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Morphine treatment selectively regulates expression of rat pituitary POMC and the prohormone convertases PC1/3 and PC2

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Abstract

The prohormone convertases, PC1/3 and PC2 are thought to be responsible for the activation of many prohormones through processing including the endogenous opioid peptides. We propose that maintenance of hormonal homeostasis can be achieved, in part, via alterations in levels of these enzymes that control the ratio of active hormone to prohormone. In order to test the hypothesis that exogenous opioids regulate the endogenous opioid system and the enzymes responsible for their biosynthesis, we studied the effect of short-term morphine or naltrexone treatment on pituitary PC1/3 and PC2 as well as on the level of pro-opiomelanocortin (POMC), the precursor gene for the biosynthesis of the endogenous opioid peptide, beta-endorphin. Using ribonuclease protection assays, we observed that morphine down-regulated and naltrexone up-regulated rat pituitary PC1/3 and PC2 mRNA. Immunofluorescence and Western blot analysis confirmed that the protein levels changed in parallel with the changes in mRNA levels and were accompanied by changes in the levels of phosphorylated cyclic-AMP response element binding protein. We propose that the alterations of the prohormone processing system may be a compensatory mechanism in response to an exogenous opioid ligand whereby the organism tries

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[1]At the 6th Gordon Research Conference on Proprotein Processing, Trafficking and Secretion (2004), the leading researchers agreed to use the terminology, PC1/3 to describe the identical PC1 and PC3 prohormone convertase.

to restore its homeostatic hormonal milieu following exposure to the opioid, possibly by regulating the levels of multiple endogenous opioid peptides and other neuropeptides in concert.

Keywords

Post-translational processing; opioids; endorphin; prohormone convertase; pituitary; drug addiction

1. Introduction

Drug addiction is now viewed as a brain disease [22], rather than merely a social problem, as previously thought. Consequently, there has been an attempt to understand which neurochemical systems are altered by drug use. Although much research has focused on opioid use altering various neurotransmitter systems, other evidence has found abnormalities in neuroendocrine systems, such as corticotrophin-releasing hormone (CRH), pro-opiomelanocortin (POMC) and dynorphin in animals and humans exposed to drugs of abuse [14, 37, 47, 52]. Although several studies have shown that exogenous opioid administration down-regulates both brain POMC mRNA and plasma β-endorphin levels [4, 14, 18], other studies using different opioid paradigms have been unable to reproduce this finding [53, 54]. However, little is known if the changes in neurohormone(s) with drug intake are isolated or is a part of an altered hormonal environment, which may account for some of the pharmacological effects of abused substances.

Most prohormones, including precursors of opioid peptides, are synthesized in an inactive form and are converted to the biologically active hormone by cleavage at paired basic residues. PC1/3[1] and PC2, two members of the family of prohormone convertases (PCs), are located primarily in neuroendocrine tissues [8, 16, 43, 44] and can activate many neurohormones [42]. PC1/3 null mice are small due to impaired proGHRH processing and are hyperglycemic due to impaired proinsulin processing [55, 56]. PC2 null mice have absent proglucagon processing leading to hypoglycemia and also have impaired proinsulin processing [13]. POMC, proenkephalin and prodynorphin processing is blunted [1, 21]. The PCs have been shown to be regulated by several factors, including by dopamine receptor agonists/antagonists [2, 3, 8], CRH [3], glucose [41], suckling [33], glucocorticoids [8] and thyroid hormone [8]. Our laboratories has extensively studied the regulation of the PCs by cytokines [25], hyperglycemia [31, 32], thyroid status [10, 23, 26, 45, 46] and fasting/feeding [39]. Opioid withdrawal was associated with increased PC2 protein levels in the midbrain periaqueductal gray matter [34] and we recently demonstrated that short-term morphine exposure down-regulated hypothalamic PC1/3 and PC2 protein levels, while long-term morphine exposure up-regulated expression of these proteins [11].

Indirect experiments demonstrated that expression of PC1/3 is dependent on activation of the cyclic AMP response element binding protein (CREB)/cAMP system [19, 20]. Given that these PCs have the potential to process a wide variety of prohormones, alterations in the levels of these enzymes in areas rich in neuropeptides would be expected to change the ratio of active hormone to inactive precursor for many hormonal systems.

Morphine, a plant alkaloid, as well as the endogenous opioid peptide, β-endorphin, bind with high affinity to the mu-opioid receptor [50]. Furthermore, mu-opioid receptor expression has been detected in the pituitary [5, 6], an endocrine gland that is the site of hormonal biosynthesis, including the bioactive peptides adrenocorticotropic hormone (ACTH) and β-endorphin from the POMC precursor. Thus, we tested the hypothesis that activation of the opioid receptor by its respective agonist as well as the blockade of the receptor by the opioid

antagonist naltrexone, can alter the levels of PC1, PC2, the POMC precursor and potentially alter the amount of active hormones in the pituitary. We also studied the effect of short-term opioid exposure on the level of phosphorylated CREB (P-CREB) as this transcription factor regulates PC expression [19, 20].

2. Materials and methods

2.1 Animals

Male Sprague-Dawley (275-300 g) rats, housed in a room with controlled light, temperature and humidity and unrestricted access to food and water. All experimental protocols and animal procedures were performed in compliance with the NIH Guidelines for the Use of Animals in Research and approved by the Institutional Animal Care and Use Committees of Charles Drew University of Medicine & Sciences and Cedars-Sinai Medical Center.

2.2 Animal procedures

Rats were implanted with either a 75 mg morphine or placebo pellet [National Institute on Drug Abuse (NIDA)], or two naltrexone (60 mg total, NIDA) or placebo pellets (NIDA) in the nape of the neck under methoxyflurane anesthesia. Morphine pellets have been used for more than 40 years to study the effects of chronic opioids [51]. We have recently submitted a manuscript comparing the regulation of P-CREB in mouse brain regions by different paradigms of morphine administration and found that morphine pellets have a much more robust effect on P-CREB levels than daily morphine injections (Ren et al., submitted). Six to eight animals per group were used. Previous studies using rat hypothalamus [11] have shown that the stress of surgery/pellet implantation did not affect PC1/3 or PC2 mRNA levels compared to untreated rats (data not shown). Rats were euthanized by cervical decapitation 6 h after implantation for ribonuclease protection assay (RPA) or 24 h later for measurement of ACTH and β-endorphin. Western blot analysis or perfused with paraformaldehyde and used for immunohistochemical studies, as preliminary data demonstrated these times showed optimal regulation by morphine. Pellet implantation and euthanasia occurred at the same time between 1300 and 1500. For RPAs, the whole pituitary was removed from animal, frozen in liquid nitrogen and then stored at −80°C until analysis. For Western blot analysis, the anterior and neurointermediate lobes of the pituitary were carefully dissected under a dissection microscope, rapidly frozen in liquid nitrogen and then stored at −80°C.

