Effect of Opioid Receptor Antagonism on Proopiomelanocortin Peptide Levels and Gene Expression in the Hypothalamus

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In order to determine how brain β-endorphin (β-EP) and its precursor proopiomelanocortin (POMC) adapt to chronic opioid blockade we have examined the effects of treatment with the opioid receptor antagonist naltrexone (NTX) on POMC gene expression and peptide levels in the hypothalamus. Male rats were treated with NTX by daily injection or constant minipump infusion. RNA was isolated from the medial basal hypothalamus (MBH) after an aliquot was removed for peptide RIA and the amount of POMC mRNA was measured by solution hybridization SI nuclease protection assay. β-EP and several other POMC-derived peptides including α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP) or γ-MSH were measured in the MBH and anterior hypothalamus (AH) by RIA. In an initial experiment POMC peptide levels were measured after 7 days of NTX (4.8 mg/day) infusion. There was a marked fall in the concentrations of β-EP, α-MSH, and CLIP; levels in the MBH declined by more than 60% (P < 0.001). In the next experiment NTX (1 mg) was injected daily and POMC peptides and mRNA were measured after 2 and 5 days of treatment. β-EP and α-MSH levels fell progressively in the MBH and AH and were significantly less than those of the controls by 5 days of treatment (P < 0.02). POMC mRNA levels, however, did not change after 2 or 5 days. When NTX was infused for 3 weeks there was a decrease in the concentrations of β-EP, α-MSH, and CLIP; levels in the MBH declined by more than 60% (P < 0.001). The concentration of POMC mRNA in the MBH, however, was significantly higher in the NTX-treated animals, 0.99 ± 0.06 pg/µg RNA vs 0.81 ± 0.05 pg/µg RNA (P < 0.05). Since NTX can affect LH and testosterone release, the study was repeated in castrated rats. POMC peptide levels again fell after 3 weeks of NTX. POMC mRNA levels were higher in the castrated rats than in the intact rats, 1.14 ± 0.06 pg/µg RNA vs 0.85 ± 0.09 pg/µg RNA (P < 0.05), consistent with our previous findings in long-term castrated rats. However POMC mRNA increased further to 1.40 ± 0.09 pg/µg RNA in the castrated animals treated with NTX (P < 0.05). We conclude that opioid receptor blockade has significant effects on POMC peptide levels and gene expression in the MBH. The increase in POMC gene expression associated with a fall in peptide levels is consistent with a compensatory increase in brain β-EP synthesis and release in the setting of chronic opioid receptor blockade.

INTRODUCTION

Endogenous opioid peptides that are synthesized in the brain have numerous biological effects including many neuroendocrine effects that are important in modulating anterior pituitary function (1-9). β-Endorphin (β-EP), one of the three distinct classes of endogenous opioids, has been shown to play a role in suppressing gonadotropin release and reproductive function under different physiological and pathological conditions (4,5). Thus treatment with opioid antagonists has been suggested for disorders of LH secretion associated with high levels of β-EP (10-12). Similar treatments have also been suggested for a variety of other pathological conditions in which endogenous opioids have been implicated (10). Currently the long-acting opiate antagonist naltrexone is being used on a chronic basis to treat opiate addiction (12). Little is known, however, about how brain β-EP and its precursor proopiomelanocortin (POMC) adapt to chronic opioid blockade.

We have previously shown in the rat that treatment with naltrexone for 1 month resulted in a significant fall in the β-EP concentration of the medial basal hypothalamus (MBH) where POMC is synthesized, as well as in the thalamus and amygdala where POMC neurons project (13). Other studies had shown that opiate antagonists increase opiate receptor binding (14-17). These results were consistent with the hypothesis that chronic opiate receptor blockade causes a compensatory increase in brain β-EP release and an enhanced ability to bind this endogenous opioid peptide. It was unclear however how the fall in brain β-EP content that we measured after naltrexone
related to changes in β-EP biosynthesis or turnover. In this study we have therefore examined the effects of treatment with naltrexone on POMC gene expression and peptide levels in the hypothalamus. In addition to β-EP, α-melanocyte-stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP), and γ3-MSH levels were also measured since they represent the major POMC-derived peptide products in the hypothalamus.

