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Stimulation of mitogen-activated protein kinase kinases (MEK1/2) by μ-, δ- and κ-opioid receptor agonists in the rat brain: Regulation by chronic morphine and opioid withdrawal

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Abstract

Opioid addiction modulates the extracellular signal-regulated kinase (ERK) leading to synaptic plasticity in the brain. ERK1/2 are stimulated by mitogen-activated protein kinase kinases (MEK1/2), but little is known about the regulation of MEK activity by opioid drugs. This study was designed to assess the acute effects of selective μ-, δ-, and κ-opioid receptor agonists, as well as those induced by chronic morphine and opioid withdrawal, on the content of phosphorylated MEK1/2 in the rat brain. Sufentanil (1–30 μg/kg, 30–120 min) induced dose- and time-dependent increases in MEK1/2 phosphorylation in the cerebral cortex and corpus striatum (30–177%) through a naloxone-sensitive mechanism. Morphine (100 mg/kg, 2 h) also augmented MEK1/2 phosphorylation in the both brain regions (50–70%). Similarly, the selective δ-opioid receptor agonist SNC-80 (10 mg/kg, 30 min) increased MEK1/2 activity in the cortex (60%) that was antagonized by naltrindole. In contrast, the selective κ-opioid receptor agonist (−)-U50488H (10 mg/kg, 30–120 min) did not modify significantly MEK1/2 phosphorylation in the cortex. Chronic morphine (10–100 mg/kg, 5 days) was not associated with alterations in the content of phosphorylated MEK1/2 in the brain (induction of tachyphylaxis to the acute effects). In morphine-dependent rats, however, naloxone (2 mg/kg)-precipitated withdrawal (2–6 h) induced robust increases in MEK1/2 phosphorylation in cortex (27–49%) and striatum (83–123%). Spontaneous opioid withdrawal (24 h) in morphine-dependent rats did not alter MEK1/2 activity in the brain. The findings may be relevant in the context of the pivotal role played by the MEK/ERK pathway in various long-lasting forms of synaptic plasticity associated with opioid addiction.

Keywords: MEK1/2 activity; Sufentanil; SNC-80; U50488H; Morphine addiction; Rat brain

1. Introduction

The opioid receptors modulate many signaling pathways in the brain, including several kinase cascades such as those containing protein kinases A (PKA) and C (PKC), calmodulin kinase II and, more recently, the extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase system (Law et al., 2000; Williams et al., 2001). The core module of the ERK signaling pathway consists of three kinases that phosphorylate each other sequentially. Thus, ERK1/2 are activated by p45-MEK1 and p46-MEK2 (MAP/ERK kinases or MAP kinase kinases) (review in Yoon and Seger, 2006), which in turn are phosphorylated by c-Raf-1, a kinase of the Raf family (Baccarini, 2005). C-Raf-1 is initially stimulated by different small GTP-binding proteins likewise Ras (Grewal et al., 1999, Baccarini, 2005). Activated ERK1/2 phosphorylate and regulate many cytosolic and nuclear targets (Yoon and Seger, 2006) to alter gene expression and modulate, among other events, synaptic plasticity in the brain (Pearson et al., 2001; Pouysségur and Lenormand, 2003). It is generally accepted that MEK1/2 (dual-specificity protein kinases that share 78% amino acid identity) are equally competent activators of ERK1/2 (Chang and Karin, 2001; Robinson et al., 2002), which are the only known substrates of MEK1/2 (Pearson et al., 2001). However, increasing

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evidences suggest that ERK1/2 can be also activated via some MEK-independent pathway (Band and Posner, 1997; Bapat et al., 2001; Barry et al., 2001; Tapinos and Rambukkana, 2005), as well as by other direct activators (Tapinos and Rambukkana, 2005). Transgenic mice models expressing an active form of MEK1 (tissue/organ-restricted) have revealed a number of substantial effects in various tissues related to proliferation and differentiation phenomena (Scholl et al., 2005), which illustrates the physiological relevance of the MAP/ERK kinases.

Opioid-induced activation of ERK1/2 was first described in cells expressing the transfected opioid receptors (Li and Chang, 1996; Fukuda et al., 1996; Belcheva et al., 1998; Schulz et al., 2004). Human neuroblastoma SK-N-SH and SH-SY5Y cells, which endogenously express μ/δ-opioid receptors, also exhibited a rapid and transient opioid-mediated phosphorylation of ERK1/2 enzymes (Trapaidze et al., 2000; Ferrer-Alcón et al., 2004). In these in vitro studies, the main mechanism for coupling opioid receptors with the ERK pathway was shown to involve the activation of Ras via Gβγ subunits (Belcheva et al., 1998) that are associated with Gi/o protein activity (i.e., sensitive to pertussis toxin) (Audet et al., 2005). Other mechanisms by which opioid receptors could regulate the ERK pathway involve the modulation of PKA and PKC signaling systems (Luttrell and Luttrell, 2003; Belcheva et al., 2005).

