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RESEARCH PAPER

Activation of the ERK signalling pathway contributes to the adaptive changes in rat hearts during naloxone-induced morphine withdrawal

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Background and purpose: We have previously demonstrated that morphine withdrawal induced hyperactivity of the heart by activation of noradrenergic pathways innervating the left and right ventricle, as evaluated by noradrenaline turnover and c-Fos expression. The extracellular signal-regulated kinase (ERK) has been implicated in drug addiction, but its role in activation of the heart during morphine dependence remains poorly understood. Here, we have looked for activation of ERK during morphine withdrawal and if this activation induced gene expression.
Experimental approach: Dependence on morphine was induced by s.c. implantation of morphine pellets for 7 days. Morphine withdrawal was precipitated on day 8 by injection of naloxone (2 mg kg^{-1}, s.c.). ERK1/2, their phosphorylated forms and c-Fos were measured by western blotting and immunohistochemistry of cardiac tissue.
Key results: Naloxone-induced morphine withdrawal activated ERK1/2 and increased c-Fos expression in cardiac tissues. c-Fos expression was blocked by SL327, a drug that prevents ERK activation.
Conclusions and implications: These results indicate that signalling through the ERKs is necessary for morphine withdrawal-induced hyperactivity of the heart and suggest that this pathway may also be involved in activation of immediate-early genes in both cytosolic and nuclear effector mechanisms that have the potential to bring about long-term changes in the heart.
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Keywords: heart; morphine withdrawal; ERK; c-Fos; SL327; naloxone

Abbreviations: ABC, avidin-biotin complex; BSA, bovine serum albumin; DAB, 3′, 3′-diaminobenzidine; DMSO, dimethylsulphoxide; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulphate; TBST, Tris-buffered saline-Tween 20; TH, tyrosine hydroxylase

Introduction

The development of dependence on opiates continues to be a significant clinical and social problem. Psychological dependence is the key point in the study of dependence and the centre of most experimental studies. However, physical dependence is a prominent cause of compulsive drug-taking behaviour and short-term relapse. Thus, elucidation of the various mechanisms involved in physical dependence is required to develop treatment strategies to attenuate or overcome opioid dependence.

Accumulating evidence has demonstrated that, upon repeated exposure to morphine, long-lasting neurochemical alterations occur in discrete brain regions. Changes in gene expression are likely to mediate these adaptations in brain neurochemistry, thereby contributing to dependence and drug addiction (Nestler, 2004). Previous studies from our laboratory have shown that morphine withdrawal results in activation of immediate-early genes in the central nervous system (CNS) and in the heart (Laorden *et al*., 2002a, b; González-Cuello *et al*., 2003, 2004a). These effects are accompanied by an increase in tyrosine hydroxylase (TH) activity and noradrenaline turnover in the paraventricular nucleus (Laorden *et al*., 2000; Benavides *et al*., 2003) and in different cardiac tissues (González-Cuello *et al*., 2004b). However, it remains unclear what are the key intracellular signalling molecules that participate in regulating the alterations in gene expression induced by chronic opiate exposure. Extracellular signal-regulated kinases (ERKs), members of the mitogen-activated protein kinases (MAPK) family, transduce a broad range of extracellular stimuli into diverse intracellular responses and are involved in various processes in the CNS, including drug addiction (Berhow *et al*., 1996; Eitan *et al*., 2003; Mazzucchelli *et al*., 2002).

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The ERK pathway is among numerous signal transduction pathways that could alter gene expression in distinct brain regions in response to repeated opioid exposure. The drug-induced changes in gene expression are considered to be the main reason for the long-lasting alterations in brain neuronal plasticity responsible for the state of addiction (Nestler, 2004). Recently, several studies have shown that the ERK pathway contributes to naloxone-precipitated withdrawal in morphine-dependent rats (Ren *et al*., 2004; Cao *et al*., 2005).

Although the involvement of the ERK pathway in morphine dependence has been reported, no data are available on the characteristics and functional disturbances of the ERKs in the heart after chronic morphine treatment and upon drug withdrawal. Therefore, the purpose of the present study was to assess the changes in the activity of signal transduction and gene expression in the heart after chronic morphine administration and following morphine withdrawal. To this end, we first evaluated in the left and right ventricle the changes in phosphorylation of ERK in morphine-dependent rats, in addition to total levels of ERK. We then investigated if the activation of ERK during morphine withdrawal induces c-Fos expression in the heart. For this purpose, we used SL327, a drug that prevents ERK activation (Atkins *et al*., 1998).

Material and methods

Animals and treatments

Male Sprague–Dawley rats (220–240 g at the beginning of the experiments) were housed (4–5 rats per cage) under a 12-h light/dark cycle (light, 0800–2000 hours) in a room with controlled temperature ($22 \pm 2^{\circ}C$), humidity ($50 \pm 10\%$), food and water available *ad libitum*. The animals were handled for several days preceding the experiment to minimise stress. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the local Committee.

Experimental procedure

Rats were rendered tolerant/dependent on morphine by s.c. implantation of morphine pellets (75 mg morphine base), one on day 1, two on day 3 and three on day 5, under light ether anaesthesia. Control animals were implanted with placebo pellets containing lactose instead of morphine, on the same time schedule. These procedures have repeatedly been shown to induce both tolerance and dependence as measured behaviourally and biochemically (Rabadán *et al*., 1997; Milanés and Laorden, 2000; Milanés *et al*., 2000; Benavides *et al*., 2003). On day 8, morphine- and placebo-treated rats were injected either with saline s.c. or naloxone ($2\,mg\,kg^{-1}$, s.c.). 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 a lower caloric intake (Berhow *et al*., 1995). In addition, body weight loss was determined as the difference between the weight determined immediately before saline or naloxone injection and a second determination made 60 or 90 min later.

To determine the effect of inhibiting protein phosphorylation on the changes in c-Fos expression induced by morphine withdrawal, in the right and left ventricle, c-Fos was determined in morphine-dependent and control rats treated with SL327, a selective inhibitor of MAPK/ERK kinase (MEK) (Atkins *et al*., 1998) 1 h before the administration of naloxone or saline. This inhibitor was dissolved in dimethylsulphoxide (DMSO) 100% and injected intraperitoneally (i.p.) at an injection volume of $1\,ml\,kg^{-1}$ and at doses of 50 and $100\,mg\,kg^{-1}$. On the basis of our initial experiments of SL327-induced inhibition of ERK phosphorylation, the $100\,mg\,kg^{-1}$ dose was chosen for our experiments.

