Conditioned aversive memory associated with morphine withdrawal increases brain derived neurotrophic factor in dentate gyrus and basolateral amygdala

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ABSTRACT

Morphine has been shown to increase the expression of brain-derived neurotrophic factor (BDNF) in the brain. However, little is known about the effect of conditioned naloxone-precipitated morphine withdrawal on BDNF and its precursor protein, proBDNF. We used the conditioned-place aversion (CPA) paradigm to evaluate the role of corticotropin-releasing factor (CRF)/CRF1 receptor signalling on the BDNF expression and corticosterone plasma levels after CPA expression and extinction. Male mice were rendered dependent on morphine and injected acutely with naloxone before paired to confinement in a naloxone-associated compartment. The expression of BDNF and proBDNF in the dentate gyrus (DG) and basolateral amygdala (BLA) was measured in parallel with the corticosterone plasma levels with and without CRF1 receptor blockade. Mice subjected to conditioned naloxone-induced morphine-withdrawal showed an increased expression of BDNF (in DG and BLA) in parallel with an enhancement of corticosterone plasma levels. These results demonstrated that BDNF expression together with the increased activity of hypothalamic-pituitary-adrenocortical (HPA) axis are critical to the acquisition of aversive memory. However, we have observed a decrease in corticosterone plasma levels and BDNF expression after CPA extinction reaffirming the importance of BDNF in the maintenance of aversive memory. In addition, the pre- treatment with the CRF1 receptor antagonist CP-154,526 before naloxone conditioning session impaired morphine withdrawal-induced aversive memory acquisition, the increased corticosterone plasma levels and the expression of BDNF observed after CPA expression in the DG and BLA. Altogether present results suggesting a clear connexion between HPA axis and BDNF in the formation and extinction of aversive memory.

Key words

Brain-derived neurotrophic factor (BDNF), hypothalamic-pituitary-adrenocortical (HPA) axis, corticotropin-releasing factor (CRF) 1 receptor, conditioned place aversion (CPA), aversive memory acquisition and extinction.

INTRODUCTION

Conditioned place aversion (CPA) has been widely used to assess dysphoric or aversive aspects of withdrawal (Tzschentke, 2007). In rodents, the negative affective component of opioid dependence could be reflected by CPA, in which opioid withdrawal is paired with a particular environment, which triggers the association between negative affective consequences of withdrawal with context. When the animals are re-exposed to the paired environment in a drug-free state, they avoid the paired environment due to the association between the context and aversive effects of drug withdrawal. The aversive memory associated with drug withdrawal can evoke motivational and/or emotional states that lead to compulsive drug taking (Koob, 2000; Hutcheson et al., 2001). Extinction of such memory has been proposed as a therapeutic strategy for the treatment of drug addiction (Barad, 2005; Davis et al., 2006; Hofmann et al., 2006). Among the limbic structures that are likely to mediate these components of drug addiction, the basolateral amygdala (BLA) and the hippocampus represent critical neural substrates (Frenois et al., 2005; Lucas et al., 2008). It has been stablished that the hippocampus is crucially important for memory encoding/consolidation but also for episodic memory retrieval. In this context, much experimental evidence has demonstrated that the granular zone of the hippocampal dentate gyrus (DG) has a critical role in learning and memory function (Kee et al., 2007; Aimone et al., 2011;). In addition, the BLA is correlated with the acquisition and recall of opiate-related associative memories (Frenois et al., 2005), indicating a critical role for this area during opioid-related learning and memory (Fuchs and See, 2002).

Most commonly abused drugs share a common action, they stimulate the hypothalamic-pituitary-adrenocortical (HPA) axis when there is a withdrawal through the activation of corticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus,

resulting in a strong release of adrenocorticotropic hormone (ACTH) and corticosterone (Ueno et al., 2011). Corticosterone mediates somatic and negative affective-like components of withdrawal (Contarino and Papaleo, 2005; Harris and Aston-Jones, 2007; Papaleo et al., 2007; Koob, 2008). It also been reported that brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, regulates neuron synaptic plasticity and play a vital role in learning and memory in multiple brain areas (Linnarsson et al., 1997; Hall et al., 2000; Tokuyama et al., 2000; Santi et al., 2006; Rex et al., 2007). Furthermore, increasing evidence shows that BDNF can modulate glucocorticoid receptor signalling and HPA axis function in depression through a crosstalk mechanism (Kunugiet al., 2010; Lambert et al., 2013; Egeland et al., 2015). However, the possible relationship between HPA axis and BDNF in the formation and extinction of aversive memories associated with conditioned morphine withdrawal remains not understood. Therefore, we studied the role of HPA axis and BDNF in modulating morphine-withdrawal-induced aversive memories and its extinction. In addition, we have evaluated the role of CRF/CRF1 receptor signalling (by injected CP-154,526, a selective CRF1R antagonist) in the BDNF expression and corticosterone plasma levels after CPA expression and extinction in order to establish a possible connexion between HPA and BDNF during conditioned morphine withdrawal.

MATERIAL AND METHODS

Subjects

Male Swiss mice weighing 25-30 g from the Animal Facilities of the University of Murcia were housed 4–6/standard cage in a temperature-controlled environment, received ad libitum access to food and water and were maintained on a 12-h/12-h light/dark cycle. Mice were habituated to the testing room for at least 1 week prior to the experimental manipulations. All animals received humane care according to the guidelines provided by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and were approved by Comité Ético de Experimentación Animal (CEEA, Universidad de Murcia, RD 53/2013).

Drug treatment

Mice were randomly divided into two groups: chronic saline-treated and chronic morphine-treated mice. Morphine was injected i.p. in the mice's home-cages with a chronic escalating-dose regimen, every 12 h (at 8 am and 8 pm), starting on day 1, 10 mg/kg; day 2, 30 mg/kg; day 3, 50 mg/kg and day 4, 60 mg/kg (only one injection in the morning). This pattern of morphine administration, which involves ascending drug doses, has been used extensively to study opioid tolerance and dependence (Liang et al., 2006, 2007; Goeldner et al., 2011; Ziólkowska et al., 2012). The doses of morphine were selected based in previous studies form our laboratory (Almela et al., 2012; Valero et al., 2018). The chronic saline-treated groups were administered with saline (i.p.) using the same protocol. To determine the effect of CRF 1 receptor on morphine withdrawal aversive memories, other set of mice were acutely pretreated with the selective CRF1 receptor antagonist CP-154,526 [N-butyl-N-ethyl-2,5-dimethyl-7-(2,4,6-trimethylpheny) pyrrolo [3m2e]pyrimidin-4-amine] (30 mg/kg i.p.) 30 min before the acute administration of naloxone.