Chronic morphine in rats and opioid addiction in humans have been shown to be associated with down-regulation of ERK1/2 activity in the various brain regions (Schulz and Höllt, 1998; Ferrer-Alcón et al., 2004; Muller and Unterwald, 2004). In contrast, little attention has been paid to the in vivo short-term regulation of ERK1/2 in brains of laboratory animals after the activation of μ-, δ- or κ-opioid peptide (MOP-, DOP-, KOP-) receptors. Thus, acute morphine treatment in mice was shown to induce opposite brain region-specific modulations of phosphorylated ERK1/2 (Eitan et al., 2003; Valjent et al., 2004). Acute morphine in rats has been associated with up-regulation of ERK1/2 phosphorylation in the cerebral cortex (Ferrer-Alcón et al., 2004), but other studies with this opioid failed to modulate the content of phosphorylated ERK1/2 in various rat brain regions, including the cortex and the ventral tegmental area (Berhow et al., 1996; Schulz and Höllt, 1998). In addition, acute morphine was reported to induce down-regulation of phosphorylated ERK1/2 in the rat nucleus accumbens but not in the caudate–putamen (Muller and Unterwald, 2004). Notably, these conflicting in vivo studies have exclusively dealt with the effects of morphine, a low efficacy μ-opioid receptor agonist (Williams et al., 2001), on the modulation of ERK1/2, the classical MAP kinase, and very little is known (see Ortiz et al., 1995; Lesscher et al., 2003) about the short- and long-term regulation of MEK1/2 activity by opioid drugs in the brain.

Therefore, this study was designed to assess the acute effects of selective μ-, δ-, and κ-opioid receptor agonists, as well as those induced by chronic morphine and opioid withdrawal, on the content of phosphorylated MEK1/2 in the rat cerebral frontal cortex and corpus striatum, two brain regions relevant to opioid addiction (see Ferrer-Alcón et al., 2004; Valjent et al., 2005).

2. Materials and methods

2.1. Animals and treatments

Adult male Sprague-Dawley rats (250–300 g) were used. The rats were housed under controlled environmental conditions (20±2 °C, 70% humidity and 12 h light/dark cycle) with free access to standard diet and tap water. The rats were treated in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/EEC), and in agreement with the Bioethical Committee of the University of the Balearic Islands. The animals were handled in the previous days of the experiments to reduce stress on the day of drug administration.

For the acute drug treatments, the rats received single subcutaneous (s.c.) or intraperitoneal (i.p.) injections of the various opioids as follows: dose–response for sufentanil (1, 2.5, 5, 15 and 30 μg/kg, s.c., for 30 min); time–course for sufentanil (15 μg/kg, s.c., for 5, 10, 15, 30, 60 and 120 min); dose–response for morphine (10, 30 and 100 mg/kg, i.p., for 120 min); time–course for the selective δ-opioid receptor agonist SNC-80 (10 mg/kg, i.p., for 30, 60 and 120 min); time–course for the selective κ-opioid receptor agonist (−)-U50488H (10 mg/kg, i.p., for 30, 60 and 120 min). In another series of experiments, groups of rats received single doses of the nonselective opioid receptor antagonist naloxone (10 and 100 mg/kg, i.p.) for 120 min, or naloxone (10 mg/kg, i.p.) 90 min before sufentanil (15 μg/kg, s.c., for 30 min), or a single dose of the selective δ-opioid receptor antagonist naltrindole (5 mg/kg, i.p.) 30 min before SNC-80 (10 mg/kg, i.p., for 30 min). Control rats received 0.9% saline vehicle (1 ml/kg) or dimethyl sulfoxide (DMSO, 1 ml/kg, in the case of SNC-80), and the animals were killed by decapitation at the indicated times. The doses of the different opioids (sufentanil, morphine, SNC-80 and U50488H) were chosen from previous studies that reported clear neurochemical effects in rats (García-Fuster et al., in press) with little alteration in gross behavior (e.g. doses of SNC-80 higher than 10 mg/kg induce convulsant activity and catalepsy; see Broom et al., 2002). The doses of sufentanil used (except 1 μg/kg) are extra-analgesic in the clinical setting, and the opioid induced short-lived sedation and frozen postures in most rats (see also Serra et al., 2003).

For the chronic treatment with morphine, groups of rats were injected (i.p.) three times (at 8:00, 14:00 and 20:00 h) during 5 consecutive days with increasing doses of the opioid (day 1: 10, 10 and 10 mg/kg; day 2: 10, 20, and 20 mg/kg; day 3: 20, 20 and 40 mg/kg; day 4: 40, 40 and 80 mg/kg; day 5: 80 and 100 mg/kg) (see Boronat et al., 2001), and the animals were killed 2 h after the last dose of morphine. After this chronic treatment, naloxone (2 mg/kg, i.p., for 2 h or 6 h)-precipitated withdrawal or spontaneous (24 h) opiate withdrawal was induced, which resulted in the standard behavioral reaction (data not shown; see Gabilondo and García-Sevilla, 1995; Miralles et al., 2005). Control rats received chronic 0.9% saline (1 ml/kg) in parallel.

