Neurofilament proteins and cAMP pathway in brains of \( \mu \)-, \( \delta \)- or \( \kappa \)-opioid receptor gene knock-out mice: effects of chronic morphine administration

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Abstract

Opiate addiction is associated with abnormalities of neurofilament (NF) proteins and upregulation of cAMP signaling in the brain, which may modulate neuronal plasticity. This study investigated, using gene-targeted mice lacking \( \mu \)-, \( \delta \)- or \( \kappa \)-opioid receptors, the role of these receptors in modulating the basal activity and the chronic effects of morphine on both intracellular targets. In WT mice, chronic treatment (5 days) with morphine (20–100 mg/kg) resulted in decreases in the immunodensity of neurofilament (NF)-L in the cerebral cortex (14–23%). In contrast, chronic morphine did not decrease NF-L in cortices of \( \mu \)-, \( \delta \)-, and \( \kappa \)-KO mice, suggesting the involvement of the three types of opioid receptors in this effect of morphine. Also, the marked increase in phosphorylated NF-H induced by chronic morphine in WT mice (two-fold) was abolished in \( \mu \)-KO mice. In cortex and/or striatum of \( \mu \)-, \( \delta \)- and \( \kappa \)-KO mice, the basal immunodensities of G\(_{i1/2}\) proteins, the catalytic isoform (C\(_{a}\)) of protein kinase A (PKA) and the total content of cAMP response element-binding protein (CREB, the nuclear target of PKA) were not different from those of WT mice. In contrast, phosphorylated CREB (the active form of this transcription factor) was reduced in cortex and/or striatum (23–26%) of \( \mu \)- and \( \delta \)-KO mice, but not in \( \kappa \)-KO animals. These results suggest that the endogenous opioid tone acting on \( \mu \)-/\( \delta \)-receptors tonically stimulate CREB activation in the brain. In cortex and/or striatum of WT mice, chronic morphine did not induce upregulation of the main components of the cAMP signaling pathway. In contrast, chronic morphine treatment in \( \mu \)-KO mice, but not in \( \delta \)- or \( \kappa \)-KO, resulted in a paradoxical upregulation of G\(_{i1/2}\) (12–19%), PKA (19–21%) and phosphorylated CREB (21–73%), but not total CREB, in cortex and/or striatum. The induction of heterologous receptor adaptations in \( \mu \)-KO mice may explain this paradoxical effect of morphine.

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1. Introduction

In recent years, the neurobiology of opiate addiction has focused on the molecular and cellular mechanisms associated with the regulation of synaptic structures (e.g. cytoskeletal proteins) and specific signaling systems (e.g. cAMP pathway) leading to neuronal plasticity in specific regions of the brain, including the neocortex (for review, see Simonato, 1996; Nestler
and Aghajanian, 1997; Nestler, 2001; Williams et al., 2001). The neurofilament (NF) proteins (triplet NF-L, NF-M, NF-H) are the major elements of the neuronal cytoskeleton. The triplet proteins are co-assembled in vivo and NF-L appears to be the core polypeptide essential for filament assembly. NF phosphorylation (mainly of NF-H) appears to be a major mechanism for NF assembly/disassembly, a crucial process for the integrity of the neuronal cytoskeleton. NF proteins are associated with various functions such as axon caliber and axonal transport, axonal regeneration and plasticity, and the determination of neuronal morphology (for review, see Julien and Mushynski, 1998). Previous studies have demonstrated that chronic morphine treatment in rats is associated with marked reductions in the immunodensity of NF proteins in brain regions relevant to opioid addiction (Beitner-Johnson et al., 1992; Boronat et al., 1998, 2001; Jaquet et al., 2001). The content of nonphosphorylated NF proteins was also shown to be decreased in postmortem brains of chronic heroin abusers (García-Sevilla et al., 1997; Ferrer-Alcón et al., 2000, 2003). Moreover, aberrant hyperphosphorylation of NF-H and NF-M was demonstrated in brains of opioid addicts (Ferrer-Alcón et al., 2000, 2003) and in brains of morphine-dependent rats (Jaquet et al., 2001). These abnormalities of NF proteins may play a significant role in the molecular mechanisms of opiate addiction which lead to neuronal plasticity in the brain (Ferrer-Alcón et al., 2000).

