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# Enkephalin is essential for the molecular and behavioral expression of cocaine sensitization

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## ABSTRACT

Behavioral sensitization to cocaine is associated to neuroadaptations that contribute to addiction. Enkephalin is highly expressed in mesocorticolimbic areas associated with cocaine-induced sensitization; however, their influence on cocaine-dependent behavioral and neuronal plasticity has not been explained. In this study, we employed a knockout (KO) model to investigate the contribution of enkephalin in cocaine-induced behavioral sensitization. Wild-type (WT) and proenkephalin KO mice were treated with cocaine once daily for 9 days to induce sensitization. Additionally, to clarify the observations in KO mice, the same procedure was applied in C57BL/6 mice, except that naloxone was administered before each cocaine injection. All animals received a cocaine challenge on days 15 and 21 of the treatment to evaluate the expression of locomotor sensitization. On day 21, microdialysis measures of accumbal extracellular dopamine, Western blotting for GluR1 AMPA receptor (AMPAR), phosphorylated ERK2 (pERK2), CREB (pCREB), TrKB (pTrkB) were performed in brain areas relevant for sensitization from KO and WT and/or naloxone- and vehicle pre-treated animals. We found that KO mice do not develop sensitization to the stimulating properties of cocaine on locomotor activity and on dopamine release in the nucleus accumbens (NAc). Furthermore, pivotal neuroadaptations such as the increase in pTrkB receptor, pERK/CREB and AMPAR related to sensitized responses were absent in the NAc from KO mice. Consistently, full abrogation of cocaine-induced behavioral and neuronal plasticity after naloxone pre-treatment was observed. We show for first time that the proenkephalin system is essential in regulating long-lasting pivotal neuroadaptations in the NAc underlying behavioral sensitization to cocaine.

**Keywords** Behavioral sensitization, cocaine, dopamine, neuroadaptations, naloxone, proenkephalin KO.

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## INTRODUCTION

Repeated intermittent exposure to cocaine produces a progressively greater locomotor response to the drug (behavioral sensitization) (Robinson & Berridge 1993), which is generally coupled to a greater drug-induced dopamine efflux in the nucleus accumbens (NAc) (Kalivas & Duffy 1993). The process underlying behavioral sensitization involves a complex interplay between dopamine and other neurotransmitters and neuropeptides, such as glutamate (Vanderschuren & Kalivas 2000), opioid peptides (DuMars, Rodger & Kalivas 1988) and neurothrophins (Bahi *et al.* 2008).

Behavioral sensitization to psychostimulants is probably mediated by converging extracellular signals that give rise to a limited number of specific molecular and cellular events, such as the activation of the extracellular signal-regulated kinase (ERK) pathway (Lu *et al.* 2006). In the NAc, activated ERK controls the state of phosphorylation of transcription factors, including cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), and thereby initiates a gene transcription program that leads to the long-term effects of repeated exposure to psychostimulants and opiates (Robison & Nestler 2011). Two pivotal neuroadaptations with consequences for behavioral responses to cocaine have been consistently identified following repeated cocaine administration: an enhancement of GluR1 AMPA receptor (AMPAR) and of brain-derived neurotrophic factor/tyrosine kinase B receptor (BDNF/

TrkB) receptor signaling within the NAc (Boudreau *et al.* 2007; Crooks *et al.* 2010). It is thought that these changes are critically involved in cocaine-induced sensitization and other behavioral responses related to drug addiction (Thomas, Kalivas & Shaham 2008).

Proenkephalin-derived opioid peptides and opioid receptors are widely expressed in brain areas relevant to cocaine-induced sensitization (Le Merrer *et al.* 2009). It has been reported that the enkephalinegic system increases mesoaccumbal dopamine neurotransmission (Latimer, Duffy & Kalivas 1987). Cocaine increases NAc dopamine levels by binding to dopamine transporter and inhibits dopamine uptake. This increase contributes to the psychomotor stimulant and rewarding effects of cocaine. In addition to the direct effects on monoamine reuptake, cocaine can alter levels of endogenous opioid peptides. More specifically, an increase in the expression of enkephalin in mesocorticolimbic areas associated with the expression of cocaine-induced sensitization after acute and chronic psychostimulant treatment has been reported (Crespo *et al.* 2001; Assis *et al.* 2009).

Enkephalin can activate delta-opioid receptors (DOPr) and mu-opioid receptors (MOPr). Pharmacologic evidence demonstrates that proenkephalin-derived opioid peptides showed high affinity for DOPr, but also good affinity for MOPr (Mansour *et al.* 1995). Studies using pharmacologic tools have demonstrated that MOPr and DOPr contribute to the cocaine-induced elevation in dopamine release in the NAc (Van Ree *et al.* 2000; Shippenberg & Chefer 2003). Consistently with this neurochemical data, pharmacologic approaches demonstrate a role of MOPr and DOPr in the development and expression of cocaine sensitization. Naloxone, a non-selective opioid receptor antagonist, naltrindole, a DOPr and CTAP (D-Phe-*cyc*(Cys-Tyr-D-Trp-Arg-Thr-Pen)-Thr-NH2), a selective MOPr, block or attenuate the development of cocaine sensitization in rats (Heidbreder, Shoaib & Shippenberg 1996; Schroeder *et al.* 2007) and mice (Kim *et al.* 1997; Hummel *et al.* 2004). However, MOPr knockout (KO) mice did not show a significant influence of this receptor in cocaine sensitization although the behavioral evaluation after short-term cocaine withdrawal could have masked a possible influence of MOPr (Yoo, Kitchen & Bailey 2012). In spite of all this evidence, so far, the literature has not explained the influence of enkephalin in cocaine-induced neuronal plasticity underpinning long-term sensitization.

In this study, we employed a KO model to investigate the contribution of proenkephalin and opioid receptors in cocaine-dependent behavioral and neuronal plasticity. Proenkephalin KO mice were tested for cocaine-induced locomotion and dopamine release from NAc, as well as cocaine-dependent activation of the ERK/CREB signaling pathway, GluR1 AMPAR surface, and BDNF/TrkB expression in reward-processing areas relevant for sensitization to cocaine. Additionally, as enkephalin binds to MOPr and DOPr, and naloxone is a promising drug to treat cocaine addiction, the effect of this opiate antagonist on behavioral sensitization to cocaine and associated neuroadaptations in NAc were studied in C57BL/6 mice. We show for the first time that the proenkephalin system is critical in regulating long-lasting pivotal neuroadaptations underlying behavioral sensitization to cocaine. This study is highly relevant to understand how modifications in the proenkephalin system can influence the vulnerability to psychostimulant addiction. Additionally, our data provide insights on neurobiological basis of the clinical usefulness of opiate antagonists in the treatment of cocaine addiction.

## MATERIALS AND METHODS

### Animals

The generation of mice lacking the preproenkephalin gene (KO Penk −/−) has been described previously (Konig *et al.* 1996). Animals were imported from Germany to Argentina. Wild-type (WT) and homozygous KO mice were obtained from heterozygous breeding. The mice used in the present study were on a C57BL/6 background after 10 backcrossing to C57BL/6 mice (Facultad de Ciencias Veterinarias, La Plata, Argentina). Male mice 8–12 weeks old were housed four per cage in a temperature- (21 ± 1°C) and humidity- (55 ± 10%) controlled room with a 12-hour light/dark cycle (lights on between 8:00 AM and 8:00 PM). Food and water were available *ad libitum*. All mice were genotyped by polymerase chain reaction (PCR) to identify WT, KO and heterozygous animals and matched by age, genotype and sex. WT (Penk +/+) littermate mice were used as controls for all experiments. For naloxone experiments, C57BL/6 male mice were purchased from the Facultad de Ciencias Veterinarias, La Plata, Argentina. Mice were habituated to their new environment for 1 week before starting the experimental procedure and tested during the light cycle, between 9:00 AM and 4:00 PM. All procedures were handled in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina.

