Regulation of pleiotrophin, midkine, receptor protein tyrosine phosphatase β/ζ and their intracellular signaling cascades in the nucleus accumbens during opiate administration

Regular research article

Daniel García-Pérez* M.D., M. Luisa Laorden M.D. Ph.D. and M. Victoria Milanés M.D. Ph.D.

Group of Cellular and Molecular Pharmacology, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain. IMIB, Instituto Murciano de Investigación Biosanitaria, Murcia, Spain.

* Corresponding author at: Department of Pharmacology, Faculty of Medicine, Campus de Espinardo, 30100 Murcia, Spain. Phone number: +34868887183. E-mail address: daniel.garcia9@um.es (D. García-Pérez)

Abstract: 224

Manuscript text: 4841

References: 59

Figures: 8

Short title: Morphine regulates pleiotrophin and midkine.

Abbreviations: Pleiotrophin (PTN); midkine (MK); receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ); nucleus accumbens (NAc); glial fibrillary acidic protein (GFAP); GFAP-immunoreactivity (GFAP-IR); dopamine (DA).
Abstract

**Background:** Most classes of addictive substances alter the function and structural plasticity of the brain reward circuitry. Midkine (MK) and pleiotrophin (PTN) are growth/differentiation cytokines which, similarly to neurotrophins, play an important role in repair, neurite outgrowth and cell differentiation. PTN or MK signaling through receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), leads to the activation of extracellular signal-regulated kinases (ERKs) and thymoma viral proto-oncogene (Akt). This activation induces morphological changes and modulates addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines.

**Methods:** In the present work we studied the effect of acute morphine, chronic morphine and morphine withdrawal on PTN, MK, and RPTPβ/ζ expression and on their signaling pathways in the nucleus accumbens (NAc).

**Results:** Present results indicated that PTN, MK and RPTPβ/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminoceptive neurons expressed RPTPβ/ζ.

**Conclusions:** All these observations suggest that the neurotrophic and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by cytokines.

**Key words:** Morphine; withdrawal; astrocyte; pleiotrophin; midkine; glial fibrillary acidic protein.
Introduction

The action of many addictive substances converges on the mesolimbic dopaminergic reward pathway, inducing increased firing of dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and a subsequent increase of dopamine (DA) release in the nucleus accumbens shell [NAc(shell)] (Di Chiara & Imperato, 1988; Ikemoto, 2007). Additionally, drugs of abuse produce widespread effects on the structure and function of neurons throughout the brain reward circuitry, which are believed to underlie the long-lasting behavioral phenotypes that characterize addiction (Russo et al., 2009). However, the molecular mechanisms regulating the neuronal remodeling are not fully understood yet.

The role of glial cells in providing structural, metabolic and trophic support to neurons has been well established (Kettenmann & Ransom, 1995). Moreover, glial cells are considered the immune competent cells of the central nervous system (CNS) as well as crucial components of synaptic plasticity (Huang et al., 2000; Ransohoff & Brown, 2012). There is increasing evidence that drugs of abuse produce alterations in CNS immunology. For example, opioids induce profound changes in glial cellular morphology and phenotypic immunohistological marker expression (GFAP: glial fibrillary acidic protein, a cell surface marker of astrocyte reactivity) in specific brain areas (Beitner-Johnson et al., 1993). Importantly, the actions of opioids through glial reactivity are involved in the development of opioid dependence (Hutchinson et al., 2008; Hutchinson et al., 2009; Watkins et al., 2009).

It has been suggested that midkine (MK), a secreted heparin binding growth factor/cytokine (Kadomatsu et al., 1988) and pleiotrophin (PTN), also known as heparin binding-growth associated molecule (HB-GAM) (Deuel et al., 2002) could be involved in addiction to drugs of abuse. PTN mRNA and/or MK mRNA levels are up-regulated after acute amphetamine administration (Le Grevès, 2005) and after injection of delta-9-
tetrahydrocannabinol (Mailleux et al., 1994) or morphine (Ezquerra et al., 2007) in brain areas related to addiction, such as the NAc(shell), the prefrontal cortex and the hippocampus, respectively. Likewise, increased mRNA and protein levels were found in the prefrontal cortex of alcoholics and tobacco smokers (Flatscher-Bader & Wilce, 2008). Given that these cytokines exert effects that are similar to those of neurotrophins, these findings support the hypothesis that these two cytokines are up-regulated in order to induce neurotrophic or neuroprotective effects during drug consumption (Herradón & Pérez-García, 2013). MK and PTN bind common receptors, including receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ) (Muramatsu, 2002), which is abundantly expressed in the CNS. The interaction of MK or PTN with RPTPβ/ζ establishes a “ligand-dependent inactivation” of RPTPβ/ζ, presumably as a consequence of RPTPβ/ζ dimerization (Deuel et al., 2002). Thus, the signaling of PTN or MK through RPTPβ/ζ leads to the activation of ERK and phosphatidylinositol 3-kinase (PI3K)-Akt (Polykratis et al., 2005; Qi et al., 2001), important axes inducing morphological changes and modulating addictive behaviors.