Naloxone-induced conditioned place aversion is a recognized paradigm of negative affective learning.

The present CPA procedure induces place aversion when the animals relate the environment with the negative effects of morphine withdrawal syndrome (García-Carmona et al., 2012). The CPA equipment used in this study was composed by two identical boxes with three polyvinylcarbonate (PVC) chambers (Valverde et al., 1996) connected to a computer. Two large side chambers (20 length, 18 cm width and 25 cm height) were separated by a smaller chamber (20 cm, length 7 cm width and 25 cm height). The two larger chambers differed in their wall paint and floor texture (i.e., grey striped wall with black smooth floor or black spotted wall with grey rough floor, respectively) and provided distinct contexts that were paired to naloxone injections. Three distinct chambers were separated by manual guillotine doors which were removed during the test. The CPA training procedure was used in previous experiments (García-Carmona et al., 2012; Gómez-Milanés et al., 2012). The protocol consisted of five phases: pre-test, drug treatment, conditioning, post-test and extinction. In the pre-test (day 0), mice were placed in the middle chamber and allowed to shuttle between the three chambers in the apparatus for 15 min. The time spent in each chamber was recorded, and the animals that spent less than 360 s in either chamber were considered not to be neutral in preference for either side and were excluded from further study (n = 1). During days 1–4, animals were treated with morphine or saline as described above. To study the role of CRF1 receptor in the acquisition of opioid withdrawal-associated memories, on day 4 and 90 min after the last morphine injection, CP-154,526 (30 mg/kg i.p.) or its vehicle (Tween 80, 10%) were acutely injected, and 30 min later naloxone was administered subcutaneously (s.c.) at a dose of 1 mg/kg, in order to precipitate the morphine withdrawal syndrome, and mice were immediately confined to one of the chambers during 18 min. On day 5, CPA expression was tested in a drug-free state (post-test). The testing

procedure was the same that previously for the pre-test. CPA score represents the time spent in the drug naloxone-paired chamber during the testing phase minus that recorded during the preconditioning phase.

Extinction of conditioned place aversion

Briefly, extinction training began 24 h after the post-training test. CPA extinction was performed for 12 consecutive days (day 6–12) under identical conditions that pre-test and post-test.

Radioimmunoasay

Sixty min after the end of the post-test or the CPA extinction, mice were decapitated at the same time (10:00–11:00 h) and blood samples were collected. Plasma corticosterone concentrations were measured by using commercially available kit for mice (125 I-corticosterone radioimmunoassay; MP Biomedicals, USA). The sensitivity of the assay was 7.7 ng/ml for corticosterone.

Preparation of tissue extract

Sixty min after the end of the post-test or the CPA extinction, mice were decapitated at the same time (10:00–11:00 h) and sacrificed by decapitation and the brains were rapidly removed and stored at -80 °C for Western blot analyses. Brains were sliced on a cryostat and kept at −20 °C until each region of interest comes into the cutting plane. For dentate gyrus (DG) and basolateral amigdala (BLA) study, three consecutive 500 µm coronal slides were made corresponding to an area between -1.34 and -2.18 mm (DG) or -0.58 and -1.58 (BLA) posterior to the Bregma according to the mice brain atlas of Franklin and Paxinos, 2008. Tissues of interest were dissected using a punch with a 1-mm internal diameter. Punches of the DG and BLA were collected in Eppendorf tubes, according to the method of Leng, Feldon and Ferger (2004).

Electrophoresis and Western blotting

Punches from DG and BLA were placed in homogenization buffer. Samples were sonicated, vortexed and sonicated again prior to centrifugation. Each sample, which contained equal quantities of total proteins (20 µg), was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), which were blocked in 1% bovine serum albumin for 60 minutes at room temperature (RT). Incubations with the primary antibodies were made at 4 °C overnight: anti-pERK 1/2 (1:750, Santa Cruz Biotechnology); rabbit polyclonal anti-pCREB (1:750, Millipore), rabbit polyclonal anti-precursor form of BDNF (proBDNF, 1:250, Santa Cruz Biotechnology); and rabbit polyclonal anti-mature BDNF (BDNF, 1:250, Santa Cruz Biotechnology). Goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked (1:5000, Santa Cruz Biotechnology) or goat anti-rat IgG HRP-linked (1:5000, Santa Cruz Biotechnology) were used as secondary antibodies. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent Western blot detection system (ECL Plus, GE Healthcare, LittleChalfont, Buckinghamshire, UK) and visualized by a ImageQuant LAS 500 imager (GE Healthcare). Blots were incubated with stripping buffer (glycine 25 mM, SDS 1%, pH 2) for 1 hour at 37 °C and subsequently reblocked and probed with rabbit polyclonal antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; #2118, Cell Signaling Technology Inc.) or total (t) ERK (1:1000 dilution; sc-135900, Santa Cruz Biotechnology), which were used as loading control. The ratios pERK1/tERK, pERK2/tERK, pCREB/GADPH, proBDNF/GADPH and BDNF/GADPH were plotted

Drugs and reagents

Morphine HCl (Alcaliber, Madrid, Spain) and naloxone HCl (Sigma Chemical, St. Louis, MO, USA), were dissolved in physiological saline and all injections were administered in a volume of 0.01 ml/g body weight. CP-154,526 [N-butyl-N-ethyl-2,5-dimethyl-7-(2,4,6-trimethylpheny) pyrrolo [3m2e]pyrimidin-4-amine] (selective CRF1 receptor antagonist), was kindly provided by Pfizer (New York, NY) and was dissolved in Tween-80 (10%, Sigma-Aldrich).

Statistical analyses

Data is expressed as mean ± standard error of the mean (SEM). Hormonal plasma concentrations and proteins expression after CPA were analyzed by one-way analysis of variance (ANOVA) and Newman-Keuls test for multiple comparisons. Unpaired Student's t test was used when comparisons were restricted to two experimental groups. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Differences with a $P<0.05$ were considered significant.