### Drugs

Cocaine hydrochloride (Verardo Laboratory, Buenos Aires, Argentina) and naloxone dihydrochloride (Sigma-Aldrich) were dissolved in sterile physiological saline (0.9% NaCl), that was also used for vehicle control injections.

 

### Repeated cocaine injections and behavioral analysis

Following the drug administration protocol used by De Jong, Steenbergen & De Kloet (2009), mice were randomly assigned to one of two treatments: vehicle or cocaine [15 mg/kg, intraperitoneal (i.p.) ]. The sensitization paradigm consisted of a treatment phase (days 1–9), a withdrawal phase, a vehicle challenge (day 14), and two different cocaine challenges (days 15 and 21). Locomotor activity was measured on days 1, 5 and 9. On these days, animals received the treatment in the test setting while on the remaining days, injections were given in the home cage. The treatment period was followed by a 5-day withdrawal interval. On day 14, all animals received a vehicle challenge, and on days 15 and 21, a 7.5-mg/kg cocaine challenge. For naloxone experiments, the same procedure was applied in C57BL/6 male mice, except that naloxone (1 mg/kg) or vehicle was subcutaneously injected 15 minutes before each cocaine or vehicle injection in the treatment phase (days 1–9).

Locomotor responses were measured using individual locomotor activity boxes (40 cm in diameter), constructed of opaque plastic walls with two transverse photocells positioned 1 cm above the floor coupled to a computer interface. Following a 2-hour habituation period, animals were injected and activity was monitored using a video camera for 30 minutes by an investigator blind to the genotype condition. Cages were carefully cleaned with a 20% ethanol solution between tests to minimize odor cues.

### Preparation of brain homogenates and cellular fractionation

For the biochemical studies, animals injected with 7.5 mg/kg cocaine on day 21 (challenge injection) were used. After 30 minutes of the cocaine challenge, mice were killed by decapitation. Brains were rapidly removed and the NAc, dorsal striatum, prefrontal cortex (Pfc) and hippocampus dissected. After the dissections, bilateral NAc and dorsal striatum tissue blocks from two animals were pooled to obtain enough material for each experiment. The procedures used for protein isolation, subcellular fractionation, AMPAR cell surface expression, Western blotting and antibody detection were based on Gorosito & Cambiasso (2008) and Esparza *et al.* (2012), and were described in Supporting Information Appendix S1.

### Tissue preparation

After 30 minutes of cocaine (7.5 mg/kg) or vehicle challenge on day 21, mice were transcardially perfused with heparin (15 000 UI/L) in 0.9% saline and fixative solution (4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.2). Brains were removed and postfixed in the same fixative solution overnight at 4°C and cryoprotected in 30% sucrose for 24–72 hours. Brain sections were cut with a cryostat (CM1510S, Leica Microsystems, Wetzlar, Germany) and kept floating in phosphate-buffered saline (PBS) (for immunohistochemistry) or mounted on gelatinized slides (for BDNF immunofluorescence).

### Immunohistochemistry

Met-enkephalin detection was performed on 30-µm thick free-floating sections using a standard avidin-biotin peroxidase method (Elite Vectastain Kit; Vector Laboratories, Burlingame, CA, USA). Briefly, after reducing endogenous peroxidase activity and blocking non-specific binding, floating sections were incubated for 15–16 hours with anti-met-enkephalin (1:500; Millipore, Bioscience Research Reagents, Temecula, CA, USA) at 4°C. Primary antibodies were detected with biotinylated goat anti-rabbit secondary antibodies (1:500; Jackson Laboratories, West Grove, PA, USA). Diaminobenzidine (Sigma-Aldrich, Saint Louis, MO, USA) was used as a chromogen. The sections were mounted on gelatinized slides, and coverslipped with Distrene-80 plasticizer xylene (DPX mounting medium) (Sigma-Aldrich). Some sections were counterstained with cresyl violet.

### Image acquisition and counting protocol

Met-enkephalin-expressing cells were counted manually on images acquired using light-field microscopy (Leica DM 4000 B) with a magnification of 20×. NAc and dorsal striatum were analyzed. Positive met-enkephalin cells were identified and counted in the selected brain areas using the Image J Program [Image J is a National Institutes of Health (NIH) Image Software] by a treatment-blind investigator using identical area size (0.35 $mm^2$) of the same shape for each brain region.

### Surgery and microdialysis procedure

Mice were anesthetized with ketamine and xylazine solution (5 mg/kg xylazine-55 mg/kg ketamine i.p.) and mounted into a Stoelting stereotaxic instrument with a mouse adaptor. Dialysis probes were implanted unilaterally in the NAc (AP: +1.2; ML: ±1.0; DV: −4.9) according to the coordinates of Franklin & Paxinos (2007).The probe was secured in place with dental cement. After surgery, all mice were placed individually in plastic cages and allowed to recover for at least 18–22 hours. The day following surgery, the dialysis membrane was perfused with Ringer's solution (NaCl 145 nM, KCl 4.0 nM, $CaCl_2$ 2.2 nM) at a constant flow rate of 1 µl/minute. Samples of the dialysate were automatically collected every 30 minutes. Baseline samples were taken during 2 hours. Subsequently, mice were injected with vehicle and samples were collected for 1.5 hours. Then, they were

injected with cocaine 7.5 mg/kg and the dialysates collected for 3 hours. Baseline data for consecutive samples differed by no more than 10%.

### Microdialysis probe construction

A vertical concentric dialysis probe was prepared with AN69 (Hospal, Bologna Italy), following Di Chiara's design (Di Chiara *et al.* 1993) with some minor modifications (Pacchioni *et al.* 2007). The length of the active dialyzing area for the NAc was 1.0 mm.

### High-performance liquid chromatography (HPLC) system for dopamine quantification

The perfusate was assayed for dopamine content by reverse-phase HPLC coupled with electrochemical detection (Model 582, solvent delivery model; ESA, Chelmsford, MA, USA). The mobile phase was composed of 50 mM $NaH_2PO_4$; 5 mM $Na_2HPO_4$; 0.1 mM EDTA-Na; 0.5 mM n-octyl-sodium sulphate; and 12% methanol; pH was adjusted to 5.5. The mobile phase was delivered at a flow of 1 ml/minute through a RP 18 column (C18, 125-4.6 mm, 5 mm). Samples were injected via a 20-µl injection loop. Dopamine was detected using a coulometric detector (ESA Coulochem II) consisting of three electrodes: a guard cell (+350 mV), an oxidation analytical electrode (+175 mV), and a reduction analytical electrode (−175 mV). Peaks were recorded and height measured by a computer using an ESA Chromatography Data System (ESA, Inc., Chelmford, MA, USA). The values obtained were compared with an external standard curve.

### Histology

At the end of the microdialysis experiments, animals were anesthetized with chloral hydrate (400 mg/kg i.p.), perfused transcardially with saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed in the same fixative overnight at 4°C. They were then placed in 30% sucrose in PBS, sectioned in a cryostat (Leica CM1510S) into 30-µm thick coronal slices and stained with cresyl violet. The histologic sections were examined under light microscope to check the position of the probe. Only those animals that had been implanted correctly were included in the study.