MATERIALS AND METHODS

Animals and Treatment Schedules

Adult male Sprague–Dawley rats (200–225 g) were obtained from Charles River (Wilmington, MA) and used in all studies. Naltrexone HCl was kindly donated by DuPont Pharmaceuticals (Wilmington, DE). Methohexital sodium was used as the anesthetic for all surgical procedures. Four experiments were performed. The first experiment was performed to determine the effect of naltrexone on the concentrations of several POMC-derived peptides in the hypothalamus. Two experiments were performed to determine the time course of the naltrexone effects on POMC mRNA and peptide levels in the hypothalamus. A final experiment was performed in castrated rats to eliminate potential complicating effects of naltrexone on testosterone release (6). In one short-term experiment naltrexone was administered by daily subcutaneous injection. However in the other three experiments naltrexone was infused by subcutaneously implanted osmotic minipumps to ensure continuous high levels of drug over a 1- to 3-week period. Similar chronic minipump infusion of naltrexone has been shown to cause opiate receptor upregulation and functional supersensitivity (16).

Experiment 1. In an initial experiment eight rats were infused with naltrexone 4.8 mg/day via subcutaneously implanted osmotic minipumps (Alza, Palo Alto, CA) for a total of 7 days. Naltrexone was dissolved in 8.5% lactic acid for infusion. Seven rats with empty Silastic implants served as controls. In this study β-EP, α-MSH, and CLIP were measured in the MBH and β-EP and α-MSH were measured in the anterior hypothalamus (AH).

Experiment 2. In this study 1 mg of naltrexone was administered daily by subcutaneous injection. Eight rats were injected with naltrexone for 5 days, eight rats received saline for 3 days and naltrexone for 2 days, and eight rats received saline for 5 days. Animals were sacrificed 18 h after their last injection. β-EP and α-MSH were measured in the MBH and AH. POMC mRNA was measured in the MBH.

Experiment 3. Naltrexone was infused constantly sc by osmotic minipump at increasing doses for 3 weeks in 10 rats (1.2 mg/day, first week; 2.4 mg/day, second week; 3.6 mg/day, third week). Ten rats served as controls. β-EP, α-MSH, and γ3-MSH were measured in the MBH and AH. POMC mRNA was measured in the MBH.

Experiment 4. Ten rats were orchietomized and infused with naltrexone at increasing doses of 1.2–2.4 mg/day for 3 weeks. Nine rats were orchietomized and received only Silastic implants, and 9 rats were sham orchietomized. β-EP, α-MSH, and γ3-MSH were measured in the MBH and AH. POMC mRNA was measured in the MBH.

Tissue Dissection and Extraction of Peptides and RNA

Rats were sacrificed by decapitation and the brain was quickly removed and placed on ice. Trunk blood was collected in some experiments to measure plasma testosterone levels. The brain was then placed in a luteine brain holder, ventral side up, with razor blades positioned at 2-mm intervals for cutting sections. The MBH was dissected from a 2-mm coronal section cut immediately caudal to the optic chiasm. The dissection was limited laterally by the hypothalamic sulci and dorsally by the mammillothalamic tract. The anterior hypothalamus/preoptic area was dissected from the next rostral 2-mm section, which was bordered dorsally by the anterior commissure, and it was homogenized in 1 ml of 0.2 N HCl. The MBH was then homogenized in 1 ml of cold sterile AT buffer (10 mM Tris, pH 8.0, 3 mM CaCl2, 2 mM MgCl2, 0.5 mM DTT, 0.15% Triton X-100) containing 0.3 M sucrose and 50 units of human placental ribonuclease inhibitor. A 25-μl aliquot was removed for protein determination and a 200-μl aliquot was removed for β-EP and α-MSH and either γ3-MSH or CLIP peptide content. The 200-μl aliquot removed for peptide determinations was sonicated in 400 μl of 0.2 N HCl and centrifuged at 4000g for 30 min. The supernatant was then kept frozen until the time of peptide RIA. Protein content was determined in a 25-μl aliquot of the supernatant by the Bradford (18) method using bovine serum albumin as the standard.

Radioimmunoassays

The β-EP content in the MBH extracts was assayed with an antiserum to human β-EP raised in this laboratory, which cross-reacts equally with camel β-EP, N-acetyl β-EP, and β-EP (1–27) and 30% on a molar basis with human β-lipotropin (19). Synthetic camel β-EP (Penin-
Experiment 1

When naltrexone was infused at a dose of 4.8 mg/day for 7 days, there was a marked fall in the concentration of all POMC peptides measured in both the MBH and the AH (Fig. 1). In the MBH, β-EP fell from 4.96 ± 0.41 (SE) in the control rats to 1.82 ± 0.12 ng/mg protein in the naltrexone-treated animals, α-MSH fell from 3.02 ± 0.13 to 1.16 ± 0.07 ng/mg protein, and CLIP fell from 6.34 ± 0.49 to 2.35 ± 0.16 ng/mg protein (P < 0.001). In the AH, β-EP fell from 3.98 ± 0.46 to 1.93 ± 0.29, and α-MSH fell from 2.04 ± 0.17 to 1.27 ± 0.14 ng/mg protein (P < 0.005). Mean plasma testosterone levels were not significantly different in the control (1.58 ± 0.55 ng/ml) vs the naltrexone-treated animals (1.29 ± 0.61 ng/ml).

