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Long-term regulation of signalling components of adenylyl cyclase and mitogen-activated protein kinase in the pre-frontal cortex of human opiate addicts

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Abstract

Opiate addiction involves the development of chronic adaptive changes in μ-opioid receptors and associated pathways (e.g. cAMP signalling) which lead to neuronal plasticity in the brain. This study assessed the status of cAMP and mitogen-activated protein kinase (MAPK) pathways in brains (pre-frontal cortex) of chronic opiate addicts. In these subjects ($n = 24$), the immunodensities of adenylyl cyclase-I, PKA Cα, total and phosphorylated CREB were not different from those in sex-, age- and PMD-matched controls. Moreover, the ratio pCREB/tCREB was similar in opiate addicts (0.74) and controls (0.76), further indicating that opiate addiction in humans is not associated with an upregulation of several key components of cAMP signalling in the pre-frontal cortex. In contrast, the components of MAPK cascade (Ras/c-Raf-1/MEK/ERK) were decreased in the same brains. Notably, pronounced down-regulations of phosphorylated MEK (85%) and ERK1/2 (pERK1: 81%; pERK2: 80%) were quantitated in brains of opiate addicts. Chronic morphine treatment in rats (10–100 mg/kg for 5 days) was also associated with decreases of pERK1/2 (59–68%) in the cortex. In SH-SY5Y cells, morphine also stimulated the activity of pERK1/2 (2.5-fold) and the MEK inhibitor PD98059 blocked this effect (90%). The abnormalities of MAPK signalling might have important consequences in the long term development of various forms of neural plasticity associated with opiate addiction in humans.

Keywords: cAMP signalling, human post-mortem brain, MAPK signalling, opiate addiction, rat brain, SH-SY5Y cells.
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Opiate addiction in humans is a chronic disorder characterized by drug tolerance and dependence, sensitization, long-term vulnerability to relapse and high mortality. The development of opiate addiction involves the establishment of chronic adaptive changes in μ-opioid receptors and associated signalling systems (e.g. cAMP pathway) leading to neuronal plasticity in specific regions of the brain, including the neocortex (Simonato 1996; Nestler and Aghajanian 1997; Law *et al*. 2000; Nestler 2001, 2002; Williams *et al*. 2001).

Chronic opiate agonist exposure has been shown to result in upregulation of cAMP signalling in the rat brain, the best established molecular adaptation to chronic morphine to date, and this compensatory response develops probably to oppose the acute opiate inhibition of the cAMP pathway (Nestler and Aghajanian 1997). In the rat locus coeruleus, this upregulation of cAMP signalling involves increased concentrations of Gi/o proteins, adenylyl cyclases, protein kinase A (PKA) and its downstream nuclear target CREB (Nestler and Aghajanian 1997; Williams *et al*. 2001). Notably, the activity of CREB plays a relevant role in opiate addiction (Maldonado *et al*.

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Abbreviations used: AC, adenylyl cyclase; BSA, bovine serum albumin; CREB, cAMP response element-binding protein; DMSO, dimethyl sulphoxide; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; Gi/o, inhibitory coupling protein; IOD, integrated optical density; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase and ERK kinase; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase A; PMD, post-mortem delay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

1996; Blendy and Maldonado 1998) and, in this way, chronic morphine can stimulate the expression of numerous genes through PKA-mediated CREB phosphorylation. Modulation of transcription factors such as CREB represents one potential mechanism for persisted opiate-induced plasticity in the brain (Nestler and Aghajanian 1997; Nestler 2001, 2002).

More recently, numerous investigations have also shown the involvement of MAPK pathway in the effects of opiate drugs (Law *et al*. 2000; Liu and Anand 2001; Williams *et al*. 2001), but most studies have dealt only with the modulation of the best-characterized ERK1/2 (Ortiz *et al*. 1995; Berhow *et al*. 1996; Gutstein *et al*. 1997; Schulz and Höllt 1998; Mazzucchelli *et al*. 2002; Eitan *et al*. 2003; Lesscher *et al*. 2003). The MAPK pathway is a key signalling mechanism that transduces extracellular signals to the nucleus and regulates many aspects of cellular functions (e.g. cell growth, differentiation and apoptosis) (Cobb 1999; Grewal *et al*. 1999; Pearson *et al*. 2001; Sweatt 2001). The first component of this pathway Raf-1 (a serine/threonine kinase, MAPKKK) relays the extracellular signal from the receptor/Ras (a small G protein) complex to a cascade of cytosolic kinases by phosphorylating and activating MEK (MAPKK), which in turn phosphorylates and activates (exclusively) ERK1/2 (p44 and p42 MAPK) that finally regulate various cytoplasmic (e.g. cdk5, cytoskeletal proteins) and nuclear proteins (e.g. CREB, Elk-1) to alter gene expression (Pearson *et al*. 2001; Pouysségur and Lenormand 2003). ERK1/2 are expressed in mature neurones being the most abundant kinases in the cascade, which provides a mechanism of signal amplification (Derkinderen *et al*. 1999; Pouysségur and Lenormand 2003) that plays a key role in various long-lasting forms of synaptic plasticity (Grewal *et al*. 1999; Sweatt 2001; Adams and Sweatt 2002; Mazzucchelli *et al*. 2002). In the context of opiate addiction, a striking increase in sensitivity to the rewarding properties of morphine (mediated by an upregulation of ERK2) has been observed in mice lacking ERK1 (Mazzucchelli *et al*. 2002).

Although several studies in laboratory animals have clearly demonstrated the involvement of cAMP and MAPK signalling pathways in the chronic effects of opiates, nothing is known about similar or dissimilar adaptive changes of these key systems in brains of human opiate addicts. This approach is particularly relevant because animal models, besides the obvious differences between animal and human brain, cannot fully reproduce the features and framework of opiate intake in humans. Therefore, the aim of this study was to evaluate in parallel the status of the various components of the cAMP (AC/PKA/CREB) and MAPK (Ras/Raf/MEK/ERK) pathways in a large series of brains of well-defined chronic opiate abusers who had died of an opiate overdose. The main findings demonstrate an apparent normal functioning of cAMP signalling and the existence of a pronounced downregulation of the MAPK pathway in the pre-frontal cortex of chronic opiate addicts.