### Statistical analysis

Data were analyzed using the Statistica 7.1 program (Statsoft, Inc., Tulsa, OK, USA). Statistical analyses of molecular and behavioral data were performed using two-way ANOVA (genotype × treatment). Statistical analyses of neurochemical data were performed using two-way ANOVA with repeated-measures over time. To evaluate the stimulating effect of acute administration of cocaine on dopamine release in KO animals, unpaired *t*-test was performed comparing the dopamine levels at 180 and 210 minutes of the microdialysis experiment. Significant main effects indicated by the ANOVA were further analyzed through Fisher's least significant difference or Bonferroni *post hoc* test.

## RESULTS

### Chronic cocaine treatment is associated with increases in met-enkephalin levels in the NAc

To explore the effect of chronic cocaine treatment on the enkephalin system, we evaluated the expression of met-enkephalin at day 21 of our treatment protocol. As shown in Fig. 1, cocaine-treated WT mice showed an increased met-enkephalin expression in the NAc (treatment $F_{(1,16)} = 22{,}20$; $P < 0.01$) associated with chronic cocaine treatment, i.e. not dependent on whether or not cocaine challenge was administered on day 21. Similar results regarding cocaine-induced increases in met-enkephalin levels were observed in the dorsal striatum (Supporting Information Fig. S1).

### Preproenkephalin KO and naloxone-pre-treated mice did not show sensitization to the behavioral effects induced by cocaine

On day 1 of treatment, mice locomotor activity was monitored after a cocaine injection (15 mg/kg i.p.) or vehicle. Acute response to cocaine was similar in both genotypes [treatment $F_{(1,31)} = 86{,}80$; $P < 0.01$; genotype $F_{(1,31)} = 1{,}37$; not significant (NS) ] (Fig. 2a). Mice then received daily administrations of cocaine or vehicle at days 1–9, followed by a 5-day withdrawal interval. Figure 2b depicts locomotor responses to the cocaine challenge (7.5 mg/kg i.p.) observed at day 15. Cocaine-treated WT mice displayed a sensitized response, which was not observed in KO mice [treatment ($F_{(1,31)} = 38{,}32$; $P < 0.01$); interaction ($F_{(1,31)} = 10.74$; $P < 0.01$]. Bonferroni *post hoc* comparisons showed an increase in horizontal activity when cocaine challenge was administered in WT mice previously treated with cocaine 15 mg/kg compared with vehicle-treated WT ($P < 0.01$) and KO mice ($P < 0.01$). Similar results were observed on day 21 [treatment $F_{(1,31)} = 16{,}28$; $P < 0.01$; interaction $F_{(1,31)} = 13{,}13$; $P < 0.01$] (Fig. 2c). Bonferroni *post hoc* comparisons showed an increase of horizontal activity when cocaine challenge was administered in WT mice previously treated with cocaine compared with vehicle-treated WT ($P < 0.01$) and KO mice ($P < 0.05$). It is important to note that locomotor responses to the vehicle challenge (day 14) were similar in all treatment groups

 

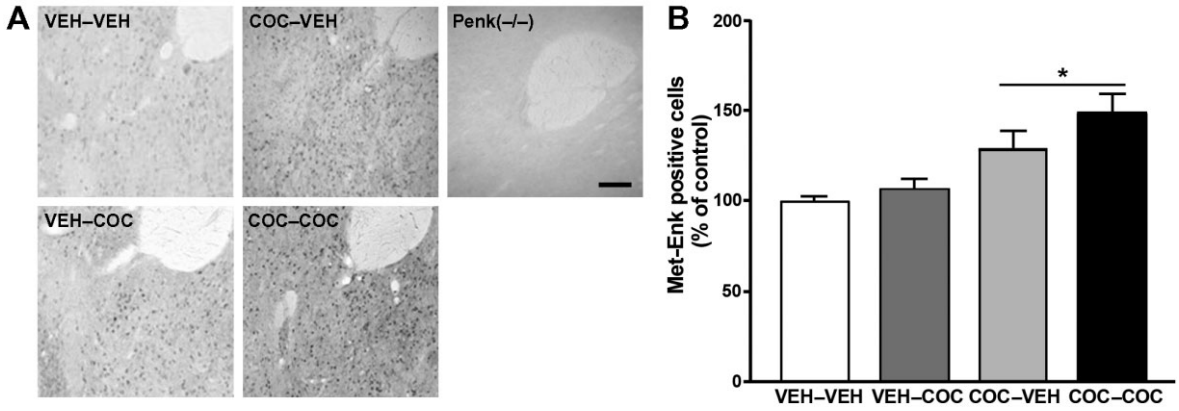


**Figure 1** Enkephalin elevations in animals treated chronically with cocaine. (a) Photomicrographs (20×) of met-enkephalin immunoreactivity in nucleus accumbens (NAc) mice were daily treated with cocaine during 9 days and challenged with the drug 21 days after the beginning of the treatment. Animals were anesthetized and transcardially perfused 30' after the challenge dose was administered; 30-μm thick brain slices were subjected to immunohistochemistry (see Materials and Methods section) to visualize met-enkephalin expression in the NAc. Scale bar represents 100 μm. (b) Quantification of positive met-enkephalin cells (number of positive cells/0.35 $mm^2$) from the different treatment groups (VEH–VEH $n = 5$–6; VEH–CO $n = 5$–6; CO–VEH $n = 6$; CO–CO $n = 6$). Data are represented as mean ± standard error of the mean (SEM) (expressed as the percentage of VEH–VEH). *$P < 0.01$ indicates difference comparing treatment groups (vehicle and cocaine)