On the other hand, the upregulation of the cAMP signaling pathway in the brain is the best established molecular adaptation associated with chronic opiate agonist (morphine) exposure, and this compensatory response develops gradually to oppose the acute morphine inhibition of the pathway. The upregulation of cAMP signaling induced by chronic morphine in rats involves increased concentrations of Gi/o proteins, adenylyl cyclase, cAMP-dependent protein kinase A (PKA), and its target cAMP response element-binding protein (CREB) (for review, see Nestler and Aghajanian, 1997). The activity of the transcription factor CREB is regulated by phosphorylation and plays a relevant role in opiate addiction (for review, see Blendy and Maldonado, 1998). In this way, chronic morphine can stimulate the expression of numerous genes through PKA-mediated phosphorylation of CREB at Ser 133. Modulation of transcription factors such as CREB is clearly one potential mechanism for persisted opiate-induced plasticity in the brain (Nestler and Aghajanian, 1997).

Mice lacking opioid receptors (gene knock-out technology) represent useful models to evaluate the biological effects and molecular mechanisms of action of opiate drugs in intact animals and in different experimental conditions (Kieffer, 1999; Kieffer and Gavériaux-Ruff, 2002). In particular, the absence of an opioid receptor type in knock-out mice has permitted to address the issue of which opioid receptor mediates the specific functions of opiate agonists. In this context, the aim of the present study was to investigate using gene-targeted mice lacking functional μ-, δ- or κ-opioid receptors the role of these receptors in modulating the basal activity and the chronic effects of morphine on key NF proteins (NF-L and phosphorylated NF-H) and components of the cAMP signaling pathway (Gζi proteins, PKA and CREB) in the brain. The cerebral cortex was mainly selected for examination because it expresses the highest content of CREB (Blom et al., 2002) and includes important areas functionally related to the mesolimbic dopaminergic reward system which are relevant to opiate addiction (Simonato, 1996; Williams et al., 2001).

2. Methods

2.1. Animals

Homzygous knock-out (KO) mice without μ (MOP), δ (DOP) or κ (KOP)-opioid receptor and their respective wild-type (WT) litters were used in this study. The μ-KO mice were generated by disruption of exon two in the receptor gene (Matthes et al., 1996). The δ-KO (Filliol et al., 2000) and κ-KO (Simonin et al., 1998) mice were created by deletion of the first coding exon in the corresponding receptor gene. Homzygous animals for each mutation were between 16 to 20 weeks old at the beginning of the experiments, and were housed between five per cage in a temperature-controlled room (21 °C) with a 12 h light:dark cycle (light on between 8:00 and 20:00 h). Food and water were available ad libitum. Mice were acclimated to handling for 1 week before the experiments.

2.2. Morphine treatment of mice and brain samples

Opioid dependence was induced by repeated morphine administration (Matthes et al., 1996). Morphine was dissolved in saline (0.9%) and injected intraper-
between the opiate doses (see also Ruiz et al., 1996). Not associated with spontaneous abstinence symptoms Three hours after the last morphine administration, the composition: group I (5 WT saline-treated, 5 WT morphine-treated), group II (5 WT saline-treated, 5 WT morphine-treated and 5 μ-KO saline-treated, 5 μ-KO morphine-treated), group II (5 WT saline-treated, 5 μ-KO saline-treated, 5 WT morphine-treated and 5 μ-KO morphine-treated), and group III (5 WT saline-treated, 5 μ-KO saline-treated, 5 WT morphine-treated and 5 μ-KO morphine-treated). Each set of experiments was performed 2–3 times which allowed the quantification of 10–15 samples for each subgroup of mice (see section 2.4 below). These experiments in mice were performed according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals) and approved by the local ethical committees.