RESULTS

CPA to morphine withdrawal

The sequence of events during CPA assay can be conceptualized as follows: 1) The morphine-dependent mice were administered with naloxone and develop naloxone-induced precipitated morphine withdrawal (unconditioned stimulus); 2) animals experienced a subjective aversive dysphoric effect (unconditioned response); 3) mice learnt to associate this unconditioned response with a specific environmental context (conditioned stimulus); and 4) when subsequently presented with conditioned stimulus the animals exhibited avoidance behaviour (conditioned response) to the earlier withdrawal-paired conditioned stimulus. The animals treated with saline+CP-154,526+naloxone did not show significant differences in score when compared with chronic saline-treated mice injected with saline+vehicle+naloxone (data not shown). According to previous studies (Garcia-Carmona et al., 2015a), present results showed that naloxone administration to mice dependent on morphine evoked an increased in body weight loss (0.79 ± 0.04 g) compared with the control group receiving saline instead morphine (0.12 ± 0.06 g; t_{14}=8.369, *P*<0.0001; Student t test).

One-way ANOVA examined the effects of morphine on place aversion induced by naloxone-precipitated morphine withdrawal. We observed significant effects of treatment (*F* (2,42) = 16.42, *P*< 0.0001). The Newman-Keuls *post hoc* test showed that naloxone induced a significant (*P* < 0.001) place aversion in the morphine-treated groups versus the saline-treated mice. (Fig. 1A). There was a significant difference between the scores exhibited by morphine dependent mice receiving vehicle before naloxone versus those presented by mice receiving the CRF1 receptor antagonist before naloxone (t_{27}=5.034, P<0.0001; Student t test) (Fig. 1A), indicating that the blockade of CRF1 receptor before conditioning prevented the avoidance behaviour seen during naloxone-induced withdrawal. We examined extinction of CPA score in withdrawn mice

for aversion from day 6 to 11. The aversion score is shown in figure 2B. After day 11 all the animals extinguished their aversion (Fig. 1B).

Corticosterone plasma levels

We measured plasma corticosterone levels after naloxone-induced CPA expression and after CPA extinction (Fig. 2). One-way ANOVA for corticosterone plasma levels after CPA expression revealed a significant effect of treatment ($F_{(2,24)} = 6.570$, $P<0.0053$). As shown in figure 2, naloxone induced an increase ($P < 0.01$) in corticosterone plasma levels after CPA expression in morphine-treated mice versus the saline+naloxone group. CP-154,526 administration to mice treated chronically with saline and injected with naloxone showed a decrease in the corticosterone plasma levels versus the group injected with vehicle instead CP-154,526 ($F_{18}=3.006$, $P=0.0076$, student t test) (Fig. 2A). Regarding corticosterone after CPA extinction, unpaired t test revealed significant effect of treatment ($t_{14}=4.908$, $P=0.0002$; Student t test). Extinct morphine-withdrawn mice showed a decreased ($P < 0.001$) in corticosterone plasma levels versus the same mice treated with saline (Fig. 2B).

Changes in the expression of pERK 1/2, pCREB, proBDNF and BDNF in the DG and BLA after CPA expression

Figures 3 and 4 show the effects of CPA on ERK 1/2, pCREB, pro BDNF and BDNF expression in the DG and BLA nuclei. In the DG one-way ANOVA for pERK1 and pERK2 expression revealed a significant effect of the treatment ($F(2,11)=11.05$, $P=0.0023$) and ($F(2,23)=19.76$, $P<0.0001$). A Newman-Keuls post-hoc analysis revealed that pERK1 and PERK2 expression in the DG was decreased ($P<0.05$ and $P<0.01$, respectively) after CPA expression compared with their controls (Fig. 3). On the other hand, one-way ANOVA for pCREB and proBDNF expression did not reveal significant differences ($F(2,15)=0.4317$, $P=0.6572$ and $F(2,12)=1.129$, $P=0.3555$, respectively) (Fig.

3). However, one-way ANOVA for BDNF revealed a significant effect of treatment (F(2,12)=20.39, P<0.0001). A post-hoc test revealed an increased (p<0.01) expression of BDNF after CPA expression versus control groups. The administration of CRF1 receptor antagonist blocked the increased expression of BDNF showed after CPA expression in naloxone withdrawn mice (t_9 =7.862, *P*<0.0001, student t test) (Fig. 3). Similar results were obtained in BLA. Thus, the one-way ANOVA showed a significant effect of treatment on ERK 1 and ERK 2 (F(2,15)=8.610, *P*=0.0032; F(2,15)=6.436, *P*=0.0096, respectively), without no significant effects on pCREB and proBDNF (F(2,12)=0.06061, *P*=0.9415; F(2,12)=0.3254, *P*=0.7284). However, there is a significant effect of treatment on BDNF (F(2,13)=14.10, P=0.0006). The Newman-Keuls post hot test showed a significant (*P*<0.001) enhancement of BDNF after naloxone-induced morphine withdrawal versus its control group. The BDNF expression was significantly reduced after CP-154,526 administration (t_{10}=5.134, *P*=0.0004; Student t test) (Fig. 4)

Changes in the expression of pERK 1/2, pCREB, proBDNF and BDNF in the DG and BLA after CPA extinction

As can be seen in Fig. 5 there were not changes in the pCREB or proBDNF expression after CPA extinction in morphine withdrawn animals (t_6=1.210, *P*=0.2720 and t_6=0.5459, *P*=0.6048, respectively; Student t test) in the DG. However, the group treated chronically with morphine and injected with vehicle+naloxone showed a significant decrease in the ERK 1/2 (ERK1: t_{10}=4.242, P=0.0017; ERK2: t_8=2.518, *P*=0.0359; Student t test) and BDNF expression (t_{10}=4.242, P=0.0017; Student t test) when compared with the group treated chronically with saline.

Concerning to BLA we have obtained similar results, no changes in the pCREB or proBDNF expression (t_6=0.4427, *P*=0.6735; t_6=0.04395, *P*=0.9664, respectively; Student t test) and a

decrease in the expression of ERK1, ERK2 and BDNF (ERK1: t_8=2.677, *P*=0.0281; ERK2: t_8=3.068, P=0.0154; BDNF: t_6=3.032, *P*=0.0230; Student t test) (Fig. 6).

