dependent animals infused with vehicle, suggesting that activation of the GR pathway might positively modulate the somatic expression of opiate withdrawal.

NA has been implicated in addiction and in particular in acute opiate withdrawal (Delfs *et al.*, 2000); for review, see (Maldonado, 1997; Smith and Aston-Jones, 2008). The present findings demonstrate that administration of naloxone to morphine-treated rats significantly elevated MHPG production and NA turnover in the PVN, which project from the noradrenergic NTS-A_2 cell group. This is in agreement with previous data from our laboratory showing that morphine withdrawal stimulates NA turnover in the PVN (Navarro-Zaragoza *et al.*, 2010; 2011) as well as the activity of NTS-A_2 TH-positive neurons (as reflected by c-Fos expression; (Laorden *et al.*, 2002a,b;; Benavides *et al.*, 2003). In the present study, mifepristone blocked the morphine withdrawal- induced increase in MHPG production in the PVN and attenuated NA turnover, which provides evidence that glucocorticoids, released during morphine withdrawal, enhance ascending catecholaminergic afferents to the stress-related hypothalamic PVN. In accord with present findings, TH (the rate-limiting enzyme in catecholamine synthesis) protein levels were shown to be increased by glucocorticoids (Hagerty *et al.*, 2001; Núñez *et al.*, 2009). In addition, we reported that glucocorticoids affect the mRNA expression in the noradrenergic NTA-A_2 area; thus, chronic morphine administration and morphine withdrawal induced an increase in TH mRNA expression in the NTS-A_2 and increased the activity of TH in the PVN, whereas depletion of glucocorticoids by adrenalectomy reduced the activity of this enzyme in the PVN and TH gene transcription in the NTS-A_2 (Benavides *et al.*, 2003; Núñez *et al.*, 2009). Our interpretation of all these findings is that glucocorticoids have a permissive role in noradrenergic neurotransmission, and that this is a GR-mediated effect.

Recently, we reported that morphine withdrawal is associated with an increase in TH phosphorylation (activation) at Ser^{31} in the NTS-A_2 and PVN (Núñez *et al.*, 2007b; 2009). It is well known that changes in the state of phosphorylation of TH, the rate-limiting enzyme in the synthesis of catecholamines, are critically involved in the regulation of catecholamine synthesis and function. In particular, increases in the phosphorylation of Ser^{31} and Ser^{40} accelerate TH activity, thereby stimulating the production of neurotransmitters in catecholamine terminals and then their release (for review, see Kumer and Vrana, 1996; Bobrovskaya *et al.*, 2007). Information regarding the role of GR in the interaction between opiate withdrawal and TH activation is limited. We therefore assessed the effect of GR blockade with mifepristone on morphine withdrawal-induced TH phosphorylation (activation) in the NTS-A_2. Using phosphorylation state-specific antibodies directed towards Ser^{31} or Ser^{40}, in the present study, we

showed that morphine withdrawal increased the levels of TH phosphorylated at Ser^{31} and Ser^{40} in the rat NTS concomitantly with the above described enhanced NA turnover. Together, these data confirm that Ser^{31} and/or Ser^{40} phosphorylation of TH may be an important modulator of TH activity during opiate withdrawal and might be directly involved with the increased NA turnover in morphine-withdrawn rats, as has been recently described (Navarro-Zaragoza *et al.*, 2011). In the present study, we used the GR antagonist mifepristone to check the involvement of the GR in the TH phosphorylation at Ser^{31} and/or at Ser^{40} during morphine withdrawal, and we found that pretreatment with mifepristone significantly attenuated the morphine withdrawal-induced stimulation of Ser^{31} but not Ser^{40} phosphorylation in the NTS. These results suggest that morphine withdrawal, through the release of glucocorticoids, activates the GR at the NTS level, which would result in enhanced Ser^{31} phosphorylation, TH activity and catecholamine release in the PVN. On the other hand, stress and abused drugs share the ability to trigger overlapping patterns of neuronal activation within the CNS, resulting in the activation of IEGs expression. In this context, the present results show that morphine withdrawal induced c-Fos expression in the NTS. The use of mifepristone demonstrated that GR is required for c-Fos expression in NTS cells. Together, these findings suggest that the decreased neuronal activation in the NTS in animals exposed to morphine withdrawal and treated with mifepristone may underlie the decreased noradrenergic activity in the PVN.