The present study aimed to identify whether the expression of PTN, MK, RPTPβ/ζ and their intracellular signaling pathways (Akt and ERK) are altered as a result of acute and chronic morphine exposure and/or morphine withdrawal in the NAc. We further assessed the possible activation of astrocytes, that could lead to the release of astrocyte-related soluble factors. Finally, we also characterized those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTPβ/ζ in response to morphine administration or morphine withdrawal.
Methods

Subjects

Male Wistar rats (n = 65, Harlan, Barcelona, Spain) 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. All surgical and experimental procedures were performed in accordance with the European Communities 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

Rats were implanted subcutaneously (s.c.) with placebo pellets (lactose) for six days. Another set of rats were made dependent on morphine by implantation (s.c.) of two 75 mg morphine pellets under light ether anesthesia. On day 7, rats were injected intraperitoneally (i.p.) with either morphine HCl (20 mg/kg; in a volume of 1 ml/kg body weight), naloxone (1 mg/kg; 1 ml/kg body weight) or an equivalent volume of 0.9% saline and sacrificed 2 h later.

Electrophoresis and Western blotting

Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 6%, 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4°C with the primary antibody diluted in 1% BSA in TBS-T. The following primary antibodies were used: goat polyclonal anti-PTN (1:1000; AF-252-PB, R&D Systems, Minneapolis, MN, USA); rabbit polyclonal anti-MK (1:500; sc-20715, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-RPTPβ/ζ (1:750; 610180, BD
Transduction Laboratories, Erembodegem, Belgium); mouse monoclonal anti-phospho-ERK 1/2 (p-ERK 1/2; 1:1000; sc-7383; Santa Cruz Biotechnology); mouse monoclonal anti-ERK 1/2 (1:1000; sc-135900; Santa Cruz Biotechnology); rabbit monoclonal anti-phospho-Akt (p-Akt; 1:2000; #4060, Cell Signaling Technology Inc., Danvers, MA, USA); rabbit polyclonal anti-Akt (1:1000; #9272, Cell Signaling Technology Inc.). Blots were subsequently reblocked and probed with rabbit polyclonal anti-GAPDH (1:5000; #2118, Cell Signaling Technology Inc.) or rabbit polyclonal anti- α-Tubulin (1:2500; #2144, Cell Signaling Technology Inc.).

GFAP Immunohistochemistry

Sections of the NAc(shell) were used for immunohistochemistry to detect astrocytes. Immunohistochemistry was performed as described in (García-Pérez et al., 2012). We used mouse monoclonal anti-GFAP (1:400; sc-33673, Santa Cruz Biotechnology) as primary antibody. Secondary antibody was horse anti-mouse (1:500; BA-2000, Vector Laboratories, Burlingame, CA, USA).

Quantification GFAP-positive cells

Neuroanatomical sites were identified using the Paxinos and Watson (1997) atlas. Photomicrographs were captured by means of Leica microscope (DM 4000B; Leica) connected to a video camera (DFC290, Leica). GFAP-positive cell nuclei were counted using a computer-assisted image analysis system (QWIN, Leica). Positive cells were counted at 20X magnification. A square field (325 µm) was superimposed upon captured image to use as reference area. The number of astrocytes was counted bilaterally in four to five sections from each animal including the rostral NAc(shell) and caudal NA(shell), and averaged to obtain a single value for each rat. The whole histological quantification was performed blindly. Total counts for different brain regions are expressed as mean ± SEM.
GFAP Densitometric Analysis

The same conventional light microscopy described above was used for optical density (OD) study of the nuclei and processes as described in (García-Pérez et al., 2014). In addition, the area outlined in each image was also calculated to assure that there were no differences between the regions of interest analyzed in different groups.

Immunofluorescence

The characterization and specificity of antibodies used in this study have previously been established and proven to be suitable for our research by isotype, epitope, applications and species reactivity. Negative controls without the primary antibody also were used to assure lack of non-specific binding of the secondary antibodies used for immunofluorescence (García-Pérez et al., 2013). Sections were treated with citrate buffer (60°C for 20 min). Non-specific Fc binding sites were blocked with 2% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4°C, constant shaking) with primary antibodies: mouse monoclonal anti-GFAP (1:400; sc-33673, Santa Cruz Biotechnology) (Wang et al., 2013; Roberts et al., 2009), goat polyclonal anti-PTN (1:400; AF-252-PB, R&D Systems) (Marchionini et al., 2007; Gurok et al., 2004) and rabbit polyclonal anti-MK (1:250; sc-20715, Santa Cruz Biotechnology) (Lorente et al., 2011; Doi et al., 2011). Secondary antibodies were applied sequentially for 4 h: Alexa Fluor 488 anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 594 anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 405 anti-mouse IgG (1:1000; A-31553, Invitrogen).