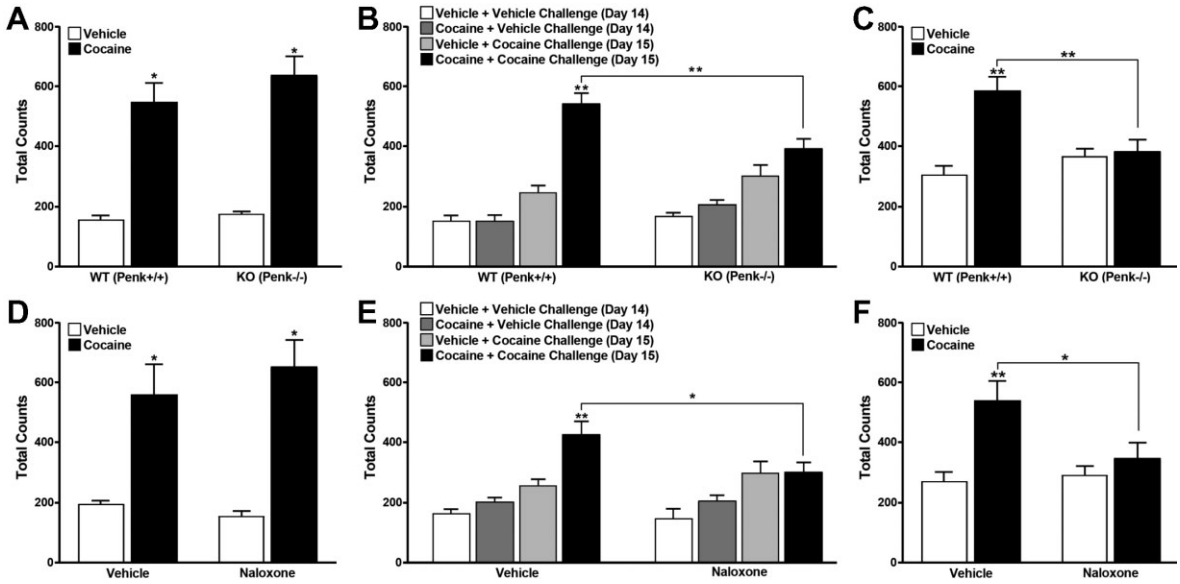


**Figure 2** Preproenkephalin knockout (KO) and naloxone pre-treated mice did not show sensitization to the behavioral effects induced by cocaine. Left panels shows locomotor activity in response to acute cocaine administration [15 mg/kg intraperitoneal (i.p.)] in KO (a) and naloxone pre-treated animals (d). Middle panels depict locomotor activity in response to a vehicle challenge (day 14) and 7.5 mg/kg cocaine challenge (day 15) in KO (b) and naloxone [1 mg/kg subcutaneous (s.c.)] pre-treated mice (e) Right panels reveal the effect of cocaine challenge administration on the locomotor activity of KO (c)and naloxone pre-treated mice (f) on day 21. Data are expressed as the average of total counts in 30 minutes ± standard error of the mean (SEM) of $n = 9$ WT–VEH, $n = 6$ WT–COC, $n = 9$ KO–VEH, $n = 8$ KO–COC and $n = 6$ VEH–VEH, $n = 7$ VEH–COC, $n = 7$ NX–VEH, $n = 6$ NX–COC animals/group. **$P < 0.01$ and *$P < 0.05$ compared with WT/VEH–VEH and/or KO/NX–COC groups, respectively (Bonferroni *post hoc* test)

and markedly lower than the cocaine challenge on days 15 and 21 [treatment $F_{(1,31)} = 0{,}89$; NS; genotype $F_{(1,31)} = 1{,}39$ NS]. Thus, the full sensitization observed in response to the cocaine challenge cannot be accounted for a conditioned responsiveness.

We next wished to determine if the blockade of MOPr/DOPr abolishes the facilitating influence of enkephalin on cocaine-induced behavioral sensitization. Therefore, mice received naloxone [1 mg/kg subcutaneous (s.c.) ] or vehicle 15 minutes prior to the administration of cocaine (15 mg/kg i.p.) during the 9 days treatment period. We first assessed the effect of naloxone on cocaine-stimulated locomotor activity on day 1 of the treatment. No differences were found between mice pre-treated with

 

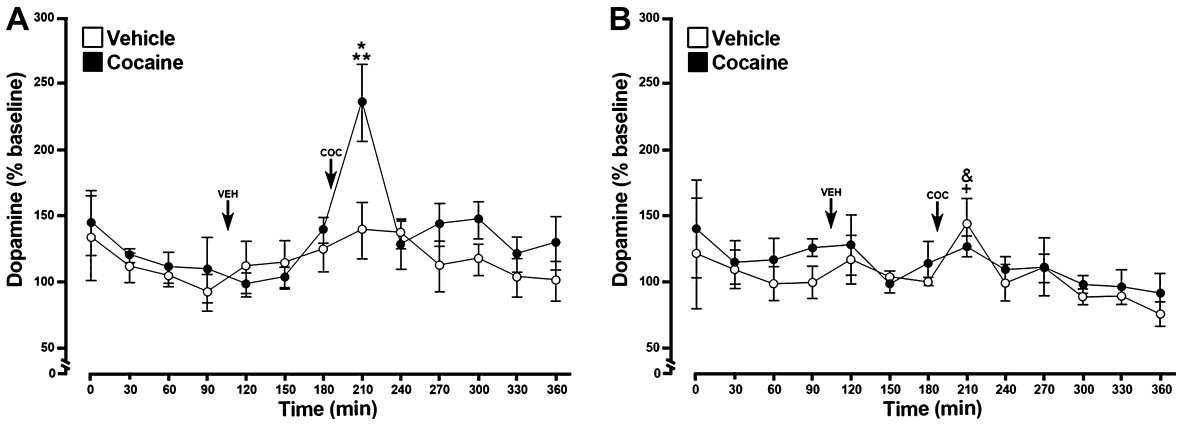


**Figure 3** Sensitization to the stimulating effect of cocaine on dopamine release in the nucleus accumbens (NAc) is absent in knockout (KO) mice. Effects of cocaine [7.5 mg/kg, intraperitoneal (i.p.) ] and vehicle on dopamine concentrations in dialysates obtained by *in vivo* microdialysis from the NAc of WT (a) and KO mice (b). The arrows indicate vehicle and cocaine challenge administration at 120 and 210 minutes, respectively. All values are expressed as mean ± standard error of the mean (SEM) and represented as a percentage from baseline levels of each treatment group $^{**}P < 0.01$ compared with dopamine baseline levels; $^{*}P < 0.05$ comparing dopamine levels follow the cocaine challenge (210 minutes) between WT–CO and KO–CO group (Fisher *post hoc* test) + and &$P < 0.01$ comparing dopamine levels prior (180 minutes) and follow the cocaine challenge (210 minutes) in KO–VEH/CO group, respectively (*t*-test). Basal levels of DA in NAc dialysates were similar in both genotypes (WT–VEH: 41.8 fmol ± 4.8 $n = 9$; KO–VEH: 45.0 fmol ± 5.7 $n = 7$; WT–CO: 45.93 fmol ± 6.0 $n = 8$; KO–CO: 47.0 fmol ± 5.7 $n = 9$)

naloxone and those pre-treated with vehicle [treatment $F_{(1,22)} = 36{,}4035$; $P < 0.01$; pre-treatment $F_{(1,22)} = 0{,}65$; NS] (Fig. 2d).

Animals treated chronically with cocaine showed expression of behavioral sensitization to cocaine when the cocaine challenge (7.5 mg/kg) was administered on days 15 or 21 of the treatment. In contrast, when naloxone (1 mg/kg s.c.) was injected 15 minutes prior to each cocaine injection during 9-day treatment, the expression of behavioral sensitization to cocaine was abrogated [treatment ($F_{(1,22)} = 6{,}00$; $P < 0.05$), interaction ($F_{(1,22)} = 5{,}19$; $P < 0.05$) (Fig. 2e and f) ]. Bonferroni *post hoc* comparisons revealed an increase in horizontal locomotor activity when the cocaine challenge was administered on day 15, compared with vehicle pre-treatment, ($P < 0.01$). Animals receiving naloxone also showed a reduced response to the cocaine challenge ($P < 0.05$). On day 21, the expression of behavioral sensitization to cocaine was also abrogated in naloxone pre-treated animals [treatment ($F_{(1,22)} = 12,\ 18$; $P < 0.05$); interaction ($F_{(1,22)} = 5{,}17$ $P < 0.05$) ]. Bonferroni *post hoc* comparisons showed an increase in horizontal activity when the cocaine challenge was administered in cocaine–vehicle group, compared with vehicle–vehicle ($P < 0.01$) and naloxone–vehicle groups ($P < 0.05$).