After these treatments, the cerebral frontal cortex and corpus striatum were dissected on ice, immediately frozen in liquid nitrogen, and stored at −80 °C for no longer than 2 weeks until use.

2.2. Sample preparation and immunoblotting

Samples of cerebral frontal cortex (100–150 mg) and corpus striatum (50–70 mg) were homogenized with an Ultraturrax homogenizer (1:15 wt./vol.) in cold 50 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA, 2% sodium dodecyl sulfate (SDS), various protease inhibitors (1.3 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 μg/ml of each leupeptin, pepstatin A and antipain, and 5 μg/ml of E64), and two phosphatase inhibitors (1 mM cantharidin and 1 mM sodium orthovanadate). Then the samples were sonicated 3× for 5 s. The protein content was determined by the biuret reaction using bicinchoninic acid for the colorimetric detection of cuprous cation (BCA Protein Assay Reagent, Pierce Chemical Co., Rockford, IL, USA). Aliquots of brain samples were combined with equal volumes of electrophoresis loading buffer (50 mM Tris–HCl, pH 6.8, 1.5% SDS, 10% glycerol, 2.5% β-mercaptoethanol and 0.1% bromophenol blue), boiled (denaturated) and stored at −80 °C until use.

In routine experiments, 50 μg protein of each brain sample was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on 15-well (6×8-cm minigels, 1 mm thickness) gels (8%). Proteins were electrophoretically transferred (110 V, 3 h, 4 °C) to 0.45 μm nitrocellulose membranes (Western blotting) and blocked at room temperature for 1 h in Tris buffer saline (TBS: 137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 5% non-fat dry milk and 0.1% Tween 20. Then the membranes were incubated overnight at 4 °C in TBS with the primary rabbit polyclonal antibody (affinity purified) anti-phospho-MEK1/2 (raised against a synthetic phospho-Ser217/221 MEK peptide of human origin), dilution 1:1000 (Cell Signalling Technology, Beverly, MA, USA). To immunodetect the total content of MEK1/2, independently of the protein phosphorylation state, the corresponding phosphorylated blots were stripped (2% SDS and 0.1 M β-mercaptoethanol in 62.5 mM Tris–HCl, pH 6.8, for 30 min at 50 °C), washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4, 1.38 mM KH_2PO_4, pH 7.4), incubated in blocking solution (PBS containing 5% non-fat dry milk, 0.5% bovine serum albumin and 0.2% Tween 20), and then processed as above with the primary rabbit polyclonal antibody (affinity purified) anti-MEK1/2, dilution 1:5000 (Cell Signalling Technology). The secondary antibody, horseradish peroxidase-linked anti-rabbit IgG (dilution 1:3000 or 1:5000) (Amersham, Buckinghamshire, UK) was incubated in the corresponding blocking solution at room temperature for 2 h. These anti-MEK antibodies labeled bands with molecular masses of about 45 kDa (phosphorylated MEK1/2 and total MEK1/2), as described previously in the human brain (Ferrer-Alcón et al., 2004). Bound antibody (immunoreactivity) was detected using the enhanced chemiluminescence Western blot detection system (ECL, Amersham) and visualized by exposure to autoradiographic film (Amersham ECL Hyperfilm) for 1–10 min (autoradiograms).

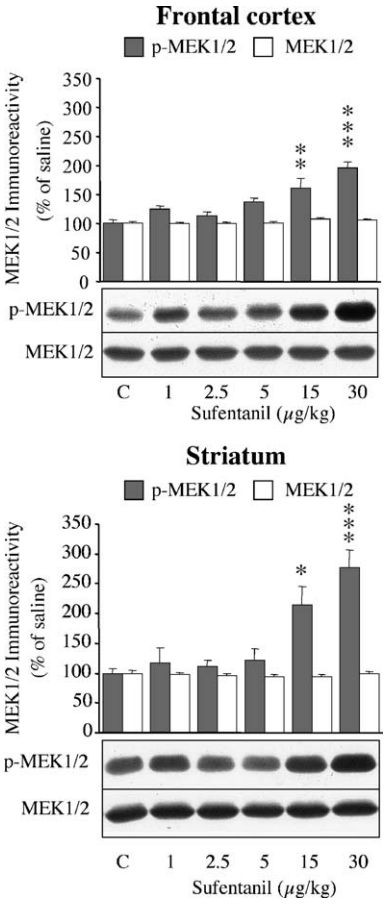

Fig. 1. Acute effects of sufentanil on phosphorylated (p-) MEK1/2 and total MEK1/2 immunoreactivities in the rat cerebral frontal cortex and corpus striatum. Groups of rats were treated (s.c.) with saline vehicle (C, $n=8–15$) or sufentanil (1–30 μg/kg, $n=5–10$ for each dose) and killed 30 min later. Columns are mean±S.E.M. of the immunoreactivity of the target proteins and expressed as percentage of the corresponding saline-treated group (C). One-way ANOVA detected significant differences between groups of treatments only for p-MEK1/2 in the cortex ($F=12.71$, $P<0.0001$) and striatum ($F=8.47$, $P<0.001$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with the corresponding control group (C) (ANOVA followed by Scheffé's test). Lower panels: representative immunoblots (50 μg protein) for the effects of sufentanil on p-MEK1/2 and total MEK1/2 in the cortex and striatum. Immunoblots for total enzyme were obtained after stripping and reprobing the corresponding p-blot with anti-total-protein antibodies.