Striatal sections containing the NAc(shell) were stained with mouse monoclonal anti-RPTPβ/ζ (1:50; 610180, BD Transduction Laboratories) (Maeda & Noda, 1998; Lorenzetto et al., 2013) and rabbit polyclonal raised against cAMP-regulated phosphoprotein of 32 kDa
(DARPP-32) phosphorylated at Threonine 34 (p-DARPP-32 Thr-34; 1:400; ab51076, Abcam, Cambridge, UK) (Kim et al., 2011; Yuste et al., 2012). Appropriate secondary antibodies were used: Alexa Fluor 488 anti-rabbit IgG (1:1000; A-21206, Invitrogen) and Alexa Fluor 594 anti-mouse IgG (1:1000; A-21203, Invitrogen). Sections were incubated in DAPI (1:100 000) for 1 min.

Confocal analysis

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images were captured from low magnification to high magnification (20X to 63X oil objective) as previously described in (García-Pérez et al., 2015). Confocal images were obtained using 405-nm excitation for Alexa Fluor 405 or DAPI, 488-nm excitation for Alexa Fluor 488 and 543-nm excitation for Alexa Fluor 594.

Materials

Morphine HCl and morphine base were supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Naloxone HCl was purchased from Sigma-Aldrich (Sigma Chemical Co, St Louis, MO, USA). Morphine HCl and naloxone HCl doses are expressed as the weight of the salt.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test. Correlations between changes in protein expression were assessed using Pearson
correlation. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).
Results

Effects of acute and chronic morphine administration and precipitated morphine withdrawal on PTN, MK and RPTPβ/ζ expression in the NAc(shell)

We focused our analysis on the NAc, a brain region that play an important role in the acute-rewarding morphine effects and in the development of morphine dependence (Di Chiara & Imperato, 1988; Frenois et al., 2002). We studied the NAc(shell) because this portion of NAc appears to be more important than the core for reward and receives strong dopaminergic innervation from the posteromedial VTA (Ikemoto, 2007). This experiment addressed whether PTN, MK or RPTPβ/ζ protein levels were altered after different treatment regimens: i) pla+mor: rats were implanted with placebo pellets and on day 7 were injected i.p. with an acute dose of morphine; ii) mor+sal: another set of rats were made dependent on morphine by implantation of two morphine pellets, and received saline on day 7; iii) mor+nx: morphine-dependent rats were injected i.p. with naloxone on day 7, and thus, developed morphine withdrawal.

ANOVA showed significant effects for PTN after morphine administration \( F(2,21) = 14.250; p = 0.0002 \) and MK \( F(2,19) = 6.408; p = 0.0084 \) in the NAc(shell). As shown in Fig. 1A,C, post hoc comparisons showed that acute morphine administration significantly elevated the expression levels of PTN \( (p < 0.01) \) and MK \( (p < 0.05) \). Such increase was not observed during chronic morphine administration \( (p < 0.01) \) compared with acute morphine injection \( (PTN: p < 0.001; MK: (p < 0.01) \). Two-way ANOVA for PTN expression showed a significant effect of acute treatment \( F(1,22) = 10.50; p = 0.0038 \), and an interaction between pretreatment and acute treatment \( F(1,22) = 9.68; p = 0.0051 \). Post hoc test revealed that PTN levels in the NAc(shell) were increased after naloxone precipitated morphine withdrawal compared with chronic morphine-treated rats \( (p < 0.001) \) and with placebo-treated rats receiving saline \( (p < 0.05) \), as shown in Fig. 1B. Two-way ANOVA for MK showed a
significant effect of acute naloxone injection \((F(1, 24) = 8.46; p = 0.0077)\). Post hoc test revealed that MK levels in the NAc(shell) were increased after naloxone precipitated morphine withdrawal \((p < 0.05)\), as shown in Fig. 1D.

Western blot analysis was developed to examine whether morphine and or morphine withdrawal affected the protein expression levels of RPTPβ/ζ. In the NAc(shell) ANOVA showed significant effect after acute morphine \((F(2, 20) = 14.590; p = 0.0002)\). As shown in Fig. 1E, post hoc comparisons showed that acute morphine administration significantly elevated RPTPβ/ζ \((p < 0.001)\) expression. However, there was a decrease in its expression during morphine dependence compared with acute morphine-treated rats \((p < 0.01)\). Two-way ANOVA for RPTPβ/ζ expression revealed main effects for chronic pretreatment \((F(1, 23) = 5.71; p = 0.0255)\), naloxone injection \((F(1, 23) = 6.99; p = 0.0145)\), and significant interaction between acute and chronic treatment \((F(1, 23) = 5.54; p = 0.0276)\). Post hoc test revealed that RPTPβ/ζ levels in the NAc(shell) were increased in morphine-withdrawn rats compared with morphine dependent animals receiving saline and with placebo treated rats receiving naloxone \((p < 0.01); \) Fig. 1F).

We next compared the expression of PTN and MK with the induction of RPTPβ/ζ protein levels by Pearson correlation. There were not significant correlations in the different experimental groups between MK expression and RPTPβ/ζ protein levels in the NAc(shell) \(\text{(data not shown).}\) In contrast, we observed that after acute morphine administration, the expression of PTN was significantly positively correlated with RPTPβ/ζ levels (Fig. 2).