2.3 In vitro transcription of riboprobes

Rat POMC (exon 3) and furin cDNA fragments subcloned into pBluescript SK-vector (Stratagene, La Jolla, CA) were obtained from Dr. Malcolm Low (University of Oregon) and Dr. Richard Mains (University of Connecticut), respectively. Rat PC1/3 and PC2 cDNA fragments subcloned into pBluescript II SK-vector (Stratagene) were obtained from Dr. Richard Mains. The cDNAs were linearized with the following restriction enzymes and transcribed with T7 polymerase (PC1/3 used T3 polymerase) to yield the following fragments: rPOMC-Ehe1-141 nucleotides (nt) (287-427 of rat POMC); rPC1/3-PstI-306 (nt) (243-548 of rat PC1/3); rPC2-Eag1-361 (nt) (353-713 of rat PC2); rfurin-BalI-179 (nt) (263-441 of rat furin). For the PC1/3 probe, the cDNA was treated with Klenow after linearization to remove the 3− overhang. After linearization, the cDNA fragment was purified by phenol/chloroform extraction and ethanol precipitation.

In vitro transcription was carried out according to the protocol described in Riboprobe *in vitro* transcription systems' manual (Promega, Madison, WI). Linearized cDNA (1 μg) was transcribed with 20 units of either T3 or T7 RNA polymerase and ^{32}P-UTP. All probes were

gel purified on TBE mini-gels (Novex, San Diego, CA), stored at −20° C and used within 3 days.

2.4 Ribonuclease protection assay

RPA was used due to its ability to accurately quantify low levels of mRNA. Tissues were homogenized in Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) to prepare mRNA prior to use. Quality of mRNA was checked on a 1% agarose/TBE gel. The tissue POMC, PC1/3, and PC2 and furin mRNA levels were measured by RPA according to the protocol described in the RPAIITM Kit (Ambion, Austin, TX). For each ^{32}P-labeled probe, a control tube with yeast tRNA instead of tissue RNA and a probe only tube were used. Samples were hybridized at 55°C for 16 h and then treated with 1:100 RNase A/RNase T1 mixture for 30 min at 37°C and electrophoresed on a 6% denaturing polyacrylamide gel at 60 W for 2 h. Following electrophoresis, the gel were dried and exposed to X-ray film at −80°C. The POMC, PC1/3, and PC2 and furin mRNA levels were quantified on an AlphaImager 2000 densitometry (Alpha Innotech Corporation, San Leandro, CA) using β-actin as an internal control. The intensity of the signal was found to be linear with the amount of RNA.

2.5 Tissue preparation for immunohistochemistry

Rats (n= 4/group) were anesthetized with an intraperitoneal injection of ketamine (40 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) and xylazine (5 mg/kg, Phoenix Scientific Inc, St. Joseph, MO, USA) and then perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were carefully removed and post-fixed in 4% paraformaldehyde overnight at 4°C, then immersed in 30% sucrose until the tissues sunk and were then frozen on dry ice. Ten μm-thick coronal sections of the pituitary were cut using a cryostat and mounted onto slides (Superfrost ®*/Plus, Fisher-Scientific, Pittsburg, PA, USA) and stored at −80°C until processing.

2.6 Immunofluorescence and quantification by Image Analysis System

This procedure was performed similar to published methods [15]. Pituitary ACTH, PC1/3 and PC2 protein levels were determined by immunohistochemistry with specific antibodies. For determining the ACTH expression, we used antiserum 2952, a rabbit polyclonal antiserum against full-length ACTH generated in the laboratory of Y. Peng Loh, (NIH) similar to the DP6 antiserum previously described [12]. This antibody recognizes ACTH, 21-23K ACTH and POMC. The PC1/3 antibody and PC2 antibodies used for immunohistochemistry were made in rabbits against amino acids 113-158 of human preproconvertase PC1 and 112-159 of human preproconvertase PC2, respectively (Millipore Corp., Billerica, MA, USA). The tissue sections were incubated with first antibody at a concentration of 1:1000 for POMC, 1:100 for PC1/3 and 1:150 for PC2, then incubated with a goat anti-rabbit IgG (H+L) Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Eugene, OR) at a concentration of 1:2000. They were mounted with Vectashield mounting medium for fluorescence with propidium iodide (Vector Laboratories, Burlington, CA) as a counterstain. Negative control slides were performed by incubation with only the secondary antibody (without the addition of the primary antibody) and did not show any staining.

Immunofluorescence images were obtained using an Olympus microscope (Temple Valley, PA) (TM)-B35V, equipped with digital camera and Olympus software BX-61. After setting the parameters of exposure time, brightness and contrast on the first slide, all pictures were taken at the same exposure and no further adjustments were done. Quantitative image analysis was performed by computerized densitometry using the ImagePro 5.01 software (Media Cybernetics, Silver Spring, MD). The number of positive cells for ACTH, PC1/3 and PC2 over the total number of cell were analyzed in a computerized grid and expressed as %

of positive cells in the anterior and intermediate lobes. The intensity of the staining was measured as the total integrated optical density per area (Total IOD). The total number of cells was counted by propidium iodide red counterstaining (PI) and the ACTH, PC1/3 and PC2 immunoreactive levels were determined by the green fluorescence of the Alexa-labeled protein. In all cases, six fields of the anterior lobe and four fields of the intermediate lobe at 200x were analyzed per tissue section, with at least four matched sections per animal and four animals per group.

2.7 Protein analysis by immunoblotting

Following morphine and placebo implantation (n=4 rats/ group), the pituitary of each rat was removed and the anterior and neuro-intermediate lobes were homogenized immediately in ice-cold RIPA buffer (50 nmol/L Tris-HCl, 150 mmol/l NaCl, 1% sodium deoxycholate, 0.1% SDS, 100 mmol/L sodium orthovanadate, 1% Triton X-100, and EDTA-free protease inhibitors). Homogenates were then centrifuged at 4°C at 12,000*g* for 15 min and supernatants were collected. Protein concentration of the supernatants was measured with the Bradford assay (Bio-Rad protein assay kit, Hercules, CA, USA). Fifty micrograms of total protein were loaded and separated on 10% SDS polyacrylamide gels and then transferred to Hybond-ECL nitrocellulose membranes (Millipore Corporation, Bedford, MA, USA) in buffer containing 0.02% SDS and 20% methanol. Membranes were blocked with blocking solution (5% skim milk in PBS-T), washed three times (5 min each), and proteins detected using either polyclonal anti-phospho-CREB antibody (antibody raised against a synthetic phosphopeptide corresponding to residues 123-136 of rat cyclic AMP response element binding protein, Upstate, Lake Placid, NY, USA) at a dilution of 1:1000, anti-PC1/3 or anti-PC2 antibodies at a dilution of 1:2000. The anti-phospho-CREB antibody cross-reacts with phosphorylated forms of CREB, CREM and ATF-1, but not with non-phosphorylated forms. Anti-PC1/3 and anti-PC2 antibodies were raised against either a PC1/3-glutathione fusion protein or a PC2-glutathione fusion protein [17] and generously provided by Nigel Birch, Ph.D. (University of Auckland, NZ). Blots were then washed five times (5 min each) with PBS-T and incubated with anti-rabbit conjugated with horseradish peroxidase secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at a dilution of 1:2000 for 2 h. Bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). Band intensities were measured by optical densitometry of the auto-radiographs relative to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin.