2.3. Quantitation of specific MEK1/2 immunoreactivity

The autoradiograms (MEK1/2 immunoreactivity) were scanned in the transmittance mode with a resolution of 600 dpi with an Agfa SnapScan 600 scanner, and the analysis of the images were performed with a Power Macintosh G3 computer using a public domain image program (developed at the National Institutes of Health, USA, and available on Internet at http://rsb.info.nih.gov/nih-image/). The amount of MEK1/2 in the cerebral frontal cortex or corpus striatum of rats treated with opioid drugs and that of control rats, which received saline or DMSO, were compared in the same gel using standard curves [i.e., total protein loaded versus integrated optical density

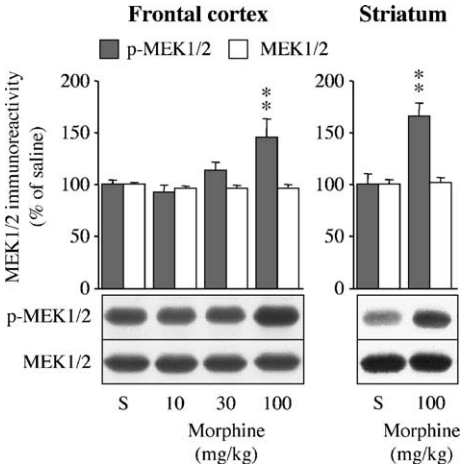

Fig. 2. Acute effects of morphine on phosphorylated (p-) MEK1/2 and total MEK1/2 immunoreactivities in the rat cerebral frontal cortex and corpus striatum. Groups of rats were treated (i.p.) with saline vehicle (C, $n=8$–20) or morphine (10–100 mg/kg, $n=10$ for each dose, frontal cortex; and 100 mg/kg, $n=4$, striatum) and killed 2 h later. Columns are mean±S.E.M. of the immunoreactivity of the target proteins and expressed as percentage of the corresponding saline-treated group (C). One-way ANOVA detected significant differences between groups of treatments only for p-MEK1/2 in the cortex ($F=6.51$, $P<0.001$). $^{**}P<0.01$, when compared with the corresponding control group (C) (ANOVA followed by Scheffé's test or unpaired t-test for data in the striatum). Lower panels: representative immunoblots (50 μg protein) for the effects of morphine on p-MEK1/2 and total MEK1/2 in the cortex and striatum. Immunoblots for total enzyme were obtained after stripping and reprobing the corresponding p-blot with anti-total-protein antibodies.

(IOD)], which consisted of at least five points of different protein content (usually 15–90 μg of protein) from naive control rats. For the quantitation of MEK1/2 immunoreactivity, samples from saline- and opioid-treated rats and the standard curve were loaded in the same gel and, for every sample, a theoretical amount of protein loaded in the gel (Pt) was obtained by intrapolation of its IOD in the standard curve. The percentage of target protein immunoreactivity of a given sample respect to the standard (saline-treated) sample was calculated as (Pt/Pr)×100; where Pr is the real amount of protein loaded in the gel well. This quantification procedure was assessed 4–5 times for each rat brain sample in different gels, and the mean value of the different gels was used as a final estimate.

2.4. Data analyses and statistics

All series of data were analyzed with the program GraphPad Prism™, version 3.0. Results are expressed as mean±S.E.M. values. One-way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test or two-tailed Student's *t*-test was used for the statistical evaluations. The level of significance was chosen as $P=0.05$.

2.5. Drugs and chemicals

Sufentanil HCl (Janssen Cylag S.A., Madrid, Spain) was kindly provided by Dr. María A. Hurlé (University of Cantabria, Santander, Spain). Morphine HCl was from the Servicio de Restricción de Estupefacientes, Dirección General de Farmacia y Productos Sanitarios, Ministerio de Sanidad y Consumo (Madrid, Spain). SNC-80 ((+)-4-[(α*R*)-α-(2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*-*N*, diethylbenzamide) was purchased from Tocris Cookson Ltd. (Avonmouth, UK). (−)-U50488H HCl (1*S*-*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) was from Sigma/RBI (St Louis, MO, USA). Naloxone HCl and naltrindole HCl were from Sigma. All other chemicals were from Sigma Chemical Co.