\textit{Astrocytes were activated by morphine and morphine withdrawal in the NAc(shell)}

We investigated the possible activation of astrocytes by acute morphine injection, morphine dependence and/or morphine withdrawal in both rostral and caudal NAc(shell). Rostral and caudal NAc(shell) were examined separately based on studies suggesting a
possible dichotomy of their activity according to emotional valence (Reynolds & Berridge, 2002). No differences were found for GFAP-positive cells or GFAP-IR between rostral and caudal NAc(shell). ANOVA showed significant effect of morphine administration for GFAP-positive cells \( (F(2,12) = 17.07; p = 0.0006) \) and GFAP-IR \( (F(2,13) = 14.23; p = 0.0009) \) in the rostral NAc(shell). Post hoc test revealed an increase of the number of GFAP-positive cells \( (p < 0.00; \text{Fig. 3G}) \) after chronic morphine administration and elevation of GFAP-IR after acute morphine injection \( (p < 0.001) \) and in morphine-dependent rats \( (p < 0.01; \text{Fig. 3I}) \).

Two-way ANOVA for number of GFAP-positive cells revealed significant effects of chronic pretreatment \( (F(1,14) = 73.52; p < 0.0001) \). Post hoc test indicated an increase \( (p < 0.001) \) of GFAP-positive cells during morphine dependence and withdrawal (Fig. 3H). Two-way ANOVA for GFAP-IR revealed significant effects of chronic pretreatment \( F(1,16) = 14.08; p = 0.0017 \). Post hoc test indicated an increase \( (p < 0.05) \) of GFAP-IR during morphine dependence and withdrawal (Fig. 3J).

At NAc(shell) caudal level, ANOVA showed significant effect of morphine administration for GFAP-positive cells \( (F(2,12) = 15.58; p = 0.0008) \) and GFAP-IR \( (F(2,13) = 11.01; p = 0.0024) \). Post hoc test revealed an increase of the number of GFAP-positive cells \( (p < 0.01; \text{Fig. 3S}) \) after chronic morphine administration and elevation of GFAP-IR after acute morphine injection and in morphine-dependent rats \( (p < 0.01; \text{Fig. 3U}) \). Two-way ANOVA for number of GFAP-positive cells revealed significant effects of chronic pretreatment \( (F(1,14) = 73.52; p < 0.0001) \) and acute treatment \( (F(1,14) = 4.82; p = 0.0456) \). Post hoc test indicated an increase \( (p < 0.001) \) of GFAP-positive cells during morphine dependence and withdrawal (Fig. 3T). Two-way ANOVA for GFAP-IR revealed significant effects of chronic pretreatment \( F(1,16) = 21.96; p = 0.0002 \). Post hoc test indicated an increase \( (p < 0.05) \) of GFAP-IR during morphine dependence and withdrawal (Fig. 3V).
PTN but not MK was overexpressed in astrocytes during acute morphine administration and morphine withdrawal in the NAc(shell)

Triple immunofluorescence study revealed that acute morphine (Fig. 4B-B‴‴) or morphine withdrawal (Fig. 4C-C‴‴) mediated the activation of astrocytes that expressed high levels of PTN protein, but not MK protein in the NAc(shell). Colocalization between an activated astrocyte and PTN protein was detected both in the nuclei (white arrows) and in the processes (yellow arrows; Fig. 4D-D‴). Fig. 4E-E‴ represents a cell that expresses MK and is surrounded by astrocytic processes expressing PTN.

RPTPβ/ζ was expressed in striatal neurons

DARPP-32 is a dual-function protein selectively expressed in all medium-sized spiny neurons (MSNs) and therefore a good marker for MSNs. Moreover, it is well established that acute administration of morphine results in an increase in the state of phosphorylation of DARPP-32 at Thr-34 in the NAc, without affecting phosphorylation at Thr-75, through a DA D1 receptor (D1R)-mediated activation (Borgkvist et al., 2007). Interestingly, we observed in rats injected with acute morphine, that RPTPβ/ζ immunoreactivity in the NAc(shell) was distributed homogeneously over the whole structure, on the membranes and proximal projections of neurons. RPTPβ/ζ staining colocalized with p-DARPP-32 Thr-34, suggesting its presence on D1R MSNs (Fig. 5A-A‴″).

Effects of morphine administration and precipitated morphine withdrawal on Akt and ERK pathways in the NAc(shell)

Previously, it has been described that PTN or MK signaling through RPTPβ/ζ leads to activation of ERK and Akt pathways (Polykratis et al., 2005; Qi et al., 2001). In each experiment, the specific signal of p-Akt or p-ERK proteins was normalized to the
corresponding Akt or ERK signals, respectively, and then to the level of GAPDH measured in the same preparation.