2.8 RIA analyses

The assay used for ACTH and β-endorphin peptides was developed in the laboratory of E.A.N. using commercially available peptides and primary antibodies developed in his laboratory [35]. ACTH and β-endorphin were iodinated with ^{125}I using the Chloramine T oxidation-reduction method followed by HPLC separation, and the purified peptide was used as tracer. The ACTH and β-endorphin RIA assay was performed in 0.5 ml of RIA buffer containing anti-ACTH antiserum (1:30,000), anti-β-endorphin (1:40,000) and 5000 cpm of ^{125}I-ACTH or ^{125}I-β-endorphin. The sensitivity of the assays was approximately 10 pg/tube, and the intra- and inter-assay variability were approximately 5-7% and 10-11%, respectively. The ACTH assay detects 100% of CLIP and ACTH forms; however, this assay does not cross react with any form of α-MSH or β-endorphin. The ACTH assay cross-reacts with the POMC precursor, although the amount of cross-reactivity is unknown. The β-endorphin antiserum does not cross react with either ACTH or α-MSH assays.

2.9 Statistical analyses

The data were expressed as the mean ± SEM. Unpaired Student's t-test was used to compare two groups and one-way analysis of variance with Tukey-Kramer *post-hoc* test was used to compare more than two groups. Significance level was set at $p< 0.05$.

3. Results

3.1 Short-term morphine or naltrexone treatment regulated rat pituitary POMC, PC1/3 and PC2 mRNA levels

To test the hypothesis that exogenous opioids regulate the endogenous β-endorphin system and the enzymes responsible for its biosynthesis, we studied the effect of morphine or naltrexone treatment on pituitary POMC, PC1/3 and PC2 expression. Short-term (6 h) morphine treatment resulted in a decrease in whole pituitary POMC mRNA levels (corrected for β-actin levels) compared to placebo-treated animals (54.7±9.9%; $p<0.01$). On the other hand, short-term naltrexone exposure increased pituitary POMC mRNA levels compared to placebo-treated animals (142±9.0%; $p<0.05$) (Fig. 1A and B).

Morphine treatment for 6 h decreased PC1/3 mRNA levels (corrected for β-actin levels) in pituitary (44.2±2.4%; $p <0.0005$) compared to placebo treatment (Fig. 2A, 2B). PC2 mRNA levels were also reduced by morphine compared to placebo treatment in pituitary (40.1±2.1%; $p <0.0001$) (Fig. 2C, 2D). On the other hand, short-term naltrexone treatment (60 mg; sc pellet implantation) enhanced PC1/3 mRNA levels (123.2±3.9%; $p <0.005$) compared to placebo treatment (Fig. 2A, 2B). PC2 mRNA levels were also increased with naltrexone compared to placebo treatment (218±7.1%; $p <0.0001$) (Fig. 2C, 2D).

3.2 Short-term morphine or naltrexone treatment regulated pituitary PC1/3, PC2 and ACTH expression as determined by immunofluorescence

We then used fluorescent immunohistochemistry to examine the levels of the POMC-derived peptide ACTH, PC1/3 and PC2 protein expression in the pituitary gland. Placebo-treated rats, as expected, showed high levels of POMC and PC2 in the intermediate lobe, with lower levels in the anterior lobe (Figs. 3 and 5). In contrast, PC1/3 expression was higher in the anterior lobe than in the intermediate lobe (Fig. 4). Similar to mRNA changes, we found that short-term (6 h) morphine exposure decreased expression of ACTH immunoreactivity as determined by % positive cells ($p < 0.01$, anterior lobe, $p < 0.05$, intermediate lobe), and by IOD ($p < 0.05$, both lobes) (Fig. 3). Naltrexone treatment, on the other hand, increased expression of ACTH immunoreactivity in the anterior lobe as determined by IOD ($p < 0.05$) and in the intermediate lobe as determined by % positive cells ($p < 0.05$) and IOD ($p < 0.01$) (Fig. 3).

PC1/3 expression was decreased with morphine treatment (% positive cells, $p < 0.001$, IOD, $p < 0.05$) in the anterior lobe but was decreased in the intermediate lobe only as determined by % positive cells (Fig. 4). Naltrexone treatment increased PC1/3 expression in both lobes as seen by % positive cells ($p < 0.01$) and in the intermediate lobe as determined by IOD (Fig. 4). The finding of lower levels of PC1/3 in the intermediate lobe by IOD compared to % positive cells suggests that PC1/3 staining in this lobe occurs in many cells, but with low intensity per cell.

In the intermediate lobe, PC2 expression was decreased with morphine treatment (% positive cells, $p < 0.01$, IOD, $p < 0.05$) and increased with naltrexone treatment as determined by % positive cells ($p < 0.001$) (Fig. 5). Expression of PC2 was minimal in the anterior lobe and not regulated by morphine or naltrexone (Fig. 5).

3.3 Morphine treatment decreased the mature forms of PC1/3 and PC2 as well as P-CREB as determined by Western blot

We confirmed our protection assay and immunohistochemistry experiments with Western blot experiments that are excellent for quantitation of protein levels and can also be used to determine the size of the protein detected. In western blotting experiments, we detected the 66 kDa form of PC1/3 and the 64 kDa form of PC2 in both the anterior and neuro-intermediate lobes of the pituitary (Fig. 6). We found a reduction in the levels of PC1/3 (Fig. 6A; $p < 0.005$) and PC2 (Fig. 6B; $p < 0.05$) in the anterior lobe of the pituitary of rats subjected to 24-h morphine treatment compared to placebo treatment. We also found a reduction in the levels of PC1/3 ($p < 0.005$, Fig. 6D) and PC2 ($p < 0.05$, Fig. 6E) in the neuro-intermediate lobe of the pituitary in rats treated with morphine compared to placebo.