Western blotting

Animals were killed by decapitation 60 (for total ERKs determination and phosphorylated ERK1/2 evaluation), or 90 min (for total ERKs determination, phosphorylated ERK1/2 evaluation and c-Fos analysis) after administration of naloxone or saline. The hearts were rapidly removed, and the right and left ventricles were dissected, fresh frozen and stored at $-80^{\circ}C$ until analysis.

Samples were placed in homogenisation buffer (phosphate buffered saline (PBS), 2% sodium dodecylsulphate (SDS) plus protease and phosphatase inhibitors, Boehringer Mannhein, Germany) and homogenised for 50 s before centrifugation at $6000\,g$ for 20 min at $4^{\circ}C$. Total protein concentrations were determined spectrophotometrically using the bicinchoninic acid method. The optimal amount of protein to be loaded was determined in preliminary experiments by loading gels with increasing protein contents (25–100 μg) from samples of each experimental group. Equal amounts of protein (50 μg per lane) from each sample were loaded on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), electrophoresed, and transferred onto polyvinylidene difluoride membrane (Millipore Corp, Bedford, MA, USA) using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA, USA). Parallel gels were stained with Coomassie blue to verify loading, sample integrity and protein separation. Similar transfer was ascertained by cutting the lower portion of the blot and staining for total protein with Amido Black. Nonspecific binding of antibodies was prevented by incubating membranes in 1% bovine serum albumin (BSA) in Tris-buffered saline containing Tris-buffered saline-Tween 20 (TBST: 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20). The membranes were incubated overnight, at $4^{\circ}C$, with the following primary antibodies: polyclonal anti-ERK (1:1000 dilution; sc-154, Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-phospho-ERK1/2 (1:1000 dilution; sc-7383, Santa Cruz) or polyclonal anti-(c-Fos) (1:2000 dilution; non cross-reactive with Fos-B, Fra-1 or Fra-2, according to the manufacturer's instructions; sc-52, Santa Cruz) 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-rabbit sc-2004 for total ERKs and c-Fos; anti-mouse sc-2005 for phospho-ERK1/2, Santa Cruz) at 1:5000 dilution.

After washing, immunoreactivity was detected with an enhanced chemiluminescence Western blot detection system (ECL, Amersham-Pharmacia-Biotechnology, Madrid, Spain) and visualised by Amersham Hyperfilm-ECL. 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. Blots were subsequently reblocked and probed with 1:8000 anti-actin (mouse clone 5C5, Sigma). Quantification of immunoreactivity corresponding to total ERK and phosphorylated ERKs (42 and 44 kDa) and c-Fos (62 kDa) bands was carried out by densitometry (Gel Doc, BioRad, San Diego, CA, USA). The integrated optical density of the bands was normalised to the background values. Relative variations between the bands of the experimental samples and the control samples were calculated in the same image.

Immunohistochemistry

Rats were killed with an overdose of pentobarbital (100 mg kg^{-1}, i.p.) for c-Fos and phospho-ERK1/2 determination 90 min after naloxone or saline administration. Following anaesthesia, rats were perfused through the descending aorta with 300 ml of PBS (pH 7.4) followed by 500 ml of cold, 4% paraformaldehyde in PBS. Following perfusion, hearts were removed, postfixed in the same fixative and stored at 4°C overnight. The samples were then processed routinely and embedded in paraffin wax. The sections (5 μm thick) were obtained on a microtome (HM310, Microm, Barcelona, Spain) and were immunostained. The sections were deparaffinised in xylene and hydrated in a graded ethanol series. Endogenous peroxidase activity was destroyed by 30 min treatment with 0.3% hydrogen peroxide in PBS. Sections were washed in three 5 min changes of PBS and treated with NSS-PBS (PBS containing 1% normal swine serum; Dako, Glostrup, Denmark; and 0.5 Triton X-100) for 30 min. c-Fos immunohistochemistry was performed as described previously, using the same primary antibody as in western blot analysis, at a dilution 1:3000 for 36 h at 4°C. Then the bound primary antibody was localised by biotinylated secondary anti-rabbit IgG (diluted 1:200 in NSS-PBS; Vector, Burlingame, CA, USA) and subsequently with the avidin–biotin complex (ABC kits; Vector) at room temperature for 1 h each. Visualisation of antigen-antibody reaction sites used 0.033% 3′, 3′-diaminobenzidine (DAB; Sigma) and 0.014% H_2O_2 in 0.05 M Tris-HCl buffer for 7 min. The reaction was stopped in PBS. The same immunohistochemistry procedure as described above was followed to determine phospho-ERK1/2 except that mouse monoclonal anti-phospho-ERK1/2 antibody (diluted 1:1500 in NSS-PBS, overnight) was used as a primary antibody and horse antimouse IgG (1:400, 1 h; Vector) as a secondary antibody. All sections were dehydrated through graded alcohols, cleared in xylene and coverslipped with DPX.

Statistical analysis

Data are presented as mean ± s.e.m. Data were analysed by analysis of variance (ANOVA) followed by the Newman–Keuls *post-hoc* test. Body weight gain and loss in naive and morphine-dependent rats and phospho-ERK1/2 inhibition by SL327 in controls and in morphine dependent rats were analysed by unpaired Student's *t*-test. One-way ANOVA followed by Dunnett's multiple comparison test was used when required. Differences with $P<0.05$ were considered significant.

Drugs and chemicals

Pellets of morphine base (Alcaliber Laboratories, Madrid, Spain) or lactose were prepared by the Department of Pharmacy and Pharmaceutic Technology (School of Pharmacy, Granada, Spain); Naloxone HCl was purchased from Sigma Chemical Co. (St Louis, MO, USA) dissolved in

