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Pathways Involved in the Cardiac Adaptive Changes Observed During Morphine Withdrawal

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1. Introduction

The development of opioid addiction involves complex adaptive changes in opioid receptors and associated signalling systems, leading to neuronal plasticity in the brain regions projecting to different systems including the cardiovascular system. So, adaptive changes also occur in peripheral tissues and cells expressing opioid receptors, such as in the heart (Pugsley, 2002).

The effects of drugs of abuse, especially cocaine, on the cardiovascular system, have been extensively documented in animal model and in human. There is emerging evidence that drug abuse might trigger a variety of cardiac disorders from arrhythmias to acute myocardial infarction, heart failure and even sudden cardiac death (Lippi et al., 2010). Thus, various types of cardiac arrhythmias have been described in heroin addicts. Moreover, street heroin addicts frequently die suddenly, and there is evidence that this is an arrhythmia-related event (Nerantzis et al., 2011).

The majority of studies dealing with morphine on the field of cardiology are oriented on clinical usage of this drug and current cardiovascular research has been limited to the evaluation of factors or pathways believed to contribute to its physiological actions, such as delta- and kappa-opioid receptors, cyclooxygenase-2, inducible nitric oxide synthase or reactive oxygen species (Huh et al., 2001; Wang et al., 2001; Jiang et al., 2006; Xu et al., 2011).

Given the importance of morphine in clinical practice for the treatment of pain, investigation of its impact on the heart at the molecular levels requires more attention. Therefore, in this chapter we will discuss our recent discoveries about the implication of different molecular pathways in the cardiac adaptive changes that occur during morphine withdrawal.

The noradrenergic pathways and the hypothalamo-pituitary-adrenocortical (HPA) axis, a system largely controlled by corticoticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus, comprise two major adaptation mechanisms to stress. Like stressors, morphine withdrawal activates HPA axis in rats, which results in neuronal activation of stress-related neurosecretory neurons in the parvocellular neurons of the PVN. The PVN is anatomically divided into three magnocellular and five parvocellular subdivisions. The parvocellular subdivisions comprise the dorsal, lateral, medial periventricular and anterior parvocellular subnuclei (fig. 1).
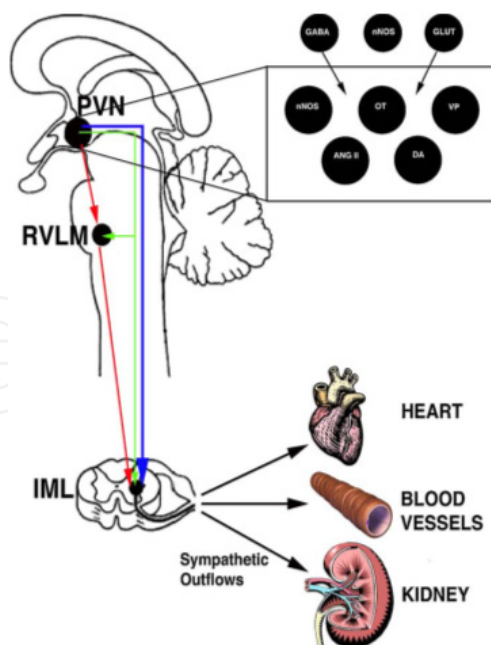
Fig. 1. Schematic illustrating the three main pathways by which the paraventricular nucleus of the hypothalamus (PVN) can influence sympathetic activity. Rostral ventrolateral medulla (RVLM), spinal intermediolateral cell column (IML). (Taken from Pyner, 2009).

These regions project to autonomic nuclei in the brain stem and spinal cord and are responsible for the activation of the sympathetic nervous system including cardiovascular regulation (Sawchenko and Swanson, 1982). In addition, the PVN receives afferent projections from several limbic structures that are implicated in behavioural and cardiovascular control, such as the medial amygdale, the prefrontal cortex and the lateral septum (Ongur et al., 1998; Risold and Swanson, 1997).

2. Hemodynamic variables during chronic morphine treatment and its withdrawal

Previous studies have demonstrated that chronic µ-opiod receptor stimulation decreases muscle sympathetic nerve activity (Kienbaum et al., 2001; 2002), NA plasma concentration (Kienbaum et al., 2001) and dopamine turnover in the heart (Rabadán et al., 1997). According to these data, we have demonstrated that chronic morphine treatment decreases two baseline cardiovascular parameters, mean arterial blood pressure (MAP) and heart rate (HR). However, µ-opioid receptor blockade by naloxone unmasks these effects, resulting in markedly increases in both parameters (fig. 2 and 3). In agreement with these data, naloxone administration to patients with chronic opioid abuse or to morphine dependent rats results in markedly increased muscle sympathetic nerve activity, NA plasma concentrations (Peart and Gross, 2006), NA and dopamine turnover (Almela et al., 2008; Milanés et al., 2000b) and total tyrosine hydroxylase (TH) expression (Almela et al., 2008). Altogether, these results suggest that an up-regulation of TH would be expected to increase the capacity of noradrenergic neurons to synthesize NA, which could contribute to the increase in NA turnover and in the hemodynamic changes seen in the heart during morphine withdrawal.
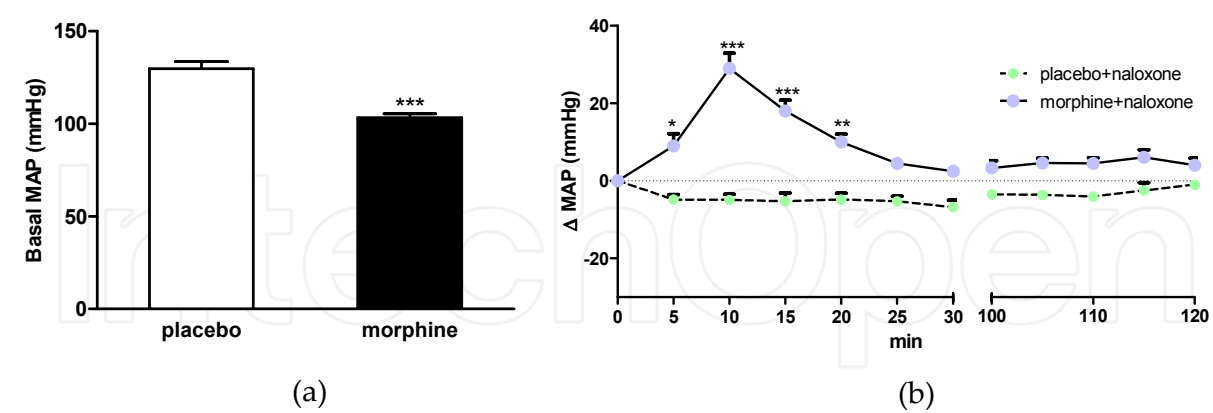
(a)   (b)