As prior experiments have suggested that expression of PC1/3 and PC2 is dependent on activation of the CREB/cAMP system [19, 20], we also examined P-CREB expression in the pituitary. We found exclusively a 43 kDa band corresponding to the size of P-CREB in the anterior (Fig 6C) and neuro-intermediate (Fig. 6F) lobes of the pituitary. We found a reduction in the levels of P-CREB in the anterior (Fig 6C; $p < 0.05$) and neuro-intermediate ($p < 0.05$, Fig 6F) lobes of the pituitary in rats implanted with morphine for 24 h compared to placebo pellets.

3.5 Morphine treatment reduced β-endorphin levels

Since a key objective of this study was to demonstrate that morphine treatment reduces the production of an endogenous opioid, we determined the levels of β-endorphin in both anterior and intermediate pituitary in rats treated with morphine and naltrexone. A significant decrease was observed in the levels of β-endorphin in both anterior and intermediate lobe (Fig. 7), which was well correlated with a decrease in POMC message, ACTH peptide, and processing enzymes following short-term morphine treatment. Naltrexone did not alter β-endorphin levels. The data also showed that the production of β-endorphin in the intermediate lobe was more than ten times higher as compared with the anterior lobe, consistent with the finding that the major products derived from POMC processing in the anterior pituitary are the 16kDa amino terminal fragment, ACTH and β-LPH, whereas in the intermediate lobe ACTH is cleaved to α-MSH and CLIP, while β-LPH is cleaved to β-endorphin and γ-LPH [28].

4. Discussion

Opioids alter a series of physiological processes including nociceptive information, respiration, gastrointestinal motility, reproductive processes, carbohydrate metabolism and food intake; many of these processes may be regulated by changes in endogenous neuropeptides [30]. Understanding how exogenous opioids regulate the prohormone convertases involved in neuropeptide biosynthesis is likely to elucidate mechanisms related to opioid addiction as well as help explain some of the consequences of opioid exposure.

POMC mRNA is present primarily in the hypothalamus (arcuate nucleus) and pituitary, with lesser amounts in the amygdala, midbrain and cerebral cortex [7, 27]. Interestingly, high levels of the prohormone convertases PC1/3 and PC2 are found in the pituitary, while moderate levels are found in brain regions such as the hypothalamus, midbrain and hippocampus [36, 40], where they can process a variety of prohormones that may be altered in the condition of short-term opioid exposure. In an early study, we showed that short-term morphine exposure down-regulated, while long-term morphine exposure up-regulated P-CREB, PC1/3 and PC2 protein levels in the rat hypothalamus [11]. In the current paper, we tested the effect of exogenous morphine or naltrexone on the levels of POMC processing

enzymes, PC1/3 and PC2 in the rat pituitary. We hypothesized that short-term treatment with morphine down-regulates, whereas short-term naltrexone treatment up-regulates both POMC transcription and the POMC processing enzymes. We predicted that the down regulation induced by short-term morphine treatment would be due to end-product inhibition, where the organism no longer needs endogenous opioids when exogenous opioids are supplied.

Consistent with our hypothesis, we found that short-term morphine exposure down-regulated and short-term naltrexone treatment up-regulated pituitary POMC, PC1/3 and PC2 expression. We also showed that short-term morphine treatment decreased the POMC processing products ACTH and β-endorphin. These results obtained by RPA, Western blot analysis and immunohistochemistry are in good agreement, although a less dramatic effect was seen by Western blot analysis. The PC1/3 fusion antibody can detect the 87, 74 and 66 kDa forms of PC1/3 [17]. However, the predominant form seen with this antibody in pituitary [17], AtT-20 cells [24] and spleen [31] was the 66 kDa form, a finding confirmed in the pituitary in the current study. Similarly, we detected the 64 kDa form of PC2, a finding similar to previous reports [17, 31]. P-CREB was down-regulated in a similar manner as PC1/3 and PC2 by short-term morphine as determined by Western blot (Fig. 6). This suggests that phosphorylation of pituitary CREB is involved in the regulation of the PC1/3 and PC2 by short-term opioid exposure, similar to our finding in hypothalamus [11]. The effect of morphine may be direct or indirect via hypothalamic hormones including CRH [9]. Our immunohistochemistry data demonstrates a high amount of PC2 staining in the intermediate lobe and PC1/3 staining in the anterior lobe and intermediate lobe, which agrees with the finding of Uehara *et al.* [49] in rats and Marcinkiewicz *et al.* [29] in mice.

In both Western blot and immunofluorescence experiments, PC2 protein was abundant in the neuro-intermediate lobe with weak (Western blot) to trace expression (immunofluorescence) in the anterior lobe. The minor differences in quantitation of protein levels between Western blot and immunofluorescence experiments in different experiments may be due to the use of 1) neuro-intermediate lobe in Western blots studies versus intermediate lobe in immunofluorescence studies, 2) antibody; i.e., Western blot experiments used a fusion protein antibody and immunofluorescence experiments used a peptide antibody, with the different antibodies recognizing different epitopes, and 3) there can be also a dilution effect due to the use of whole neurointermediate lobe in Western blot, whereas in immunofluorescence experiments, we study the expression of the protein per cell.

It is noteworthy that short-term naltrexone increased PC1/3 and PC2 mRNA levels. It appears that an endogenous substance capable of binding to the μ-receptor (possibly β-endorphin) maintains PC1/3 and PC2 levels in a suppressed state. When this action is blocked by naltrexone, PC1/3 and PC2 levels increase. It would be interesting if animals with low β-endorphin levels [such as those with targeted truncation of the POMC gene leading to absent β-endorphin [38] or patients with excess β-endorphin [48] would have altered PC1/3 and PC2 expression.

In conclusion, this study demonstrated that exogenous opioid ligands regulated the PCs and their products, as we found that short-term morphine down-regulates and naltrexone up-regulates POMC, PC1/3, PC2 mRNA, and ACTH and β-endorphin peptide levels in rat pituitary. Activation of the cAMP/CREB system appears to be linked to this regulation as P-CREB expression is co-regulated with PC1/3 and PC2 expression. The regulation of POMC, PC1/3 and PC2 may be a potential pathway to uncover some of the actions of opioids. The alterations in these important processing enzymes may alter levels of many bioactive hormones and these changes, or the homeostatic response to these changes may explain

some of the acute and chronic effects of opioids such as neuronal adaptive changes seen in drug addiction. Further studies are needed to examine changes in PC levels following chronic opioid intake as well as in states of opioid withdrawal and to examine the physiological consequences of alterations of PC levels following opioid use.

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Highlights

- Morphine down-regulated and naltrexone up-regulated pituitary PC1/3 and PC2 mRNA.
- This regulation was accompanied by changes in P-CREB.
- The alterations of the prohormone processing system may be compensatory.
- Following exposure to opioids, the organism tries to restore its homeostatic hormonal milieu.
- This may occur by regulating the levels of multiple endogenous opioid peptides in concert.