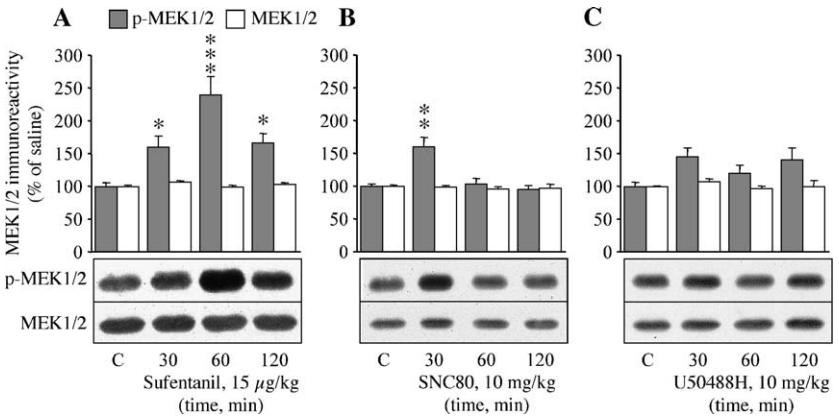

Fig. 3. Time–course for the effects of sufentanil (A), SNC-80 (B) and U50488H (C) on phosphorylated (p-) MEK1/2 and total MEK1/2 immunoreactivities in the rat cerebral frontal cortex. Groups of rats were treated with (A) saline vehicle (C, s.c., $n=15$) or sufentanil (15 μg/kg, s.c.) and killed 30 ($n=6$), 60 ($n=6$) and 120 ($n=6$) min later; (B) DMSO (C, i.p., $n=6$) or SNC-80 (10 mg/kg, i.p.) and killed 30 ($n=4$), 60 ($n=4$) and 120 ($n=4$) min later; (C) saline vehicle (C, i.p., $n=14$) or U50488H (10 mg/kg, i.p.) and killed 30 ($n=4$), 60 ($n=8$) and 120 ($n=3$) min later. Columns are mean±S.E.M. of the immunoreactivity of the target proteins and expressed as percentage of the corresponding vehicle-treated group (C). One-way ANOVA detected significant differences between groups of treatments only for p-MEK1/2 after sufentanil ($F=15.94$, $P<0.0001$), SNC-80 ($F=12.30$, $P<0.001$) and U50488H ($F=3.68$, $P=0.016$). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with the corresponding control group (C) (ANOVA followed by Scheffé's test). For U50488H treatments the modest increases did not reach statistical significance when compared with the control group (at best $P=0.11$ at 30 min; Scheffé's multiple comparison test; see also Results). Lower panels: representative immunoblots (50 μg protein) for the various drug time–courses on p-MEK1/2 and total MEK1/2 in the cortex. Immunoblots for total enzyme were obtained after stripping and reprobing the corresponding p-blot with anti-total-protein antibodies.

3. Results

3.1. Effects of acute treatments with μ-opioid receptor ligands on MEK1/2 activity in rat brain

The acute treatments of rats with the potent μ-opioid receptor agonist sufentanil (1–30 μg/kg, s.c., for 30 min), compared with saline administration, induced dose-dependent increases (significant with the highest doses) in the immunodensity of phosphorylated MEK1/2 proteins in the cerebral cortex (30–95%, Fig. 1A) and corpus striatum (113–177%, Fig. 1B), without altering the content of total MEK1/2 in both brain regions (Fig. 1). Similarly, a high dose of morphine (100 mg/kg, i.p., for 2 h) significantly increased the inmunodensity of phosphorylated MEK1/2 in the cortex (50%) and striatum (70%) (Fig. 2).

Sufentanil treatment (15 μg/kg, s.c., for 30–120 min) resulted in a rapid (30 min) and time-dependent (30–120 min) phosphorylation of MEK1/2 proteins (65–140%) in the cerebral cortex (Fig. 3A). The observed time–course for sufentanil was bell-shaped, with a maximal increase in phosphorylated MEK1/2 detected at 60 min (140%) (Fig. 3A). At shorter time points (5, 10 and 15 min), sufentanil (15 μg/kg) did not alter the content of phosphorylated MEK1/2 in the cortex (data not shown). A higher dose of sufentanil (30 μg/kg, s.c.) induced similar increases in phosphorylated MEK1/2 (95±11%, $n=5$, $P<0.001$, and 80±27%, $n=5$, $P<0.05$ at 30 min and 60 min, respectively) (data not shown).

Pretreatment of rats with naloxone (10 mg/kg, i.p.) fully prevented the acute phosphorylation of MEK1/2 proteins induced by sufentanil (15 μg/kg, s.c., 30 min) in the cerebral cortex (Fig. 4A), indicating the implication of μ-opioid receptors in MEK activation. Naloxone by itself (10 mg/kg, i.p., for 2 h) had no effect on the immunodensities of phosphorylated MEK1/2 and total MEK in the cortex (Fig. 4A). Moreover, the acute treatment with a high dose of naloxone (100 mg/kg, i.p., for 2 h) did not alter significantly the basal contents of phosphorylated MEK1/2 and total MEK in the cortex (data not shown), suggesting the absence of a tonic regulation of opioid receptors (mediated by endogenous opioid peptides) on these enzymes.