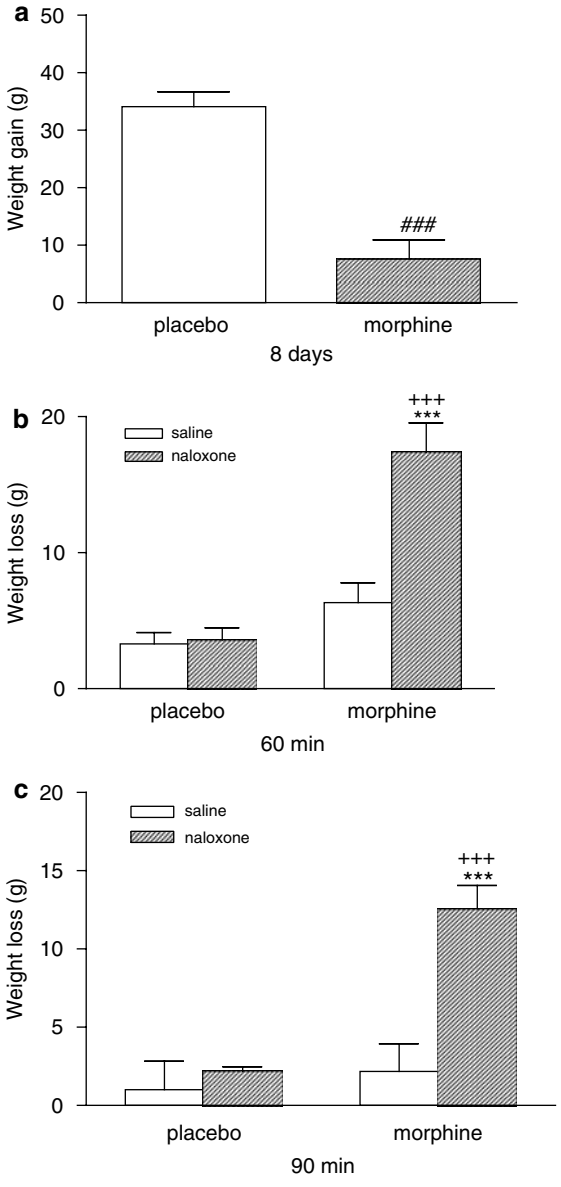

Figure 1 Effect of morphine and morphine withdrawal on the body weight of rats. The body weight was recorded on the days of pellet implantation and on the day of killing (day 8), before receiving any injection (**a**), 60 (**b**) and 90 min (**c**) after saline or naloxone administration. Data represent means ± s.e.m, $n=20$–25. ###$P<0.001$ versus placebo; ***$P<0.001$ versus placebo + naloxone; +++$P<0.001$ versus morphine + saline.

sterile 0.9% NaCl (saline) and administered in volumes of 0.1 ml $100\,g^{-1}$ body weight. SL327, kindly provided by Dr R Santos (Bristol-Myers Squibb Co, Princenton, NY) was dissolved in DMSO. DMSO was purchased from Sigma. Drugs were prepared fresh everyday. Other reagents were from: protease inhibitors (Boehringer Mannhein, Germany); phosphatase inhibitor Cocktail Set (Calbiochem, Germany); goat serum (Sigma); ABC kits (Vector).

Results

Before performing the immunodetection assays, we assessed the efficacy of chronic treatment with implanted morphine pellets, which has been previously shown to induce tolerance and dependence to the effects of morphine (Milanés *et al*., 2001; Laorden *et al*., 2002a; Martínez *et al*., 2003; González-Cuello *et al*., 2004b). For this purpose, the weight of the animals was recorded on the day of pellet implantation and on the day of killing (day 8), before receiving any injections. Rats treated with morphine showed a significantly lower body weight gain than that observed in animals receiving placebo pellets (Figure 1a), as was previously reported (Fuertes *et al*., 2000).

The time course of body weight loss after saline or naloxone injection to placebo-pelleted and morphine-dependent rats is shown in Figure 1b and c. Administration of naloxone to control rats resulted in no significant changes