ANOVA for p-Akt ($F(2,19) = 25.750; \ p < 0.0001$) and t-Akt ($F(2,19) = 4.457; \ p = 0.0278$) showed significant effect after acute morphine administration. Post hoc test revealed that p-Akt and t-Akt levels were increased after acute morphine injection ($p < 0.001; \ p < 0.05$, respectively; Fig. 6A,C), whereas chronic morphine administration decreased both p-Akt ($p < 0.001$) and t-Akt ($p < 0.05$) compared with acute administration of the opiate. Two-way ANOVA for p-Akt revealed an interaction between pre-treatment and acute treatment ($F(1,23) = 9.57; \ p = 0.0051$). Post hoc test revealed that p-Akt levels in the NAc(shell) were significantly ($p < 0.05$) elevated in morphine-withdrawn rats compared with the morphine dependent group receiving saline instead naloxone and with the placebo treated rats injected with naloxone (Fig. 6B). Two-way ANOVA for t-Akt revealed an interaction between pre-treatment and acute treatment ($F(1,22) = 4.93; \ p = 0.0370$), although post hoc test failed to show any significant effect of chronic morphine or morphine withdrawal (Fig. 6D).

ANOVA did not show significant effects after acute or chronic morphine for p-ERK 1/t-ERK 1 ($F(2,22) = 2.043; \ p = 0.1558$; Fig. 7A), whereas ANOVA for p-ERK 2/t-ERK 2 showed significant effects ($F(2,22) = 5.284; \ p = 0.0144$; Fig. 7G). Post hoc comparisons showed that chronic morphine administration significantly elevated p-ERK 2/ t-ERK 2 ($p < 0.05$) expression in the NAc(shell). Two-way ANOVA revealed that morphine pre-treatment, acute naloxone injection or the interaction between pre-treatment and acute treatment had no significant effects on p-ERK 1/t-ERK 1 (Fig. 7B). Two-way ANOVA for p-ERK 2/t-ERK 2 expression revealed main effects for chronic pretreatment ($F(1,25) = 13.30; \ p = 0.0012$). Post hoc comparisons showed that chronic morphine administration significantly ($p < 0.01$) elevated p-ERK 2/t-ERK 2 levels compared with the control group (Fig. 7H).
Similar results were obtained when considering the p-ERK 1/GAPDH and p-ERK 2/GAPDH ratio. Thus, ANOVA did not show significant effect after acute morphine for p-ERK 1/GAPDH ($F(2,22) = 1.553; p = 0.2661$; Fig. 7E). ANOVA revealed significant effect for morphine treatment on p-ERK 2/GAPDH ($F(2,22) = 7.906; p = 0.0030$). As shown in Fig. 7K, *post hoc* comparisons showed that acute morphine administration injection significantly elevated p-ERK 2/GAPDH ($p < 0.01$) expression in the NAc(shell). Two-way ANOVA showed significant effect of chronic pretreatment ($F(1,25) = 11.34; p = 0.0025$) and acute treatment ($F(1,25) = 5.46; p = 0.0278$) on p-ERK 1/GAPDH ($F(1,24) = 12.41; p = 0.0017$). As shown in Fig. 7F, there was an increase ($p < 0.01$) of p-ERK 1 after naloxone administration to morphine-dependent rats. Two-way ANOVA for p-ERK 2/GAPDH showed significant effects of morphine pretreatment $F(1,25) = 25.90; p < 0.0001$). *Post hoc* test showed that both chronic morphine and morphine withdrawal elevated ($p < 0.01; p < 0.05$, respectively) p-ERK 2 levels, compared with their corresponding control groups (Fig. 7L).

We next examined the effect of acute morphine administration, chronic morphine and morphine withdrawal on t-ERK 1 (ratio t-ERK 1/GAPDH) and t-ERK 2 (ratio t-ERK 2/GAPDH) protein levels (Fig. 7C-J). ANOVA showed no significant effects after acute morphine for t-ERK 1 ($F(2,22) = 0.6526; p = 0.5314$) or t-ERK 2 ($F(2,22) = 0.3751; p = 0.6919$; Fig. 7C,I). Two-way ANOVA for t-ERK1 expression in the NAc(shell) showed a significant effect of chronic pretreatment ($F(1,25) = 14.66; p = 0.0008$), acute treatment ($F(1,25) = 7.00; p = 0.0139$), and an interaction between pretreatment and acute treatment ($F(1,25) = 6.78; p = 0.0153$). As shown in Fig. 7D, morphine withdrawal increased t-ERK1 expression compared with morphine dependent rats ($p < 0.01$) and with placebo treated rats receiving naloxone ($p < 0.001$). Two-way ANOVA failed to detect any significant effects of chronic morphine pretreatment, acute treatment or an interaction between pretreatment and acute treatment on t-ERK1 levels in the NAc(shell) (Fig. 7L).
Relationship between PTN, MK and/or RPTPβ/ζ and p-ERK or t-ERK protein levels

We first compared the expression of PTN, MK and/or RPTPβ/ζ with the induction of p-ERK or t-ERK protein levels in control rats that were injected an acute dose of morphine. There were significant negative correlations between PTN expression and p-ERK 1 and p-ERK 2 protein levels in the NAc(shell) (Fig. 8A,B). Moreover, RPTPβ/ζ expression in acute morphine-injected rats was also negatively correlated with changes on p-ERK 1 (Fig. 8E). On the other hand, when we investigated the group of rats that were exposed to chronic morphine we did not identify any significant correlation in the expression of these proteins (Supplementary Table S1). During morphine withdrawal, we observed that ERK 1 phosphorylation was positively correlated with RPTPβ/ζ expression (Fig. 8Q), but negatively correlated with MK levels (Fig. 8O). In addition, we detected a positive relationship between PTN and t-ERK 1 levels in morphine-withdrawn rats (Fig. 8M).