Materials and methods

Human brain samples

Brain samples were dissected at the time of autopsy (Institute of Forensic Medicine, University of Geneva, Switzerland) and immediately stored at − 80°C until assay. Specimens of the right pre-frontal cortex (Brodmann's area 9) were collected from 24 well-defined chronic opiate abusers who had died of an opiate overdose. As in previous studies (Ferrer-Alcón *et al*. 2003, 2004), the pre-frontal cortex was chosen for examination because it includes important areas functionally related to the mesolimbic dopaminergic reward system which are relevant to opiate addiction (Simonato 1996; Williams *et al*. 2001). These subjects had been used in a previous study, which revealed abnormalities in the expression of μ-opioid receptors and receptor regulatory mechanisms (Ferrer-Alcón *et al*. 2004). Drug screening and the determination of a long-term history of opiate addiction were made as described previously (Ferrer-Alcón *et al*. 2004). Drug screening in blood samples (Table 1) showed the presence of fatal concentrations of morphine or methadone in all opiate addicts in whom a heroin or methadone overdose was the cause of death (for further details see Ferrer-Alcón *et al*. 2004). Moreover, the detection in hair samples (3–10 cm from the root) of 6-monoacetylmorphine (range 0.2–13 ng/mg), morphine (range 0.2–1.3 ng/mg) or methadone (range 0.4–14.2 ng/mg) indicated a chronic abuse of opiates in these subjects (6–12 months before death; Table 1; Tagliaro *et al*. 1998). Ethanol was found in blood samples of some opiate addicts (range 0.05–2.4 g/L, $n = 10$), but no evidence of a diagnosis of ethanol dependence was obtained by medical records. Other drugs detected in blood samples of these subjects (e.g. weak concentrations of cocaine; Table 1) did not appear to alter the status of the target proteins in brains of opiate addicts (see Results). Control brain specimens (right pre-frontal cortex) were obtained at autopsy from potential healthy subjects dying in the same period and who met specific criteria to match the opiate addicts (including a negative toxicological screening), as described previously (Ferrer-Alcón *et al*. 2004). Ethanol was detected in some control subjects (range 0.05–2.02 g/L, $n = 6$), but also in these cases no evidence of a diagnosis of ethanol dependence was obtained. The control group consisted of specimens from 18 subjects with an age at death (mean ± SEM: 33 ± 2 years) and a post-mortem delay (PMD: 34 ± 4 h) that did not differ from those in opiate addicts (Table 1). The influence of PMD (range 2–62 h) and age (range 18–50 years) on MAPK signalling components in the brain was assessed in a similar group of control subjects (see Results). These variables did not alter the expression of AC-I in the human brain (data not shown). The influence of age (15–52 years) and PMD (3–81 h) on other components of cAMP signalling (PKA, CREB, pCREB) had been assessed with negative results in a previous study (Odagaki *et al*. 2001). The definitive pairs of subjects (opiate addicts and their respective matched controls) are given in Table 1. This study was approved by the research and ethical review board of the Department of Psychiatry, University of Geneva.

Opiate drug treatment of rats and brain samples

Male Sprague–Dawley rats (250–300 g) were used. The animals were housed under controlled environmental conditions (22°C, 70% humidity, and 12-h light/dark cycle) with free access to food and

Table 1 Demographic characteristics and toxicological data of individual opiate addicts

Sex/Age* (years)	PMD (h)	Total blood opiates (μg/mL)	Other drugs (μg/mL) Ethanol (g/L)	Hair opiates (ng/mg)
M/18 (M/20)	21 (35)	Met (1.1)	Chl (1.5) + Qua (0.2)	Met (0.4) + MAM (4.8)
M/19 (M/20)	26 (35)	Mor (0.2)	None	NT
M/23 (M/15)	66 (70)	Mor (1.7)	None	NT
M/23 (M/15)	55 (70)	Met (1.5)	Thio (1.4)	Mor (0.4) + MAM (2.2)
M/39 (M/40)	56 (48)	Mor (1.1)	Eth (2.4)	MAM (2.5)
M/30 (M/37)	60 (48)	Mor (1.5)	None	MAM (1.2)
M/25 (M/23)	41 (38)	Met (0.7)	None	NT
M/25 (M/20)	26 (35)	Mor (1.1)	Eth (0.4)	MAM (0.9)
M/26 (M/28)	44 (36)	Mor (0.3) + Cod (3.6)	Eth (0.05)	Mor (0.3) + MAM (1.3)
M/25 (M/28)	50 (36)	Met (0.5)	Coc (0.05)	Mor (0.4) + MAM (1.1)
M/26 (F/34)	73 (67)	Mor (1.7)	Coc (0.04)	Mor (1.3) + MAM (13.0)
M/23 (M/31)	52 (62)	Met (1.3)	Eth (0.06)	Met (0.5) + MAM (0.2)
M/29 (M/22)	25 16	Met (0.5)	Cit (3.4)	Met (1.1)
M/26 (M/27)	20 22	Met (0.3)	Eth (0.12)	Met (0.6)
M/26 (M/27)	13 22	Mor (2.0)	Eth (0.28)	MAM (0.4) + Met (4.0)
M/40 (M/48)	16 24	Met (0.8)	Qui (1.2)	Met (0.8)
M/33 (M/40)	16 24	Mor (1.5)	Eth (0.87)	MAM (0.4) + Met (14.2)
M/44 (M/44)	19 19	Mor (0.6)	None	Mor (0.2)
M/32 (M/36)	29 29	Mor (1.6) + Cod (0.5)	Eth (0.15)	Mor (0.7) + Coc (1.0)
M/42 (M/44)	28 19	Met (0.4)	None	Met (13.5)
M/42 (F/50)	18 10	Mor (0.8)	Eth (1.64)	Mor (0.4)
M/46 (F/50)	13 10	Mor (0.8)	Coc (0.2)	Mor (0.2) + Coc (6.5)
F/28 (M/23)	13 17	Met (1.4)	Coc (0.02)	Met (4.7) + Coc (0.9)
M/29 (M/35)	38 29	Met (0.4)	Eth (0.36)	Met (1.3) + Mor (0.8)
Addict group				
23M/1F	34 ± 4 h	Mor (1.2 ± 0.2, $n = 13$)		
30 ± 2 years		Met (0.8 ± 0.1, $n = 11$)		