We recently demonstrated that the activation of the CRF_2 but not CRF_1 receptor subtype is responsible, in part, for the elevation of NA neurotransmission innervating the PVN (Navarro-Zaragoza *et al.*, 2010; 2011). The present results support the idea that, in addition to CRF receptors, the morphine withdrawal-mediated activation of noradrenergic neurons in the NTS is dependent at least partially on GR. Together, these results suggest that noradrenergic activity in morphine-withdrawn rats is modulated by GR signalling, such that blockade of GR effectively reduces excessive TH activation and NA turnover in the PVN, expanding the literature by showing that CRF receptors and GR signalling are recruited during morphine withdrawal. These conclusions are based on the following observations: (1) The dose of mifepristone used in this study has been shown to inhibit GR-mediated CNS effects in different studies (Koenig and Olive, 2004; O'Callaghan *et al.*, 2005). (2) Manipulation of the HPA axis independent of CRF antagonism (i.e. blockade of glucocorticoid receptors or inhibition of corticosterone synthesis with adrenalectomy) attenuated morphine withdrawal-induced noradrenergic pathways activation, as has been stated above. (3) Brainstem NA cell groups express high levels of GR, and it has been shown that glucocorticoids have a permissive role in noradrenergic neurotransmission (Roozendaal *et al.*, 2006) (Fuxe *et al.*, 1987; Sah *et al.*, 2005). (4) A putative glucocorticoid response element (GRE) has been identified in the regulatory region of the TH gene (Kumer and Vrana, 1996). Consistent with this finding, glucocorticoids are one of several hormones that regulate levels of TH mRNA and TH protein levels (Hagerty *et al.*, 2001; Núñez *et al.*, 2009).

Next, the expression of GR in the NTS was determined. Morphine withdrawal resulted in an up-regulation of GR immunoreactivity. According to our results, blockade of GR is expected to result in an increase of GR expression,. However, it has been shown that GR mRNA expression is decreased in the PVN after chronic treatment with mifepristone (Schmidt *et al.*, 2005). Although the functional consequences of increased GR expression in response to opiate withdrawal have not been identified, one possibility is that up-regulation of GR may contribute to the hyperactivation of noradrenergic transmission that was seen in morphine-withdrawn rats. Furthermore, these data are consistent with an increased vulnerability to stress during opiate dependence.

The present results showed that mifepristone did not significantly alter the morphine withdrawal-induced corticosterone release when compared to morphine-withdrawn animals treated with vehicle. Additionally, basal corticosterone concentrations following acute mifepristone treatment are in agreement with previous findings (de Kloet *et al.*, 1988; Spencer *et al.*, 1998) reporting no difference in tonic HPA axis activity with acute GR antagonists. Other studies have shown increased basal corticosterone levels following prolonged antagonism of GR (Bachmann *et al.*, 2003). An increase in basal corticosterone following chronic treatment with mifepristone is not surprising since mifepristone acts by blocking GR, which would increase the amount of freely circulating corticosterone by partial disruption of the GR-mediated feedback (Belanoff *et al.*, 2001).

In conclusion, the results of this study indicate that noradrenergic afferents to the PVN and the GR pathways are critically involved in the physical symptoms of opiate withdrawal. The present data help in the identification of the mechanisms that underlie the complex interactions between stress responsiveness (i.e. glucocorticoids) and noradrenergic pathways during opiate withdrawal. Defining the neurobiological targets of this GR-dependent activation of noradrenergic pathways innervating the PVN should provide valuable insight into the addiction process and aid in the development new approaches for the treatment of opiate dependence. Given the known neuroanatomical and functional interactions between glucocorticoids and NA, it is possible that these systems might be important therapeutic targets that mediate the aversive effects of opiate dependence.

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Conflicts of interest

Authors have no conflict of interest.

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