### Sensitization to the stimulating effect of cocaine on dopamine release in the NAc is absent in KO mice

Extracellular dopamine was measured in the NAc of WT and KO mice to determine whether sensitization to the stimulating properties of cocaine was associated with an increased dopamine release. On day 21, all animals were injected first with vehicle and 90 minutes later with cocaine (7.5 mg/kg, i.p.). This dose challenge was selected based on the behavioral results that showed a marked expression of behavioral sensitization in WT animals and complete attenuation of the phenomenon in the KO mice when the animals were re-exposed to a lower dose of cocaine. In agreement with previous findings (Kalivas & Duffy 1993), chronic cocaine administration induced a sensitized response of dopamine release in the NAc in WT mice, as revealed by the enhanced extracellular dopamine levels in mice chronically treated with cocaine compared with those treated with vehicle [interaction $F_{(12,156)} = 1{,}92$; $P < 0.05$) ]. Fisher *post hoc* comparisons showed a significant increase in the percentage of dopamine output, 210 minutes after cocaine challenge in mice previously treated with cocaine, compared with WT controls (Fig. 3a). In contrast, KO mice did not show a sensitized response to stimulating effects of cocaine on dopamine release after cocaine challenge and repeated measurements (interaction $F_{(12,156)} = 1{,}32$; NS) (Fig. 3b). Finally, two-way ANOVA demonstrated interaction between genotype and treatment at 30 minutes after cocaine challenge ($F_{(1,26)} = 8{,}32$; $P < 0.01$). Fisher *post hoc* comparisons showed that the dopamine levels in WT mice treated chronically with cocaine were the highest of all the remaining groups at the 210 minutes time-point. However, the stimulating effect of acute cocaine administration on dopamine release in the NAc was observed in KO mice treated chronically with vehicle or cocaine

 

after the challenge injection with the drug (KO–VEH $t\text{-test}_{12} = 2.79$, $P < 0.01$; KO–COC $t\text{-test}_{12} = 4.78$, $P < 0.01$). Additionally, basal dopamine levels in NAc dialysates were similar in both genotypes (treatment $F_{(1,29)} = 0.39$; NS; genotype $F_{(1,29)} = 0.20$; NS; interaction $F_{(1,29)} = 0.04$; NS) WT/vehicle: 41.8 fmol ± 4.8; KO/vehicle: 45.0 fmol ± 5.7; WT/cocaine: 45.93 fmol ± 6.0; KO/cocaine: 47.0 fmol ± 5.7.

**Preproenkephalin KO and naloxone-pre-treated mice did not show molecular neuroadaptations associated to cocaine-induced sensitization**

The behavioral and neurochemical results previously obtained led us to ask whether long-lasting molecular events specifically associated with the sensitization phenomenon were abrogated similarly in KO and naloxone-pre-treated mice. Thus, we studied the levels of pTrkB, pERK2, pCREB and AMPAR cell surface expression in the NAc from WT and KO mice treated chronically with cocaine for 9 days and challenged with 7.5 mg/kg at day 21 from the beginning of treatment. There was a significant increase in pTrkB levels in the NAc (Fig. 4a) from WT mice chronically treated with cocaine, but this effect was not present in KO mice that received the chronic treatment with the drug (interaction $F_{(1,12)} = 21{,}58$; $P < 0.01$). Fisher *post hoc* comparisons revealed an increase in pTrkB levels from WT mice chronically treated with cocaine compared with all the remaining groups after cocaine challenge injection ($P < 0.05$). Consistently, cocaine-induced increases in BDNF levels were absent in the NAc from KO mice treated chronically with cocaine (Supporting Information Fig. S2). However, a similar increase of BDNF levels was observed in the Pfc from WT and KO mice treated chronically with cocaine compared with vehicle controls (Supporting Information Fig. S3). The differences between WT and KO in cocaine-induced BDNF levels in the NAc indicate a specific influence of enkephalin on this brain area. Also, these data indicate that the lack of cocaine-induced increases in BDNF within the NAc from KO mice cannot be attributed to an impairment in neurotrophin synthesis, because increases of BDNF levels were seen in both animal genotypes in the Pfc.

ERK is the major effector for BDNF/TrkB signaling and is also activated by dopaminergic agonisticactivity through D1 receptors. After activation, ERK1/2 proteins are translocated to the nucleus, resulting in phosphorylation and activation of transcription factors, such as CREB. Thus, we studied ERK2/CREB activation in NAc nuclear fractions from WT and KO mice. We found an increase in ERK2 phosphorylation from WT mice treated chronically with cocaine and challenged with 7.5 mg/kg of cocaine on day 21, but in KO mice, this effect was not observed (interaction $F_{(1,15)} = 4{,}73$; $P < 0.05$) (Fig. 4b). Fisher *post hoc* comparisons showed an increase of ERK2 activity when cocaine challenge was administered in WT mice chronically treated with cocaine, compared with that induced in WT mice chronically treated with vehicle ($P < 0.05$) and KO mice chronically treated with cocaine ($P < 0.05$). Figure 4c shows the pattern of changes in CREB phosphorylation induced by chronic cocaine treatment within the NAc from WT and KO animals. Two-way ANOVA showed an effect in treatment ($F_{(1,17)} = 4{,}99$; $P < 0.05$) and genotype ($F_{(1,17)} = 6{,}99$; $P < 0.05$ ). No difference was found in ERK2 activity in Pfc and hippocampus from WT and KO mice chronically treated with cocaine (data not shown).

ERK activity has been implicated in AMPAR trafficking in striatal neurons (Zhu *et al.* 2002). Additionally, animals demonstrating cocaine psychomotor sensitization showed increased ERK phosphorylation in parallel with increased AMPAR surface expression in the NAc (Boudreau *et al.* 2007), indicating that ERK signaling is directly responsible for the increase in AMPAR (GluR1) in this brain area. Based on the results obtained in pERK2, we also studied the NAc level of AMPAR cell surface expression from WT and KO mice treated with cocaine and challenged with 7.5 mg/kg of cocaine on day 21 of the treatment. We observed a main increase in the AMPAR (GluR1) expression in the NAc from WT administered chronically with cocaine, but not in KO mice that received the same treatment (genotype $F_{(1,12)} = 7{,}57$; $P < 0.05$; interaction $F_{(1,12)} = 6{,}88$; $P < 0.05$) (Fig. 4d). Fisher *post hoc* comparisons revealed an increase in AMPAR cell surface expression in the NAc, when cocaine challenge was administered in WT mice chronically treated with cocaine compared with that induced in KO mice exposed chronically to the drug ($P < 0.05$).

Similar results regarding cocaine-induced plastic changes in WT, but not in KO mice exposed chronically to the drug were observed in the dorsal striatum (Supporting Information Fig. S4).

Interestingly, naloxone pre-treatment similarly prevented the cocaine-induced neuroadaptations associated with the sensitization phenomenon. We found that naloxone pre-treatment prevented cocaine-induced increases in pTrkB levels in the NAc ($F_{(1,12)} = 6{,}26$; $P < 0.05$) (Fig. 4e) and also in the dorsal striatum (Supporting Information Fig. S3). Fisher *post hoc* comparisons revealed an increase in pTrkB levels within the NAc from the vehicle–cocaine group compared with all the remaining groups after the cocaine challenge injection ($P < 0.05$). Consistently, naloxone pre-treatment prevented cocaine-induced elevations in BDNF levels in the NAc (Supporting Information Fig. S2). Interestingly, the data obtained in Pfc from naloxone-pre-treated animals were similar to the results observed in the Pfc from KO mice. We found an increase in BDNF levels within the Pfc