2.3. Gel electrophoresis and immunoblot assays

The mouse brain samples (crude total homogenate) were prepared as described previously for the human brain (Ferrer-Alcón et al., 2000) with minor modifications. For measurements of the target proteins, 150–200 mg of cerebral cortex or 30–60 mg of striatum was homogenized (1:7.5, wt/vol) in 50 mM Tris-HCl buffer, pH 6.8, containing 1 mM EDTA, 2% 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) (Ferrer-Alcón et al., 2000), and the mixture was sonicated for 10 s. Aliquots of the previous mixture (total homogenate containing the cytoskeleton) were combined with solubilization 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 about 3 μg/μl. This mixture was boiled (denaturated) at 95 °C for 4 min and after cooling the samples were stored at −20 °C until use. Proteins were determined by the biuret reaction using bicinechonic 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 mouse brain sample was subjected to SDS-PAGE (at 100 V for 3 h) on 15-well (6 × 8 cm² gels, 1.0 mm thickness) 12% polyacrylamide minigels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were electrophoretically transferred (110 V and 4 °C for 2.5 h) to nitrocellulose membranes (western blotting) that were incubated (blocking solution) in phosphate-buffered saline (142 mM NaCl, 3.6 mM KCl, 8.8 mM Na₂HPO₄, and 1.69 mM KH₂PO₄) containing 5% nonfat dry milk, 0.2% Tween 20 and 0.5% bovine serum albumin (BSA) (Harlow and Lane, 1999) or 0.5% Tween and 3% BSA (for PKA, tCREB and pCREB; see Odagaki et al., 2001) for 1 h at room temperature with gentle rocking. Then, the membranes were incubated overnight at 4 °C in blocking solution containing the appropriate primary antibody. For the different target proteins, the following monoclonal or polyclonal antibodies were used: NF-L (anti-NF 68 kDa, Product N-5139, clone NR-4, Lot 127H4840, 1:500 dilution, Sigma BioSciences, St. Louis, MO, USA); phosphorylated NF-H (anti-NF 200 kDa, SMI-31, batch 13, 1:5000 dilution, Sternberger Monoclonals, Baltimore, MD, USA); GFAP (anti-GFAP, Product Z-0334 Lot 096, 1:5000 dilution, DAKO A/S, Glostrup, Denmark); Gizi (anti-Gzi/1,2, Product NEI-801, AS/7, 1:5000 dilution, NEN Life Science Products, Boston, MA, USA); PKA (anti-PKA Cα catalytic subunit, Product sc-903, Lot E109, 1:100,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA); CREB (anti-total CREB, amino acids 5–21 of human CREB, Product 06-863, Lot 17795, 1:5000 dilution, Upstate Biotechnology, New York, USA); phosphorylated CREB (anti-phospho-CREB, amino acids 123–136 of rat CREB, Product 06-519, Lot 17926, 1:5000 dilution, Upstate Biotechnology, New York, USA). The secondary antibody, horseradish peroxidase-linked sheep anti-mouse IgG or donkey anti-rabbit IgG, was incubated at 1:5000 dilution in blocking solution at room temperature for 2 h. Immunoreactivity was detected with an enhanced chemiluminescence (ECL) Western blot detection system (Amersham, Buckinghamshire, UK), followed by exposure to Amersham Hyperfilm ECL for 1–10 min (autoradiograms).

The antibodies used labelled bands with molecular mass of 68 kDa (NF-L protein), 200 kDa (phosphorylated NF-H protein), 49 kDa (GFAP), 40 kDa...
(Gαi1/2 proteins), 40 kDa (PKA Cz subunit) and 43 kDa (tCREB and phosphorylated CREB) in good agreement with previous studies in rat and human brains (Escribá et al., 1994; García-Sevilla et al., 1997; Boronat et al., 1998, 2001; Ferrer-Alcón et al., 2000; Jaquet et al., 2001; Odagaki et al., 2001). The 40 kDa PKA immunoreactivity was always detected as a doublet, with a greater optical density in the lower band (used for quantification) of a slightly smaller molecular mass (see Fig. 4). As this antibody is known to be partially cross-reactive with Cβ and Cγ catalytic subunits, the upper band may represent PKA C subunits other than Cz (see Odagaki et al., 2001). Immunodetection of total CREB was performed by stripping and reprobing the same nitrocellulose membranes that had been used for the immunodetection of phosphorylated CREB. Anti-phospho-CREB antibody detects CREB phosphorylation at the activator-site residue Ser 133.

2.4. Quantification of target protein contents

The autoradiograms were quantitated by densitometric scanning (GS-700 Imaging Densitometer, resolution: 42 μm, Bio-Rad, Hercules, CA, USA), by measuring the IOD units of the immunoreactive bands. Prior to analyses, the linearity of protein concentration (total homogenate) for Western blotting was ascertained by resolution of selected concentrations of protein (i.e., total protein loaded versus IOD units, consisting of 4–6 points of different protein content, usually 10–100 μg, resulting in good linear relations). In a given experiment and for a direct comparison, two groups of 5 mice were run together in the same gel to assess for differences in opioid receptor genotypes and/or in the chronic effects of morphine (i.e., WT saline versus KO saline; WT saline versus WT morphine; KO saline versus KO morphine) and 40 μg of protein were loaded in each lane for the different blots. Then the experiment was quantitated and repeated 1–2 times to confirm the results. This procedure allowed the quantification of 10–15 samples for each subgroup of mice and/or protein. Finally, percent changes in immunoreactivity with respect to control saline (WT or KO) samples (100%) were calculated for each experimental sample in the various gels and the mean value was used as a final estimate. The contents of phosphorylated NF-H in brains of WT and μ-KO mice were assessed and compared in the same gel with a standard curve built in each gel using samples from saline-treated mice (see Boronat et al., 2001), and the experiments were repeated twice and quantitated as above. In the Figures, representative immunoblots are shown for each protein comparison together with the mean data values of the different gels. To strengthen the data of chronic morphine on the various target proteins in WT mice, the results of the three independent series of experiments (i.e., saline WT, n = 15 mice vs. morphine WT, n = 15 mice) were combined and the mean results also reported.