Fig. 2. Baseline mean arterial blood pressure (MAP) (mmHg) (A) in rats implanted with morphine or placebo pellets. Effects of naloxone (2 mg/kg s.c.) on changes in MAP (B). Naloxone was injected at time 0. Data are the mean±S.E.M. (n=5–7). ***P<0.001, **P<0.01, *P<0.05 versus placebo+naloxone.
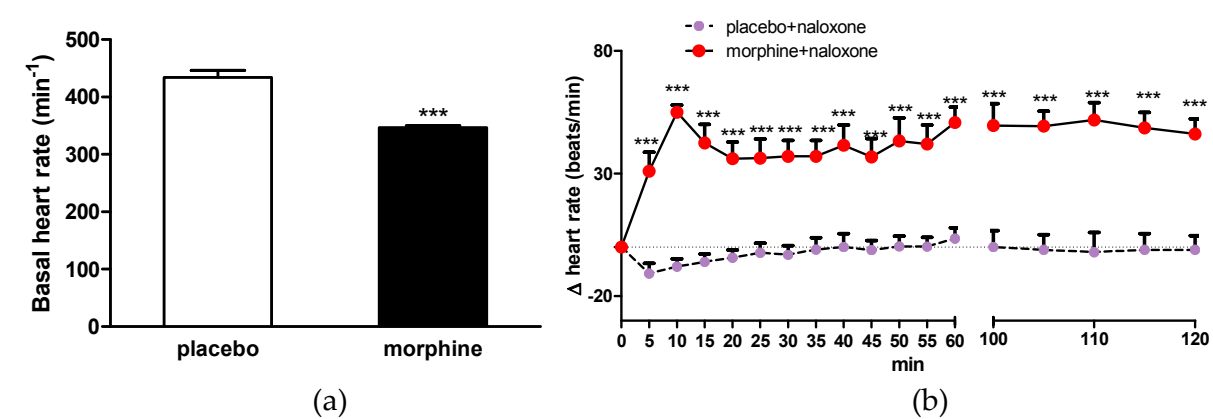

(a)   (b)

Fig. 3. Baseline heart rate (min−1) (A) in rats implanted with morphine or placebo pellets. Effects of naloxone (2 mg/kg s.c.) on changes in heart rate (B). Naloxone was injected at time 0. Data are the mean±S.E.M. (n=5–7). ***P<0.001, **P<0.01, *P<0.05 versus placebo+naloxone.

3. Evaluation of changes in pERK1/2 during morphine withdrawal

Extracellular signal-regulated kinase (ERK), one member of mitogen-activated extracellular kinase (MAPK) family, transduces a broad range of extracellular stimuli into diverse intracellular responses. ERK signalling pathway could be important as regulator of cardiac function (Michel et al., 2001) and neuronal plasticity (Adams et al., 2002). Recently, several studies have shown that this pathway contributes to naloxone-precipitated withdrawal in morphine dependent rats (Ren et al., 2004; Almela et al., 2007, 2008, 2011).

Our time course study showed that there was a significant elevation of phospho(p)ERK1 and phospho(p)ERK2 levels in the right (fig. 4) and left ventricle 30, 60, 90 or 120 min

after naloxone administration to morphine dependent rats. We also studied the distribution of these proteins by immunohistochemical procedures and we observed high levels of pERK1/2 immunoreactivity in the right and left ventricle after naloxone administration to morphine-treated rats (fig. 5). 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. These immunohistochemical results were consistent with western blot analyses (figure 4).
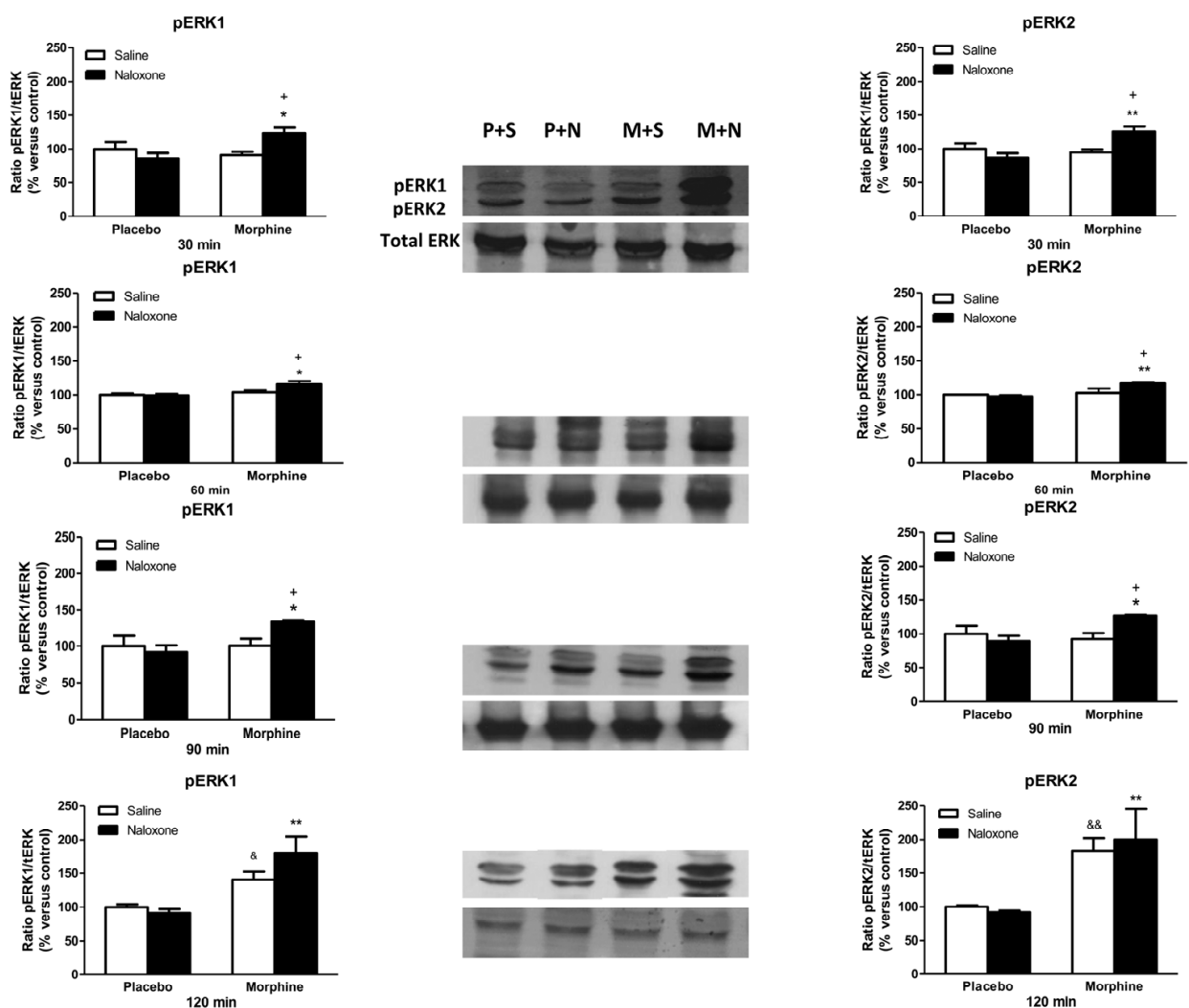

Fig. 4. Western-blotting analysis of phospho(p)ERK1 and phospho(p)ERK2 in the right ventricle 30, 60, 90 and 120 min after saline (S) or naloxone (N) administration to placebo- (P) or morphine- (M) pretreated rats. The immunoreactivity corresponding to pERK1 or pERK2 is expressed as a percentage of that in the control group defined as 100% value. Data are the mean±S.E.M. (n=5–6). **P<0.01, *P<0.05 versus the placebo group injected with naloxone; +P<0.05 versus the dependent group injected with saline instead of naloxone; &&P<0.01, &P<0.05 versus the placebo group receiving saline.
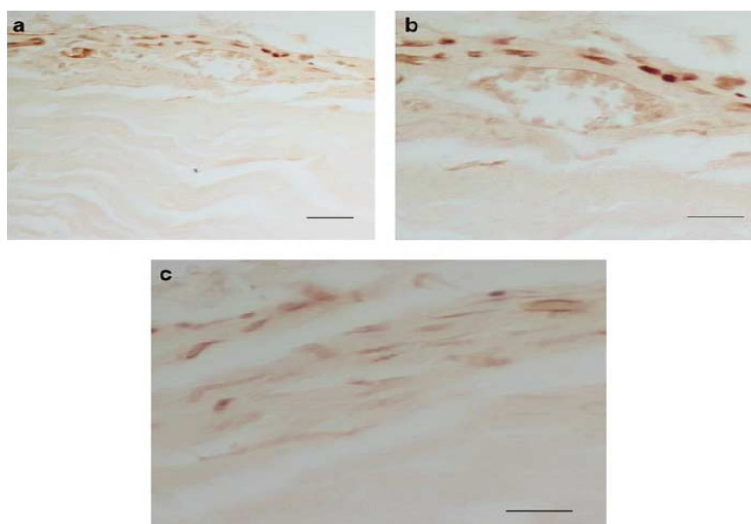
Fig. 5. Immunohistochemical detection of phospho(p)ERK1/2 in the left ventricular wall. Rats were made dependent on morphine for 7 days and on day 8 were injected with naloxone (2 mg/kg s.c.). 90 min after injections, rats were perfused and the right and left ventricle was processed for pERK1/2 immunohistochemistry. Scale bar 30 μm (a), 20 μm (b, c).