*Data of the respective matched control subject presented in parentheses. PMD, post-mortem delay (time period from death to the storage of the brain specimen). NT: not tested. Cause of death for all opioid addicts was an overdose of heroin [detection of 6-monoacetylmorphine (MAM) and free morphine (Mor) in blood] or methadone (Met, detection of Met and its metabolite 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidone in blood). Quantitative determinations of opiate drugs and other drugs in blood and hair samples were performed as indicated in Materials and methods. Chl, chloroquine; Cit, citalopram; Coc, cocaine; Cod, codeine; Eth, ethanol; Qua, methaqualone; Qui, quinine; Thio, thioridazine. Some subjects were also positive for benzodiazepines and cannabinoids in urine specimens.

water. For the acute treatment, the rats received a single intraperitoneal (i.p.) injection of morphine (30 mg/kg i.p. for 2 h). For the chronic treatment with morphine, the rats were injected i.p. three times daily (at 08:00, 14:00 and 20:00 h) during 5 days with increasing doses of the opiate (10–100 mg/kg; Ventayol *et al.* 1997), and the animals were killed 2 h after the last dose of morphine. These morphine treatments in rats were well-tolerated and no lethality was observed. Control rats received 0.9% saline vehicle (1 mL/kg i.p. for 2 h). The rats were killed by decapitation and specimens of cerebral cortex were dissected and stored at −80°C until use. These experiments in rats were performed according to the guidelines of the University of the Balearic Islands, Spain.

SH-SY5Y cell culture, induction of ERK activity by morphine and cell lysates

Human neuroblastoma SH-SY5Y cells (a SK-N-SH cell clone which expresses high densities of μ- and δ-opioid receptors, proportion 4 : 1; Yu *et al.* 1986) were grown in monolayer (incubator at 37°C and 5% CO_2/95% humidified air) in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were passaged every 4 days. Confluent cells were released in DPBS (150 mM NaCl, 1.5 mM KH_2PO_4, 3 mM KCL, 7.9 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4), centrifuged (300 *g*, 5 min), resuspended in RPMI-1640 medium and counted. To assess the induction of ERK activity (pERK1/2) by morphine, aliquots of SH-SY5Y cells were exposed to various morphine concentrations (1 nM–100 μM for 5 min) or to the chosen 1 μM morphine for 1–60 min (time-course experiments). To block the basal activation of ERK1/2 by MEK and the stimulatory effect of morphine on the former enzyme isoforms, cells were also pre-incubated with PD98059 (2-amino-3-methoxyflavone, 50 and 100 μM), a selective inhibitor of MEK (Alessi *et al.* 1995), or vechicle control (0.1% dimethyl sulphoxide, DMSO), for 60 min prior to basal assessment or drug stimulation. Drug-treated and control SH-SY5Y cells were finally resuspended in homogenization buffer and cell lysates prepared as for the brain samples (see below). For these *in vitro* experiments, freshly prepared membrane homogenates were used.

Human and rat brain sample preparations and immunoblot assays

Target proteins in the brain were assessed by quantitative immunoblotting as described previously (Ferrer-Alcón *et al*. 2003, 2004). Briefly, 200–250 mg of human pre-frontal cortex was homogenized in 50 mM Tris–HCl buffer, pH 6.8, containing 1 mM EDTA, 2% sodium dodecyl sulphate (SDS) and various protease inhibitors (1.3 mM pefabloc, 10 μg/mL leupeptin, 5 μg/mL E64, 10 μg/mL antipain and 10 μg/mL pepstatin A). Aliquots of this mixture were combined with solubilization loading buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol and 0.1% bromophenol blue] to reach a final protein concentration of 3 μg/μL. The mixtures were denatured at 95°C for 5 min and stored at −80°C. For the rat brain, 80–100 mg of cerebral cortex was homogenized in 40 mM Tris–HCl buffer, pH 7.5, containing 1% Triton X-100, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM NaCl, and the protease inhibitors phenylmethyl–sulphonyl fluoride (1 mM) and leupeptin (40 μg/mL). The samples were centrifuged at 40 000 *g* for 45 min and aliquots of the resulting supernatant were mixed with loading buffer as above. Then the samples were denatured and stored at −80°C until use. Protein concentrations were determined by the biuret reaction using bicinchoninic acid for colorimetric detection of cuprous cation (BCA, Protein Assay Reagent, Pierce Chemical Company, Rockford, IL, USA).

In routine experiments, 40 μg protein of each human or rat brain sample (total homogenate) or SH-SY5Y cell lysate was subjected to SDS–PAGE on 15-well 12% polyacrylamide minigels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes and incubated (blocking solution) in phosphate-buffered saline containing 5% non-fat dry milk, 0.2% Tween-20, and 0.5% bovine serum albumin (BSA) or 0.5% Tween and 3% BSA (for PKA, tCREB and pCREB; Odagaki *et al*. 2001). Then, the membranes were incubated (overnight at 4°C) in blocking solution containing the appropriate primary antibody. The following primary polyclonal (affinity-purified) or monoclonal antibodies were used: AC-I (anti-A cyclase I, Product sc-586, 1 : 1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA); PKA (anti-PKA Cα catalytic subunit, Product sc-903, 1 : 100 000 dilution, Santa Cruz Biotechnology); CREB (antitotal CREB, Product 06–863, 1 : 5000 dilution, Upstate Biotechnology, New York, NY, USA); phosphorylated CREB (antiphospho-CREB, p^+Ser 133, Product 06–519, 1 : 5000 dilution, Upstate Biotechnology); Ras (anti-Ras, Product R02120, 1 : 1000 dilution, Transduction Laboratories, Lexington, KY, USA); c-Raf-1 (anti-Raf, Product R19120, dilution 1 : 1000, Transduction Laboratories); MEK (antitotal MEK1/2, Product 9120 Antibody Kit, 1 : 3000 dilution, New England Biolabs, Beverly, MA, USA); phosporylated MEK (antiphospho MEK1/2, p^+Ser 217/221, Product 9120 Antibody Kit, 1 : 1000 dilution, New England Biolabs); phosporylated MEK (antiphospho MEK1/2, p^+Ser 217/221, Product 9121, 1 : 1000 dilution, Cell Signalling Technology, Beverly, MA, USA); ERK (antitotal ERK1/2, Product 442704, 1 : 1000 dilution, Calbiochem-Novabiochem Corporation, San Diego, CA, USA); phosphorylated ERK (antiphospho ERK1/2, p^+Tyr 204, Product 442705, 1 : 1000 dilution, Calbiochem); phosphorylated ERK (antiphospho ERK1/2, p^+Thr 202/p^+Tyr 204, Product 9106S, 1 : 1000 dilution, New England Biolabs), and antiα-internexin (Product AB1515, 1 : 1000 dilution, Chemicon International Inc., Temecula, CA, USA). For CREB, MEK and ERK the samples were first analysed with antiphosphoprotein antibodies (active forms) and then stripped and reprobed with antitotal-protein antibodies. The secondary antibody was horseradish peroxidase-linked anti-rabbit IgG or sheep anti-mouse IgG (1 : 5000 dilution). Bound antibody (immunoreactivity) was detected using the enhanced chemiluminescence (ECL) western blot detection system (Amersham, Buckinghamshire, UK) and visualized by exposure to autoradiographic film (Amersham ECL Hyperfilm) for 1–15 min (autoradiograms).