2.5. Statistical analysis

All series of data were analyzed with the program GraphPad Prism® version 2.0. Results are expressed as mean ± SEM values. Student’s two-tailed t test or one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison test, was used for the statistical evaluations. The level of significance was chosen as P = 0.05.

2.6. Drugs and reagents

Morphine hydrochloride was provided by the Ministerio de Sanidad y Consumo (Spain). Acrylamide (Protogel) was from BDH Brunschwig (Dorset, UK). Pefabloc was from Boehringer (Rotkreuz, Switzerland). Other materials such as the secondary antibodies, ECL reagents and autoradiography films were purchased from Amersham International (UK) or Santa Cruz Biotechnology (USA). All other chemicals were from Sigma Chemie or Fluka Chemie (Buchs, Switzerland).

3. Results

3.1. Immunodensities of NF proteins and GFAP in brains of wild-type (WT) and opioid receptor knock-out (KO) mice: Effect of chronic treatment with morphine

In the cerebral cortex of μ, δ, and κ-opioid receptor KO mice, the basal immunodensities of NF-L (the core peptide for neurofilament assembly) did not significantly differ from those in brains of their respective WT littermates (Fig. 1(A)–(C)) (In A note that NF-L in KO 5 was abnormally reduced and not used for calculations). In the three groups of WT mice, chronic treatment (5 days) with morphine (increasing doses from 20–100 mg/kg), compared with saline solution administration, resulted in decreases in the immunodensity of NF-L in the cerebral cortex (23, 14 and 15%, P < 0.05) (Fig. 1(A)–(C)). The combined results of the three morphine treatments in WT mice clearly indicated that the opiate decreased the immunodensity of NF-L in the mouse brain (17 ± 3%, n = 15, t = 5.69, P < 0.0001) (see Boronat et al., 1998; Jaquet et al., 2001). In contrast, similar chronic morphine treatments did not decrease the immunodensity of NF-L in the cerebral cortex of μ-, δ-, or κ-opioid receptor KO mice (Fig. 1(A)–(C)).
In the cerebral cortex of WT and μ-opioid receptor KO mice, the basal immunodensities of phosphorylated NF-H (a major mechanism for NF assembly and disassembly) were also similar (Fig. 2). In WT mice, chronic morphine treatment induced a marked increase in the immunodensity of phosphorylated NF-H proteins in the cerebral cortex (105%, \( P < 0.01 \)) (Fig. 2). In contrast, chronic morphine did not alter significantly the content of phosphorylated NF-H in this brain region of μ-opioid receptor KO mice (19% increase, \( P > 0.05 \)) (Fig. 2).

In the same WT and μ-opioid receptor KO mice, the basal immunodensity of GFAP (a specific glial cytoskeletal protein, used as a negative control) was not significantly altered by opioid receptor genotype or chronic treatment with morphine in the cerebral cortex (Fig. 3) and corpus striatum (data not shown).