4. Tyrosine hydroxylase phosphorylation

TH, the rate limiting enzyme in the synthesis of catecholamines, plays important roles in the regulation of sympathetic nervous system and its impact on cardiac function (Rao et al., 2007). In particular, increases in the phosphorylation of Ser40 and Ser31 accelerate TH activity, thereby stimulating production of neurotransmitter in catecholamines terminals (Kumer and Vrana, 1996; Dunkley et al., 2004). TH expression is subjected to intricate regulation by a number of mechanisms, including transcriptional and post-transcriptional processes (Kumer and Vrana, 1996; Mallet, 1999). Short-term regulation of catecholamine biosynthesis occurs through the modulation of the state of phosphorylation of TH. TH phosphorylation and activation is the primary mechanism responsible for the maintenance of catecholamine levels in tissues after catecholamine secretion. TH can be phosphorylated at serine (Ser) residues 8, 19, 31 and 40 by a variety of PKs (Campbell et al., 1986). PKA and PKC phosphorylate TH only at Ser40 (Roskoski et al., 1987; Funakoshi et al., 1991). ERK1 and ERK2 were shown to phosphorylate Ser31 in situ (Haycock et al., 1992). The phosphorylation of Ser40 increases the enzyme's activity in vitro, in situ and in vivo. Phosphorylation at Ser31 also increases the activity but to a much lesser extent than Ser40 phosphorylation. The phosphorylation of TH at Ser19 or Ser8 has no direct effect on TH activity (Dunkley et al., 2004) (fig. 6).

Previous studies have shown that naloxone-induced morphine withdrawal results in an increased NA turnover at heart level (Milanés et al., 2000a). This enhancement in NA turnover could be due to changes in the state of phosphorylation of TH, which are critically involved in the regulation of catecholamines synthesis and function. Therefore, we have studied the expression and phosphorylation at Ser31 and Ser40 during morphine withdrawal at different time points. Rats withdrawn from morphine presented an increase in total TH expression (fig. 7) and in TH phosphorylated at Ser31 (fig. 8) and Ser40 (fig. 9), together with an enhancement of TH activity (fig. 10). This activation of TH could be responsible for the increase in the hemodynamic parameters described above.
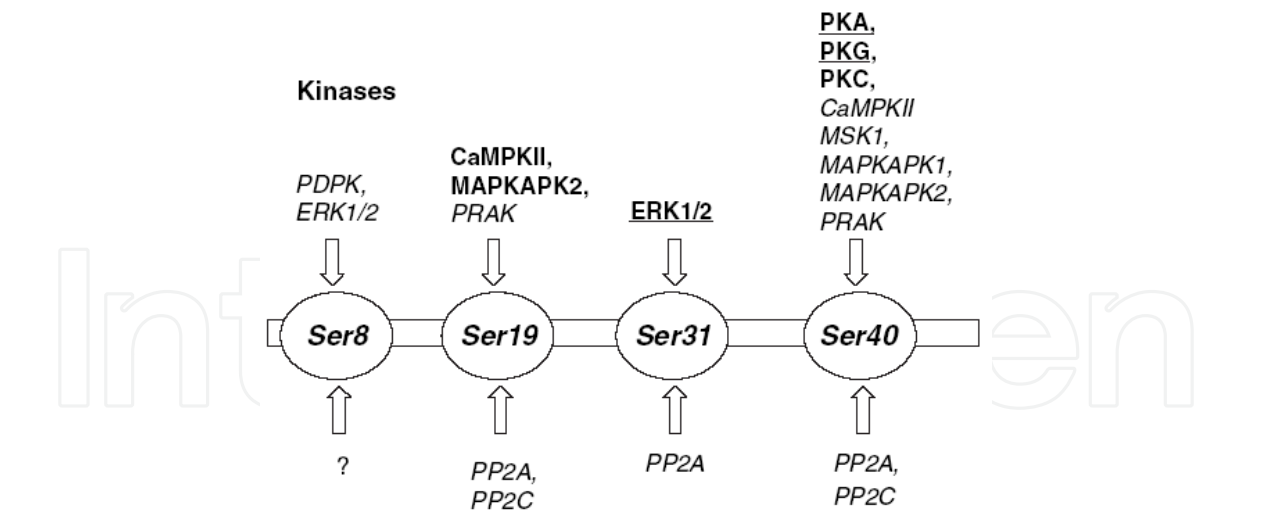
Fig. 6. The protein kinases (PK) and protein phosphatases (PP) capable of modulating tyrosine hydroxylase (TH) phosphorylation in vitro and in situ
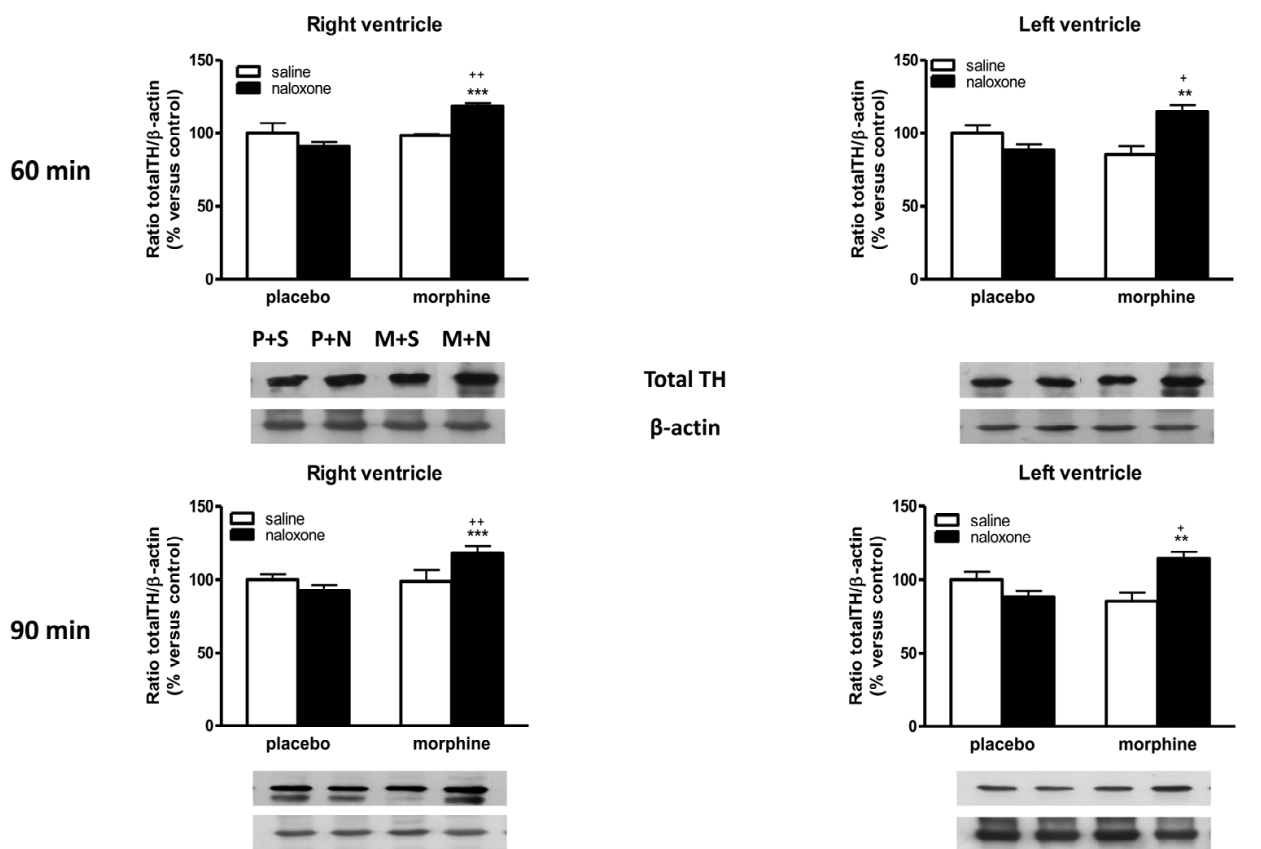(Taken from Dunkley et al., 2004).