3.2. Effects of acute treatments with δ- and κ-opioid receptor ligands on MEK1/2 activity in rat brain

The acute treatment of rats with the selective δ-opioid receptor agonist SNC-80 (10 mg/kg, i.p.) induced a rapid (30 min) and significant increase in the phosphorylation of MEK1/2 (60%), which disappeared by 60–120 min (Fig. 3B). SNC-80 did not alter the total content of MEK1/2 in the cortex (Fig. 3B). Pretreatment of rats with naltrindole (a selective δ-opioid receptor antagonist, 5 mg/kg, i.p.) fully prevented the acute activation of phosphorylated MEK1/2 induced by SNC-80 (Fig. 4B), which clearly revealed the involvement of δ-opioid receptors in MEK1/2 activation in the brain.

The acute treatments of rats with the selective κ-opioid receptor agonist (−)-U50488H (10 mg/kg, i.p., 30, 60 and 120 min) also showed a clear tendency to augment the phosphorylation of MEK1/2 in the cerebral cortex (Fig. 3C) (ANOVA, $F=3.68$, $P=0.016$), but the quantitated increases (20–46%) did not reach statistical significance when compared with the saline control group (multiple comparison post test). However, the individual comparisons of the effects of (−)-U50488H (30–120 min) against the saline control group

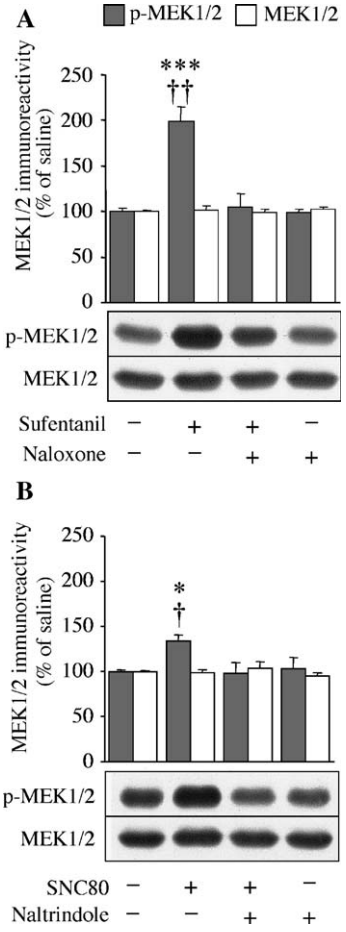

Fig. 4. Specificity of the opioid receptor involved in the acute regulation of phosphorylated (p-) MEK1/2 by opioid drugs in the rat cerebral frontal cortex. (A) Antagonism by naloxone of the effect of the μ-opioid receptor agonist sufentanil. Groups of rats ($n=5$ per group) were treated (i.p.) with saline or naloxone (10 mg/kg) 60 min before a second administration (s.c.) of saline or sufentanil (15 μg/kg) and killed 30 min after the last injection. Columns are mean±S.E.M. of the immunoreactivity of the target proteins and expressed as percentage of the corresponding saline-treated group (−,−). One-way ANOVA detected significant differences between groups of treatments only for p-MEK1/2 ($F=18.53$, $P<0.0001$). ***$P<0.001$ when compared with the corresponding control group (−,−); $^{\dagger\dagger}P<0.01$ when compared with the naloxone plus sufentanil group (+,+) (ANOVA followed by Scheffé's test). (B) Antagonism by naltrindole of the effect of the δ-opioid receptor agonist SNC-80. Groups of rats ($n=5$–9 per group) were treated (i.p.) with saline or naltrindole (5 mg/kg) 30 min before a second administration (i.p.) of DMSO or SNC-80 (10 mg/kg) and killed 30 min after the last injection. One-way ANOVA detected significant differences between groups of treatments only for p-MEK1/2 ($F=5.86$, $P<0.01$). *$P<0.05$ when compared with the corresponding saline-treated group (−,−); $^{\dagger}P<0.05$ when compared with the naltrindole plus SNC-80 group (+,+). Other details as in (A). Lower panels: representative immunoblots for each set of experiments (sample: 50 μg protein). Immunoblots for total enzyme were obtained after stripping and reprobing the corresponding p-blot with anti-total-protein antibodies.

(two-tailed Student's *t*-test) indicated the induction of a significant drug effect at 30 min (46±14% increase, $n=4$; $t=2.36$, $P=0.046$). This selective κ-opioid receptor agonist did not modify significantly the total content of MEK1/2 in the cerebral cortex (Fig. 3C).

3.3. Effects of chronic morphine treatment and opioid withdrawal on MEK1/2 activity in rat brain

Chronic (5 days) treatment with morphine (10 to 100 mg/kg, i.p.), compared with saline administration, did not alter significantly the immunodensity of phosphorylated MEK1/2 or total MEK in the cerebral cortex and corpus striatum (2 h posttreatment) (Fig. 5), which suggested the induction of tachyphylaxis to the repeated and increasing doses of the opioid drug (see the effects of 100 mg/kg in Fig. 2). In morphine-dependent rats, however, naloxone (2 mg/kg)-precipitated withdrawal (2 h and 6 h after the last dose of morphine) induced rapid and robust increases in the density of phosphorylated MEK1/2, but not in total MEK, in the cortex (27% and 49%, respectively) and striatum (83% and 123%, respectively) (Fig. 5). In morphine-dependent rats, the spontaneous opioid withdrawal (24 h after the chronic treatment) did not modify significantly the immunodensities of phosphorylated MEK1/2 and total MEK in the cortex or striatum (Fig. 5).