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**Fig. 1.** Effects of opioid receptor genotype (WT versus KO) and of chronic treatment with morphine (20–100 mg/kg i.p. for 5 days) on the immunodensity of neurofilament-L (NF-L) in the mouse cerebral cortex. *Left:* representative immunoblots (40 μg protein) for NF-L in wild type (WT) and μ-KO (A), δ-KO (B) and κ-KO (C) mice treated with saline or chronic morphine (\( n = 5 \) for each group). In A note that NF-L in KO 5 was abnormally reduced and not used for NF-L calculations. The apparent molecular mass of NF-L (68 kDa) was determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. *Right:* the columns are mean ± SEM values (% immunoreactivity) of 3 experiments per group (i.e., 12–15 samples analyzed for each comparison in each subgroup of mice, see Methods) and expressed as percentage of saline-treated (WT or KO) mice [WT saline (S) vs. KO saline (S); WT: saline (S) vs. morphine (M); KO: saline (S) vs. morphine (M)]. * \( P < 0.05 \) when compared with the corresponding saline (S) group (Student’s two-tailed \( t \)-test).
In the cerebral cortex and corpus striatum of µ-opioid receptor KO mice, the basal immunodensities of inhibitory Gz1/2 proteins, PKA Cα and total CREB in the cerebral cortex did not differ from those in brains of WT animals (Fig. 5). In contrast, the basal immunodensity of phosphorylated CREB was decreased in the cerebral cortex (23%, t = 2.94, P < 0.05) of δ-deficient mice (Fig. 5).

In κ-opioid receptor KO mice, compared with WT animals, the basal immunodensities of these signaling proteins (Gz1/2, PKA Cα and total and phosphorylated CREB) were found unchanged in the cerebral cortex (Fig. 5).

3.3. Immunodensities of Gz1 proteins, PKA Cα and total and phosphorylated CREB: effect of chronic morphine in WT and mutant mice

In one series of WT mice, chronic treatment (5 days) with morphine (20–100 mg/kg), compared with saline solution administration, increased slightly the immunodensity of inhibitory Gz1/2 proteins in the cerebral cortex (17%, t = 2.40, P < 0.05) (Fig. 6) and corpus striatum (13%, t = 2.27, P = 0.06) (not shown). However, this modest effect of chronic morphine on Gz1/2 proteins was not observed in other series of WT mice (Figs. 7 and 8). Similarly, the immunodensities of PKA Cα and total and phosphorylated CREB were not modulated significantly by chronic morphine treatment in the cerebral cortex (Figs. 6–8) or corpus striatum (not shown). The combined results of the three morphine treatments in WT mice clearly indicated that chronic opiate administration did not induce upregulation of the main components of the cAMP signaling pathway in the mouse cerebral cortex (Gz1, saline: 99 ± 5%, n = 15, morphine: 105 ± 4%, n = 15; PKA, saline: 100 ± 4%, n = 15, morphine: 103 ± 5%, n = 15; tCREB, saline: 101 ± 5%, n = 15, morphine: 101 ± 7%, n = 15; pCREB, saline: 101 ± 7%, n = 15, morphine: 102 ± 9%, n = 15) (Figs. 6–8).

In µ-opioid receptor KO mice, chronic morphine treatment increased the immunodensity of inhibitory Gz1/2 proteins (19%, t = 2.68, P < 0.05) and also the contents of PKA Cα (21%, t = 3.60, P < 0.01) and phosphorylated CREB (73%, t = 5.17, P < 0.001), but not total CREB, in the cerebral cortex (Fig. 6). Chronic morphine also induced modest increases of these signaling proteins in the corpus striatum of µ-opioid receptor KO mice (Gz1/2: 12%, t = 2.14, P = 0.07; PKA: 19%, t = 2.27 P = 0.06; phosphorylated CREB: 21%, t = 2.72, P < 0.05) (not shown).

In δ- and κ-opioid receptor KO mice, the immunodensities of Gz1/2, PKA Cα and total and phosphory-
lated CREB in the cerebral cortex were not modulated significantly by chronic morphine treatment (Figs. 7 and 8).