Fig. 7. Western blotting analysis of TH immunoreactivity levels in the right and left ventricle 60 or 90 min after saline (S) or naloxone (N) administration to placebo– (P) or morphine- (M) treated rats. The immunoreactivity corresponding to total TH is expressed as a percentage of that in the control group (P+S; defined as 100%). Data are the mean±S.E.M (n=4–6). **P<0.01, ***P<0.001 versus the group receiving saline instead of naloxone; +P<0.05, ++P<0.01 versus the group pretreated with placebo instead of morphine.
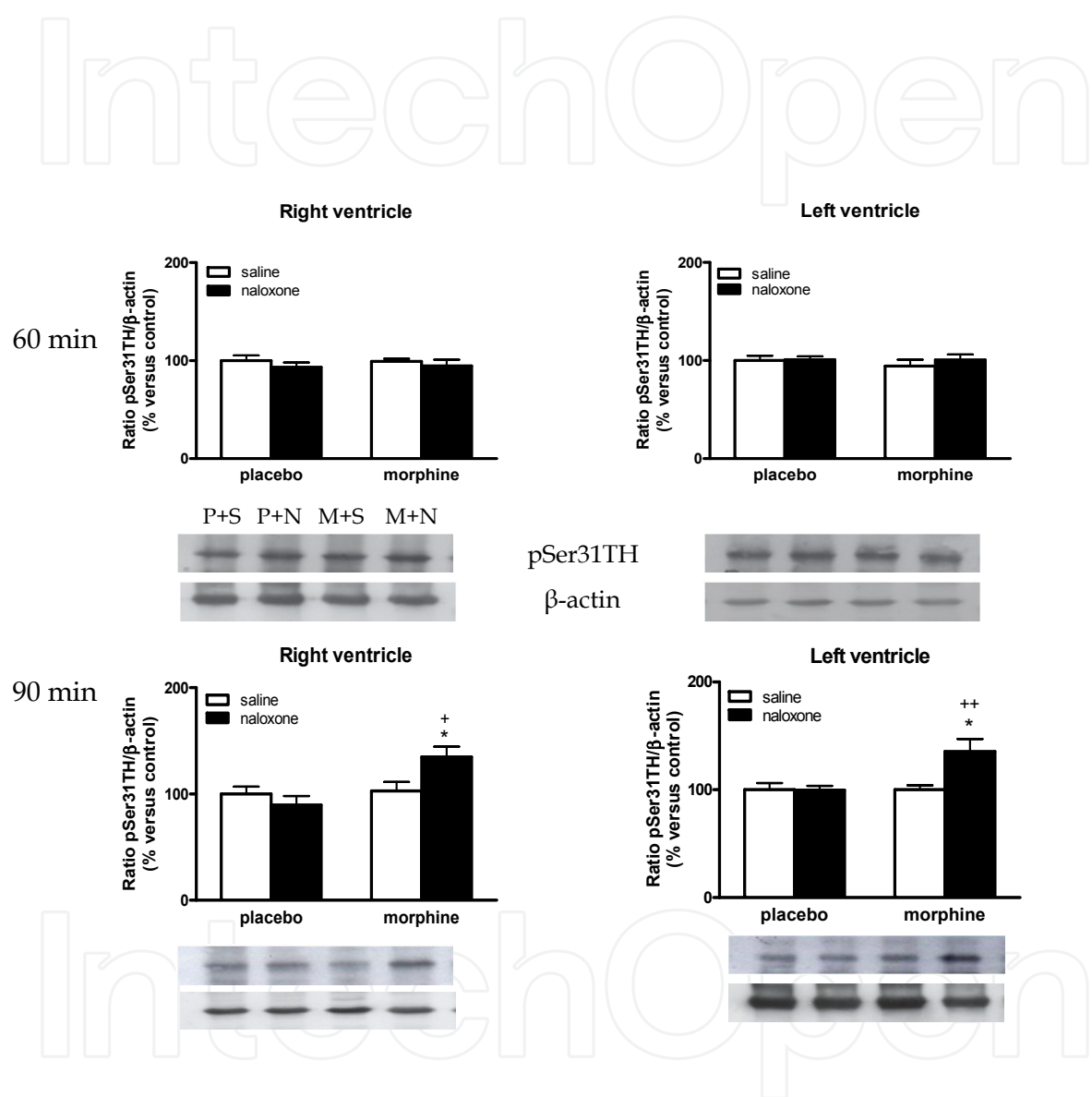
Fig. 8. Western blotting analysis of phospho(p)Ser31TH in the right and left ventricle 60 or 90 min after saline (S) or naloxone (N) administration to placebo- (P) or morphine- (M) treated rats. The immunoreactivity corresponding to pSer31TH is expressed as a percentage of that in the control group (P+S; defined as 100%). Data are the mean±S.E.M. (n=4–6). *P<0.05 versus the group receiving saline instead of naloxone; ++P<0.01, +P<0.05 versus the group pretreated with placebo instead of morphine.
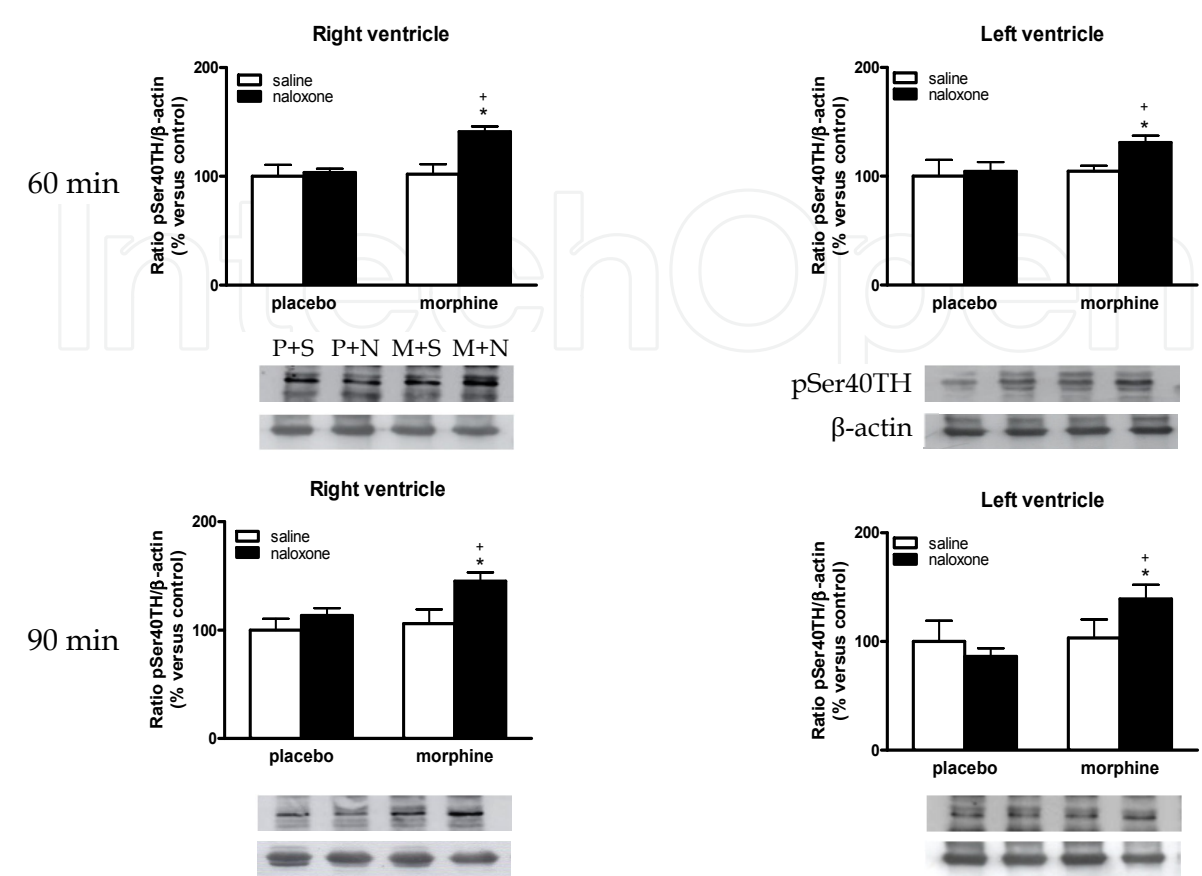
Fig. 9. Western blotting analysis of phospho(p)Ser40 TH in the right and left ventricle 60 or 90 min after saline (S) or naloxone (N) administration to placebo- (P) or morphine- (M) treated rats. The immunoreactivity corresponding to pSer40TH is expressed as a percentage of that in the control group (P+S; defined as 100%). Data are the mean±S.E.M. (n=4–6). *P<0.05 versus the group receiving saline instead of naloxone; +P<0.05 versus the group pretreated with placebo instead of morphine.
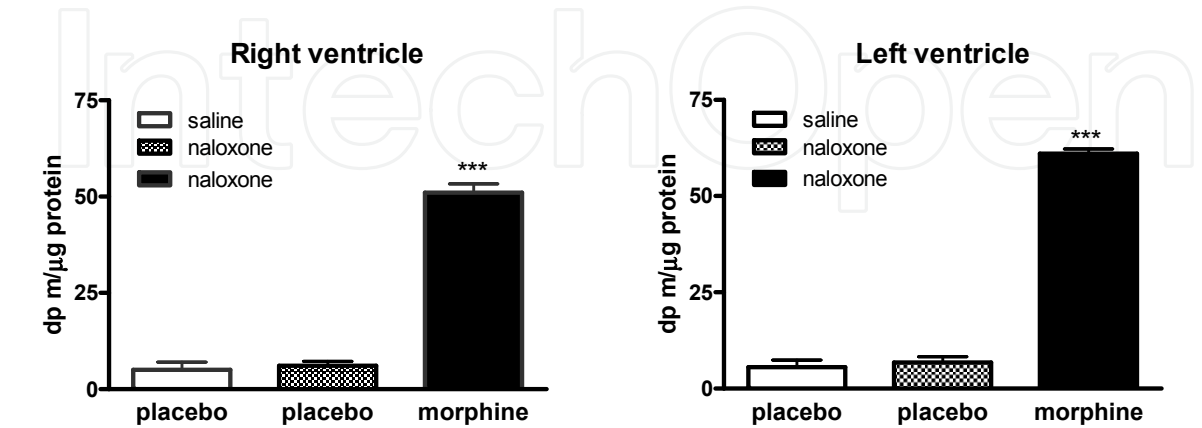