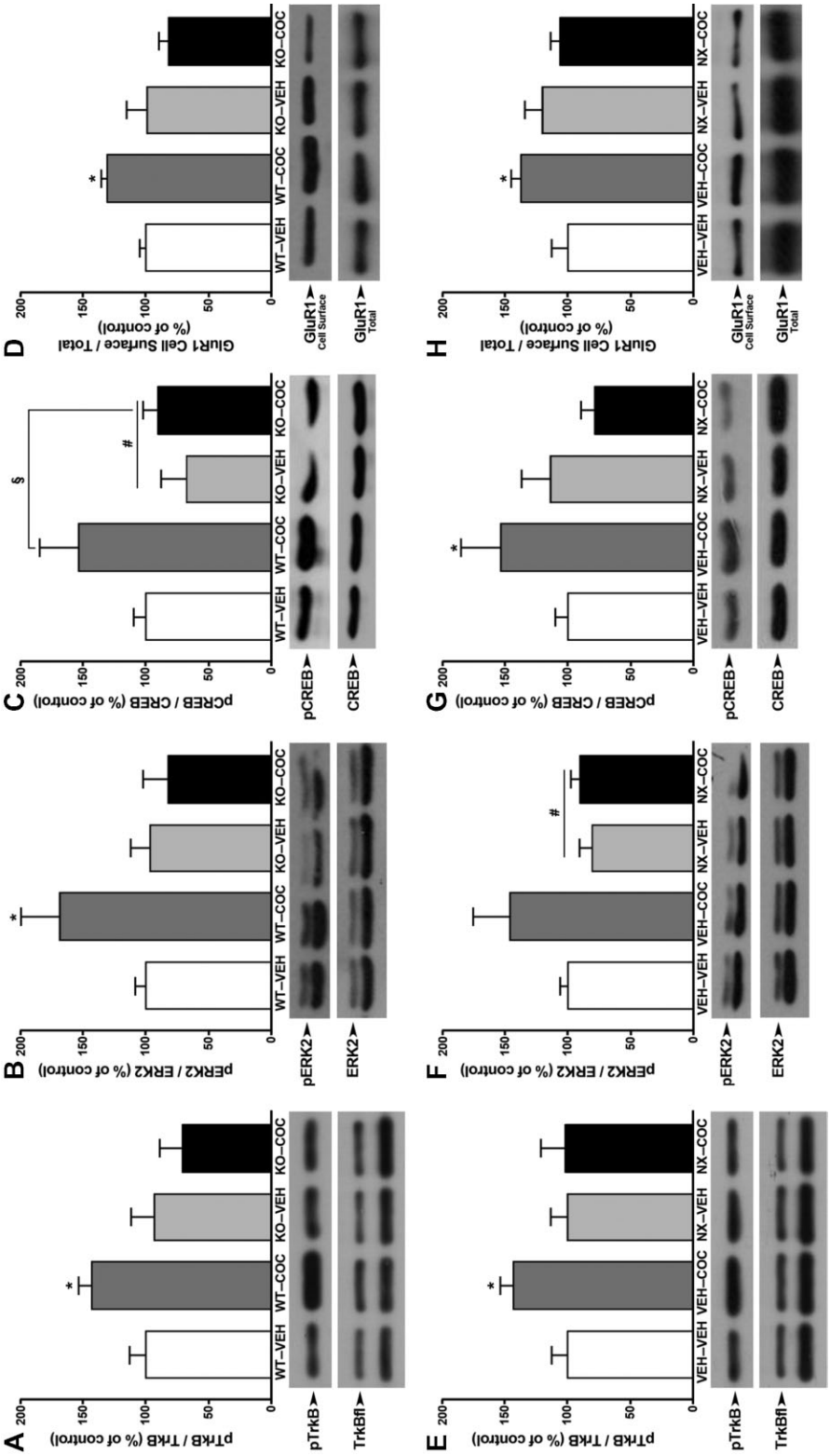


**Figure 4** Preproenkephalin knockout (KO) and naloxone pre-treated mice failed to show cocaine-induced increases in pTrkB, pERK2, pCREB and AMPA cell surface expression levels in the nucleus accumbens (NAc) associated to repeated cocaine administration. At the beginning of the treatment, all groups received nine daily injections of cocaine. Naloxone pre-treated-mice were administered with naloxone [1 mg/kg subcutaneous (s.c.)] or vehicle before each cocaine injection. Animals were sacrificed at day 21 of treatment, 30 minutes before receiving a 7.5 mg/kg cocaine challenge. (a and d) NAc pTrkB levels were not modified in KO and naloxone-pre-treated mice treated chronically with cocaine (WT–VEH $n = 4$–5, WT–COC $n = 4$, KO–VEH $n = 3$, KO–COC $n = 4$–5 and VEH–VEH $n = 4$, VEH–COC $n = 4$, NX–VEH $n = 4$, NX–COC $n = 4$ animals/group). KO and naloxone pre–treated animals did not show an increase in pERK (b and f) or pCREB (c and g) associated with repeated cocaine administration (WT–VEH $n = 4$–5, WT–COC $n = 4$–5, KO–VEH $n = 5$–6, KO–COC $n = 5$–6 and VEH–VEH $n = 4$–5, VEH–COC $n = 4$–5, NX–VEH $n = 5$–6, NX–COC $n = 5$–6) d and h). Cocaine-induced increase in AMPA cell surface expression was not evidenced in NAc from KO and naloxone pre-treated mice (WT–VEH, $n = 4$; WT–COC, $n = 4$; KO–VEH, $n = 4$; KO–COC, $n = 4$ and VEH–VEH $n = 4$, VEH–COC $n = 4$, NX–VEH $n = 4$, NX–COC $n = 4$–6). Histograms depict pTrkB, pERK2, pCREB and GluR1 in the cell surface (beads eluent) levels normalized to total TrkB, ERK2, CREB and GluR1 (lysate) levels, respectively (pools of two mice in each experiment). Data are presented as mean ± standard error of the mean (SEM) percentage of WT–VEH or VEH–VEH *$P < 0.05$ compared with KO or NX–COC group §$P < 0.05$ indicates significant difference comparing VEH vs. COC (treatment); #$P < 0.05$ indicates significant difference comparing WT vs. KO (genotype) (Fisher *post hoc* test)

from the vehicle–cocaine and naloxone–cocaine groups compared with the vehicle–vehicle group (Supporting Information Fig. S3). No changes in BDNF levels were observed in dorsal striatum (Supporting Information Fig. S2).

As shown in Fig. 4f and g, we found that naloxone prevented the cocaine-induced increases in pERK2 (pre-treatment $F_{(1,16)} = 7{,}52$; $P < 0.05$) and pCREB (interaction $F_{(1,16)} = 4{,}97$; $P < 0.05$), respectively. Fisher *post hoc* comparisons revealed a difference in pCREB levels between vehicle-cocaine group compared with naloxone–cocaine group ($P < 0.05$).

Finally, naloxone pre-treatment was also able to abrogate the increased AMPAR cell surface expression in mice chronically treated with cocaine (interaction $F_{(1,12)} = 4{,}851$; $P < 0.05$) (Fig. 4h). *Post hoc* comparisons revealed a significant difference between the vehicle–cocaine and naloxone–cocaine groups ($P < 0.05$).

## DISCUSSION

This study demonstrates that enkephalin is essential for the development of neuroadaptive changes leading to cocaine-induced psychomotor sensitization. We found that mice lacking the proenkephalin gene do not develop sensitization to the stimulating properties of cocaine on locomotor activity and on dopamine release in the NAc. Furthermore, pivotal neuroadaptation such as the increase in pTrkB receptor (Crooks *et al.* 2010), ERK/CREB transcriptional activity (Mattson *et al.* 2005; Kim *et al.* 2011) and AMPAR GluR1 surface expression (Boudreau *et al.* 2007), all related to sensitized responses were absent in the NAc of KO mice. Consistently, a persistent increase of met-enkephalin immunoreactivity was observed within the NAc of cocaine-treated WT mice as well as a full abrogation of cocaine-induced behavioral and neuronal plasticity after naloxone pre-treatment.