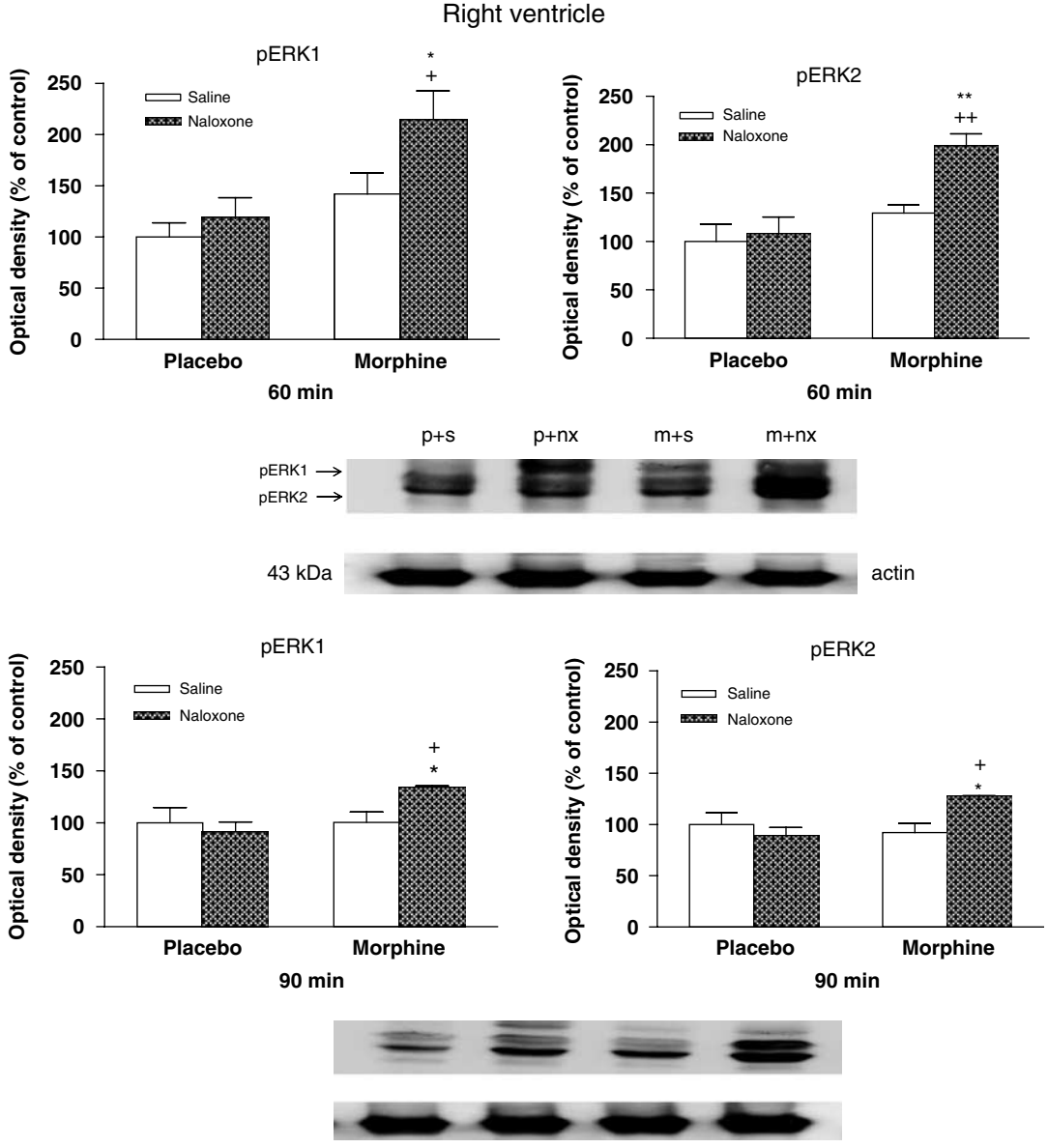

Figure 2 Morphine withdrawal stimulates ERK1/2 phosphorylation in the right ventricle. Representative immunoblots of phospho (p)ERK1 and pERK2 in ventricular tissue isolated from placebo or morphine-dependent rats after subcutaneous administration of saline or naloxone at the time indicated. Actin was used as an internal loading control. For quantification, optical densities of pERK1 and pERK2 immunoreactive bands were measured, normalised to the background values, and expressed as percentages of controls. Data represent means ± s.e.m. ($n = 3$–4). $^{+}P<0.05$; $^{++}P<0.01$ versus morphine + saline. $^{*}P<0.05$, $^{**}P<0.01$ versus placebo + naloxone. ERK, extracellular signal-regulated kinase; m, morphine; nx, naloxone; P, placebo; s, saline .

in body weight when measured 60 or 90 min after drug injection, as compared to control rats receiving saline. However, and in agreement with our previous results (Laorden *et al.*, 2002a), chronically morphine-treated animals showed a significant weight loss 60 or 90 min after naloxone injection, when compared with placebo-pelleted group also receiving naloxone. All the animals undergoing morphine withdrawal displayed characteristic abstinence symptoms: wet-dog shakes, teeth chattering, ptosis, tremor, piloerection, lacrimation, rhinorrhea, chromodiacryorrhea and spontaneous jumping.

The injection of naloxone in rats pretreated with SL327 induced a weight loss similar to that described in the group chronically pretreated with vehicle plus morphine (data not shown). In addition, this MEK inhibitor did not significantly modify the abstinence symptoms observed after naloxone administration to rats chronically treated with morphine pellets.

ERK1/2 activation after naloxone-induced morphine withdrawal in the heart

We tested the influence of morphine dependence and withdrawal on the immunoreactivity of total and phosphorylated ERK1/2 in right and left ventricles. ERK1/2 activation was monitored by western blot analysis using anti-phospho-ERK antibody and quantified in an image analyser. The influence of morphine dependence and withdrawal on phosphorylated ERK1/2 immunoreactivity was examined in the heart at different time points after s.c. injection of naloxone or saline to control rats and to animals considered dependent on morphine. Our time course study shows that chronic morphine treatment did not induce any significant change in phospho-ERK1 or phospho-ERK2 levels in right and left ventricles, at 60 or 90 min after saline injection (Figures 2 and 3). No significant differences in phospho-ERK1 or phospho-ERK2 levels were observed at any time.

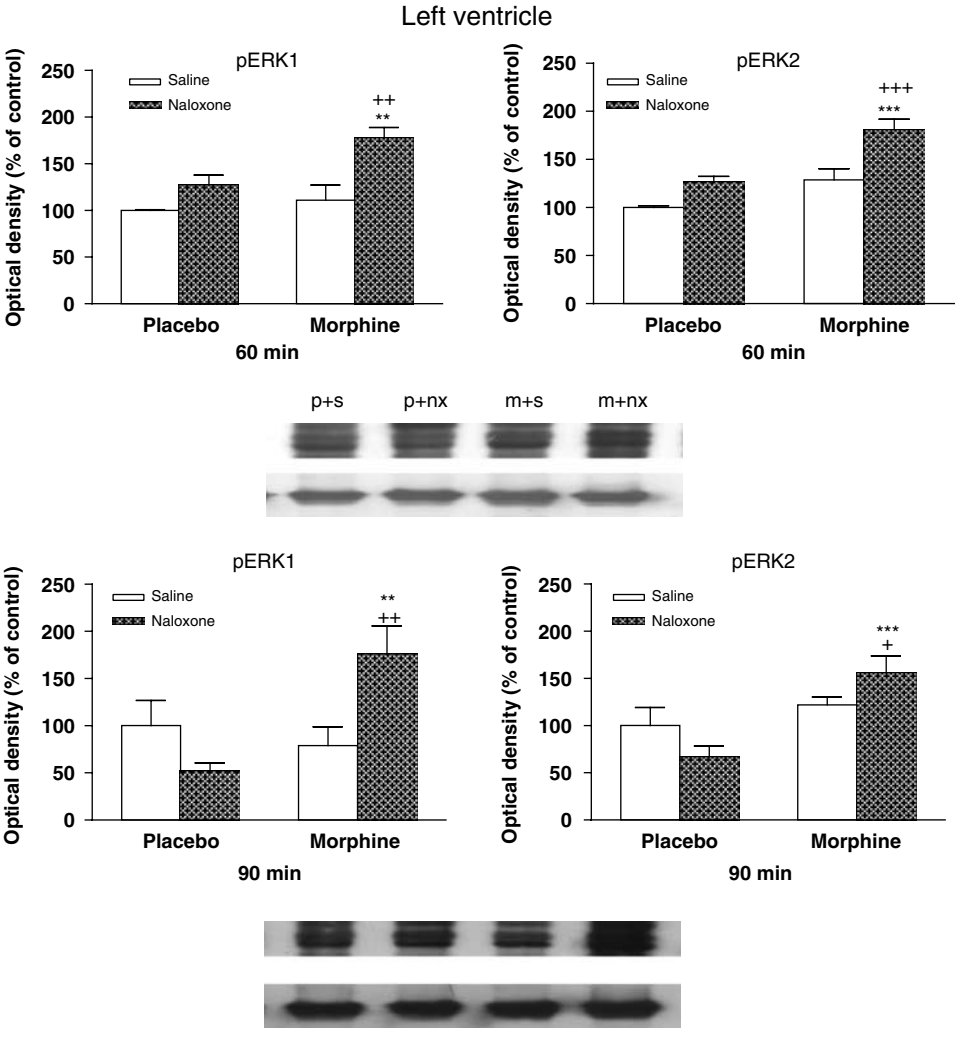

Figure 3 Morphine withdrawal stimulates ERK1/2 phosphorylation in the left ventricle. Representative immunoblots of phospho (p)ERK1 and pERK2 in the left ventricle tissue isolated from placebo- or morphine-dependent rats after subcutaneous administration of saline or naloxone at the time indicated. Actin was used as an internal loading control. For quantification, optical densities of pERK1 and pERK2 immunoreactive bands were measured, normalised to the background values, and expressed as percentages of controls. Data represent means ± s.e.m. ($n = 4$–6). $^{+}P<0.05$; $^{++}P<0.01$, $^{+++}P<0.001$ versus morphine + saline. $^{**}P<0.01$, $^{***}P<0.001$ versus placebo + naloxone. ERK, extracellular signal-regulated kinase; m, morphine; nx, naloxone; P, placebo; s, saline.