Fig. 10. TH activity in right and left ventricle from placebo or morphine dependent rats 90 min after s.c. administration of saline or naloxone. Data are the mean±S.E.M. (n=4–6). ***P<0.001 versus the group pretreated with placebo instead of morphine.

5. Changes in c-Fos expression

c-Fos immunoreactivity was examined by western blot and immunohistochemistry. Western blot analysis showed that after naloxone injection to rats chronically treated with morphine, there was a significant induction of c-Fos immunoreactivity in the right and left ventricle. Immunohistochemical analysis corroborated these results. Thus, rats dependent on morphine and given naloxone showed a significant induction of c-Fos immunoreactivity in the right ventricle, septum and left ventricle (fig. 11). This increase of c-Fos could contribute to activate TH synthesis through its activity on the AP-1 sequence present in the TH gene promoter region.
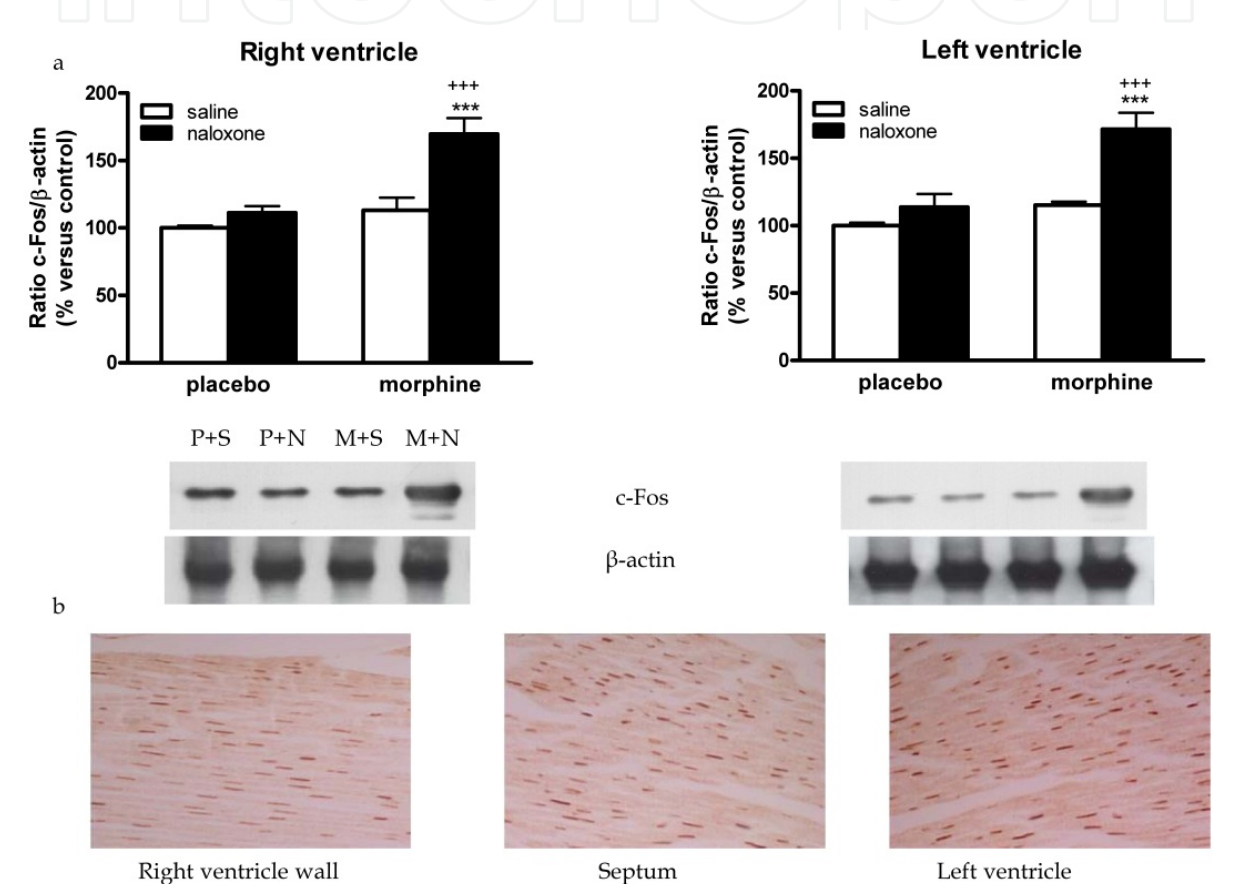