4. Discussion

4.1. The role of opioid receptors in morphine-induced abnormalities in NF proteins in the mouse brain

The NF are the major elements of the neuronal cytoskeleton being NF-L (the core peptide for protein assembly) and NF-H phosphorylation (the mechanism for assembly and disassembly) crucial elements to maintain its integrity (Julien and Mushynski, 1998). In this context, no opioid receptor genotype differences were observed in the contents of NF-L and phosphorylated NF-H in the mouse brain. The current data from gene-targeted mice clearly demonstrate that chronic morphine-induced decreases in the content of NF-L proteins in WT animals were abolished in the cerebral cortex of \( \mu \)-, \( \delta \)-, and \( \kappa \)-opioid receptor KO mice, indicating the involvement of the three types of opioid receptors in this effect of morphine. Moreover, the increase in the phosphorylation of NF-H induced by chronic morphine treatment also was ablated in the cerebral cortex of \( \mu \)-opioid receptor KO mice. Due to the limited availability of samples, the effects on NF-H were only evaluated in these KO mice since \( \mu \)-opioid receptor has been reported to be the most important for mediating chronic morphine effects (Matthes et al., 1998). In contrast, \( \mu \)-opioid receptor genotype and chronic morphine did not alter the expression of GFAP (used as a negative control) in the mouse cerebral cortex. Although there exists a distinct anatomical distribution of \( \mu \)-, \( \delta \)-, and \( \kappa \)-opioid receptors in the mouse brain, the cerebral cortex is a region enriched with the three opioid receptor types (Kitchen et al., 1997). It should be noted that morphine is a weak \( \mu \)-selective agonist in vitro (Raynor et al., 1994) and may partially act via \( \delta \)- and \( \kappa \)-opioid receptors in vivo, especially after the high doses of the opiate used in the chronic administration (up to 100 mg/kg). In fact, the functional activity of the \( \kappa \)-receptor is preserved and that of the \( \delta \)-receptor only slightly reduced in \( \mu \)-opioid receptor deficient mice (Matthes et al., 1998). In this context, chronic stimulation of \( \mu \)-receptors but not \( \delta \)-receptors in SH-SY5Y cells induced upregulation of dynamin, a cytoskeletal-associated protein that also plays a role in neuroplasticity (Noble et al., 2000). Moreover, chronic morphine administration in mice upregulated dynamin content in the caudate/putamen of WT animals but not in \( \mu \)-deficient mice, further indicating the relevance of \( \mu \)-opioid receptors in this novel effect of morphine (Noble et al., 2000). Therefore, most if not all effects of morphine investigated so far, including the decrease in the content of NF-L and the increase in the phosphorylation of NF-H in the brain, are nullified in \( \mu \)-opioid receptor deficient mice (Kieffer, 1999; Kieffer and Gavéraux-Ruff, 2002).

Previous studies have shown that opiate addiction in rats and humans is associated with marked reductions in NF (NF-H, NF-M, NF-L) proteins and with aberrant hyperphosphorylation of NF-H and NF-M in various brain regions including the cerebral cortex (Beitner-Johnson et al., 1992; García-Sevilla et al., 1997; Boronat et al., 1998, 2001; Ferrer-Alcón et al.,...
However, the decreases induced by chronic morphine on the content of NF-L in mouse brain (14–23%) were less pronounced than those observed in rat brain (40–60%) (Boronat et al., 2001) or in postmortem brains of chronic opiate addicts (45%) (García-Sevilla et al., 1997; Ferrer-Alcón et al., 2000), suggesting different species sensitivities for this effect of morphine. In line with the current results, the concurrent chronic administration (13 days) of naloxone and morphine also antagonized completely the morphine-induced decrease in NF-L immunoreactivity in the rat cerebral cortex (Boronat et al., 2001). Altogether, these data indicate that abnormalities in NF proteins may represent a neurochemical marker of opiate addiction associated with the induction of changes in neuronal plasticity by chronic opiates (Nestler, 2001; Ferrer-Alcón et al., 2003).

4.2. The role of opioid receptors in modulating the basal activity of Gαi/PKA/CREB pathway in the mouse brain

WT and mutant (µ-, δ-, or κ-KO) mice were first compared, in the absence of exogenous opiates, to assess if any endogenous opioid tone regulates the basal activity of the different components of the cAMP signaling pathway in the brain. No opioid receptor genotype differences were observed in the basal expression of Gαi/2 (inhibitory receptor coupling proteins), PKA Cα (the main target of cAMP formed from adenyl cyclase) and total CREB (the downstream target of PKA) in the cerebral cortex and/or corpus striatum of mice. The transcription factor CREB is one of the best characterized nuclear target proteins for phosphorylation by PKA and plays an important role in

Fig. 4. Effect of µ-opioid receptor genotype (WT versus µ-KO) on the immunodensity of inhibitory G coupling proteins (Gαi1/2), protein kinase A Cα (PKA), total cAMP response element-binding protein (tCREB) and phosphorylated CREB (pCREB) in the mouse cerebral cortex and corpus striatum. Left: representative immunoblots (40 µg protein) for the various target proteins in WT and µ-KO mice (n = 5 for each group and protein). The apparent molecular masses of target proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. Right: the columns are mean ± SEM values (% immunoreactivity) of 3 experiments per group and protein (i.e., 15 samples analyzed for each protein comparison, see Methods) and expressed as percentage of control WT mice (white columns: WT mice; black columns: µ-KO mice). *P < 0.05; **P < 0.02 when compared with the corresponding WT group (Student’s two-tailed t-test).