Additional experiments were performed to determine whether naloxone-induced morphine withdrawal would activate the ERK pathways in the heart. These experiments demonstrated that acute naloxone treatment had no effect on animals chronically treated with placebo. However, rats chronically treated with morphine and given naloxone showed a significant elevation of phospho-ERK1 and phospho-ERK2 levels in the right and left ventricle 60 or 90 min after administration of the opioid antagonist (Figures 2 and 3). By contrast, phospho-ERK1 and phospho-ERK2 immunoreactivity was not modified after naloxone injection to placebo-pelleted rats compared with the control rats receiving saline.

Total ERK proteins in the right and left ventricle of rats treated with morphine did not significantly differ from that of placebo control rats 60 or 90 min after naloxone administration (Figure 4), indicating that the amount of activated protein, but not total protein, was increasing during chronic morphine treatment and after naloxone-induced morphine withdrawal.

Localization of phospho-ERK1/2 by immunohistochemistry. Regulation by withdrawal from chronic morphine administration

To gain more information concerning the cellular localization of the phospho-ERKs in the heart, we studied the distribution of these proteins by immunohistochemical procedures using the same phospho-ERK1/2 antibody. Rats were killed 90 min after saline or naloxone injection. As shown in Figure 5, high levels of phospho-ERK immunoreactivity were observed in the right and left ventricle after naloxone administration to morphine-dependent rats. The immunolabelling was mainly present in cytoplasmic compartments, suggesting a local activation of the protein. A nuclear staining was also observed in some myocytes, supporting a nuclear translocation of activated ERK proteins. By contrast, there was no staining in the right and left ventricle from control rats given naloxone or dependent rats injected with saline (data not shown). These immunohistochemistry results are consistent with western blot analysis in the present study.

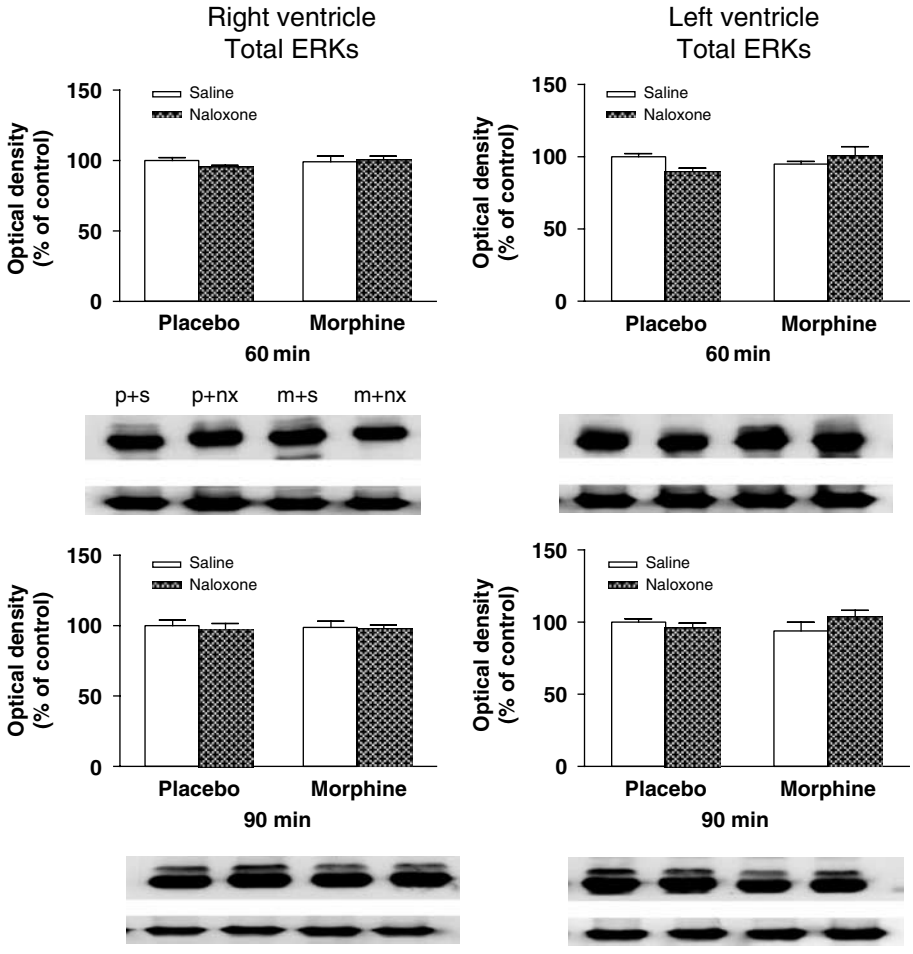

Figure 4 Time course of total ERK during morphine withdrawal. Representative immunoblots of total ERK in the right and left ventricle isolated from placebo or morphine-dependent rats after subcutaneous administration of saline or naloxone at the time indicated. Actin was used as an internal loading control. For quantification, optical densities of total ERK immunoreactive bands were measured, normalised to the background values, and expressed as percentages of controls. Data represent means ± s.e.m. ($n = 4$). ERK, extracellular signal-regulated kinase; m, morphine; nx, naloxone; P, placebo; s, saline.