4. Discussion

The results demonstrate that acute treatments with μ- and δ-opioid receptor agonists (and to a lesser extent with a selective κ-opioid receptor agonist) induced the activation (phosphorylation) of MEK1/2 (MAP/ERK kinases) in the rat brain. Thus, the high efficacy μ-opioid receptor agonist sufentanil (Cox et al., 1998) induced dose- and time-dependent up-regulations of phosphorylated MEK1/2, through a naloxone-sensitive mechanism, in the cerebral cortex and corpus striatum. A high dose of morphine, a low efficacy μ-opioid receptor agonist (Williams et al., 2001), also activated MEK1/2 in the cortex and striatum. Similarly, the selective δ-opioid receptor agonist SNC-80 increased the content of phosphorylated MEK1/2 in the cortex, an effect that was blocked by the selective antagonist naltrindole. In contrast, a high dose of (−)-U50488H (García-Fuster et al., in press), a selective κ-opioid receptor agonist, did not consistently induce the activation of MEK1/2 in the cerebral cortex. These findings indicated that μ- and δ-opioid receptors have a preponderant role in the acute in vivo regulation of MEK1/2 in the brain.

Although opioid-induced activation of ERK1/2 has been extensively studied in vitro (Fukuda et al., 1996; Li and Chang, 1996; Belcheva et al., 1998; Trapaidze et al., 2000; Ferrer-Alcón et al., 2004; Schulz et al., 2004), MEK1/2 stimulation by opioid agonists was only described for the δ-opioid receptor agonist deltorphin in Jurkat T lymphocytes (Hedin et al., 1999). Recently, fentanyl (a potent μ-opioid receptor agonist) was shown to induce a naloxone-sensitive activation of ERK1/2, without altering MEK1/2 activity, in the rat ventral tegmental area in vitro (Lesscher et al., 2003). In the present in vivo study, however, acute treatments with sufentanil and morphine clearly stimulated the activity of MEK1/2 in the rat cerebral cortex and the corpus striatum. In fact, the in vitro and in vivo stimulation of ERK1/2 by morphine and SNC-80 have been shown to be dependent on MEK1/2 activity, mainly because specific MEK inhibitors (PD98059; SL-327) blocked the opioid-induced ERK1/2 phosphorylation in SH-SY5Y cells (Ferrer-Alcón et al., 2004) and the rat brain (García-Fuster et al., in press). These findings ruled out the possibility that the activation of ERK1/2 signaling by opioid drugs might be caused by a MEK1/2-

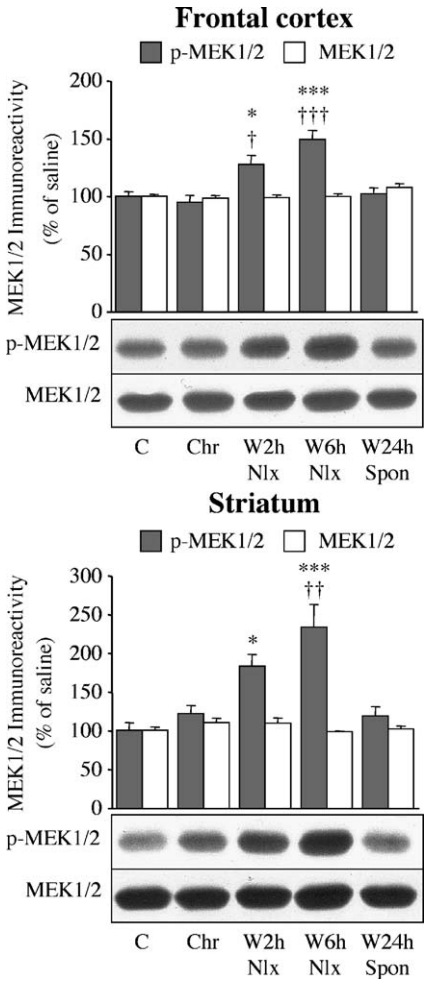

Fig. 5. Effects of chronic morphine and opioid withdrawal on phosphorylated (p-) MEK1/2 and total MEK1/2 immunoreactivities in the rat cerebral frontal cortex and corpus striatum. Groups of rats were treated chronically (i.p.) with saline vehicle (C, $n=7$–15) and morphine (Chr, 10–100 mg/kg for 5 days; $n=5$), or with chronic morphine followed by naloxone (Nlx, 2 mg/kg, i.p.)-precipitated (W2h–W6h, $n=4$–5) or spontaneous (Spon, W24h, $n=4$–5) opioid withdrawal. Columns are mean±S.E.M. of the immunoreactivity of the target proteins and expressed as percentage of the corresponding saline-treated group (C). One-way ANOVA detected significant differences between groups of treatments only for p-MEK1/2 in the cortex ($F=11.82$, $P<0.0001$) and striatum ($F=13.42$, $P<0.0001$). $^{*}P<0.05$, $^{***}P<0.001$ when compared with the corresponding control group (C); $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$, when compared with the corresponding chronic morphine-treated group (Chr) (ANOVA followed by Scheffé's test). Lower panels: representative immunoblots for each set of experiments (sample: 50 μg protein). Immunoblots for total enzyme were obtained after stripping and reprobing the corresponding p-blot with anti-total-protein antibodies.