The fact that the acute stimulating properties of the drug on motor activity and dopamine release within the NAc were not modified in KO mice, evidences an explicit role of enkephalin in establishing the long-lasting neuroadaptations within the NAc that underlie the expression of cocaine sensitization.

Chronic cocaine-induced enhancement of ERK signaling, which has been shown to contribute to persistent activation of CREB and of CREB-mediated gene expression (Mattson *et al.* 2005), was consistently confirmed in the NAc of WT and vehicle pre-treated animals. The proenkephalin gene is CREB-regulated (Monnier & Loeffler 1998), thus psychostimulant drugs that modulate CREB signaling also influence proenkephalin mRNA and the opioid peptide met-enkephalin throughout the mesolimbic system (amphetamine: Assis *et al.* 2009; Smith & McGinty 1994; cocaine: Crespo *et al.* 2001). The proenkephalin gene has been implicated in several forms of drug- and stress-induced behavioral effects. Pharmacologic studies utilizing delta and MOPr antagonists, as well as proenkephalin or MOPr KO mice, have elegantly shown the involvement of the endogenous opioid system in dopamine-related behaviors (Sala *et al.* 1995; Heidbreder *et al.* 1996; Magendzo & Bustos 2003; Hummel *et al.* 2004). As a prominent role has been attributed to met-enkephalin and MOPr within ventral tegmental area (VTA) in the initial step of sensitization (Kalivas *et al.* 1983; DuMars *et al.* 1988; Soderman & Unterwald 2008), and considering the abrogating effect of naloxone on the cocaine-induced behavioral plasticity observed in the NAc in this study, it is likely that MOPr and met-enkephalin are important players not only in the dopaminergic brain areas related to development of sensitization, but also in those underpinning the expression of sensitization to cocaine.

In good agreement with Berrendero *et al.* (2005), we found that the lack of proenkephalin does not affect dopamine basal levels, indicating that enkephalin does not tonically regulate dopamine levels in the NAc. However, there is a profound effect on cocaine-induced dopamine sensitization. It has been demonstrated that enkephalin and its receptors facilitate dopamine release at the NAc level (Spanagel, Herz & Shippenberg 1992; Hirose *et al.* 2005), through an indirect mechanism affecting GABAergic or cholinergic transmission (Jiang & North 1992; Brundege & Williams 2002). Moreover, opioid receptors are involved in the release and actions of glutamate at the level of the NAc (Rawls & McGinty 2000), suggesting that enkephalin may also facilitate glutamate transmission in this brain area. In contrast to the actions of enkephalin on the dopaminergic transmission, the endogenous opioid peptide dynorphin inhibits dopamine release in the NAc by binding to kappa opioid receptors (KOPr) on the terminal buttons of dopamine neurons (Spanagel, Herz & Shippenberg 1990). This was also confirmed in the KOPr KO mice, which have higher basal levels of dopamine in the NAc compared with WTs (Chefer *et al.* 2005). Consistently with our current findings, studies using prodynorphyn KO mice found an enhancement of cocaine-induced locomotor sensitization (Bailey *et al.* 2007). Regarding MOPr and DOPr, pharmacologic studies have demonstrated that these receptors contribute to the cocaine-induced elevation in dopamine release in the NAc (Van Ree *et al.* 2000; Shippenberg & Chefer 2003). However, genetic manipulation of MOPr demonstrated no significant influence of this receptor on cocaine-induced dopamine elevations, whereas the ability of cocaine to increase dopamine levels was reduced in DORr KO mice (Chefer, Kieffer & Shippenberg 2004). Notwithstanding, there are studies demonstrating a decrease in the locomotor stimulating

and sensitizing effects of cocaine in mice lacking MOPr (Chefer *et al.* 2004; Hummel *et al.* 2004), but there are no published studies evaluating cocaine-induced behavioral sensitization in DOPr KO mice. Although other evidence indicate no significant effect of MOPr deletion on cocaine-induced sensitization, it is necessary to address that these data were obtained after short-term cocaine withdrawal periods (Lesscher *et al.* 2005). Related to this, there is evidence demonstrating that naloxone blockade of the expression of behavioral sensitization to psychostimulants is observed after long-, but not short-term withdrawal (Magendzo & Bustos 2003), clearly suggesting that MOPr would be involved in the long-lasting effects induced by psychostimulants. Thus, given the modulatory role on cocaine-induced behavioral effects exerted by MOPr within the VTA–NAc (Soderman & Unterwald 2008), and the dose of naloxone used (Gutstein & Akil 2001), the facilitatory role of enkephalin on cocaine-induced long-term behavioral and neuronal plasticity evidenced in this study could be attributed mainly to their action on MOPr. Supporting this hypothesis, there is evidence demonstrating that MOPr agonistic activity induced persistent neuronal changes by modulating AMPAR expression at hippocampal synapsis from animals expressing context-dependent behavioral sensitization (Xia *et al.* 2011). In addition, opiate withdrawal from chronic morphine-induced ERK/CREB and GluR1 AMPAR subunit phosphorylation in brain areas related to the reward pathway (Haghparast *et al.* 2014). This evidence would address the importance of a common molecular mechanism mediated by the endogenous MOPr/enkephalin system to induce the neuronal plasticity that underlies the long-term behavioral effects of opiates and psychostimulants. Notwithstanding, considering that there is some pharmacologic evidence that the DORr is involved in the development of cocaine-induced sensitization (Heidbreder *et al.* 1996), we cannot rule out that enkephalin, through its agonistic activity on DOPr, could also contribute to its facilitatory effect on cocaine-induced behavioral and neuronal plasticity.

Related to this, ERK signaling pathway activation by acute or chronic psychostimulant treatment has been independently attributed to stimulation of D1 dopamine, glutamate and MOPr/DOPr (Asensio, Miralles & García-Sevilla 2006), as well as the activation of TrkB receptor by BDNF. These molecular events seem to govern the expression of psychostimulant-related behaviors (Kim *et al.* 2011). CREB is an important downstream mediator for, among others, BDNF. Elegant evidence has implicated BDNF/TrkB signaling in neuroadaptations within the NAc, VTA and Pfc underlying behavioral processes such as cocaine sensitization and reinstatement (McGinty, Whitfield & Berglind 2010). An increase in BDNF and the activated form of its receptor, TrkB, was shown in the NAc after cocaine exposure (Crooks *et al.* 2010). We also found an increase in BDNF/pTrkB levels within the NAc following cocaine in WT and vehicle pre-treated animals, but this effect was not observed in KO and naloxone pre-treated mice administered chronically with the drug. In this context, the lack of activation of ERK2/CREB signaling pathway in the NAc of KO and naloxone-pre-treated mice could be attributed to the absence of cocaine-induced increases in BDNF/pTrkB levels in this brain area as well as the lack of dopamine sensitization in KO mice.

Previous studies found that enhanced activation of TrkB in dopamine axon terminals within the NAc may promote increased release of this monoamine, contributing to cocaine-induced behavioral changes (Bahi *et al.* 2008). Elevated BDNF is associated with enhancement of dopamine neurotransmission (LeFoll & Diaz 2005), motor activation and goal-directed behavior, while reduced activity-dependent BDNF/TrkB signaling within the VTA–NAc circuit may result in an attenuated ability of cocaine to induce addiction-promoting pathologic changes in the NAc (Lobo *et al.* 2010). Consistently, the lack of dopamine sensitization, as well as of a cocaine-induced increase in BDNF/TrkB signaling, identified in KO and naloxone pre-treated mice buttresses an important role of both enkephalin and BDNF on dopamine-sensitized behaviors. There is evidence supporting the role of AMPAR in the expression of cocaine-induced behavioral sensitization and drug-seeking (Suto *et al.* 2004; Boudreau *et al.* 2007). Interestingly, Li & Wolf (2011) showed a close relationship between changes in BDNF and the insertion of AMPAR in the NAc following chronic cocaine. Related to this, it was also shown that ERK phosphorylation contributes to the increase of AMPAR surface expression in the NAc following chronic cocaine (Boudreau *et al.* 2007). These results are consistent with our current findings showing lack of ERK phosphorylation and cocaine-induced increases in AMPAR cell surface within the NAc from KO mice, as well as the absence of cocaine-induced upregulation of BDNF/TrkB signaling in this brain area.