Quantitation of target protein contents

The autoradiograms were quantitated by densitometric scanning (GS-700 Imaging Densitometer, resolution: 42 μm, Bio-Rad), by measuring the integrated optical density (IOD) units of the immunoreactive bands. In a given experiment, the amount of a target protein in the pre-frontal cortex of one or two opiate addicts was compared with that of a control subject matched for age (± 8 years), PMD (± 15 h), and whenever possible for sex, and these subjects were run together on the same gel. Prior to analyses, the linearity of protein concentration for western blotting was ascertained by resolution of selected concentrations of protein (i.e. total protein loaded versus IOD units, consisting of 5 points of different protein content, usually 20–60 μg, resulting in good linear relations; data not shown). Experiments in opiate addicts and matched controls were performed by using a protein concentration (40 μg in duplicate) known to be within the linear range for immunolabeling of the target proteins. This quantification procedure was assessed three to five times in different gels. Finally, percentage changes in immunoreactivity with respect to control samples (100%) were calculated for each opiate addict in each gel, and the mean value of the different gels was used as a final estimate. Similarly, the content of ERK (total and phosphorylated) in the cerebral cortex of rats treated with morphine was compared in the same gel with that of control rats which received saline solution. The quantification procedure was assessed three times in different gels. Finally, percentage changes in immunoreactivity with respect to control saline samples (100%) were calculated for each rat treated with morphine in the various gels and the mean value used as a final estimate. A similar procedure was used to quantitate the effect of morphine on ERK activity in SH-SY5Y cells.

Data analysis and statistics

Results are expressed as mean ± SEM. The two-tailed one-sample *t*-test (human data), one-way ANOVA followed by Bonferroni's multiple comparison test (rat data) and Student's *t*-test (SH-SY5Y cell data) were used for the statistical evaluations. Pearson's correlation coefficients were calculated by the method of least squares to test for possible association among variables. The level of significance was chosen at $p = 0.05$.

Reagents

The SH-SY5Y cell line was kindly provided by Dr Beat M. Riederer (IBCM, University of Lausanne, Switzerland). RPMI-1640 medium was purchased from Gibco BRL (Basel, Switzerland). Morphine HCl was from Unión-Químico-Farmacéutica S.A.E. (Madrid, Spain). PD98059 (2-amino-3-methoxyflavone) was from Calbiochem (La Jolla, CA, USA). Acrylamide (Protogel) was from BDH Brunschwig (Dorset, UK), Pefabloc from Boehringer (Rotkreuz,

Switzerland) and nitrocellulose membranes from Schleicher & Schuell (Dassel, Germany). Other materials such as horseradish peroxidase-linked secondary antibody, ECL reagents, and autoradiography films were purchased from Amersham. All other chemicals were from Sigma Chemie or Fluka Chemie (Buchs, Switzerland).

Results

Long-term regulation of AC/PKA/CREB pathway in brains of opiate addicts

In the pre-frontal cortex of opiate addicts ($n = 24$), the immunodensities of AC-I (95 ± 11%), PKA Cα (99 ± 6%), total content of CREB (95 ± 2%) and phosphorylated CREB (105 ± 7%; the active form of this transcription factor by phosphorylation at the activator-site residue Ser 133) were not significantly different from those in sex-, age-, and PMD-matched controls (100%) (Fig. 1). In these brains the ratio pCREB/tCREB was similar to that in control subjects (controls: 0.76 ± 0.06, $n = 12$; addicts: 0.74 ± 0.04, $n = 24$; Fig. 2a), further indicating that the content of the active form of CREB is not altered in chronic opiate addicts. As would be expected, however, the immunodensities of PKA showed a positive and significant correlation with those of pCREB ($r = 0.72$, $n = 24$, $p < 0.0001$), but not of tCREB ($r = 0.18$, $n = 24$, $p > 0.05$), in the same brains of opiate addicts (Figs 2b and c), which indicated the existence of a close relation between the activation of PKA (Cα catalytic subunit) and the phosphorylation of CREB in the human brain.

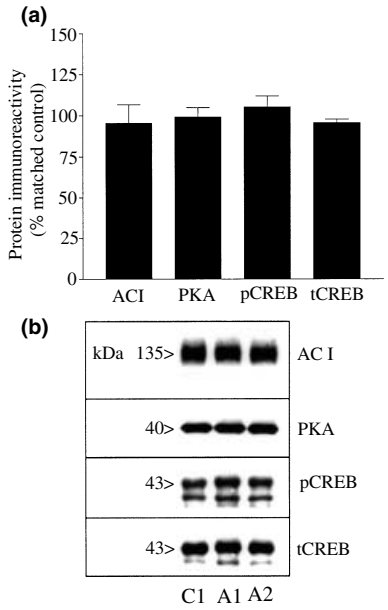

Fig. 1 (a) Immunodensities of adenylyl cyclase type I (AC-I), protein kinase A Cα (PKA), total cAMP response element-binding protein (tCREB) and phosphorylated CREB (pCREB) in the pre-frontal cortex (Brodmann area 9) of chronic opiate addicts as mean ± SEM (bars) percentages ($n = 24$) of the contents in matched controls. (b) Representative immunoblots for the various cAMP signalling components in the pre-frontal cortex of two opiate addicts (A1, heroin overdose: 44-year-old man; PMD: 19 h; A2, methadone overdose: 42-year-old man; PMD: 28 h) and the matched control subject (C1, 44-year-old man; PMD: 19 h). The amount of total protein loaded per gel well was 40 μg in all cases. The apparent molecular masses of these proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. Note that in A1, but not in A2, the immunoreactivities of PKA and pCREB were increased (45%).