DISCUSSION

It is commonly accepted that affective drug withdrawal symptoms have a major motivational significance in contributing to relapse and continued drug use; thus, it is important to understand the mechanisms that mediate affective behaviours during morphine withdrawal. A previous work has suggested that morphine associated with negative affective states and place aversion to previous neutral environmental stimuli, could represent a motivational component in the maintenance of drug abuse (Budzynska et al., 2012). In the present study, we further investigated the mechanism underlying the aversive memory associated with CPA expression and extinction in morphine-withdrawn mice. Our data demonstrated that naloxone injection into morphine-treated mice produced significant CPA. However, CP-154,526 interrupts naloxone-induced CPA in morphine-treated animals suggesting that the consolidation process of opioid-withdrawn associated aversion could be linked to CRF1 receptor activation. These results are in agreement with previous studies performed in CFR1 knockout mice (Contarino and Papeleo, 2005; Garcia-Carmona et al., 20015a). In addition, CP-154,526 administration abolished the acquisition of morphine conditioned place preference (CPP) (Lasheras et al., 2014; Garcia-Carmona et al., 2015b). Altogether, these studies indicate that CRF through CRF1 receptor plays an important role in the consolidation and expression of either aversive and rewarding drug-related memories.

Previous studies have suggested that the HPA axis activation plays a role in the negative effects observed after naloxone-induced withdrawal. Thus, it has been observed that plasma corticosterone levels were increased in morphine withdrawn animals, these increased levels were normalized after the administration of CRF1 receptor antagonist (Navarro-Zaragoza et al., 2010). In addition, a role for the HPA axis and brain extra-hypothalamic CRF/CRF1R circuitry in somatic, molecular, and endocrine alterations induced by opioid withdrawal has been reported

(Papeleo et al., 2007). However, functional implication of the HPA axis activation in conditioned morphine withdrawal has not been well understood yet. Our results demonstrated that the acquisition of CPA was accompanied by increased corticosterone release, which was blocked with CP-154,526 administration, suggesting that HPA axis could contribute through CRF1 receptor to the formation of aversive memory by increasing corticosterone concentrations. The involvement of corticosterone increases after training enhancing the formation of aversive memory has been previously reported (Roozendaal et al, 2009; Barsegyan et al., 2010). Taken all together, it is suggested that glucocorticoids may be an important component for the aversive memory formation associated with conditioned morphine withdrawal.

There is much information about the neurobiological mechanisms of extinction or reward memory of drug taking (Torregrossa et al., 2010). However, little information is known about extinction of aversive memory of drug withdrawal (Myers and Carlezon, 2010). Memory impairment during drug withdrawal is a complex phenomenon that requires an understanding of the mechanisms underlying extinction of aversive memories, which could lead to pharmacological approaches for enhancing extinction, which might facilitate the treatment of drug addiction. This study is the first to specifically investigate the possible relationship between HPA axis and CPA extinction and the possible involvement of HPA in this process. In our study animals showed a reduction of corticosterone when they extinguished the aversive memory indicating that the activation or inhibition of HPA axis are implicated in these processes. In fact, the extinction memory is an active learning process that require different mechanisms to those involved in the formation of conditioned aversive memory.

The mammalian hippocampus, prefrontal cortex and the BLA share important functional and anatomical connections (McDonald and Mott, 2016) that are involved in the formation of

associative memories linked to drug reward as well as in the aversion to drug withdrawal (Rosen et al., 2015). In our study, we found that CPA induced an increased BDNF expression in the BLA and hippocampal DG, two limbic areas that play a critical role in emotional learning and memory (Lucas et al., 2008; Aimone et al., 2011). The increased expression of BDNF observed after CPA in DG and BLA was accompanied by the activation of the HPA axis resulting in an enhancement of corticosterone plasma levels. In agreement with our results it has been described that formation of aversive memories associated with conditioned morphine withdrawal requires BDNF signalling pathway in the rat amygdala (Ju et al., 2015). Moreover, morphine treatment and morphine withdrawal increased both pro-BDNF and BDNF levels in frontal cortex (Bachis et al., 2017). However, the molecular mechanism by which BDNF elicits the formation of aversive memories associated with conditioned morphine withdrawal remains poorly understood. In our present study we found that BDNF expression significantly increased after CPA in parallel with a decreased expression in ERK and no changes in CREB or proBDNF expression in DG and BLA. Although it has been demonstrated a dual and directional regulatory mechanism between BDNF/ERK/CREB (for review see Sawamato et al., 2017) other factors could be implicated in the modulation of the BDNF pathway. It is known that BDNF undergoes both retrograde and anterograde transport (Altar et al, 1997), so BNDF could be transported from VTA to BLA or DG, without changes in pCREB and ERK in these areas. Recently, it has been described retrograde BDNF transport in the mesolimbic pathway (von Diemem et al., 2016). In addition, BDNF expression is controlled in a complex manner and several aspects of its synthesis and signalling are modulated by the HPA axis (Suri and Vaidya, 2013). Thus, stress and CRF gate the neural increases of BDNF in cocaine addiction-related mesolimbic regions (Walsh et al., 2014). In our study BDNF expression in conditional morphine withdrawal mice decreased to a basal expression in mice treated with CP-154,526, indicating that BDNF is linked to HPA axis activity in the

formation of aversive memory. This increased expression of BDNF in DG and BLA was accompanied by high corticosterone levels after CPA, which were decreased after CRF1 receptor antagonist. Altogether, these results suggest that the increased HPA axis activity observed after conditioned morphine withdrawal alter BDNF expression in areas relationship with learn and memory, higher levels of BDNF after withdrawal appear to be associated with cravings and a shorter time to relapse (for revision see von Diemen et al., 2016). The aversive memory was extinguished after CPA expression when the levels of corticosterone and BDNF expression in DG and BLA were declined to basal values.

In conclusion our study has shown that CRF1 blocked the expression of CPA and the increase in corticosterone and BDNF observed after conditioned morphine withdrawal suggesting a link between HPA axis and BDNF through CRF1 receptor. These results demonstrate that BDNF and corticosterone are essential to the formation of aversive memory and suggest that the CRF1 antagonists could be a potential therapeutic target in the field of opioid addiction.

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Conflict of interest

There are no conflicts of interest to disclose

Authors Contribution

JNZ, PA, MLL were responsible for the study concept and design. EML and VGM conducted the experiments. JNZ and EML performed the statistical analysis. MLL, JNZ and PA wrote the manuscript. All the authors read and approved the final version of the manuscript.