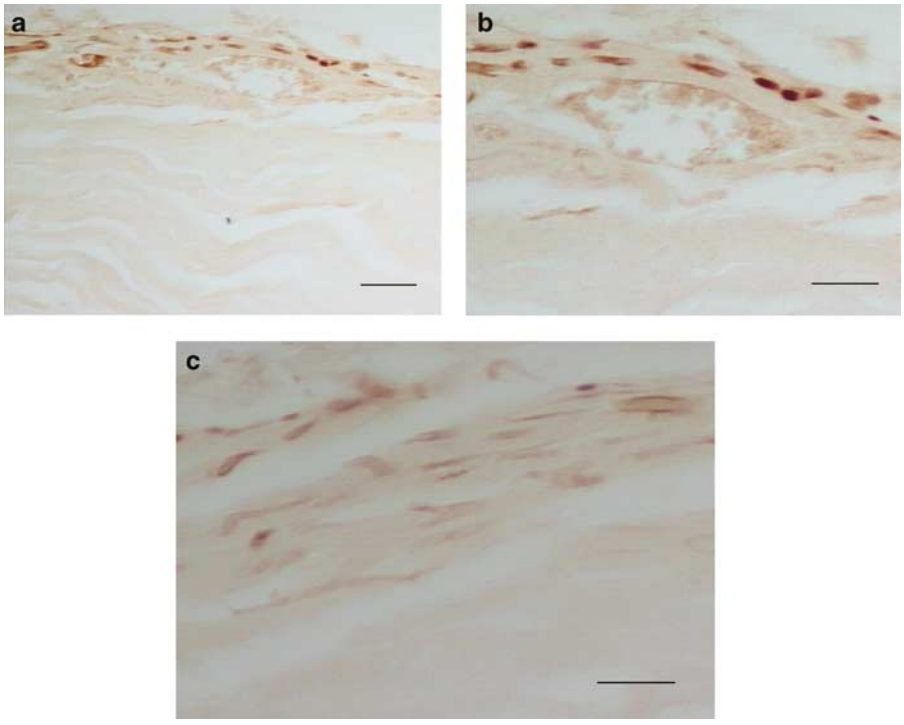

Figure 5 Morphine withdrawal activates ERK1/2 in the right and left ventricle. Rats were made dependent on morphine for 7 days and, on day 8, were injected saline or naloxone (subcutaneous). Controls received placebo pellets at the same time schedule and on day 8 were given with saline or naloxone. At 90 min after injections, rats were perfused and the right and left ventricle was processed for phospho (p)ERK1/2 immunohistochemistry. Photographs show the immunohistochemical detection of pERK1/2 in the left ventricular wall (**a, b, c**). These results are representative of four independent experiments. Normaski interference optics. Scale bar 30 μm (**a**), 20 μm (**b, c**). ERK, extracellular signal-regulated kinase.

Induction of c-Fos by morphine withdrawal is dependent on ERK activation

c-Fos protein, the product of the immediate-early gene *c-Fos* has been used as a marker for cellular activation. Because c-Fos is a prime marker of gene expression after morphine withdrawal and its transcriptional regulation is critically controlled by ERK (Monje *et al*., 2005), we measured morphine withdrawal-induced c-Fos expression and c-Fos expression after inhibition of ERK activation by SL327, a drug that prevents the activation of ERK by inhibiting MEK, the upstream kinase of ERK (Atkins *et al*., 1998). We analysed c-Fos protein in cardiac tissue by western blot and immunohistochemistry, using a specific antibody that did not recognise Fos-related antigens. As shown in Figure 6a, rats dependent on morphine and given naloxone showed significant induction of c-Fos immunoreactivity in the right and left ventricle when compared with the dependent group injected with saline instead of naloxone or the control group injected with the opioid antagonist. Figure 6b shows that administration of naloxone to morphine-dependent rats induced expression of c-Fos in the right, septum and left ventricle (Figure 6b). No c-Fos expression was observed in morphine-pelleted rats injected with saline or in placebo-pelleted rats given with saline or naloxone (data not shown).

In the second step, we determined the basal levels of phospho-ERK1/2 in the heart in control and in rats during morphine withdrawal, pretreated with SL327 at 100 mg kg^{-1} i.p. As shown in Figure 7, SL327 significantly attenuated basal levels of phospho-ERK1 and phospho-ERK2 in the right ventricle. Similar results were obtained in the left ventricle (data not shown). Next, we injected SL327 (100 mg kg^{-1} i.p.) in control rats and in animals made dependent on morphine, 1 h before saline or naloxone administration and determined c-Fos expression in the right and left ventricle. As shown in Figure 8, c-Fos expression was strongly decreased in samples from morphine-dependent rats given SL327 before naloxone, when compared to morphine-dependent rats treated with vehicle before the opioid antagonist.

Discussion and conclusions

In the present study, we found that naloxone-induced morphine withdrawal increased phosphorylated ERK1/2 in rat heart, suggesting that this treatment stimulated activity of the ERKs. Moreover, the morphine withdrawal-induced increase in ERKs activity was shown to be attributable to an enhancement in the phosphorylation state of the enzyme, without changes in total ERK immunoreactivity. This suggests that the effects of morphine withdrawal that may be mediated by ERK1/2 are likely to be affected through the activation (via phosphorylation) of ERKs. Modulation of the ERKs pathways in different brain regions was relevant to tolerance, dependence and reward (Ozaki *et al*., 2004; Valjent *et al*., 2004; Cao *et al*., 2005) after opioids or cocaine