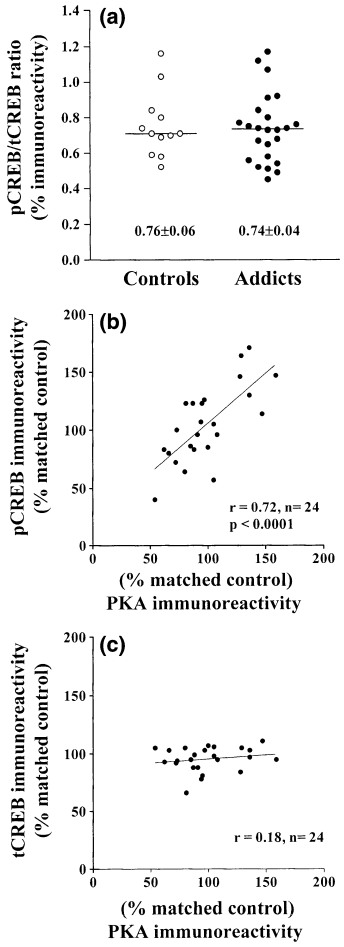

Fig. 2 (a) Ratios of phosphorylated CREB to total CREB (pCREB/tCREB) immunoreactivity in the pre-frontal cortex of control subjetcs and opiate addicts. Each circle is the ratio for a given subject. The line represents the mean value of each group (controls, $n = 12$; addicts, $n = 24$). (b, c) Scatterplots depicting the significant correlation between the immunodensities of protein kinase A Cα (PKA) and pCREB (b) or the lack of correlation between PKA and tCREB in the pre-frontal cortex (same samples) of opiate addicts. Each circle represents a different subject ($n = 24$). The solid lines are the regression of the correlations. Both axes are expressed as percentages of immunoreactivity in matched control subjects.

Similar negative results for the contents of AC/PKA/CREB were also obtained in the pre-frontal cortex of drug-free opiate addicts (no drugs other than opiates detected, $n = 16$; data not shown). Variables such as heroin or methadone overdose, plasma concentrations of opiates and presence of ethanol or other drugs (e.g. cocaine) in blood samples did not alter significantly the components of cAMP signalling pathway in brains of opiate addicts (data not shown).

Long-term regulation of Ras/Raf/MEK/ERK pathway in brains of opiate addicts

In initial studies, the influence of PMD and age on MAPK signalling components (Ras/Raf/MEK/ERK) was assessed to determine the pattern of degradation of these proteins and the effect of ageing. In brains (pre-frontal cortex) of control subjects, the PMD (range 2–62 h) and the age of subjects at death (range 18–50 years) did not alter the immunodensities of Ras, c-Raf-1 and total contents of MEK and ERK (data not shown; see Fig. 3 for tERK1/2). In the same brains, however, the immunodensities of phosphorylated MEK (p^+Ser 217/221) and ERK1/2 (p^+Tyr 204), the active forms of these enzymes, showed a marked decline with PMDs greater than 30 h (Fig. 3 for pERK1/2). Therefore, the components of the MAPK pathway were quantitated in a subset of opiate addicts (PMD: 21 ± 2 h; age: 32 ± 3 years, $n = 12$) that were matched with the appropriate control subjects (PMD: 23 ± 3 h; age: 34 ± 4 years, $n = 12$).

In the pre-frontal cortex of opiate addicts ($n = 12$), the immunodensities of Ras (96 ± 5%), c-Raf-1 (84 ± 6%, $p < 0.05$), tMEK (88 ± 2%, $p < 0.05$), pMEK (15 ± 7%, $p < 0.001$), tERK (100 ± 1%), and their active forms pERK1 (19 ± 6%, $p < 0.001$) and pERK2 (20 ± 7%, $p < 0.001$) were, in general, decreased compared with those in sex-, age- and PMD-matched controls (100%; Fig. 4). Notably, marked downregulations in the contents of active phosphorylated MEK (p^+Ser217/221; 85%) and ERK (p^+Tyr204; pERK 1 : 81%; pERK 2 : 80%) were observed in brains of chronic opiate addicts (Fig. 4). Similar results were obtained when the activities of ERK1/2 were quantitated with a different antibody that recognizes dually phosphorylated ERK (p^+Thr202/p^+Tyr204) by MEK (New England Biolabs; pERK1 : 77 ± 7% decrease, $p < 0.001$; pERK2 : 75 ± 8% decrease, $p < 0.001$, $n = 6$). It is of interest to note that the immunodensity of an unidentified peptide of ~85 kDa, detected with the antiphospho-ERK antibody (Calbiochem)

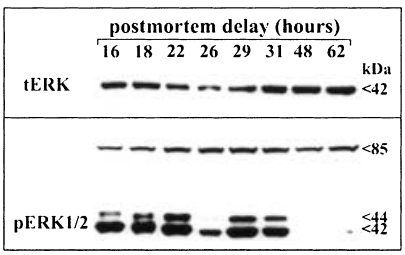

Fig. 3 Immunoblots for the effect of post-mortem delay (PMD: 16–62 h) on the immunoreactivity of total extracellular signal-regulated kinases (tERK) and phosphorylated ERK (pERK1/2, or MAPK: p44/42 MAP kinases) in the human pre-frontal cortex (eight healthy subjects). The amount of total protein loaded per gel well was 40 μg for all subjects which were run in the same gel. The apparent molecular masses of various forms of ERK were determined by calibrating the blots with prestained molecular weight markers as shown on the right hand side. Note that PMDs greater than 31 h were associated with marked reductions (> 90%) in the content of pERK1/2 but not tERK. The antiphospho-ERK antibody (Calbiochem) used also immunodetected an unidentified 85 kDa peptide which was not affected by the length of PMD (internal control).