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Figure Legends

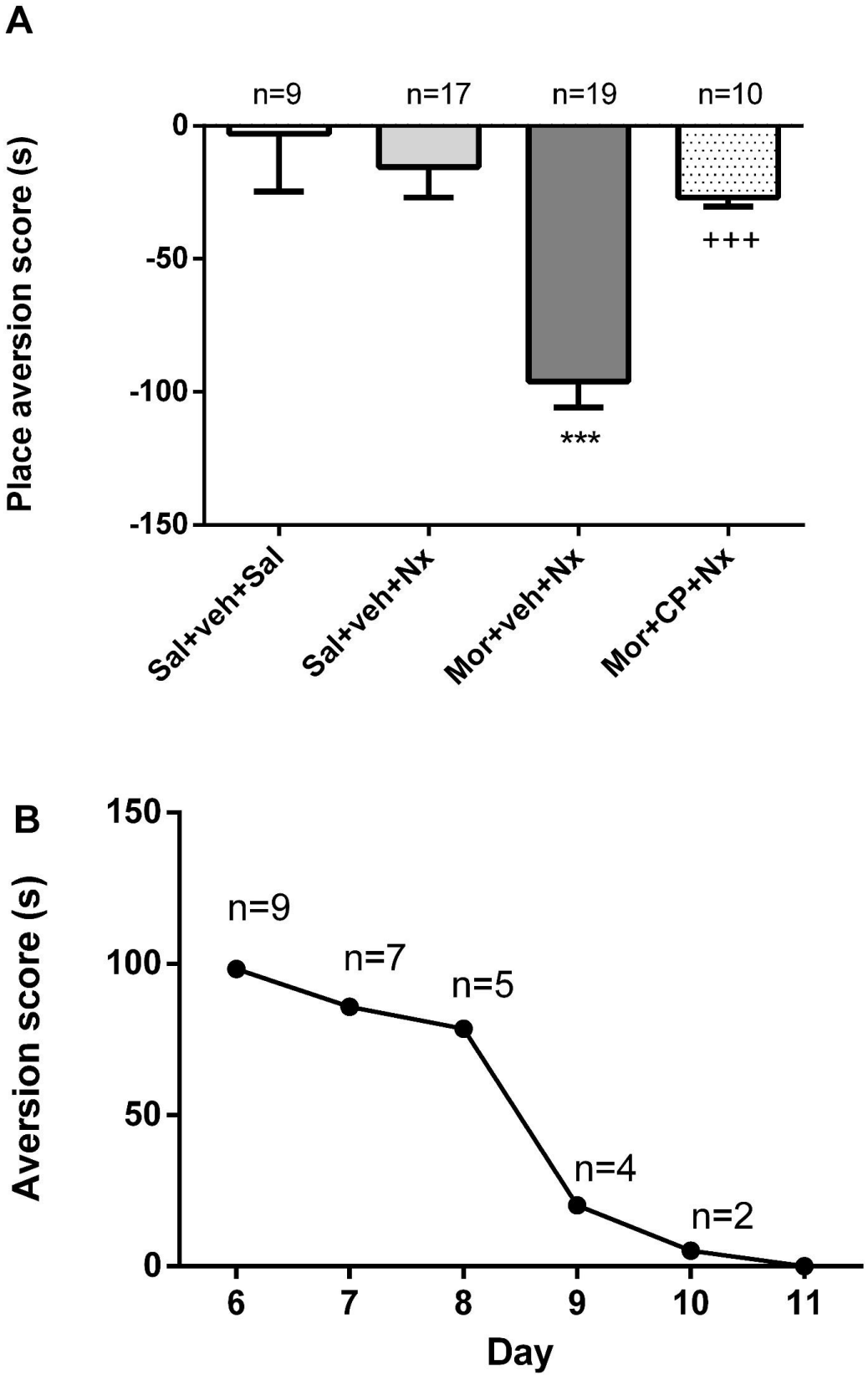

Figure 1. Conditioned place aversion (CPA) of morphine withdrawal. A) expression of CPA induced by naloxone in mice treated with morphine (Mor), saline (Sal), vehicle (veh), naloxone (Nx) or CP-154,526 (CP). The score was calculated for each mouse as the difference between the postconditioning and the preconditioning time spent in the drug-paired compartment. B) Extinction of CPA training. Aversion scores from day 10 to 11 from mice treated with Mor+veh+Nx. ***P<0.001 versus Sal+veh+Nx; +++P<0.001 versus Mor+veh+Nx