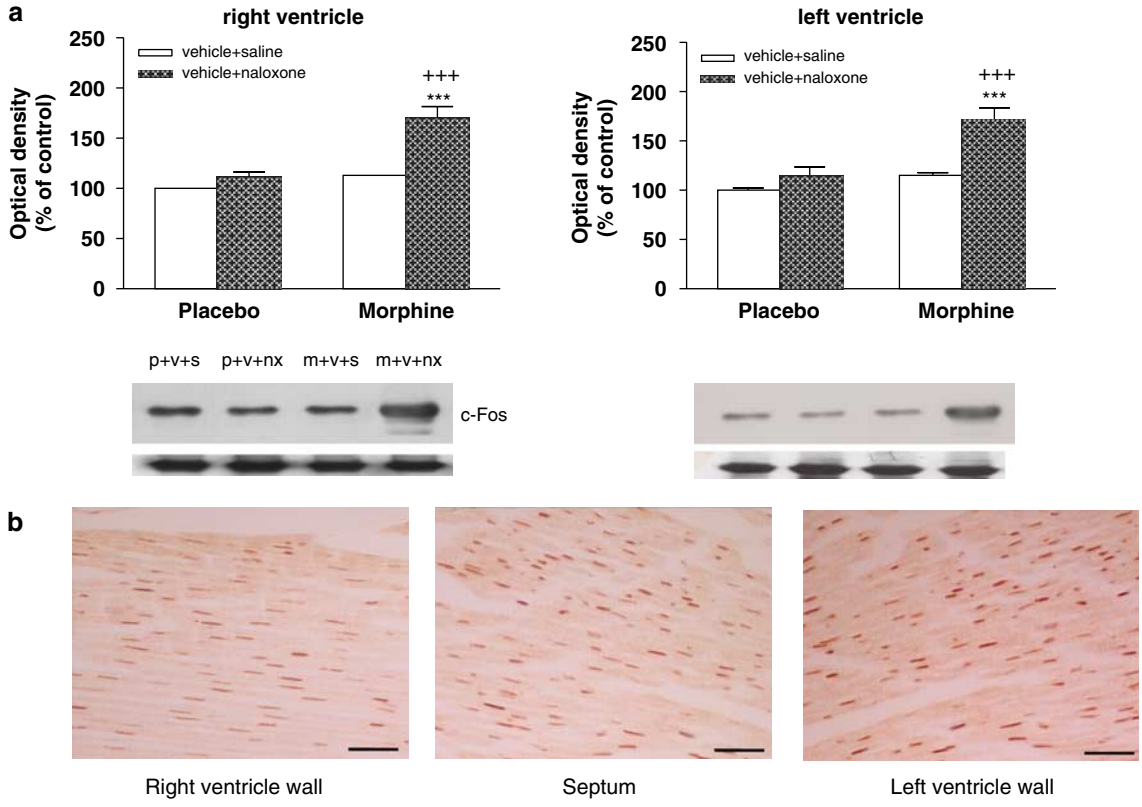

Figure 6 Morphine withdrawal stimulates c-Fos expression in the right and left ventricle. (**a**) Representative immunoblots of c-Fos in samples isolated from placebo- or morphine-dependent rats 90 min after subcutaneous administration of saline or naloxone. Actin was used as an internal loading control. For quantification, optical densities of c-Fos immunoreactive bands were measured, normalised to the background values, and expressed as percentages of controls. Data represent means ± s.e.m. ($n = 4$–6). $^{+++}P < 0.001$ versus morphine + saline.***$P < 0.001$ versus placebo + naloxone. (**b**) Represents photomicrographs of c-Fos immunoreactvity in the right and left ventricular wall and in the septum, after naloxone-precipitated withdrawal. Results are representative of four independent experiments. Normaski interference optics. Scale bar 58 μm. m, morphine; nx, naloxone; P, placebo; s, saline; v, vehicle (DMSO).

administration. The second important observation in this study is that chronic morphine treatment did not induce changes in the basal activity of ERKs in the heart. These results contrast with findings observed in several brain regions (Lesscher *et al*., 2003; Muller and Unterwald, 2004).

It is known that morphine dependence and withdrawal alter the levels and/or activity of various signalling elements. These chronic adaptive molecular mechanisms involve gene expression and/or some protein kinases, which are relevant for signalling processes involving protein phosphorylation and gene expression (Nestler and Aghajanian, 1997; Koob *et al*., 1998; Kreek and Koob, 1998; Liu and Anand, 2001). ERK is a family of serine/threonine protein kinases that have been functionally linked to addiction through phosphorylation of transcription factors leading to changes in target gene expression (Yang *et al*., 2003). ERK phosphorylates various substrates, including many enzymes, transcription factors and proteins. ERK is able to shuttle between the cytoplasm and the nucleus. Following activation, ERK dissociates from cytoplasmic anchors such as MEK and translocates to the nucleus, where it phosphorylates its nuclear substrates. Activated ERK does not always localise to the nucleus. Several transcription factors are activated by ERK in the cytoplasm and then translocate to the nucleus after phosphorylation (Ebisuya *et al*., 2005).

The present results revealed that, 90 min after naloxone-induced morphine withdrawal, phosphorylated ERKs are expressed in the heart. An interesting observation was the different subcellular localization of activated ERK proteins. The phospho-ERK staining appeared primarily cytosolic and was observed in the cytoplasm. A nuclear staining was also observed in cardiac myocytes. The ERK activation in cytoplasmic compartments suggests a local role of the protein at cytosolic effector proteins that govern specific functions. Previous studies have demonstrated that activated ERK can target a number of cytosolic proteins, including many enzymes such as TH (Haycock, 1993). It has been previously shown that morphine withdrawal activates enzymatic TH activity as well as noradrenaline turnover in the noradrenergic terminals innervating the heart (González-Cuello *et al*., 2004b).In addition, the nuclear activation in the heart samples suggests an effect in gene expression.

We have previously shown that morphine withdrawal results in c-Fos expression in the heart, an event involving activation of α-adrenoceptors (González-Cuello *et al*., 2003, 2004a). In support of our previous observations, present

