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Morphine withdrawal produces ERK-dependent and ERK-independent epigenetic marks in neurons of the nucleus accumbens and lateral septum

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ABSTRACT

Epigenetic changes such as covalent modifications of histone proteins represent complex molecular signatures that provide a cellular memory of previously experienced stimuli without irreversible changes of the genetic code. In this study we show that new gene expression induced *in vivo* by morphine withdrawal occurs with concomitant epigenetic modifications in brain regions critically involved in drug-dependent behaviors. We found that naloxone-precipitated withdrawal, but not chronic morphine administration, caused a strong induction of phospho-histone H3 immunoreactivity in the nucleus accumbens (NAc) shell/core and in the lateral septum (LS), a change that was accompanied by augmented H3 acetylation (lys14) in neurons of the NAc shell. Morphine withdrawal induced the phosphorylation of the epigenetic factor methyl-CpG-binding protein 2 (MeCP2) in Ser421 both in the LS and the NAc shell. These epigenetic changes were accompanied by the activation of members of the ERK pathway as well as increased expression of the immediate early genes (IEG) *c-fos* and activity-regulated cytoskeleton-associated protein (Arc/Arg3.1). Using a pharmacological approach, we found that H3 phosphorylation and IEG expression were partially dependent on ERK activation, while MeCP2 phosphorylation was fully ERK-independent. These findings provide new important information on the role of the ERK pathway in the regulation of epigenetic marks and gene expression that may concur to regulate *in vivo* the cellular changes underlying the onset of the opioid withdrawal syndrome.

1. Introduction

Opioids are the most powerful and effective drugs for relief of pain known to medicine. However, these substances are also widely used as drugs of abuse potentially leading to addiction, a dramatic relapsing disorder (Koob and Le Moal, 2006). Chronic morphine intake is thought to induce complex homeostatic neuronal and synaptic adaptations in the dopaminergic mesolimbic system – i.e. ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala, lateral septum (LS) and prefrontal cortex – that may be unmasked during withdrawal from drug administration or when dependent animals are treated with opioids antagonists (e.g. naloxone) to precipitate withdrawal (Erdtmann-Vourliotis et al., 1998; Koob and Kreek, 2007; Kreek, 2001; Way et al., 1969). In animal models, morphine withdrawal syndrome is characterized by both physical and negative affective components (Stinus et al., 2000) that are thought to contribute to the maintenance of addictive behaviors (Gracy et al., 2001; Koob and Le Moal, 1997; Markou et al., 1998). However, the cellular and molecular modifications triggered by cessation of chronic morphine intake are still largely unknown.

An intracellular event that is likely to play a role in these modifications is the hyperactivation of the extracellular signal-regulated kinase (ERK) cascade (Anier et al., 2010; Hofford et al., 2009; Li et al., 2010). The ERK pathway is deemed a crucial node for neuronal and synaptic modifications because of its ability to

Abbreviations: ARC, activity-regulated cytoskeleton-associated protein; ERK, extracellular signal-regulated kinase; DS, dorsal striatum; IEG, immediate early genes; LS, lateral septum; LSD, dorsal lateral septum; LSI, intermediate lateral septum; LSV, ventral lateral septum; MeCP2, methyl-CpG-binding protein 2; NAc, nucleus accumbens; MSK1, mitogen and stress kinase 1; NGS, normal goat serum; PBS, phosphate buffered saline; VTA, ventral tegmental area.