However, the correlation analysis performed between PTN, MK or RPTPβ/ζ and Akt in the NAc during the different morphine paradigms employed, showed that Akt phosphorylation was unrelated with the protein expression levels of these cytokines or their receptor (Supplementary Table S1).
Discussion

The present study showed that a single dose of morphine and morphine withdrawal increased the protein levels of PTN and MK in the NAc, but chronic morphine administration had the opposite effect, since protein levels returned to basal levels or even decreased. Our results are supported by previous findings showing that acute morphine or withdrawal but not chronic morphine promote the expression of pro-inflammatory cytokines (Campbell et al., 2013). Moreover, we detected that PTN up-regulation was restricted to astrocytes, as it has been previously described in the literature (Iseki et al., 2002;Mourlevat et al., 2005;Taravini et al., 2011). In contrast, we observed that MK was labeled in non-astrocytic cells. Since astrocytes can also express MK after kainic acid injection (Kim et al., 2010), it could be hypothesized that depending on the nature of the insult or the damage/cell death it may produce, MK can be over-expressed by neurons or astrocytes.

We also found that the expression of PTN- and MK- target receptor (RPTPβ/ζ) was regulated in the same way as these cytokines by morphine administration. Although MK and PTN bind in a similar manner to RPTPβ/ζ (Maeda et al., 1996; Maeda et al., 1999), the mechanisms triggered by the formation of the complex PTN/ RPTPβ/ζ are better known. The interaction of RPTPβ/ζ with PTN inactivates the intrinsic tyrosine phosphatase activity of RPTPβ/ζ (Meng et al., 2000). We observed that PTN direct RPTPβ/ζ-inactivation after acute morphine injection induced RPTPβ/ζ expression, which could be viewed as a homeostatic response to preserve the regulation of cellular protein tyrosine phosphorylation levels. However, the correlation between PTN - RPTPβ/ζ expression did not occur in morphine-dependent rats. Regarding the RPTPβ/ζ signal, we found a staining pattern in neurons. Our results are in line with previous studies where this protein was found to be located in neurons but not in astrocytes (Lorenzetto et al., 2013;Hayashi et al., 2005). It is well established that MSNs in the striatum of normal rats express RPTPβ/ζ (Ferrario et al., 2008). Moreover, our
findings demonstrate that RPTPβ/ζ is labeled in p-DARPP-32 Thr-34 MSNs. It has been previously described that the ability of morphine to stimulate Thr34 phosphorylation was dependent on D1R (Borgkvist et al., 2007). Thus, our data suggest that RPTPβ/ζ is expressed in D1R subtype of MSNs, which are dopaminoceptive neurons that become directly activated after morphine administration and DA release. This expression pattern supports our hypothesis of an interaction between glial and neuronal function during morphine administration and withdrawal.

Astrocytes can display both, hypertrophy and proliferation upon treatment with drugs of abuse. Daily morphine administration, using a regimen that produced tolerance and dependence, increased GFAP immunostaining in different brain regions, including the Nac (Garrido et al., 2005). Repeated morphine exposure has also been reported to increase the GFAP mRNA and protein levels in the striatum (Marie-Claire et al., 2004). We also observed an enhancement in GFAP-IR in the rostral and caudal NAc(shell) during acute morphine, chronic morphine and morphine withdrawal. Regarding to proliferation, morphine and opioid signaling have been shown to promote proliferation of astroglia in the postnatal brain (Sargeant et al., 2008). We observed a rapid astrocyte proliferation in the VTA (data not shown), the brain area where the rewarding properties of morphine are believed to be firstly mediated. Secondly, this signal causes disinhibition of dopaminergic neurons activity and increases DA release in the NAc(shell), where we showed a slower astrocytic proliferation, only after chronic morphine administration. In addition, rostral and caudal NAc(shell) were examined separately based on studies suggesting a possible dichotomy of their activity according to emotional valence (Reynolds & Berridge, 2002). Although we could not find any significant differences, we observed that the number of astrocytes tended to increase in the rostral NAc(shell) during morphine withdrawal, a subregion involved in negative emotional valence during withdrawal. On the other hand, positive or rewarding stimuli seem
to be associated with activity in the caudal NAc(shell), where astrocytes tended to decrease in morphine-withdrawn rats.

Several findings support the idea that central immune signaling contributes substantially to the pharmacodynamic actions of drugs of abuse, since different glial cell modulators are hypothesized to decrease the rewarding effects of opioids. For instance, ibudilast co-administered with morphine significantly reduced the magnitude of opioid-induced dopamine release in the NAc (Bland et al., 2009). Propentofylline also blocked the development of the effects of morphine in the conditioned place preference procedure (Narita et al., 2006). Interestingly, a role for MK and PTN in modulating drug reward behaviors has also been proposed. For example, morphine- and ethanol-induced conditioned place preference (CPP) augmented in PTN-/− mice compared to PTN+/+ mice (Vicente-Rodríguez et al., 2014; Herradón & Pérez-García, 2013). So, our increased PTN and MK levels in the NAc after an acute morphine dose may be important in limiting the acute rewarding effects of opiates.