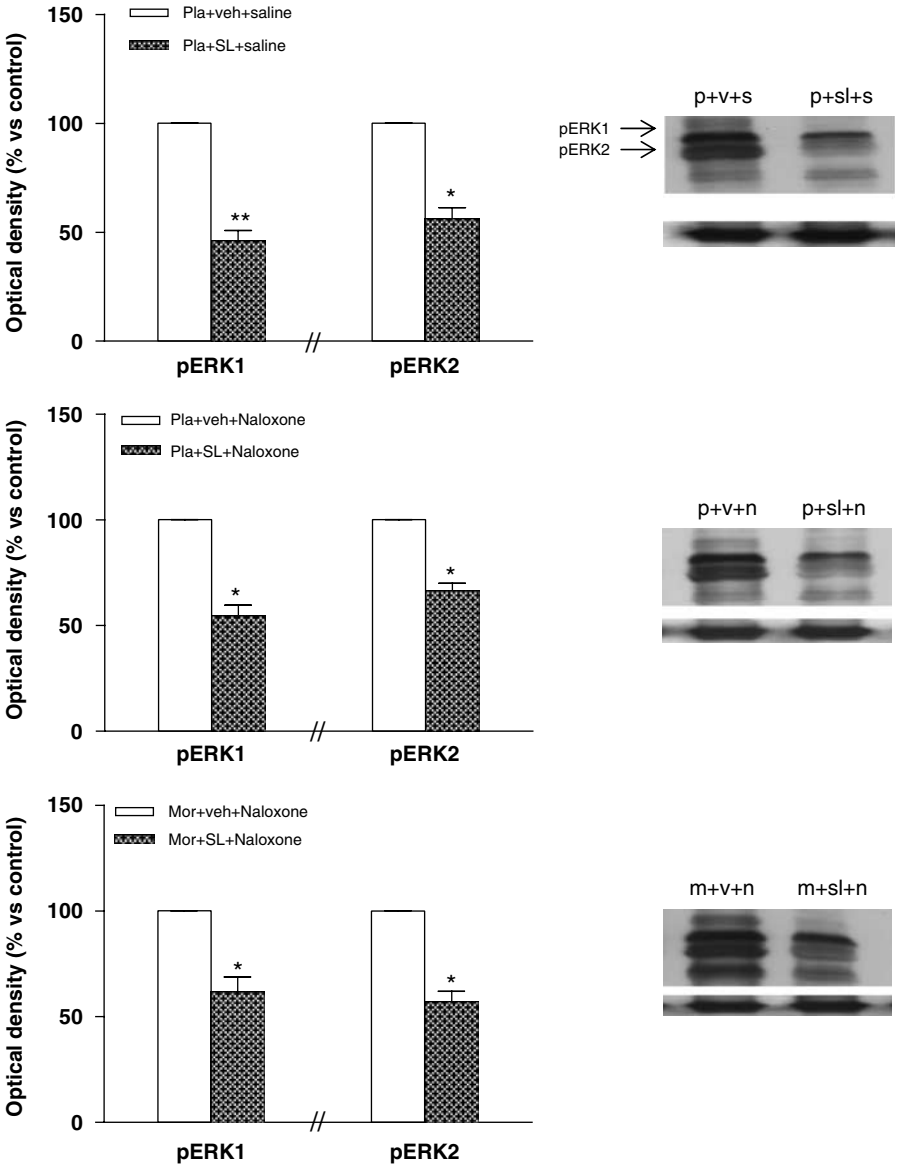

Figure 7 Immunoblots of ERK1/2 in right ventricles isolated from placebo- or morphine-dependent rats after subcutaneous administration of saline or naloxone in absence or presence of SL327 (100 mg kg^{-1}), 1 h before saline or naloxone injection. Actin was used as an internal loading control. Phospho (p)ERK1 and pERK2 immunoreactive bands were measured, normalised to the background values, and expressed as percentages of controls. Data correspond to mean$\pm$s.e.m. ($n=4$). *$P<0.05$, **$P<0.01$ versus its control group. ERK, extracellular signal-regulated kinase; m, morphine; nx, naloxone; p, placebo; s, saline; ,sl, SL327; v, vehicle (DMSO).

experiments also show that morphine withdrawal gives rise to an increase in c-Fos expression in the heart. After nuclear translocation, activated ERK controls transcription of immediate-early genes. In fact, phosphorylation of ERK is one of the major pathways for induction of c-Fos. In agreement with this, a strong inhibition of morphine withdrawal-induced c-Fos expression in the heart was found after treatment with the MEK inhibitor SL327. It suggests that phosphorylation of ERK1/2 is one of the major pathways for induction of c-Fos in the heart. However, previous results from our laboratory showed that inhibition of PKC also produced an inhibition of c-Fos expression in the heart (Almela *et al.*, 2006), suggesting that the transcriptional regulation of c-Fos in the heart seems to be under a combined control of an ERK-dependent and -independent pathway.

On the other hand, the expression of c-Fos in the heart, mainly due to phosphorylation of ERK 1/2, was not antagonised by propranolol or prazosin (González-Cuello *et al.*, 2004a). These results suggest that the activation of ERK and c-Fos expression is not due to an indirect mechanism via sympathetic activation.

In summary, our present results reveal that morphine withdrawal induced a direct activation of ERKs and suggest that the ERKs play an important role in the expression of c-Fos. These findings provide a new mechanism to explain the neurobiological substrate of morphine withdrawal-induced heart hyperactivity.

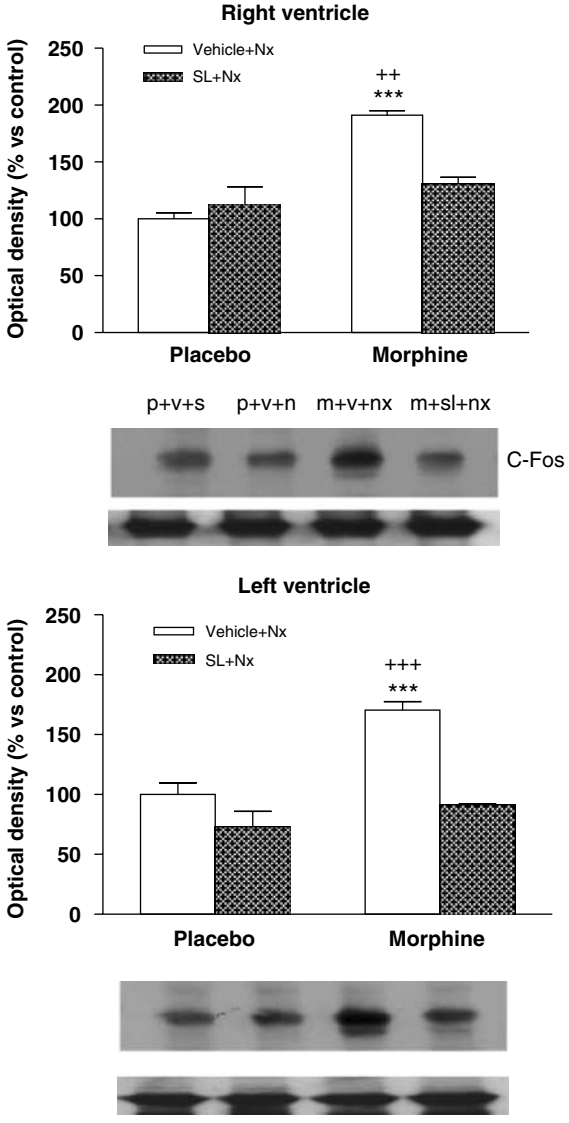

Figure 8 Morphine withdrawal stimulates c-Fos expression in the right and left ventricle. Representative immunoblots of c-Fos in the right and in the left ventricle tissue isolated from placebo- or morphine-dependent rats, 90 min after subcutaneous administration of naloxone in the absence or presence of SL327 (100 mg kg^{-1}) 1 h before naloxone. Actin was used as an internal loading control. c-Fos immunoreactive bands were measured, normalised to the background values and expressed as percentages of controls. Data correspond to mean ± s.e.m. ($n = 4$). $^{++}P<0.01$, $^{+++}P<0.001$ versus morphine + SL + saline; ***$P<0.001$ versus placebo + veh + naloxone. m, morphine; nx, naloxone; p, placebo; s, saline; ,sl, SL327; v, vehicle (DMSO).

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Conflict of interest

The authors state no conflict of interest.

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