Fig. 11. (a) Representative immunoblots of c-Fos in samples isolated from placebo (P) or morphine (M) dependent rats 90 min after s.c. administration of saline (S) or naloxone (N). For quantification, optical densities of c-Fos immunoreactive bands were measured, normalized to the background values, and expressed as percentage of controls, defined as 100% value. Data are the mean±S.E.M. (n=4–6). +++P<0.001 versus M+S; ***P<0.001 versus P+N. (b) Photomicrographs of c-Fos immunoreactvity in the right and left ventricular wall and in the septum, after naloxone-precipitated withdrawal. Scale bar 58 μm.

6. Implication of ERK and PKA in the cardiac adaptive changes observed during morphine withdrawal

To assess the relative contribution of ERK and PKA to the regulation of c-Fos and TH, we examined morphine withdrawal-induced c-Fos expression in animals receiving SL327, a

selective ERK inhibitor or HA-1004, a selective PKA inhibitor. SL327 administration before naloxone to rats chronically treated with morphine significantly diminished the increase in c-Fos levels in both ventricles (fig. 12).
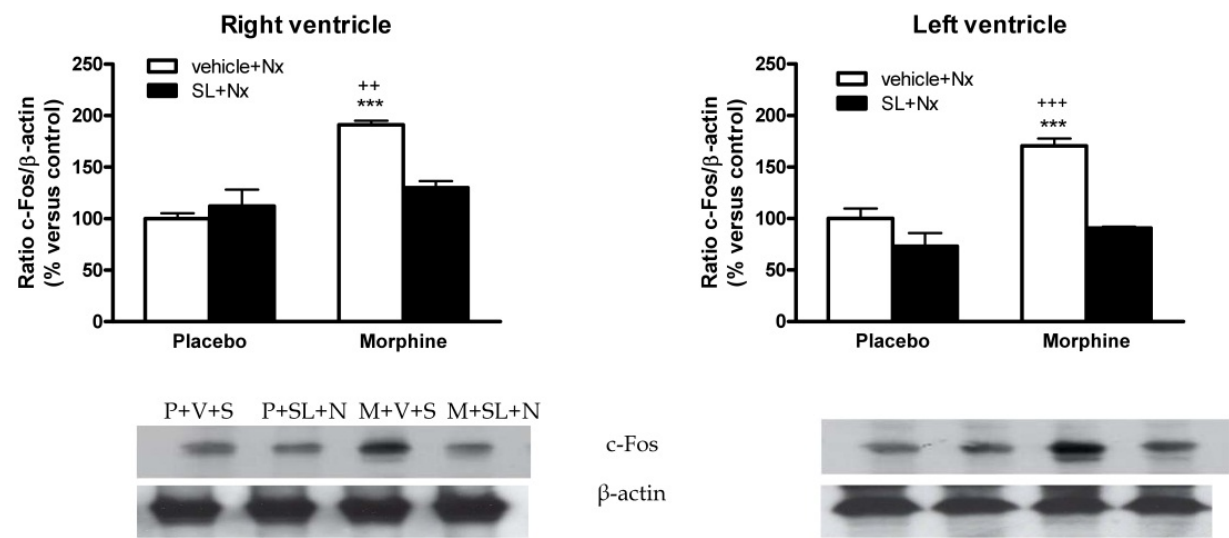

Fig. 12. Morphine withdrawal stimulates c-Fos expression in the right and left ventricle. Representative immunoblots of c-Fos in the right and left ventricle tissue isolated from placebo (P) or morphine (M) dependent rats, 90 min after s.c. administration of naloxone (Nx, N) in the absence (vehicle, veh, V, DMSO) or presence of SL327 (SL, 100 mg/kg) 1 h before naloxone. c-Fos immunoreactive bands were measured, normalized to the background values and expressed as percentages of controls. Data correspond to mean±S.E.M. (n=4). $^{+++}$P<0.001, $^{++}$P<0.01 versus M+SL+N; ***P<0.001 versus P+V+N.

However, chronic inhibition of PKA concurrently with morphine treatment did not modify c-Fos induction during morphine withdrawal (fig. 13). These results reveal that ERK but not PKA is an important pathway mediating c-Fos induction. 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 seems to be under a combined control of an ERK-dependent and-independent pathway. On the other hand, the expression of c-Fos, mainly due to phosphorylation of ERK1/2, was not antagonized by propranolol or prazosin (González-Cuello et al., 2004), suggesting that the activation of ERK and c-Fos expression is not due to an indirect mechanism via sympathetic activation.