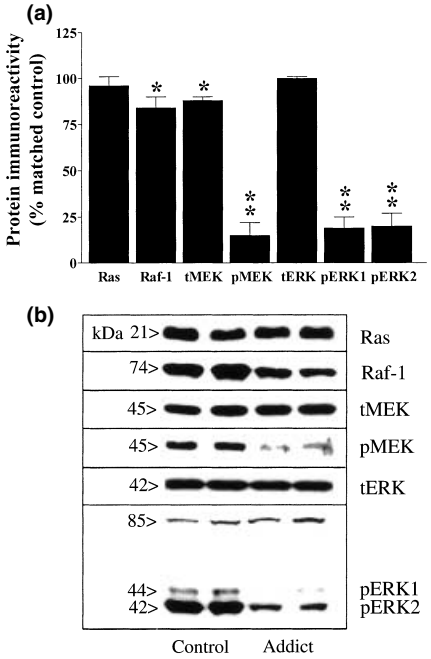

Fig. 4 (a) Immunodensities of Ras, Raf-1 (MAPKKK), total and phosphorylated MEK (tMEK, pMEK, or MAPKK) and total and phosphorylated extracelullar signal-regulated kinases (tERK, pERK1/2, or MAPK: p44/42 MAP kinases) in the pre-frontal cortex (Brodmann area 9) of chronic opiate addicts as mean ± SEM (bars) percentages ($n = 12$) of the contents in matched controls. (b) Representative immunoblots for the various MAP kinase signalling components in the pre-frontal cortex of one opiate addicts (26-year-old man; PMD: 20 h) and the matched control subject (27-year-old man; PMD: 22 h). The amount of total protein loaded per gel well was 40 μg (in duplicate) in all cases. The apparent molecular masses of these proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. Note that in the opiate addict the immunoreactivities of Raf-1, pMEK and pERK1/2 were decreased (40–82%). The antiphospho-ERK antibody (Calbiochem) used also immunodetected an unidentified 85 kDa peptide which was not reduced in the opiate addict (internal control).

and used as an internal control, was not altered in the same brains of opiate addicts (92 ± 9%, $n = 12$) compared with controls (100%; Fig. 4b). The basal activity of pERK2 (immunoreactivity) was much higher than that of pERK1, indicating that ERK2 is the predominant isoform in the prefrontal cortex of the human brain (Fig. 4b).

The marked downregulation of pMEK and pERK1/2 was also observed in brains of drug-free opiate addicts (no drugs other than opiates detected, $n = 8$; data not shown). Variables such as heroin or methadone overdose, plasma concentrations of opiates or other drugs (e.g. cocaine) in blood samples did not alter significantly the opiate-induced decreases in MAPK components in brains of opiate addicts (data not shown). The content of pERK2 in ethanol-free opiate addicts (18 ± 9%, $n = 6$) was not different from that quantitated in subjects with ethanol in blood samples (20 ± 9%, $n = 6$). Similarly, intermittent chronic ethanol exposure in rats has been shown not to alter the content of pERK2 in the frontal cortex (Sanna *et al*. 2002). In brains of opiate addicts, the immunodensity of α-internexin (a neuronal marker used as a negative control) was found unaltered (99 ± 6%, $n = 24$) compared with that in matched control subjects (100%; Ferrer-Alcón *et al*. 2004), which discounted possible effects of unspecific variables or non-specific effects of other drugs on AC and MAP kinase signalling pathways.

In vivo effects of morphine on ERK activity in the rat brain

Many G protein-coupled receptors, including the μ-opioid receptor, can effectively stimulate ERK (Van Biesen *et al*. 1996). Therefore, the acute and chronic effects of morphine on ERK activity were also investigated in the rat brain to assess whether the observed downregulation of pERK1/2 in brains of opiate addicts is a long-term effect of the abused opiates or the final acute effect of the deadly opiate overdose.

The acute treatment of rats with morphine (30 mg/kg, i.p. for 2 h), compared with saline administration, increased the immunodensity of pERK1 (88 ± 30%, $n = 5$, $p < 0.05$) and pERK2 (102 ± 23%, $n = 5$, $p < 0.01$), but not that of tERK, in the cerebral cortex (Fig. 5). In contrast, chronic treatment with morphine (10–100 mg/kg for 5 days) was associated with decreases in the contents of pERK1 (68 ± 5%, $n = 6$, $p < 0.05$), pERK2 (59 ± 5%, $n = 6$, $p < 0.05$) and tERK (19 ± 5%, $n = 6$, $p < 0.05$) in the rat brain (Fig. 5). Acute and chronic morphine did not modify significantly the immunodensity of a 85 kDa unidentified peptide (see above) in the rat brain (Fig. 5b).

In vitro effects of morphine on the induction of ERK activity in SH-SY5Y cells: blockade by the MEK inhibitor PD98059

To assess the mechanism by which morphine stimulates ERK, various *in vitro* experiments were performed in human neuroblastoma SH-SY5Y cells which are enriched in

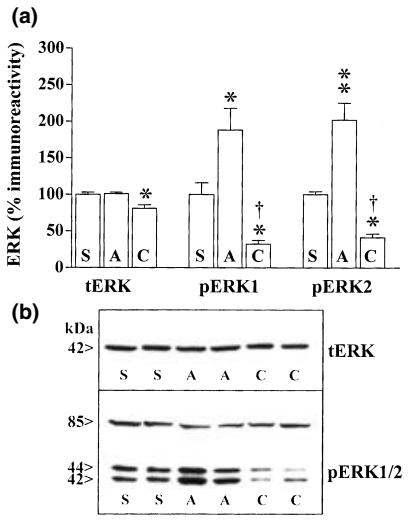

Fig. 5 Effects of acute (A, 30 mg/kg, i.p., 2 h) and chronic morphine (C, 10–100 mg/kg for 5 days) treatments on the immunodensities of total and phosphorylated extracelullar signal-regulated kinases (tERK, pERK1/2, or MAPK: p44/42 MAP kinases) in rat brain (cerebral cortex). (a) Columns are means ± SEM of 4–6 experiments per group with an animal per experiment, and expressed as percentage of saline-treated rats (S). *$p < 0.05$, **$p < 0.01$ when compared with the corresponding saline group; †$p < 0.001$ when compared with the corresponding acute effect of morphine (ANOVA followed by Bonferroni's test). (b) Representative immunoblots for the effect of acute (A) and chronic (C) morphine on tERK and pERK1/2 in the cerebral cortex. The apparent molecular masses of the various forms of ERK were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. The antiphospho-ERK antibody (Calbiochem) used also immunodetected an unidentified 85 kDa peptide which was not modulated by morphine treatment (internal control).