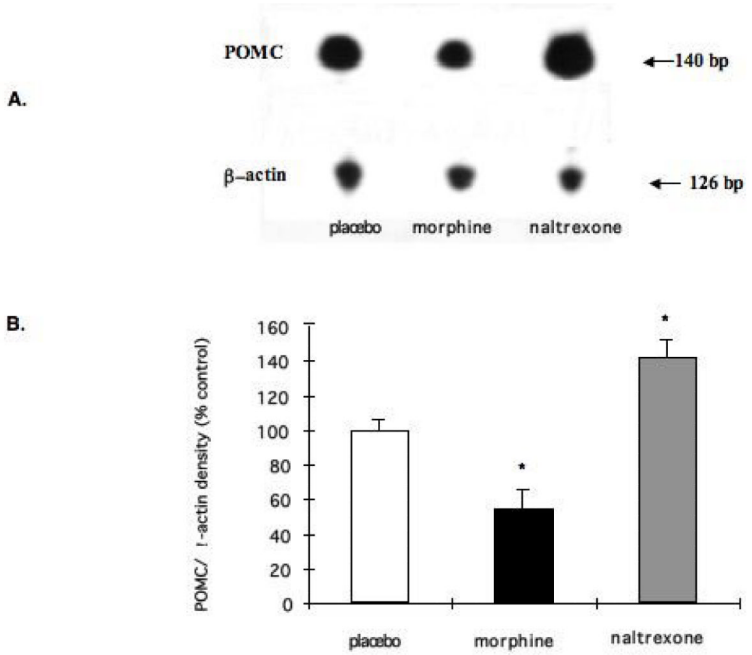

Figure 1.

Effect of six-h treatment with morphine (75 mg), naltrexone (60 mg) or placebo pellet on POMC mRNA levels in rat pituitary. Three pituitaries were pooled together per group. Total RNA was isolated from four groups for each treatment and was analyzed by RNase protection assay. RNA (5 μg) was hybridized with probes for POMC and β–actin and then digested with RNase as described in Methods. The probe sizes for POMC and β–actin were 161 and 188 bp, respectively, which were seen in protection assays without RNA or RNase. The protected band sizes for POMC and β–actin were 140 and 126 bp, respectively, and were absent when yeast tRNA (10 μg) was used instead of tissue RNA. A. Representative RNase protection assay for pituitary POMC mRNA. B. Densitometric measurement of pituitary POMC mRNA levels normalized by β–actin mRNA levels (mean ± SEM) and expressed as percentage of control. *$p<0.05$.

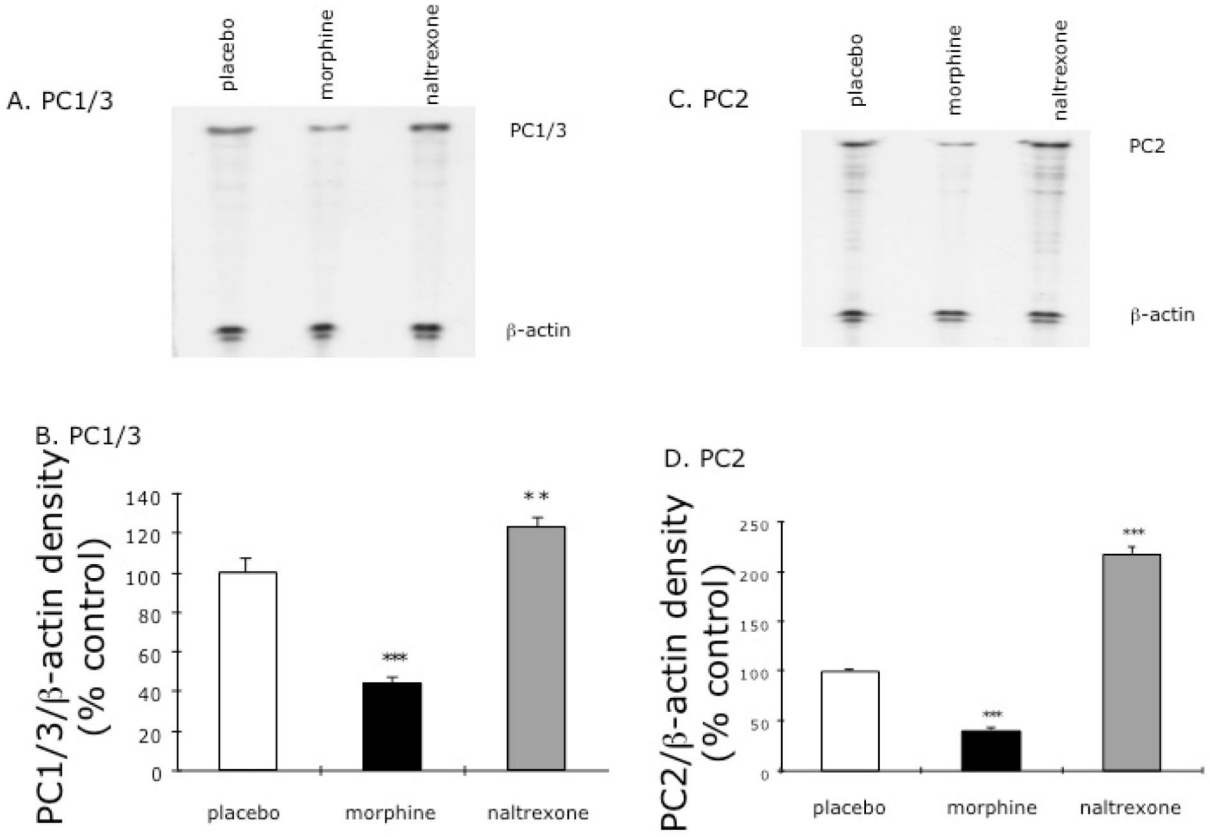

Figure 2.

Effect of six-h treatment with morphine (75 mg), naltrexone (60 mg) or placebo pellet on PC1/3 (A, B) and PC2 (C, D) mRNA levels in rat pituitary. Three pituitaries were pooled together per group. Total RNA was isolated from four groups for each treatment and was analyzed by RNase protection assay. RNA (5 μg) was hybridized with probes for PC1/3, PC2 and β–actin. The probe and protected band sizes for PC1/3 were 360 and 305 bp, respectively. The probe and protected band sizes for PC2 were 375 and 360 bp, respectively. Probes were seen in protection assays without RNA or RNase and protected bands absent when yeast tRNA (10 μg) was used instead of tissue RNA. A. Representative RNase protection assay of pituitary PC1/3 mRNA. B. Densitometric measurement of pituitary PC1/3 mRNA levels normalized by β–actin mRNA levels (mean ± SEM) and expressed as percentage of control. C. Representative RNase protection assay of pituitary PC2 mRNA. D. Densitometric measurement of PC2 mRNA levels normalized by β–actin mRNA levels (mean ± SEM) and expressed as percentage of control. **p<0.005, ***p<0.0005.