Other cytokines have also been implicated in withdrawal-related behavior. Recent findings strongly support the potential role of glial modulators, specifically ibudilast and minocycline, to decrease opioid withdrawal symptoms (Hutchinson et al., 2009; Hutchinson et al., 2010). Moreover, it was showed that corticotrophin releasing factor (CRF) and cytokines work together to worsen ethanol withdrawal phenotypes (Knapp et al., 2011). Although the role of PTN and MK in conditioned place aversion (CPA) remains uncharacterized, future studies should address this issue, since robust increased levels of PTN and MK were also observed during morphine withdrawal.

When we studied the intracellular targets of PTN/MK signaling, we observed an up-regulation of ERK phosphorylation levels only during chronic morphine treatments
(morphine-dependent and morphine-withdrawn rats) in the NAc, which agrees with previous data describing that p-ERK levels in the NAc do not change 60 min after acute morphine injection (Muller & Unterwald, 2004). Interestingly, during acute morphine administration, PTN and RPTPβ/ζ levels were negatively correlated with the phosphorylation levels of ERK 1/2. Given the prominent role of p-ERK 1/2 in the NAc in mediating morphine CPP (Lv et al., 2015) or contextual memory (Xu et al., 2012), and the importance of PTN on limiting morphine or ethanol reward (Vicente-Rodríguez et al., 2014; Herradón & Pérez-García, 2013), it is tempting to speculate that PTN limits the rewarding effects of acute morphine through limiting ERK 1/2 phosphorylation. In contrast, during morphine withdrawal, MK and RPTPβ/ζ exerted opposite roles on regulating p-ERK 1. Although RPTPβ/ζ is induced in morphine-withdrawn rats, protein levels do not reach the ones observed in rats subjected to an acute morphine injection. According to this, high PTN protein levels during morphine withdrawal may inactivate the intrinsic tyrosine phosphatase activity of RPTPβ/ζ (Meng et al., 2000), leading to increased p-ERK 1 protein.

We also detected that t-ERK levels could be altered in morphine-dependent rats. In agreement to our results, total ERK1 and ERK2 levels were increased selectively in caudate/putamen after chronic morphine treatment (Ortiz et al., 1995). Interestingly, we detected that PTN may be related with the increased t-ERK1 observed in morphine-withdrawn rats. In contrast, p-Akt levels were increased in the NAc after acute morphine injection and morphine withdrawal, which adds further evidence to the results obtained by (Muller & Unterwald, 2004). However, the control of Akt phosphorylation in the NAc during morphine administration seems to be independent of PTN, MK or RPTPβ/ζ, since we did not find any correlation.

In summary, given that PTN, MK and RPTPβ/ζ levels increase after acute morphine injection, return to basal levels during chronic opioid treatment and are up-regulated again
during morphine withdrawal in the NAc, we hypothesize a role for these cytokines in mediating, at least in part, neurotrophic and behavioral adaptations that are observed during opiate addiction.
Acknowledgments

This work was supported by grants from Ministerio de Ciencia e Innovación (SAF/FEDER 2009-07178; SAF/FEDER 2010-17907), Spain; Red de Trastornos Adictivos, Spain; Fundación Séneca (15405/PI/10) and Instituto Murciano de Investigación en Biomedicina (IMIB), Región de Murcia, Spain. Daniel García-Pérez was supported by a fellowship from Ministerio de Ciencia e Innovación (AP2009-2379). The authors declare that they have no conflict of interest.
Conflict of Interest

None.
Authors Contribution

DG-P designed and performed the research, analyzed the data and wrote the paper. MLL revised the manuscript. MVM conducted the astrocyte quantification and contributed to editing the paper.
References


Figure Legends

**Fig. 1** PTN, MK and RPTPβ/ζ protein expression are altered by acute and chronic morphine administration and during morphine withdrawal in the NAc. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of PTN (A, B), MK (C, D) and RPTPβ/ζ (E, F) protein in NAc isolated from rats receiving the above treatments. Each bar corresponds to mean ± SEM. Values are expressed as % of controls. *p < 0.05, **p < 0.01, ***p < 0.001 vs. pla+sal; +p < 0.01, +++p < 0.001 vs. pla+mor; *p < 0.05, ###p < 0.01 vs. pla+nx, &p < 0.05, &&p < 0.01, &&&p < 0.001 vs. mor+sal

**Fig. 2** Correlation between PTN and RPTPβ/ζ. The percent increase in PTN levels was positively correlated with RPTPβ/ζ protein after acute morphine injection. No significant correlation was found between PTN expression and RPTPβ/ζ levels during morphine dependence or morphine withdrawal. ###p < 0.001: PTN levels vs. RPTPβ/ζ levels.