μ-opioid receptors. In SH-SY5Y cells, morphine rapidly stimulated the phosphorylation of ERK1/2 in a concentration- dependent (1 nM-100 μM; data not shown) and time-dependent (1–60 min) manner (1.5–3.5-fold induction, $p < 0.05$), without altering the immunodensity of total ERK (Fig. 6a). With 1 μM morphine a significant increase of pERK1/2 was detected within 3 min, reached a peak at 30 min and then declined by 60 min (Fig. 6a).

Incubation (60 min) of SH-SY5Y cells with 100 μM PD98059 (a specific MEK inhibitor) almost completely abolished the expression of pERK1/2 (84–93%; Fig. 6b), which indicated that the basal activation of ERK is dependent on MEK activity (the only way in which ERK is activated by MEK-induced phosphorylation). Moreover, pre-incubation of SH-SY5Y cells with 50 μM PD98059 (60 min), a concentration that apparently did not alter the immunodensity of pERK1/2, markedly attenuated morphine (1 μM, 5 min)-induced activation of ERK1/2 (35–60%; Fig. 6b). Cell pre-incubation with 100 μM PD98059 completely blocked the stimulatory effect of morphine on pERK1/2

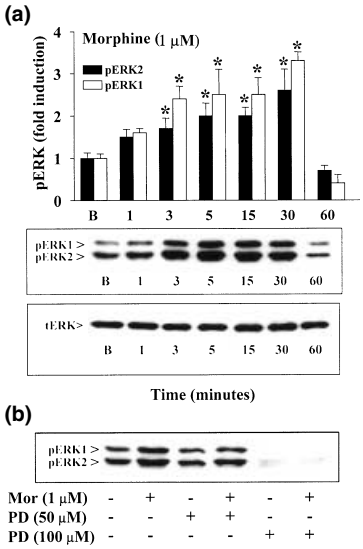

Fig. 6 (a) Time course (1–60 min) and the effect of morphine (1 μM) on the immunodensities of phosphorylated and total extracelullar signal-regulated kinases (pERK1/2, tERK or MAPK: p44/42 MAP kinases) in human neuroblastoma SH-SY5Y cells. *at least $p < 0.05$ when compared with basal values (B; Student's *t*-test). (b) Effects of the MEK (MAPKK) inhibitor PD98059 (PD, 50 and 100 μM for 60 min) on the basal activity of pERK1/2 and morphine (1 μM for 5 min)-induced activation of pERK1/2 in SH-SY5Y cells.

(88–96%; Fig. 6b), ruling out the possibility that the activation of ERK1/2 signalling by morphine might be caused by a MEK-independent pathway.

Discussion

The main results of this study demonstrate that chronic opiate addiction in humans is associated with an apparent normal functioning of several cAMP-signalling components (AC-I/PKA Cα/total and phosphorylated CREB) and a marked downregulation of key active enzyme forms of MAPK pathway (phosphorylated MEK and ERK) in the pre-frontal cortex, a brain region related to the mesolimbic dopaminergic reward system. This abnormality in MAPK signalling (decreased activation of ERK1/2) might be relevant in modulating long-lasting forms of synaptic plasticity in opiate addiction (Nestler 2001, 2002; Mazzucchelli *et al*. 2002).

Studies examining the effects of opiate drugs on cAMP signalling have produced mixed results (Liu and Anand 2001; Williams *et al*. 2001) but, in general, chronic morphine treatment has been associated with a compensatory upregulation of this system in specific regions of the rat brain. Thus, Gαi/o proteins (Lane-Ladd *et al*. 1997), AC activity (Duman *et al*. 1988; Lane-Ladd *et al*. 1997), PKA Cα (Lane-Ladd *et al*. 1997), CREB protein and the extent of CREB phosphorylation (Guitart *et al*. 1992; Widnell *et al*. 1994) were shown to be increased in the rat locus coeruleus after chronic morphine and/or opiate withdrawal. A modest enhancement of some of these components or even downregulation of CREB were also reported in other brain regions, such as the limbic system and corpus striatum of rats (see Widnell *et al*. 1996). In contrast, opiate addiction in wild-type mice, similarly to the human brain (current results), did not result in a sustained activation of cAMP signalling in the brain (Gαi1/2 proteins, PKA, pCREB; García-Sevilla *et al*. 2004). Moreover, the increases in AC activity and cAMP content induced by chronic morphine treatment were very modest, even after opiate withdrawal, in the mouse cerebral cortex (about 10%; Maldonado *et al*. 1996; Mamiya *et al*. 2001). Similarly, chronic morphine also failed to increase PKA Cα in the locus coeruleus of mice (Akbarian *et al*. 2002). It is of interest to note that the highest densities of CREB (and its active form by phosphorylation at the activator-site residue Ser133) have been quantitated in the corpus striatum and cerebral cortex (Blom *et al*. 2002). Earlier pharmacological data indicated that acute morphine treatment was associated with a decrease in the content of pCREB in the rat locus coeruleus (Guitart *et al*. 1992), although this study did not measure directly CREB phosphorylation on Ser133. More recently, morphine and the δ-agonist [D-Pen^{2,5}]-enkephalin have been shown to increase markedly the phosphorylation of CREB on Ser133 in NG108-15 cells (Bilecki *et al*. 2000). In primary cultures of rat striatum, however, acute and chronic morphine did not alter CREB (total protein content and protein phosphorylation), and naloxone-precipitated opiate withdrawal modestly enhanced the activity (phosphorylation state) of this transcription factor (Chartoff *et al*. 2003). Therefore, opiate treatments and/or opiate withdrawal might be associated with the activation of CREB in some neural cells and in rat brain tissue. In morphine-dependent mice, however, pCREB was not upregulated in the cerebral cortex (García-Sevilla *et al*. 2004) and a very modest induction of the nuclear cAMP response elements (CREs) was observed in various brain regions (including the cortex), whereas a robust CRE upregulation was induced by opiate withdrawal (Shaw-Lutchman *et al*. 2002).