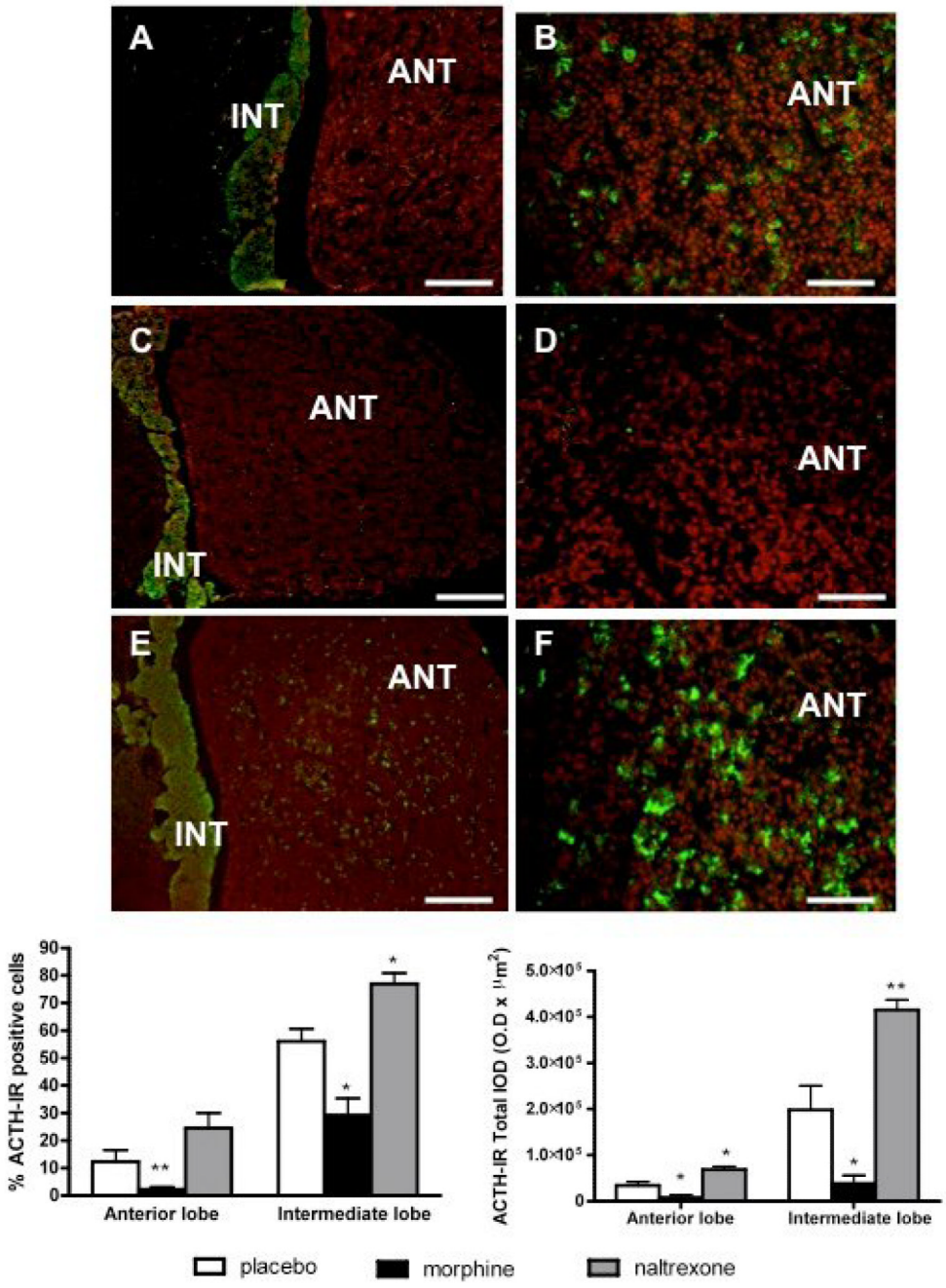

Figure 3.

Changes in ACTH-immunoreactivity (ACTH-IR, green; nucleus of pituitary cell, red) in the pituitary of rats after six h of placebo (A, B), morphine (C, D) or naltrexone treatment (E, F) as determined by immunohistochemistry. Frozen sections of the pituitary gland were immunostained with ACTH-specific antibody followed by a secondary antibody conjugated with Alexa Fluor 488. Sections were counterstained with propidium iodide. Figures are representatives of 6 rats/group. Low magnification (A, C, E) 50X. Bar = 300 μm. High magnification (B, D, F) 200×. Bar = 100 μm. Quantification of anterior lobe and intermediate lobe by % ACTH-IR positive cells (G) and the Integrated Optical Density

(IOD) of ACTH-IR positive cells (H) as determined by Image Pro analysis and expressed as the mean +/− SEM. * P<0.05, compared to control; **P<0.01 compared to control.