In conclusion, our findings demonstrate an essential role of proenkephalin-derived opioid peptides and opioid receptors in cocaine-induced behavioral sensitization and long-lasting associated molecular changes. We showed for first time that the loss of the proenkephalin gene as well as the pharmacologic opioid-receptor antagonism, are fully effective to abrogate the activation of the ERK/CREB transcriptional pathway, pTrkB levels and AMPAR cell surface expression in NAc in response to chronic cocaine treatment. Thus, our study provide insights in the neurobiological basis that explain the clinical utility of opiate antagonists in treating cocaine addiction (Schmitz *et al.* 2009; Comer *et al.* 2013; Mooney *et al.* 2013).

 

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**Disclosure**

All authors reported no biomedical financial interests or potential conflicts of interest

**Authors Contribution**

LMC designed the research and provided the funds; BMB, EZ, CG-K, MAA, MV performed the research; BMB, EZ, CGK, DHM and LMC analyzed the data; AZ provided the KO mice, revised the article and consulted on data analysis; BMB and LMC wrote the article.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Enkephalin elevations in animals treated chronically with cocaine. Left panel: Photomicrographs (20×) of met-enkephalin immunoreactivity in dorsal striatum from mice treated daily with cocaine during nine days and challenged with the drug 21 days after the beginning of the treatment. Scale bar represents 100 μm. Right panel: quantification of positive met-enkephalin cells (number of positive cells/0.35 $mm^2$) from the different treatment groups (VEH–VEH $n = 5$–6; VEH–CO $n = 5$–6; CO–VEH $n = 6$; CO-CO $n = 6$). Data are represented as mean ± standard error of the mean (SEM) (expressed as the percentage of VEH–VEH). Two-way ANOVA evidenced a significant effect in the treatment in dorsal striatum ($F_{(1,14)} = 25{,}65$ $P < 0.01$). $^*P < 0.01$ indicates difference comparing treatment groups (vehicle and cocaine).

**Figure S2.** Proenkephalin KO and naloxone pre-treated mice failed to show cocaine-induced increase in BDNF levels. All groups received nine daily injections of 15 mg/kg i.p. cocaine or vehicle. Naloxone pre-treated-animals were administered with naloxone (1 mg/kg s.c.) or vehicle before each cocaine injection. Animals were sacrificed at day 21 from starting the treatment, 30 minutes before receiving a 7.5 mg/kg cocaine challenge KO and naloxone pre-treated mice chronically treated with cocaine showed no increase in BDNF levels in NAc (a, b, e, f).No significant change was seen in BDNF levels in the dorsal striatum from the different experimental groups (c, d, g, h).(WT–VEH $n = 3$, WT–COC $n = 4$, KO-VEH $n = 3$, KO-COC $n = 4$ and VEH–VEH $n = 3$, VEH–COC $n = 4$, NX–VEH $n = 3$, NX–CO $n = 4$). Photomicrographs show BDNF expression intensity in the different brain areas. Insert shows a digital magnification of the original photo. Image was pseudo-colored to highlight fluorescence intensity differences between treatments. Scale bar: 100 um. ac = anterior commissure. $^*P < 0.05$ compared with WT–VEH/VEH (Fisher *post hoc* test).

**Figure S3.** Increased BDNF levels in the Pfc of KO (a and b) and naloxone pre-treated mice (c and d) chronically exposed to the drug was observed (WT–VEH $n = 3$, WT–COC $n = 4$, KO–VEH $n = 3$, KO–COC $n = 4$ and VEH–VEH $n = 3$, VEH–COC $n = 4$, NX–VEH $n = 3$, NX–CO $n = 4$). Photomicrographs show BDNF expression intensity in the different brain areas. Insert shows a digital magnification of the original photo. Image was pseudo-colored to highlight fluorescence intensity differences between treatments. Scale bar: 100 um. ac = anterior commissure. $^*P < 0.05$ compared with WT–VEH/VEH (Fisher *post hoc* test).

**Figure S4.** Alterations in pTrkB, pERK2, pCREB and AMPA cell surface expression levels in the dorsal striatum from KO mice and animals pre-treated with naloxone. All groups received nine daily injections of 15 mg/kg i.p. cocaine or vehicle. Naloxone–pre-treated animals were administered with naloxone (1 mg/kg s.c.) or vehicle before each cocaine injection. Animals were sacrificed at day 21 from starting the treatment, 30 minutes before receiving a 7.5 mg/kg cocaine challenge. a and d) Dorsal striatum pTrkB levels were not modified in KO and naloxone-pre-treated mice treated chronically with cocaine (WT–VEH $n = 4$–5, WT–COC $n = 4$, KO–VEH $n = 3$, KO–COC $n = 4$–5 and VEH–VEH $n = 4$, VEH–COC $n = 4$, NX–VEH $n = 4$, NX–COC $n = 4$ animals/group) KO and naloxone pre-treated mice chronically treated with cocaine showed no increase in pERK2 levels in dorsal striatum. (b and f) pCREB levels were not modified in dorsal striatum from KO mice chronically treated with cocaine (C). Naloxone pre-treatment was not able to prevent cocaine-induced increases in pCREB levels (g). (WT–VEH $n = 4$–5, WT–COC $n = 4$–5, KO–VEH $n = 5$–6, KO–COC $n = 5$–6 and VEH–VEH $n = 4$–5, VEH–COC $n = 4$–5, NX–VEH $n = 5$–6, NX–COC $n = 5$–6 animals/group). d and h) Cocaine-induced increase in AMPA cell surface expression was not evidenced in dorsal striatum from KO. Naloxone pre-treatment was not able to prevent cocaine-induced increases in AMPAR cell surface expression (WT–VEH, $n = 4$; WT–COC, $n = 4$; KO–VEH, $n = 4$; KO–COC, $n = 4$ and VEH–VEH $n = 4$, VEH–COC $n = 4$, NX–VEH $n = 4$, NX–COC $n = 4$–6 animals/group). Histograms depict the level of pTrkB, pERK2, pCREB and GluR1 in the cell surface (beads eluent) normalized to total TrkB, ERK2, CREB and GluR1 (lysate) levels, respectively (pools of two mice in each experiment). Data are presented as mean ± standard error of the mean (SEM) percentage of WT–VEH or VEH–VEH $^*P < 0.05$ compared with KO or NX–COC group §$P < 0.05$ indicates significant difference comparing VEH vs. COC (treatment); #$P < 0.05$ indicates significant difference comparing WT vs. KO (genotype) or VEH and NX (pre-treatment) (Fisher *post hoc* test).

**Figure S5.** Representative coronal section (40 μm) of mouse brain stained with cresyl violet illustrating the placement of the probe in the NAc and a diagram showing the representative probe placements between bregma 1.34 and 0.86 mm. Scale bar: 1 mm. The dashed lines represent cannula placements in vehicle group, and solid lines depict placements in cocaine group.

**Appendix S1.** Materials and Methods