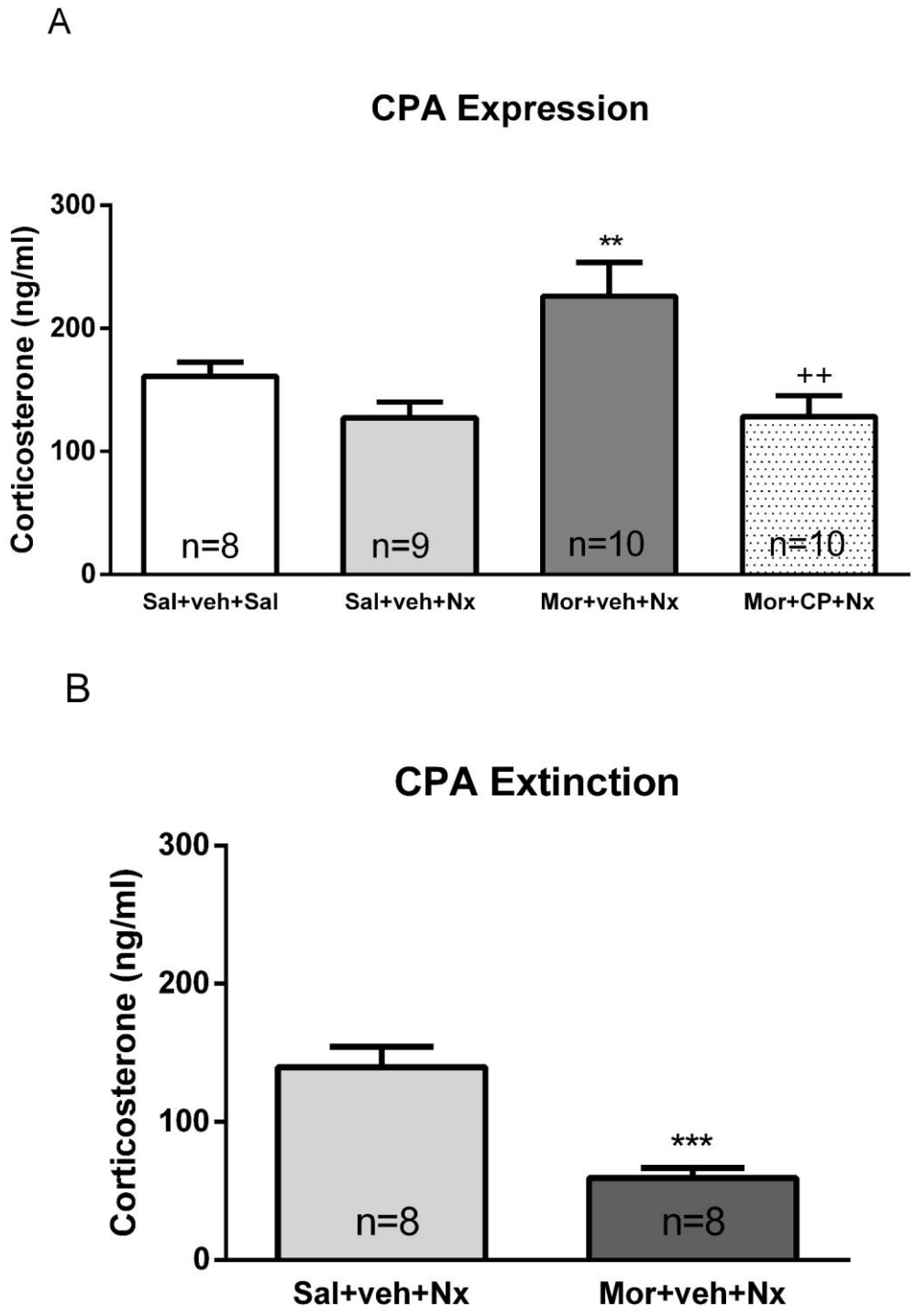

Figure 2. Corticosterone plasma levels after the expression and extinction of CPA training. A) Corticosterone plasma levels in mice treated with Sal+veh+Sal, Sal+veh+Nx, Mor+veh+Nx or Mor+CP+Nx after CPA expression. B) Corticosterone plasma levels in mice treated with

Sal+veh+Nx or Mor+veh+Nx after CPA extinction. **P<0.05, ***P<0.001 versus Sal+veh+Nx; ++P<0.01 versus Mor+veh+Nx.

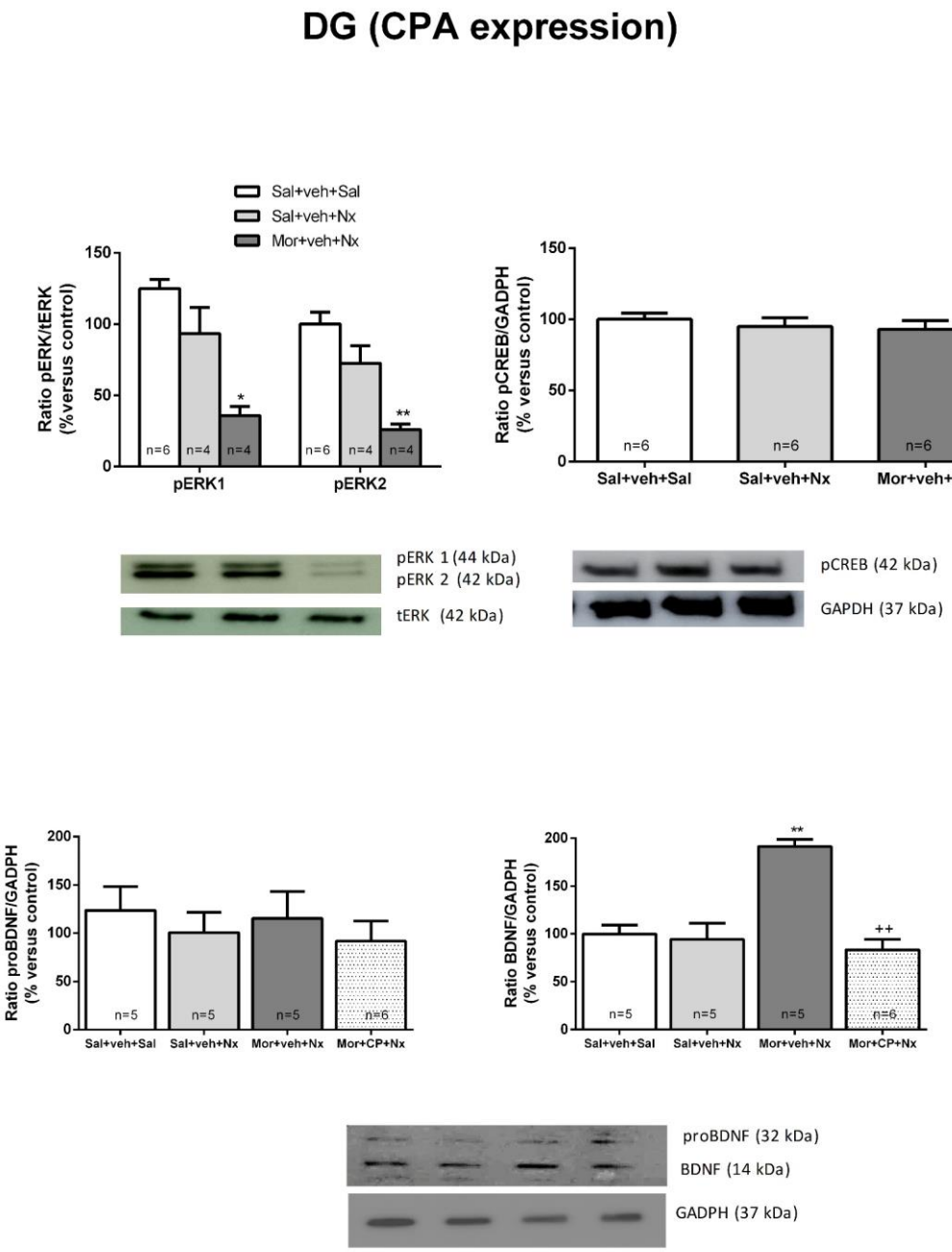

Figure 3. Western-blotting analysis of pERK, pCREB, proBDNF and BDNF in dentate gyrus (DG) after CPA expression. Each bar represents the mean optical density ± SEM; values are expressed as % of control. *P<0.05, **P<0.01 versus Sal+veh+Nx; ++P<0.01 versus Mor+veh+Nx.