Chronic opiate addiction in humans does not appear to be associated with an upregulation of several key components of cAMP signalling (AC-I/PKA Cα/pCREB) in the pre-frontal cortex (see Results for other experimental details). As mentioned, similar negative results were obtained in the mouse brain (García-Sevilla *et al*. 2004). This apparent lack of upregulation of cAMP pathway after long-term opiate exposure might be related to species sensitivity (human and mouse vs. rat) and/or differences in brain regions (greater cellular heterogeneity in the cortex), but MAPK signalling (current results) and other relevant markers of opiate addiction (Ferrer-Alcón *et al*. 2003, 2004) were clearly modulated in the pre-frontal cortex of the same opiate addicts. A recent study reported that AC-I is decreased in

temporal cortex, but not in nucleus accumbens, from brains ($n = 8$) of heroin addicts (Shichinohe *et al*. 2001), suggesting that this enzyme may be differentially regulated in different brain areas in opiate addiction. Alternatively, the apparent normalization of cAMP signalling in brains of chronic opiate addicts (current results) may suggest that this system (perhaps after an initial upregulation) has achieved a new steady-state in which the opioid receptor signalling through this pathway is normosensitive [e.g. in the pre-frontal cortex of opiate addicts a normal stimulation of [^{35}S]-GTPγS binding by the μ-agonist DAMGO (Meana *et al*. 2000) and an increased density of Gαi/o proteins (Escriba *et al*. 1994) have been observed].

In marked contrast to the cAMP pathway, the current results also demonstrate that opiate addiction in humans is associated with decreased immunodensities of the main components of MAPK cascade (c-Raf-1/pMEK/pERK) in the brain (pre-frontal cortex). The pronounced downregulation of pERK1/2 in brains of opiate addicts (75–80%) was also observed in brains of chronic morphine-treated rats (60–70%). Moreover, these opiate effects were dependent on MEK activity, because a specific MEK inhibitor (PD98059) blocked the stimulatory effect of morphine on pERK1/2 in SH-SY5Y cells. These findings are in line with previous observations indicating that MAPKs are involved in μ/δ-opioid receptor signalling in the brain (Ortiz *et al*. 1995; Berhow *et al*. 1996; Gutstein *et al*. 1997; Schulz and Höllt 1998; Eitan *et al*. 2003; Lesscher *et al*. 2003), the acute activation of pERK1/2 being mediated by Gαo inhibitory proteins (Zhang *et al*. 2003). In a recent immunohistochemical study (Eitan *et al*. 2003), acute morphine treatment in mice (10–100 mg/kg) resulted in increases in pERK1/2 in the locus coeruleus and projecting areas in the neocortex (e.g. anterior cingulate cortex), and in decreases of these pMAPKs in the nucleus accumbens and central amygdala, demonstrating brain region-specific mechanisms regulating the activation of these enzymes after the acute opiate. Chronic morphine treatment (10–40 mg/kg for 6 days) resulted in tolerance to opiate-induced pERK1/2 modulation (Eitan *et al*. 2003). Because opiate addicts died of an opiate overdose (heroin or methadone), the observed downregulation of pMEK/pERK1/2 could be related, in part, to the deadly overdose which would mimic an acute effect of the opiate. However, the acute treatment of rats with morphine was associated with upregulation of pERK1/2 (twofold), with no change in the content of tERK, in the cerebral cortex. It therefore appears that, in spite of the possible stimulating effect of the final deadly opiate overdose, the potently repressed activation of pMEK and pERK1/2 in brains of opiate addicts (pre-frontal cortex) is the net result of a chronic opiate effect (see Schulz and Höllt 1998).

The ERK cascade by co-ordinating responses to cell surface receptors and neuronal second messengers appears to play a key role in various long-lasting forms of synaptic plasticity in the brain (Derkinderen *et al*. 1999; Grewal *et al*. 1999; Sweatt 2001; Adams and Sweatt 2002; Mazzucchelli *et al*. 2002; Pouysségur and Lenormand 2003). Therefore, the observed downregulation of pMEK/pERK1/2 could be of relevance in modulating the development of the neural plasticity associated with opiate addiction in humans. In this context, the effects of ERK1/2 on two downstream targets are of special interest: the induction of phosphorylation of neurofilament (NF-H, NF-M) proteins (Veeranna *et al*. 1998) and the activation of p35, the neuron specific activator of cyclin-dependent kinase-5 (cdk5; Harada *et al*. 2001), which in turn also phosphorylates NF proteins (Pant *et al*. 1997; Grant *et al*. 2001). Moreover, cdk5 and ERK1/2 were shown to form multimeric complexes with NF proteins in the brain (Veeranna *et al*. 2000), as they assemble the functional architecture of the mature neurone. It is of special relevance that in brains of opiate addicts the content of phosphorylated NF-H was increased, as well as the ratio of phosphorylated to non-phosphorylated NF-H forms (Ferrer-Alcón *et al*. 2000, 2003). Moreover, a marked downregulation of the cdk5/p35 complex and a positive correlation between p35 and phosphorylated NF-H were also observed in the pre-frontal cortex of opiate addicts (Ferrer-Alcón *et al*. 2003). Therefore, chronic opiate addiction in humans is associated with sustained downregulations of ERK1/2 activity (current results), cdk5/p35 complex (Ferrer-Alcón *et al*. 2003) and non-phosphorylated NF (NF-H, NF-L) proteins (Ferrer-Alcón *et al*. 2000), as well as with aberrant hyperphosphorylation of NF-H (Ferrer-Alcón *et al*. 2000, 2003) in the brain. These abnormalities might have important consequences in the long term development of various forms of neural plasticity associated with opiate addiction in humans (Nestler 2001, 2002). In this context, mice disrupted in the ERK1 gene (resulting in enhanced ERK2 signalling) were more actively displaying facilitated striatal-mediated learning and memory, and showed a striking increase in sensitivity to the rewarding properties of morphine (Mazzucchelli *et al*. 2002).

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