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phosphorylate a plethora of plasticity-related intracellular targets (Boggio et al., 2007; Chwang et al., 2006; Deak et al., 1998; Vara et al., 2009). Interestingly, it has been shown that naloxone-precipitated morphine withdrawal is associated with increased levels of ERK phosphorylation in different brain areas (i.e. amygdala, locus coeruleus, solitary tract, hypothalamus, cerebral cortex, and lateral septum) (Hofford et al., 2009; Nunez et al., 2007; Schulz and Hollt, 1998). Moreover, ERK activation in limbic areas and in the spinal cord can significantly contribute to unmask both somatic and motivational symptoms of opioid withdrawal (Cao et al., 2005; Frenois et al., 2002). Nonetheless, the intracellular pathways that can be modulated by the activity of ERK signaling in the context of morphine withdrawal are still obscure. One mode of action of ERK is the modification of gene expression by the addition of specific epigenetic marks on histone proteins, thus changing chromatin structure and facilitating or preventing the recruitment of transcription factors into appropriate promoter regions (Bonasio et al., 2010; Sweatt, 2004; Zocchi and Sassone-Corsi, 2010). Current models of activity-dependent histone modifications suggest that initiation of ERK-dependent signaling in the neuronal periphery is followed by nuclear activation of the mitogen and stress kinase 1 (MSK1), resulting in histone H3 phosphorylation, followed by acetylation and changes in gene expression (Brami-Cherrier et al., 2007; Crosio et al., 2003). Interestingly, a similar mode of ERK-dependent H3 post-translational modifications has been reported for visual cortical plasticity (Putignano et al., 2007), stress response (Chandramohan et al., 2007), learning tasks (Chwang et al., 2007) and cocaine administration (Bertran-Gonzalez et al., 2008; Brami-Cherrier et al., 2005).

Recent studies have indicated that other factors may contribute to epigenetic changes in drug addiction (Renthal and Nestler, 2009). For example MeCP2 (methyl-CpG-binding protein 2), a methyl DNA-binding transcriptional regulator (Lewis et al., 1992), plays an important role in psychostimulants-induced addiction. MeCP2 expression is crucial for amphetamine-induced conditioned place preference and for the control of cocaine intake through homeostatic interactions with micro RNAs molecules (Deng et al., 2010; Im et al., 2010). Moreover, amphetamine administration induces the phosphorylation of MeCP2 at Ser421 (Deng et al., 2010; Mao et al., 2011), a Ca^{2+}/calmodulin-dependent serine–threonine protein kinase (CaMKII)-dependent activation site that regulates MeCP2 function by facilitating a genome-wide modification of chromatin in response to neuronal activity (Cohen et al., 2011; Zhou et al., 2006).

Although there is mounting evidence that chronic administration of opioids and withdrawal induce new gene expression (Ammon-Treiber and Hollt, 2005; Rodriguez Parkitna et al., 2004), the potential role of ERK-dependent epigenetic modifications, as well of other regulators of gene expression, such as MeCP2, has not been explored. Here we investigated whether naloxone-precipitated withdrawal after chronic morphine administration is associated with covalent histone H3 modifications in the mesolimbic pathway. Moreover, we analyzed whether the emergence of epigenetic marks and changes in gene expression are dependent on ERK pathway activation. Finally, we studied the involvement of MeCP2 by analyzing the effects of morphine withdrawal on the cellular localization and expression levels of its phosphorylated form.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley CD rats (Charles River, Como, Italy) with body masses of 200–250 g were utilized. After arrival at the animal facility, the rats were acclimatized to the new housing conditions for at least 1 week. They were housed under an artificial 12-h-light, 12-h-dark cycle (lights on from 08:00 to 20:00 h) and at a constant temperature of 22 ± 2 °C and a relative humidity of 65%. They had free access to water and standard laboratory food at all times. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethics committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Chronic morphine administration and morphine withdrawal

Morphine hydrochloride (S.a.l.a.r.s., Como, Italy; dissolved in physiological saline) was injected intraperitoneally in a group of rats (named morphine) twice daily (at 09:00 and 20:00 h) for 9 days according to the following schedule: 10, 15, 20, 30, 40, 50, 60, 60 and 60 mg/kg/day in a volume of 3 ml/kg as previously described (Devoto et al., 2002) (Fig. 1a). Control rats (named saline) received the same volume of saline. Animals were killed 2 h after the last morphine administration. To elicit morphine withdrawal, a group of rats (named withdrawal) treated chronically with morphine were injected with naloxone hydrochloride (4 mg/kg in a volume of 1 ml/kg, s.c., Tocris) 1 h after the last injection of morphine. The animals were observed for opioid withdrawal behaviors (wet-dog shakes, teeth chattering, rhinorrhea, lacrimation, ptosis) for 20 min h after naloxone injection. To test the effects of naloxone alone, a group of control rats (named naloxone) received saline for 9 days and naloxone (4 mg/kg, s.c.,) 1 h after the last saline injection.