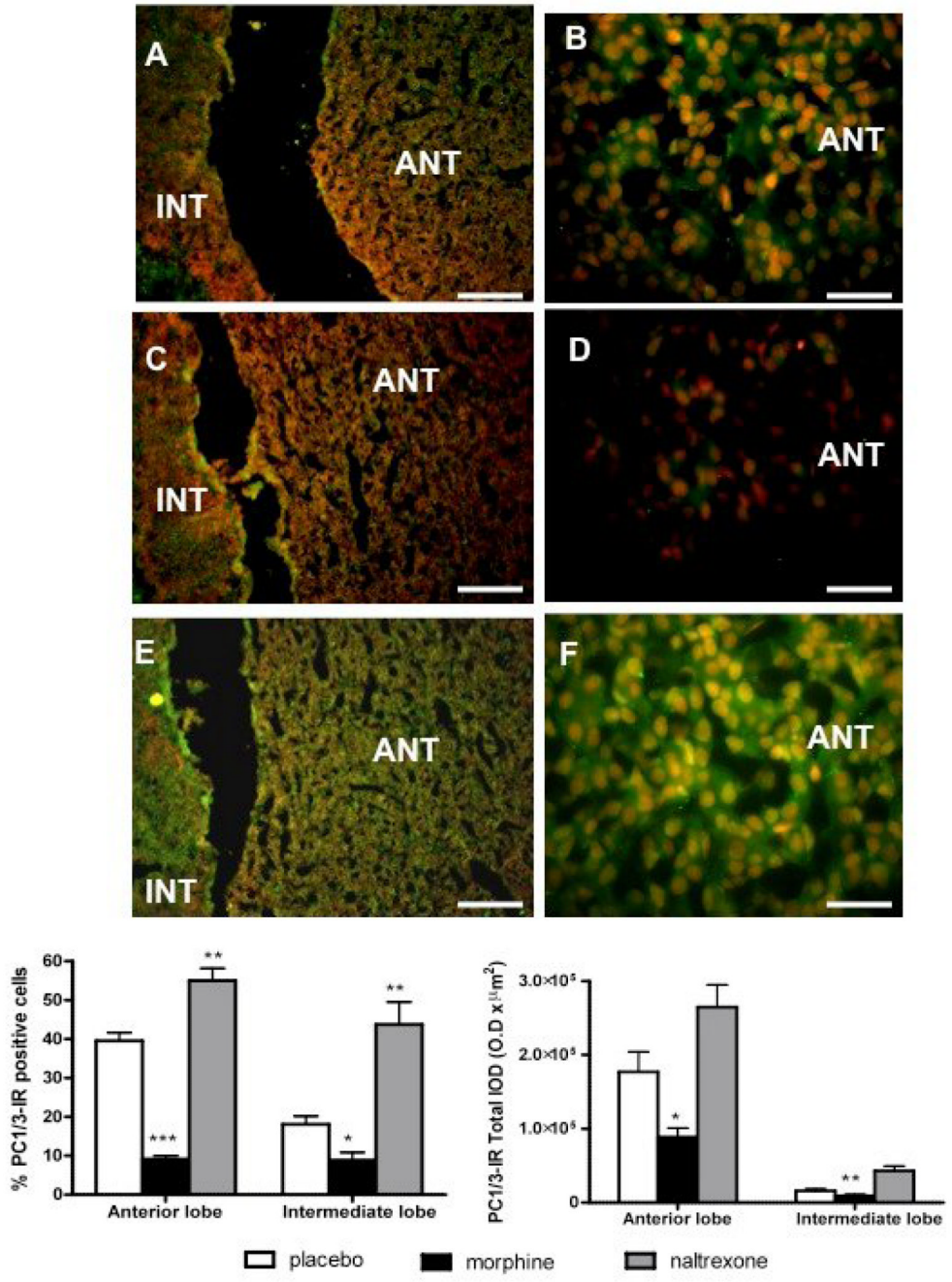

Figure 4.

Changes in PC1/3-immunoreactivity (PC1/3-IR, green; nucleus of pituitary cell, red) in the pituitary of rats after six h of placebo (A, B), morphine (C, D) or naltrexone treatment (E, F) as determined by immunohistochemistry. Frozen sections of the pituitary gland were immunostained with PC1/3-specific antibody followed by a secondary antibody conjugated with Alexa Fluor 488. Sections were counterstained with propidium iodide. Figures are representatives of 6 rats/group. Low magnification (A, C, E) 50X. Bar = 300 μm. High magnification (B, D, F) 200×. Bar = 100 μm. Quantification of anterior lobe and intermediate lobe by % PC1/3 positive cells (G) and the Integrated Optical Density (IOD) of PC1/3 positive cells (H) as determined by Image Pro analysis and expressed as the mean +/−

SEM. * P<0.05, compared to control; **P<0.01 compared to control; ***P<0.001 compared to control.

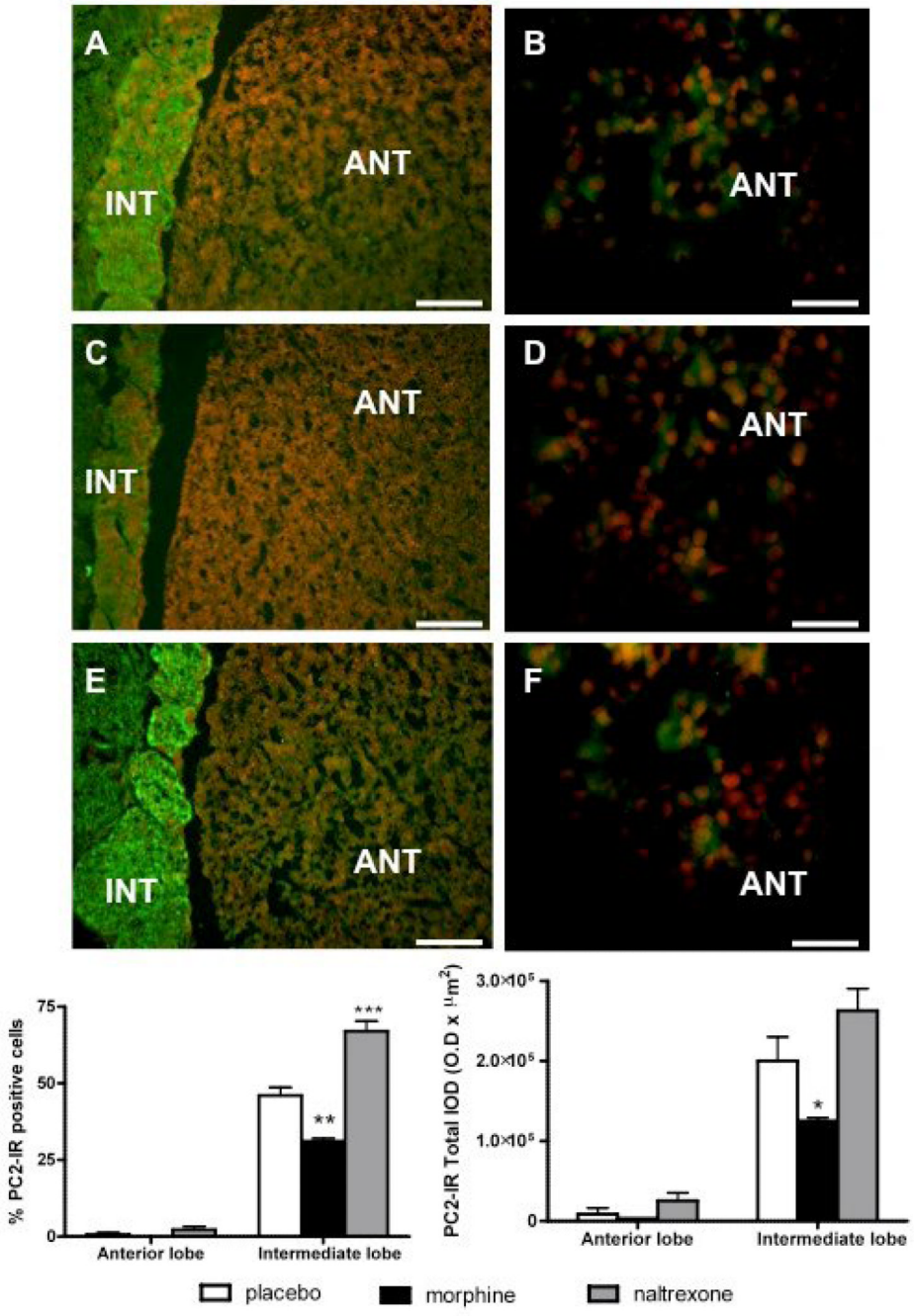

Figure 5.