Fig. 5. Effects of (A) δ- and (B) κ-opioid receptor genotypes (WT versus KO) on the immunodensities of inhibitory G coupling proteins (Gαi1/2), protein kinase A Cα (PKA), total cAMP response element-binding protein (tCREB) and phosphorylated CREB (pCREB) in the mouse cerebral cortex. Left: representative immunoblots (40 µg protein) for the various target proteins in WT and δ- or κ-KO mice (n = 5 for each group and protein). The apparent molecular masses of target proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. Right: the columns are mean ± SEM values (% immunoreactivity) of 3 experiments per group and protein (i.e., 15 samples analyzed for each protein comparison, see Methods) and expressed as percentage of control WT mice (white columns: WT mice; black columns: δ- or κ-KO mice). *P < 0.05 when compared with the corresponding WT group (Student’s two-tailed t-test).
opiate addiction (Maldonado et al., 1996; Blendy and Maldonado, 1998). In the mouse brain, the highest contents of CREB are found in the cortex and striatum (Blom et al., 2002). Unexpectedly, the basal active form of CREB (phosphorylated CREB at the activator-site residue Ser 133) was found significantly reduced (23–26%) in the cerebral cortex and/or corpus striatum of μ- and δ-opioid receptor knock-out (KO) mice. This result may suggest that μ-/δ-receptors tonically stimulate, through endogenous opioid peptides, the activation of CREB in these mouse brain structures. Recently, morphine and the δ-agonist [D-Pen²⁵]-enkephalin have been shown in vitro to increase markedly the phosphorylation of CREB on Ser 133 in NG108-15 cells (Bilecki et al., 2000). If this is also the case in vivo then the observed reduction in basal phosphorylated CREB in μ- and δ-KO mice would be the consequence of the removal of a positive tonic opioid regulation on this transcription factor. The lack of significant changes in the basal active form of CREB in κ-receptor KO mice...
indicates a low tone, if any, of κ-opioid system to control the phosphorylation state of this transcription factor. This genetic approach (mice lacking opioid receptors) also has demonstrated or confirmed the existence of an endogenous opioid tone modulating various opioid functions (Kieffer and Gavériaux-Ruff, 2002). Thus, the comparative analysis of the three opioid receptor-deficient mouse strains supports the notion that an endogenous activity of μ- and δ-opioid receptors, but not κ-receptor, modulates the phosphorylation state of the transcription factor CREB. However, this interpretation contrasts with earlier pharmacological data indicating that in the rat locus coeruleus an acute morphine treatment decreased the content of phosphorylated CREB (Guitart et al., 1992), although this early study did not measure directly CREB phosphorylation on Ser 133. Therefore, a second hypothesis would be that the observed reduction in basal phosphorylated CREB in μ- and δ-KO mice is a compensatory mechanism. Since these opioid receptors function in part to decrease the phosphorylation state of CREB, gene deletion of these receptors would result in compensatory changes of another unknown component that decreases the rate of phosphorylation.

Alternatively, the possibility of heterologous compensatory neurochemical changes after the deletion of μ- and/or δ-opioid receptors cannot be discarded. In fact, various heterologous receptor adaptations have recently been reported in brain of μ-KO mice (e.g. increased NMDA receptor expression; Jang et al., 2001), the activity of which could indirectly modulate CREB. Thus, extrasynaptic NMDA receptors have been shown to initiate a shut-off signal on CREB phosphorylation (Harsingham et al., 2002).

4.3. The effects of chronic morphine on Gαi/PKA/CREB signaling pathway in brains of WT and μ-, δ- or κ-opioid receptor KO mice

Studies examining the effects of opiates on the cAMP signaling pathway have produced mixed results (for review, see Williams et al., 2001), but in general chronic morphine treatment has been associated with a compensatory upregulation of this system in the brain. Thus, previous reports have shown that Gxi/o proteins (Escribá et al., 1994; Lane-Ladd et al., 1997), adenyl cyclase 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) are increased in the locus coeruleus of rats 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 the striatum (see Widnell et al., 1996). In morphine-dependent mice a very modest induction of the nuclear cAMP response element (CRE) was observed in various brain regions (including the cerebral cortex), whereas a robust CRE upregulation was induced by opiate withdrawal (Shaw-Lutchman et al., 2002). The current study was focussed on the mouse cortex and striatum (see Introduction) and showed that chronic morphine in WT mice did not induce upregulation of the cAMP signaling pathway (Gxi/PKA/CREB). However, these negative results do not appear to be related to the morphine protocol.