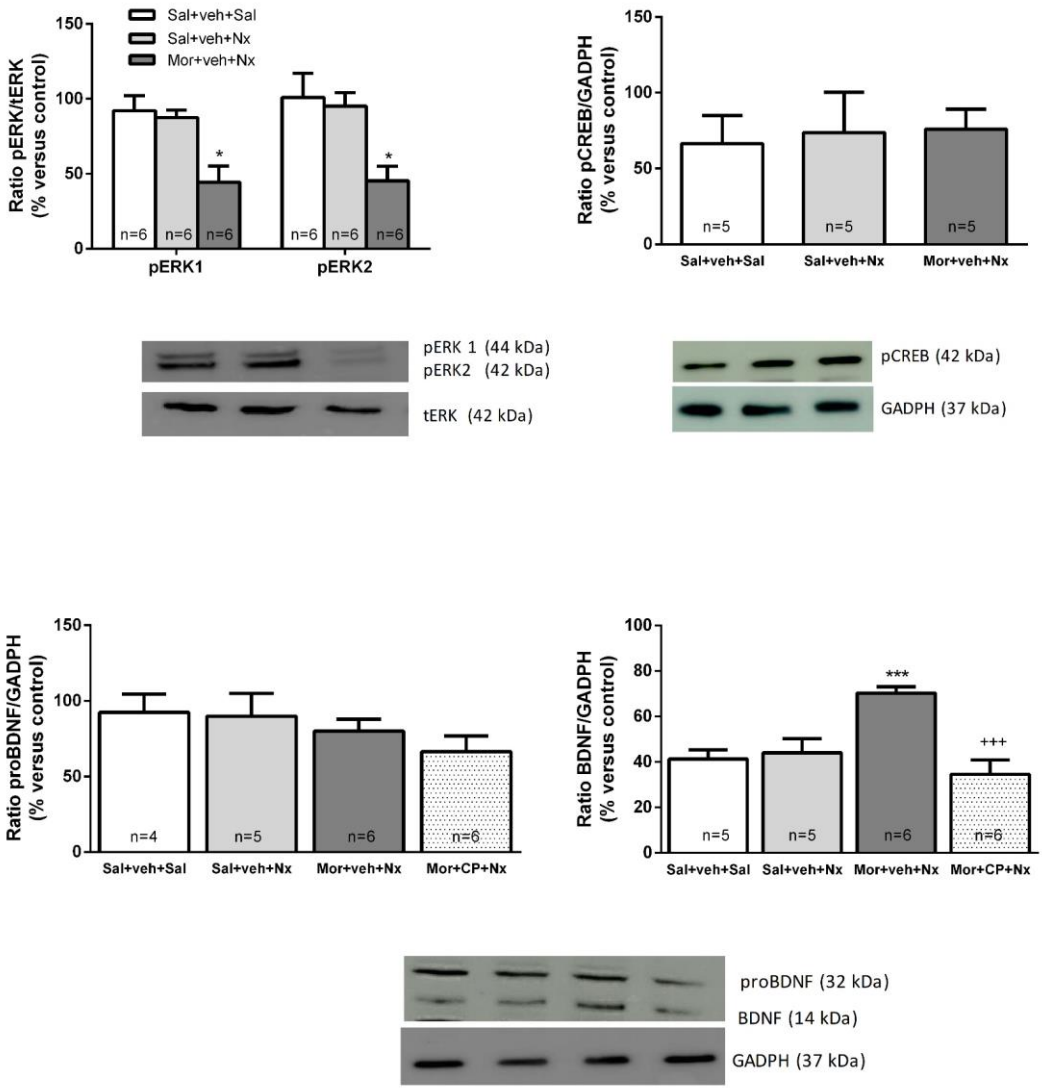

Figure 4. Western-blotting analysis of pERK, pCREB, proBDNF and BDNF in basolateral amygdala (BLA) after CPA expression. Each bar represents the mean optical density ± SEM; values are expressed as % of control. *P<0.05, ***P<0.001 versus Sal+veh+Nx; +++P<0.001 versus Mor+veh+Nx.