**Fig. 3** GFAP expression is enhanced by acute and chronic morphine administration and maintained during morphine withdrawal in the NAc(shell), while astrocyte proliferation only occurs in morphine-dependent and morphine-withdrawn rats. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. The analyzed region within the NAc(shell) rostral and NAc(shell) caudal is schematically illustrated in A and M, respectively (modified from Paxinos and Watson, 2007). A rectangle indicates the size of the photomicrographs. Representative photomicrographs showing immunohistochemical
detection of GFAP+ nuclei and fibers in coronal sections at NAc(shell) rostral level (B-F) and NAc(shell) caudal level (N-R; scale bar: 200 µm). Quantitative analysis of astrocytes in the NAc(shell) rostral (G, H) and NAc(shell) caudal (S, T). Mean optical density measurement of GFAP-immunoreactivity in the NAc(shell) rostral (I, J) and NAc(shell) caudal (U, V) from rats receiving the treatments mentioned above. (K-L, W-X): Reference area used in the densitometric analysis did not differ between groups. LV: Lateral ventricle. Each bar corresponds to mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs. pla+sal; ++p < 0.01 vs. pla+mor; #p < 0.05, ###p < 0.001 vs. pla+nx

**Fig. 4** PTN but not MK is overexpressed in astrocytes during acute morphine administration and morphine withdrawal in the NAc(shell). (A-C): Stack of confocal images from the forebrain areas immuno-stained for GFAP (blue), PTN (red) and MK (green) in control rats rats treated with acute morphine injection or morphine-dependent rats injected with naloxone. (D): High magnification shows that PTN colocalizes with GFAP (activated astrocyte) both, in the nuclei (white arrows) and in the processes (yellow arrows) in morphine withdrawal rats. (E): Panel represents a non-astrocytic cell expressing MK that is surrounded by GFAP+ processes containing PTN morphine withdrawal rats. Scale bar: A-B, 50 µm; C-D, 20 µm

**Fig. 5** RPTPβ/ζ is expressed in striatal neurons. (A): In the NAc(shell) of rats injected with acute morphine, RPTPβ/ζ immunoreactivity (red) was distributed homogeneously over the whole structure, on the membranes and proximal projections of neurons. RPTPβ/ζ staining colocalized with some p-DARPP-32 Thr-34+ neurons (green), confirming its presence on D1R MSNs. DAPI (blue) was used as a counterstaining in both nuclei. Scale bar: A, 50 µm
Fig. 6 p-Akt levels levels are enhanced during acute morphine administration and morphine withdrawal in the NAc, while t-Akt levels only increase during acute opioid injection. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of p-Akt / t-Akt ratio (A, B) and t-Akt levels (C, D) in NAc isolated from rats receiving the above treatments. Each bar corresponds to mean ± SEM. Values are expressed as % of controls. *p < 0.05, **p < 0.001 vs. pla+sal; *p < 0.05, +++p < 0.001 vs. pla+mor; #p < 0.05 vs. pla+nx; &p < 0.05 vs. mor+sal

Fig. 7 p-ERK / t-ERK ratio, t-ERK levels and p-ERK absolute levels are altered by acute and chronic morphine administration and during morphine withdrawal in the NAc. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of p-ERK 1 / t-ERK 1 ratio (A, B), t-ERK 1 levels (C, D), p-ERK 1 absolute levels (E, F), p-ERK 2 / t-ERK 2 ratio (G, H), t-ERK 2 levels (I, J), p-ERK 2 absolute levels (K, L) in NAc isolated from rats receiving the above treatments. Each bar corresponds to mean ± SEM. Values are expressed as % of controls. *p < 0.05, **p < 0.01 vs. pla+sal; *p < 0.05, +++p < 0.01 vs. pla+mor; #p < 0.05, ###p < 0.001 vs. pla+nx; &p < 0.05, &&p < 0.01 vs. mor+sal

Fig. 8 Correlation between PTN, MK and/or RPTPβ/ζ and p-ERK or t-ERK protein levels in different experimental groups. (A-F): After acute morphine injection, significant negative correlations between PTN and RPTPβ/ζ expression and p-ERK 1 and p-ERK 2 protein levels were observed. (M-R): During morphine withdrawal, ERK 1 phosphorylation was positively correlated with RPTPβ/ζ expression, but negatively correlated with MK levels. In addition, a
positive relationship between PTN and t-ERK 1 levels in morphine-withdrawn rats is
described. $p < 0.05$: PTN, MK or RPTPβ/ζ levels vs. p-ERK 1 / t-ERK 1 or p-ERK 2 / t-ERK 2 levels. $^5p < 0.05$: PTN levels vs. t-ERK 1 / GAPDH.
Figure 1
Figure 5

[Image of a figure with four panels showing immunohistochemical staining for RPTPβ/ζ, p-DARPP-32, DAPI, and RPTPβ/ζ/p-DARPP-32 in the NAc (shell).]
Figure 6

(A) NAc: p-Akt (Ser473)

(B) NAc: p-Akt (Ser473) with saline and naloxone

(C) t-Akt

(D) t-Akt with saline and naloxone

Western blots showing the expression levels of p-Akt and t-Akt in the NAc under different conditions.
Figure 8