Changes in PC2-immunoreactivity (PC2-IR, green; nucleus of pituitary cell, red) in the pituitary of rats after six h of placebo (A, B), morphine (C, D) or naltrexone treatment (E, F) as determined by immunohistochemistry. Frozen section of the pituitary gland were immunostained with ACTH-specific antibody followed by a secondary antibody conjugated with Alexa Fluor 488. Sections were counterstained with propidium iodide. Figures are representatives of 6 rats/group. Low magnification (A, C, E) 50×. Bar = 300 μm. High magnification (B, D, F) 200×. Bar = 100 μm. Quantification of anterior lobe and intermediate lobe by % PC2 positive cells (G) and the Integrated Optical Density (IOD) of PC2-IR positive cells (H) as determined by Image Pro analysis and expressed as the mean +/

− SEM. * P<0.05, compared to control; **P<0.01 compared to control; ***P<0.001 compared to control.

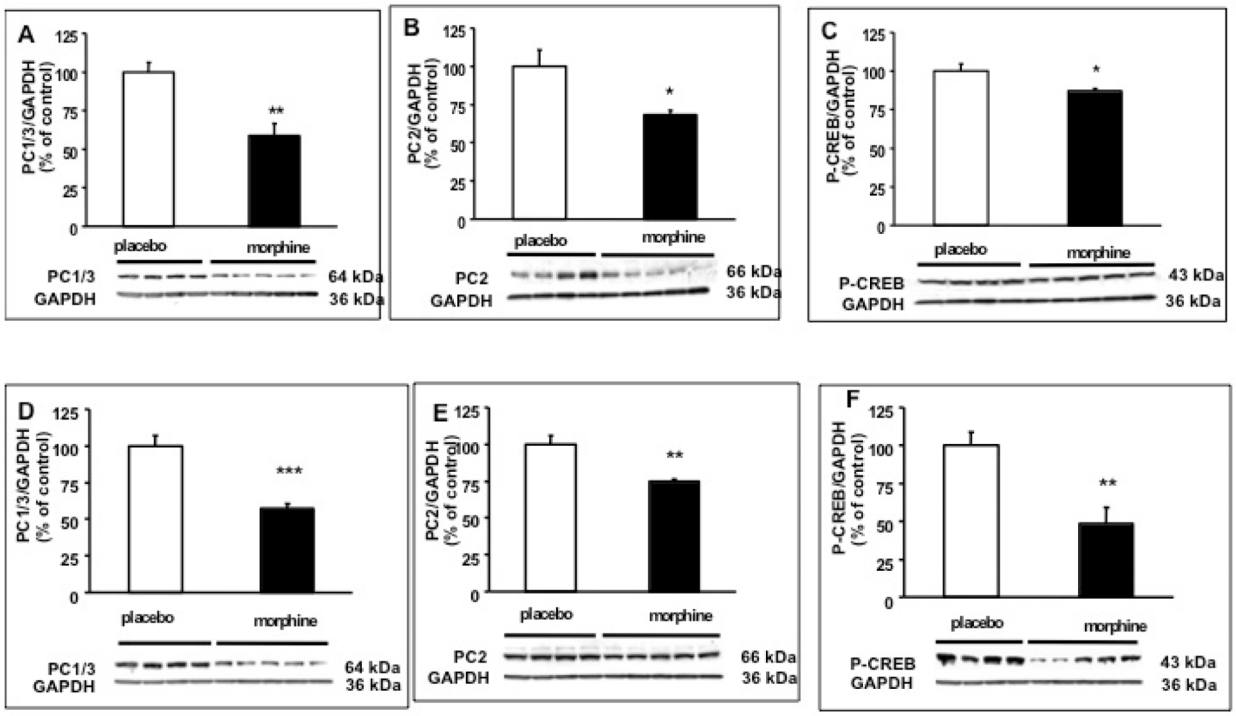

Figure 6.

Effect of 24-h morphine treatment on PC1/3 (A, D), PC2 (B, E) and P-CREB (C, F) protein expression in the anterior (A, B, C) and neuro-intermediate (D, E, F) lobe of the pituitary of morphine-treated rats as determined by Western blot. Placebo (open bars) or morphine (filled bars) pellets were implanted subcutaneously. Western blot analysis was performed on anterior pituitary extracts with PC1/3, PC2 and P-CREB antibody. The same membranes were stripped and then immunoblotted with GAPDH antibody as a control. Densitometry of the bands expressing PC1/3, PC2 and P-CREB in the anterior and neuro-intermediate lobe was compared to GAPDH for quantitation and expressed as percentage of control (mean +/− SEM). *p<0.05, **p<0.01, ***p<0.001.

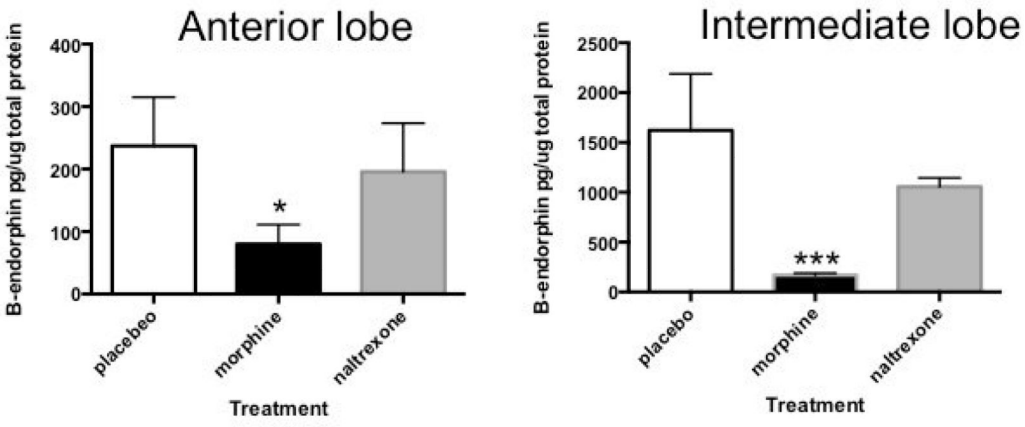

Figure 7.

Effect of 24-h morphine, naltrexone or placebo pellet exposure on β-endorphin levels, assayed with a specific β-endorphin RIA, in anterior and intermediate lobes peptide acid extracts (four per group). *$p<0.05$, ***$p<0.005$, vs placebo treated rats.