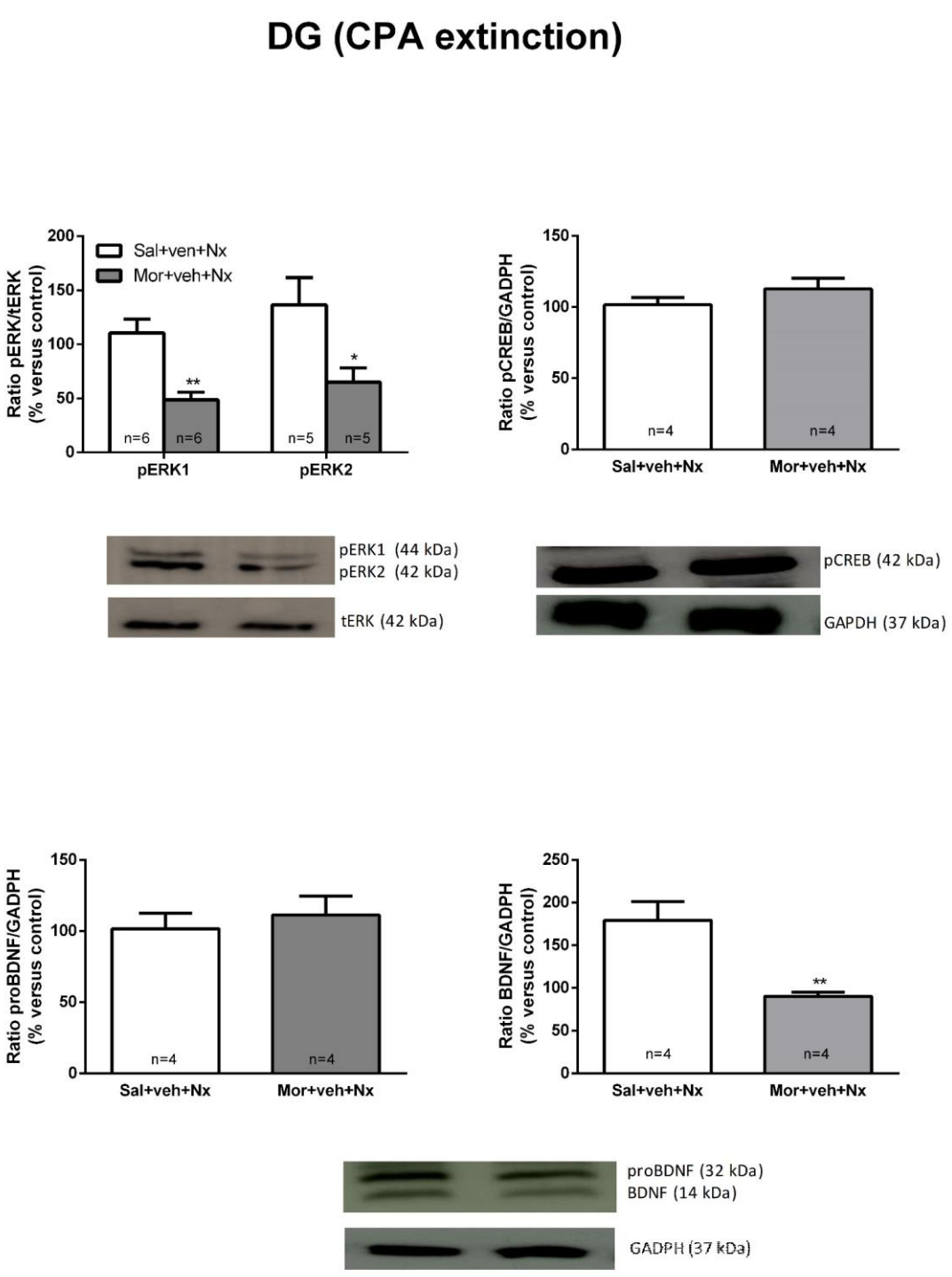

Figure 5. **Western-blotting analysis of pERK, pCREB, proBDNF and BDNF in dentate gyrus (DG) after CPA extinction**. Each bar represents the mean optical density ± SEM; values are expressed as % of control. *P<0.05, **P<0.01 versus Sal+veh+Nx.

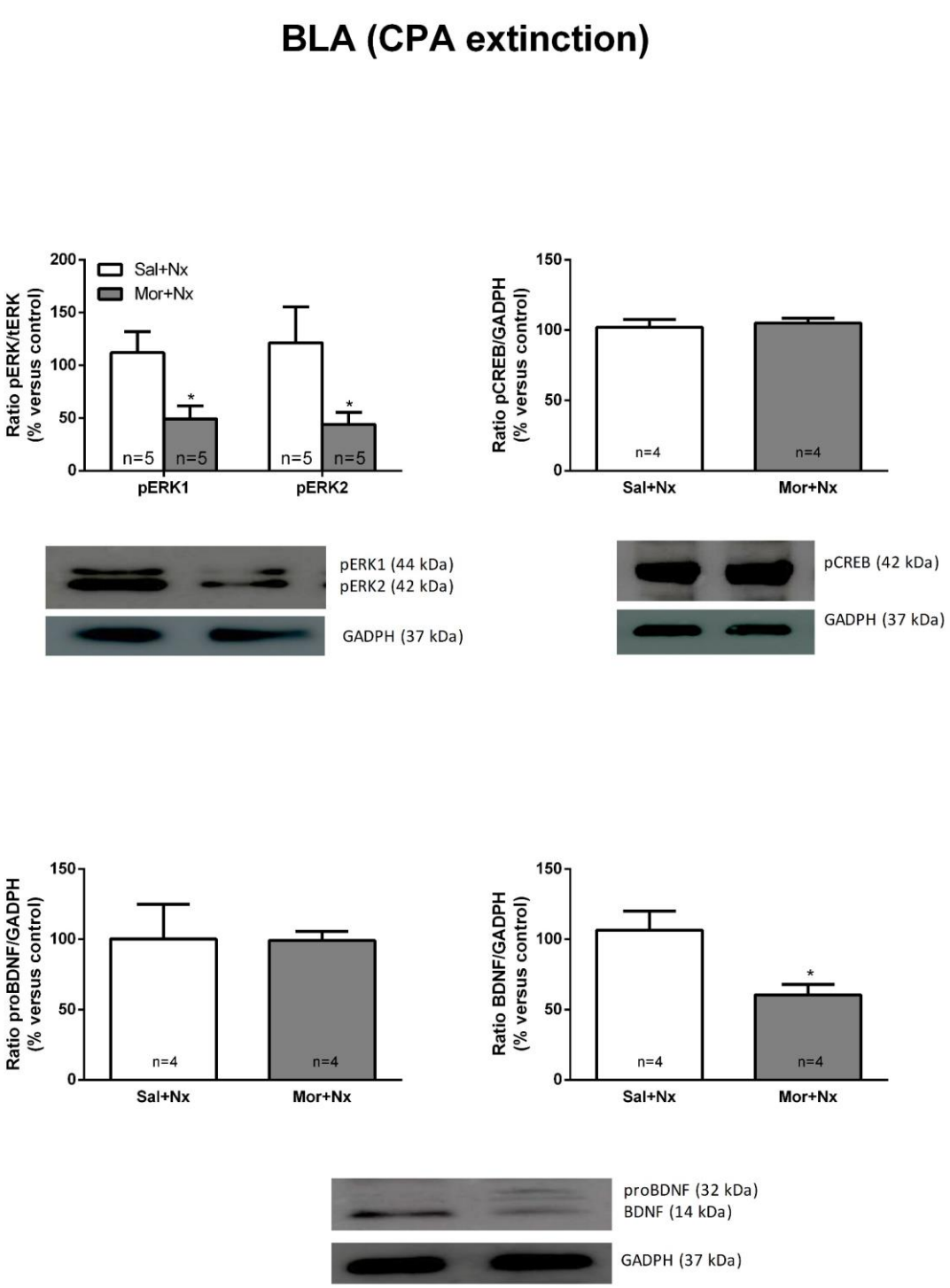

Figure 6. **Western-blotting analysis of pERK, pCREB, proBDNF and BDNF in basolateral amygdala (BLA) after CPA extinction**. Each bar represents the mean optical density ± SEM; values are expressed as % of control. *P<0.05 versus Sal+veh+Nx.