Fig. 8. Effects of chronic treatment with morphine (20–100 mg/kg i.p. for 5 days) on the immunodensities of inhibitory G coupling proteins (Gxi/2), protein kinase A Cα (PKA), total cAMP responsive element binding protein (tCREB) and phosphorylated CREB (pCREB) in the cerebral cortex of wild-type (WT) and κ-opioid receptor knock-out (KO) mice. Left: representative immunoblots (40 μg protein) for the various target proteins in WT and κ-KO (saline- and morphine-treated) mice (n = 5 for each group and protein). The apparent molecular masses of target proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. Right: the columns are mean ± SEM values (% immunoreactivity) of 2 experiments per group and protein (i.e., 10 samples analyzed for each protein comparison, see Methods) and expressed as percentage of saline-treated (WT or KO) mice (white columns: saline; black columns: chronic morphine).
same WT mice this chronic morphine treatment significantly decreased, as expected (see Ferrer-Alcón et al., 2003), the immunodensity of the complex cyclin-dependent kinase-5/p35 in the cortex, another relevant target of opiate addiction (unpublished results). It therefore appears that chronic morphine modulated, also as expected, the densities of NF-L and phosphorylated NF-H but not the main components of the cAMP signaling pathway in the mouse cerebral cortex. In this mouse brain region, the increases in adenyl cyclase activity and cAMP levels induced by chronic morphine treatment also were very modest even after opiate withdrawal (about 10%) (Maldonado et al., 1996; Mamiya et al., 2001). Similarly to the current results, chronic morphine also failed to increase PKA Cz in the locus coeruleus of WT mice (Akbarian et al., 2002). In the rat locus coeruleus, however, the compensatory upregulation induced by chronic morphine on the content of Gz1 (34%), adenyl cyclase activity (26%), PKA (41%) and CREB (42%) is clearly apparent (Lane-Ladd et al., 1997; Nestler and Aghajanian, 1997). Similarly, morphine treatments also modulated other targets of opiate addiction in the rat cerebral cortex (Boronat et al., 2001; Jaquet et al., 2001; Ferrer-Alcón et al., 2003). Therefore, differences in brain regions (greater cellular heterogeneity in the cortex) and/or species sensitivity (most probably) could explain the present negative findings (compensatory upregulation of cAMP signaling after chronic morphine) in cortex and striatum of WT mice.

In marked contrast with WT mice, chronic morphine treatment in μ-opioid receptor KO mice resulted in paradoxical upregulations of Gz1/2 proteins (12–19%), PKA Cz (19–21%) and phosphorylated CREB (21–73%), but not total CREB, in the cerebral cortex and corpus striatum. Chronic morphine did not induce upregulation of these signaling proteins in brains of δ- and κ-opioid receptor KO mice. Most of the pharmacological activities of morphine, which are classically observed in WT mice, are not detected in μ-receptor KO mice (for review, see Kieffer and Gavériaux-Ruff, 2002). Specifically, the upregulation of adenyl cyclase activity in striatal tissue during opiate withdrawal (chronic morphine followed by naloxone) did not develop in brains of mutant mice lacking the μ-opioid receptor gene (Matthes et al., 1996). The paradoxical effects of chronic morphine in μ-opioid receptor KO mice (increased PKA catalytic subunit and phosphorylated CREB) indicate that the absence of functional μ-receptors causes an abnormal upregulation of these signaling proteins which is not observed in WT mice (perhaps because adaptive changes could occur on the cAMP pathway as a consequence of μ-opioid receptor deletion), suggesting the induction of compensatory mechanisms probably on heterologous systems. Thus, a non-selective action of morphine at non-μ-opioid receptors or even non-opioid targets would be responsible for the observed effects and by a mechanism which remains to be determined.

In conclusion, this study clearly reveals the involvement of μ, δ- and κ-opioid receptors in the decrease of NF-L produced by chronic morphine on the mouse cerebral cortex. In addition, a decrease of phosphorylated CREB was observed in the cortex and striatum of μ- and δ-receptor knock-out mice suggesting that endogenous opioids acting on these opioid receptors tonically stimulate CREB activation. Finally, chronic morphine produced a paradoxical enhancement of phosphorylated CREB in the cortex of μ-receptor knock-out mice, probably due to nonspecific effects induced by this massive dose of morphine.

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