In addition, our results showed that SL327 blocks the increase in TH phosphorylated at Ser31 observed in the right and left ventricle after the injection of naloxone to morphine dependent rats (fig. 14). The only protein kinase reported to phosphorylate TH at Ser31 in vitro was ERK (Haycock et al., 1992; Lindgren et al., 2002). In situ phosphorylation of TH at Ser31 increases TH activity and catecholamine synthesis (Haycock, 1992). Given that TH is phosphorylated on a specific serine residue (Ser31) by the ERK, it is possible that activation of ERK1/2 in the heart provides a way in which TH is regulated under morphine dependence.
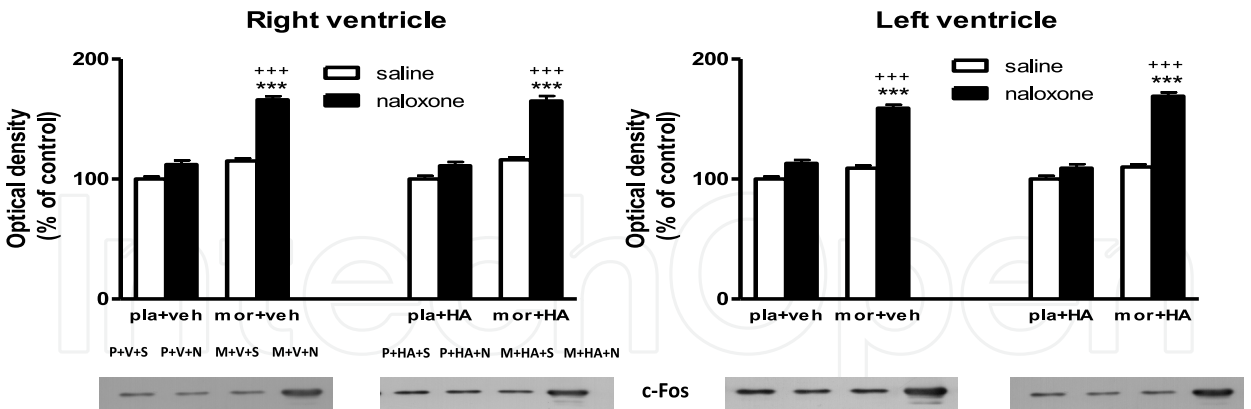
Fig. 13. Western blotting analysis of c-Fos immunoreactivity levels in the right and left ventricle after naloxone-precipitated withdrawal in vehicle- (veh, V) infused rats and in animals chronically administered with HA-1004 (HA). Animals received s.c. implantation of placebo (pla, P) or morphine (mor, M) pellets for 7 days and concomitantly were infused with vehicle or HA-1004 (40 nmol/day). On day 8, rats were injected with saline (S) or naloxone (N, 5 mg/kg, s.c.) and were decapitated 90 min later. The immunoreactivity corresponding to c-Fos is expressed as a percentage of that in the control group (P+V+S; defined as 100% value). Data are the mean±SEM (n=4–6). ***p<0.001 versus the group receiving saline instead of naloxone; +++p<0.001 versus the group pretreated with placebo instead of morphine.
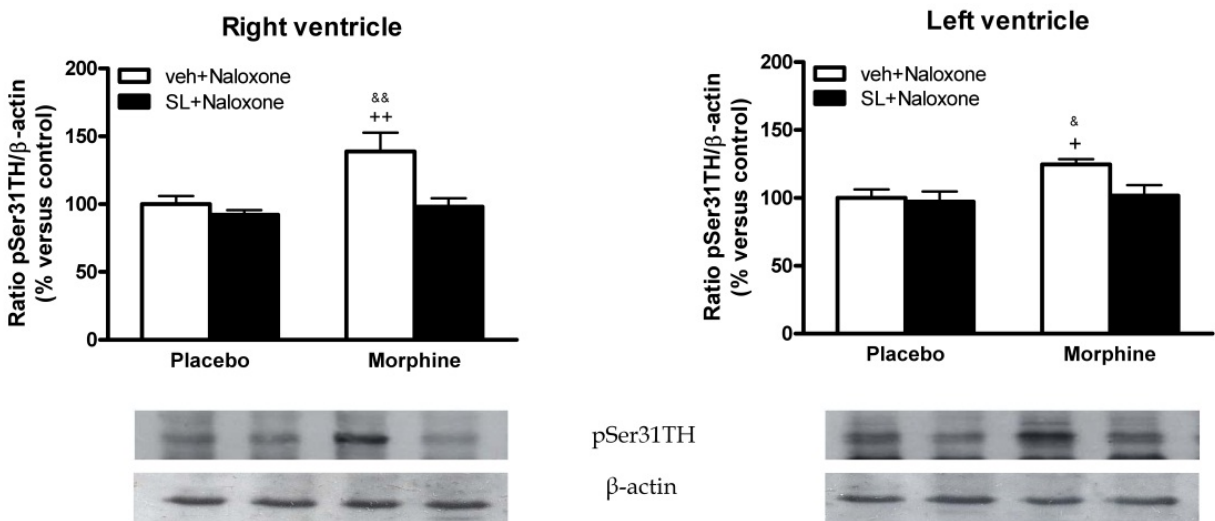

Fig. 14. Phospho(p)Ser31TH immunoblots in right and left ventricle from placebo or morphine dependent rats 90 min after s.c. administration of naloxone in the absence or presence of SL327 (SL, 100 mg/kg, i.p.), 1 h before naloxone. pSer31TH immunoreactive bands were measured, normalized to the background values and expressed as percentage of controls. Data are the mean±S.E.M. (n=4–6). ++P<0.01 versus the group pretreated with placebo instead of morphine; &&P<0.01 versus morphine+SL+naloxone.

Similarly, HA-1004 blocked the enhancement of TH phosphorylated at Ser40 in the heart after morphine withdrawal (fig. 15), suggesting that different pathways are implicated in the postranscriptional regulation of TH.
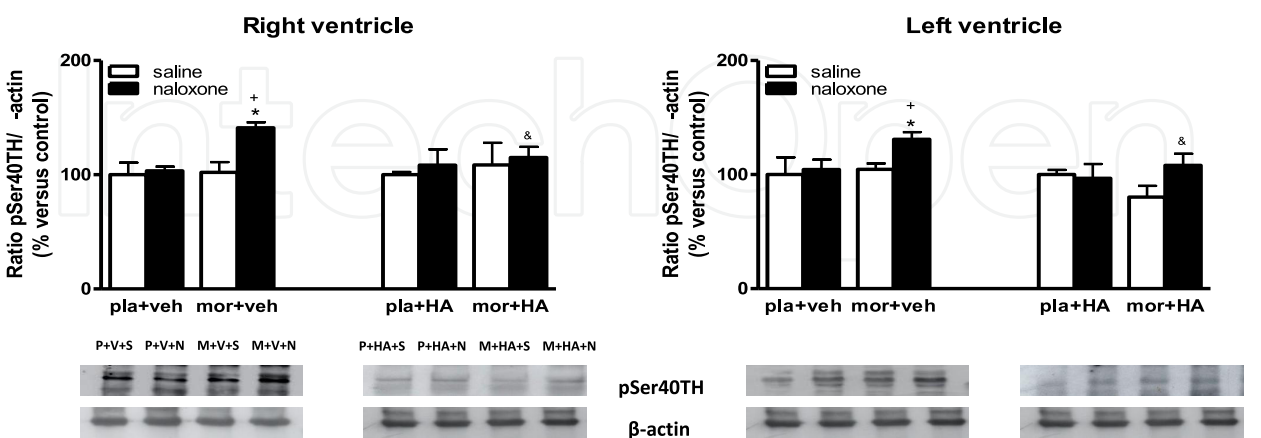

Fig. 15. Western blotting analysis of phospho(p)Ser40TH in the right ventricle 60 min after saline (S) or naloxone (N) administration to placebo- (pla, P) or morphine- (mor, M) treated rats receiving vehicle (veh, V) or HA-1004 (HA). The immunoreactivity corresponding to pSer40TH is expressed as a percentage of that in the control group (P+V+S; defined as 100%). Data are the mean±S.E.M. (n=4–6). *P<0.05 versus the group receiving saline instead of naloxone; +P<0.05 versus the group pretreated with placebo instead of morphine; &P<0.05 versus the group receiving vehicle instead of HA-1004.

7. Crosstalk between PKA and ERK

It is now appreciated that crosstalk among various signal pathways plays an important role in activation of intracellular and intranuclear signal transduction cascades. In our study, chronic treatment with HA-1004 antagonized the increase in ERK1/2 phosphorylation observed during morphine withdrawal in the heart (fig. 16). These results suggest a crosstalk between PKA and ERK pathways.

To assess the contribution of PKA to the regulation of TH, we examined TH phosphorylated at Ser31 during morphine withdrawal in animals receiving the selective inhibitor of PKA HA-1004. This inhibitor prevents the ability of naloxone-precipitated morphine withdrawal to increase TH phosphorylated at Ser31 levels in the right and left ventricle (fig. 17).