2.3. Pharmacological inhibition of ERK activation

To block ERK activity, a group of rats chronically treated with morphine (named M-W/SL327) were injected with the MEK inhibitor SL327 (55 mg/kg, i.p., Sigma, St. Louis, MO) 45 min before the naloxone injection (Fig. 4a). SL327 was dissolved in DMSO and sonicated. In this set of experiments we also utilized a group of morphine withdrawal rats (named M-W/DMSO) that received instead of SL327 the same volume of DMSO and a second group of rats (named nlx/DMSO) that received chronic saline, DMSO and naloxone. All the rats of the different experimental groups were sacrificed 1 h after naloxone injection.

2.4. Tissue preparation, immunohistochemistry and immunofluorescence

Animals were anesthetised with an intraperitoneal injection of chloral hydrate (400 mg/kg) and transcardially perfused with ice-cold paraformaldehyde (4% in 0.1 M phosphate buffer (PB), pH 7.4). After perfusion, the brains were dissected and kept in the same fixative solution overnight at 4 °C. After several washes in 0.1 M PB, brains were cryoprotected by immersion in 10%, 20%, and 30% sucrose solutions, cut in 30 μm sections with a cryostat and stored at −20 °C in a solution containing 30% ethylene glycol and 25% glycerol in 0.1 M sodium phosphate buffered saline (PBS, pH 7.2). Cryosections were subsequently processed free-floating for immunohistochemistry as described (Boggio et al., 2007). After a blocking step in a PBS solution containing 0.05% Triton X-100 and 10% normal goat serum (NGS), sections were incubated overnight at room temperature with the following primary antibodies diluted in PBS with 3% NGS and 0.05 Triton X-100: rabbit anti-phospho-H3 (S10) (1:500, Upstate/Millipore Billerica, MA), rabbit anti-phospho-MSK1 (T581) (1:50, MBL, Woburn, MA), mouse anti-phospho-ERK (ERK-1/2, T183-Y185) (1:500, Sigma, St. Louis, MO), rabbit anti-*c-fos* (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-ARC (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-MeCP2 (S421) (1:1000, generous gift from Z. Zhou, University of Pennsylvania School of Medicine; Philadelphia, PA), rabbit anti-MeCP2 (1:200, Upstate/Millipore Billerica, MA), rabbit anti-acetyl-H3 (K14) (1:100, Upstate/Millipore Billerica, MA). The sections were then washed in PBS (3 × 10 min), incubated 1 h with the appropriate biotinylated secondary antibodies (1:250; Vector Labs, Burlingame, CA) diluted in PBS with 3% NGS and 0.05% Triton X-100 and transferred to a solution containing a biotin–avidin complex (1:100, Vector Labs). The peroxidase reaction product was visualized by incubation in a solution containing 3,3′-diaminobenzidine (0.05% DAB in Tris–HCl, pH 7.6) with 0.01% H_2O_2 for 3–4 min. Sections were mounted on gelatine-coated glass slides and observed with a light microscope (Eclipse 800, Nikon, Japan) equipped with a CCD camera (Axiocam HRc, Zeiss, Germany). Immunolabelling of pMeCP2 was obtained using a secondary fluorescent antibody (goat anti-rabbit CY3, Jackson Immunoresearch, West Grove, PA) diluted 1:1000.

Double immunofluorescence was performed by simultaneous incubation with two primary antibodies as described (Vara et al., 2009). Briefly, cryosections were blocked in PBS containing 10% NGS and 0.05% Triton X-100 for 1 h, then incubated with the primary antibodies at room temperature overnight. After PBS washing, sections were incubated for 1 h with a combination of the following secondary fluorescent antibodies: goat anti-mouse CY3 (Jackson Immunoresearch, West Grove, PA) and goat anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA), both diluted 1:1000. After several PBS rinses, sections were mounted on gelatine coated glass slides and observed with a confocal microscope (Zeiss LSM-5 Pascal, Germany).

2.5. Quantitative analysis of immunohistochemical signals

We processed for immunohistochemistry 2–3 coronal brain sections for each animal (spanning between 1.60 mm and 0.70 mm from Bregma). In each section we collected a minimum of 4 micrographs of the LS and NAc (shell and core), respectively,