independent pathway. Also recently, it has been reported that i.c.v. pretreatment of mice with various MEK inhibitors (PD98059 or U0126) impaired the morphine-induced place preference paradigm (Ozaki et al., 2004), which is also in support of the current findings.

Another interesting finding of this study is that chronic morphine treatment (5 days) resulted in the induction of tachyphylaxis to the acute stimulatory effect of this opioid on the content of phosphorylated MEK1/2 in the cerebral frontal cortex and the corpus striatum, which most probably reflected the process of μ-opioid receptor desensitization demonstrated after repeated agonist exposure (Dang and Williams, 2004, 2005). In this context, an earlier study (Ortiz et al., 1995) also reported that chronic morphine treatment failed to alter the levels of MEK immunoreactivity in various brain regions of the rat, but in this investigation MEK activity (i.e., enzyme phosphorylation) was not assessed and only the total content of MEK was measured (negative data in agreement with the current results). In a recent study (Eitan et al., 2003), chronic morphine treatment in mice (resulting in analgesic tolerance) also caused tolerance to morphine-induced ERK1/2 activation in the anterior cingulate cortex and other brain regions, which is also in line with the current data. However, various in vivo investigations, dealing with the status of ERK1/2 after chronic morphine administration (tolerant state), have demonstrated the induction of decreases in the activity of these enzymes (Schulz and Höllt, 1998; Ferrer-Alcón et al., 2004; Muller and Unterwald, 2004), and only one study reported an increased activity of ERK1/2 in the brain (Ortiz et al., 1995). The reasons for these discrepancies are not readily apparent and they may be related to the marked different experimental conditions used in the reported studies (e.g. opiate dose, duration of treatment, cellular complexity of the brain region, and/or antibodies used).

Notably, naloxone-precipitated withdrawal in morphine-dependent rats (resulting in an intense behavioral reaction; Gabilondo and García-Sevilla, 1995) induced a robust stimulation of MEK1/2 in the cerebral cortex and corpus striatum, indicating that morphine addiction (dependent state) is associated with activation of the MAP/ERK kinase pathway. In fact, naloxone-precipitated withdrawal was shown to increase ERK1/2 activity in brains of morphine-dependent rats (Schultz and Höllt, 1998). Moreover, recent data from this laboratory also indicate the induction of up-regulation in the content of phosphorylated ERK1 (20±6%, $n=5$, $P<0.05$) and phosphorylated ERK2 (42±6%, $n=5$, $P<0.01$) in a similar group of morphine- (naloxone-) withdrawn rats (data not shown). Together, these results clearly indicate that the regulation of MEK1/2 and ERK1/2 followed a similar and parallel pattern of activation in the brain of morphine-dependent rats during the state of opioid withdrawal (induced by naloxone). This concomitant activation of MEK1/2 and ERK1/2 could be the result, in part, of an increase in the release of endogenous enkephalins induced by opioid withdrawal (Mas Nieto et al., 2002), which would promote a new stimulus on MEK/ERK phosphorylation acting the opioid peptides as μ/δ-opioid receptor agonists. Compared to naloxone-precipitated opioid withdrawal, spontaneous morphine withdrawal induced a slow and less intense behavioral reaction (maximal at 24 h; Gabilondo and García-Sevilla, 1995) that was not associated with significant changes in MEK1/2 activation in the cortex and striatum, which suggests that the increased phosphorylation of MEK1/2 during opioid withdrawal vanished with time and/or with the intensity of the abstinence syndrome. The current findings may be relevant in the context of the known role of the MEK/ERK signaling pathway in various long-lasting forms of synaptic plasticity (Grewal et al., 1999; Adams and Sweatt, 2002), including those associated with the chronic adaptive changes induced by morphine addiction in the brain (Nestler, 2002; Mazzucchelli et al., 2002).

To sum up, the acute treatment of rats with exogenous opioids (mainly μ-opioid receptor agonists) increased the phosphorylation of MEK1/2 in the cerebral cortex and corpus striatum, then MEK activity gradually recovered toward normal levels during the course of repeated morphine administration, and finally removal of the opioid in these morphine-dependent animals induced a new and robust increase in the phosphorylation of MEK1/2 in the brain. These new findings expand our understanding of the regulation of MAP kinase/MEK/ERK pathway by opioid drugs in the brain.

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