Although the mechanism of crosstalk between PKA and ERK pathways has not yet been clarified, it is possible that PKA pathway facilitates MEK1/2 that activates the ERK1/2 pathway (Obama et al., 2007; Stork and Schmitt, 2002). The activated ERK pathway increases the phosphorylation of proteins related to morphine dependence, including TH. Using phosphorylation state-specific antibodies directed toward TH at Ser31, we have shown that HA-1004 blocked the increase in the level of TH phosphorylation at Ser31 induced after naloxone injection to morphine dependent rats in the right and left ventricle. These data suggest that crosstalk between PKA and ERK pathways is a key regulatory design necessary to regulate the Ser31 phosphorylation of TH.
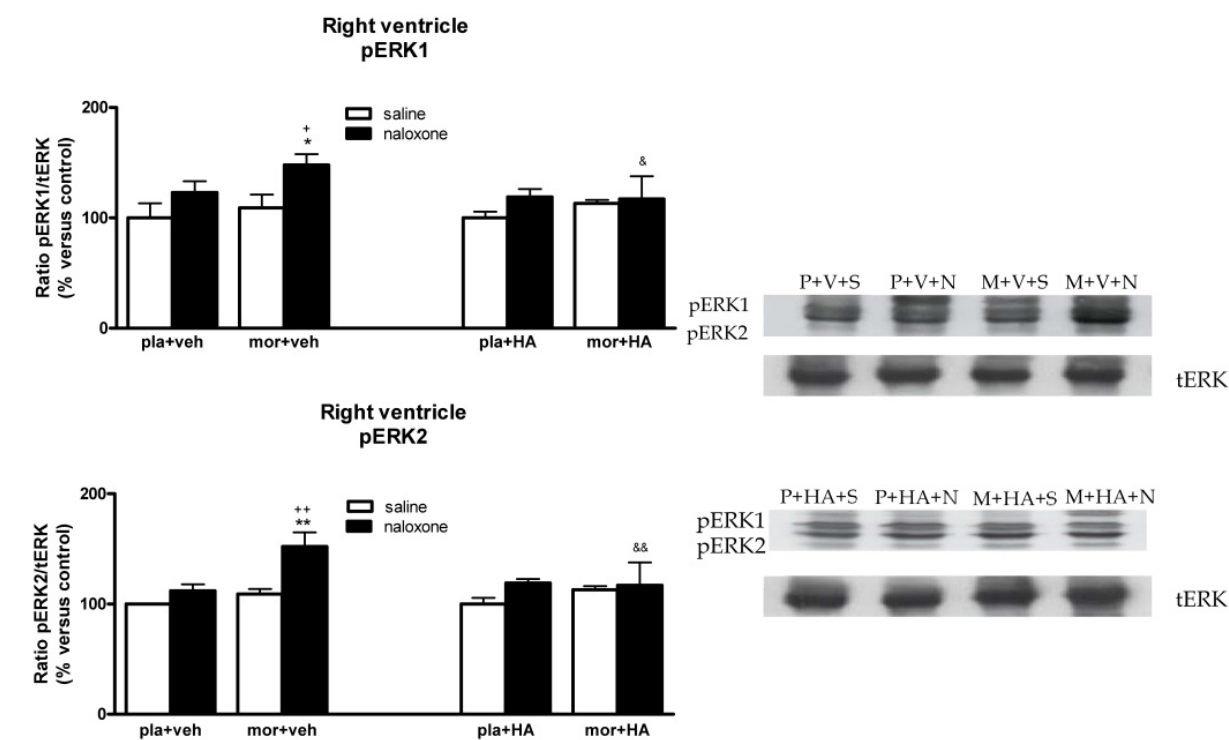
Fig. 16. Western blotting analysis of phospho(p)ERK1 and phospho(p)ERK2 immunoreactivity levels in the right ventricle 60 min after saline (S) or naloxone (N) administration to placebo- (pla, P) or morphine- (mor, M) treated rats receiving vehicle (veh, V) or HA-1004 (HA). The immunoreactivity corresponding to pERK1 or pERK2 is expressed as a percentage of that in the control group (P+V+S; defined as 100% value). Data are the mean±S.E.M. (n=4-6). **P<0.01, *P<0.05 versus the dependent group receiving saline instead of naloxone. ++P<0.01, +P<0.05 versus the group pretreated with placebo instead of morphine injected with naloxone. &&P<0.01, &P<0.05 versus the group receiving vehicle instead of HA.
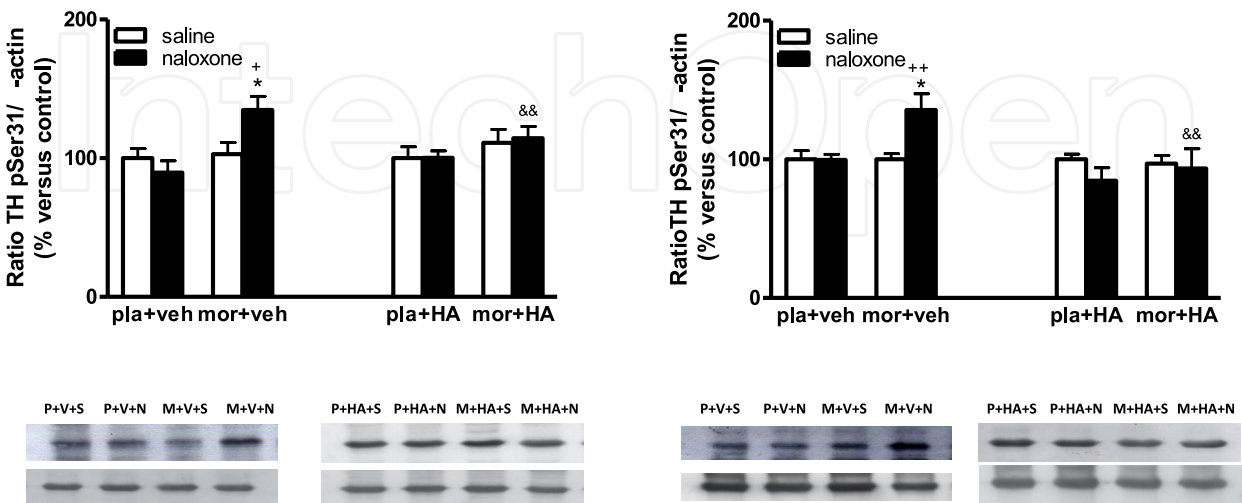

Fig. 17. Western blotting analysis of phospho(p)Ser31TH in the right and left ventricle 90 min after saline (S) or naloxone (N) administration to placebo- (pla, P) or morphine- (mor, M)

treated rats receiving vehicle (veh, V) or HA-1004 (HA). The immunoreactivity corresponding to pSer31TH is expressed as a percentage of that in the control group (P+V+S or P+HA+S; defined as 100% value). Data are the mean±S.E.M. (n=4–6). *$p<0.05$ versus the dependent group receiving saline instead of naloxone; ++P<0.01, +P<0.05 versus the group pretreated with placebo instead of morphine injected with naloxone. &&P<0.01 versus the group receiving vehicle instead of HA.

8. Conclusion

Naloxone administration after chronic morphine treatment, triggers neurochemical adaptations in the noradrenergic system and enhances PKA and ERK pathways. The functional consequences of this activation include an increase in TH activation and NA turnover and an enhancement in c-Fos expression. Many pathways implicated in the adaptive changes observed during withdrawal are subject to feedback mechanisms that can either amplify or suppress their own signalling and there is considerable signalling from one pathway to another, a phenomenon known as crosstalk. Consequently, the responses that cells mount to specific environmental conditions depend on the sum of the intensity and duration of signals from several pathways and how they interact with each other. Although the mechanism of crosstalk between PKA and ERK pathways has not yet been clarified, it is possible that PKA pathway facilitates MEK1/2 that activates the ERK1/2 pathway (Obama et al., 2007; Stork and Schmitt, 2002). The activated ERK pathway increases the phosphorylation of proteins related to morphine dependence, including TH. Our data suggest that crosstalk between PKA and ERK pathways is a key regulatory design necessary to regulate the phosphorylation of TH. These findings provide a new explanation to understand the complex mechanisms implicated in the adaptive changes observed during morphine withdrawal and could be useful for future treatment strategies.

9. Acknowledgment

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