Experiment 2

When 1 mg of naltrexone was injected daily, β-EP and α-MSH levels fell progressively in the MBH and were
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significantly less than those in the saline-treated controls by 5 days of treatment (Fig. 2) \((P < 0.05)\). The concentration of \(\beta\)-EP was \(5.77 \pm 0.50 \, \text{ng/mg protein}\) after saline compared to \(4.56 \pm 0.41\) and \(4.21 \pm 0.22 \, \text{ng/mg protein}\) after 2 and 5 days of naltrexone, respectively. The concentration of \(\alpha\)-MSH was \(3.13 \pm 0.29\) compared to \(2.52 \pm 0.20\) and \(2.20 \pm 0.13 \, \text{ng/mg protein}\). Parallel changes in \(\beta\)-EP and \(\alpha\)-MSH were also seen in the AH. \(\beta\)-EP fell from \(2.43 \pm 0.21\) to \(1.82 \pm 0.16\) and \(1.65 \pm 0.14 \, \text{ng/mg protein}\) \((P < 0.05\) at 2 and 5 days NTX vs saline); \(\alpha\)-MSH fell from \(1.45 \pm 0.13\) to \(0.97 \pm 0.08\) and \(0.85 \pm 0.07 \, \text{ng/mg protein}\) \((P < 0.01\) at 2 and 5 days NTX vs saline). POMC mRNA levels, however, did not change after 2 or 5 days of treatment (Fig. 2). The concentration of POMC mRNA in the MBH was \(0.79 \pm 0.10 \, \text{pg/µg RNA}\) in the controls compared to \(0.85 \pm 0.08\) and \(0.85 \pm 0.05 \, \text{pg/µg RNA}\) after naltrexone.

Experiment 3

When naltrexone was infused constantly at increasing doses of 1.2-3.6 mg/day for 3 weeks there was a significant decrease in the concentrations of \(\beta\)-EP, \(\alpha\)-MSH, and \(\gamma\)-MSH in the MBH \((P < 0.001)\) (Fig. 3). \(\beta\)-EP fell from \(5.15 \pm 0.31\) to \(3.47 \pm 0.21 \, \text{ng/mg protein}\), \(\alpha\)-MSH fell from \(2.94 \pm 0.07\) to \(1.83 \pm 0.08 \, \text{ng/mg protein}\), and \(\gamma\)-MSH fell from \(12.2 \pm 0.50\) to \(6.61 \pm 0.51 \, \text{ng/mg protein}\). In the AH, \(\beta\)-EP fell from \(2.94 \pm 0.22\) to \(2.17 \pm 0.23\), \(\alpha\)-MSH fell from \(1.13 \pm 0.07 \, \text{ng/mg protein}\), and \(\gamma\)-MSH fell from \(4.46 \pm 0.36\) to \(3.52 \pm 0.20 \, (P < 0.05)\). In contrast, the concentration of POMC mRNA in the MBH rose significantly from \(0.81 \pm 0.05\) to \(0.99 \pm 0.06 \, \text{pg/µg RNA}\) in the naltrexone-treated animals \((P < 0.05)\). Mean plasma testosterone levels were again not significantly different in the control (1.17 ± 0.16 ng/ml) vs the naltrexone-treated animals (2.59 ± 1.06 ng/ml).

Experiment 4

Similar effects on POMC peptide and mRNA levels were seen in castrated rats after 3 weeks of naltrexone. \(\beta\)-EP in the MBH fell from \(4.5 \pm 0.26\) in castrated control rats to \(3.43 \pm 0.14 \, \text{ng/mg protein}\) in castrated naltrexone-treated rats \((P < 0.01)\); for comparison sham-castrated animals had a \(\beta\)-EP level of \(3.97 \pm 0.28\). \(\alpha\)-MSH fell from \(2.02 \pm 0.043\) to \(1.51 \pm 0.061 \, \text{ng/mg protein after naltrexone}\) \((P < 0.001)\); sham-castrated animals had a level of \(1.80 \pm 0.09 \, \text{ng/mg protein after naltrexone}\) \((P < 0.005)\); sham-castrated animals had a level of \(8.09 \pm 0.73\). The concentrations of \(\beta\)-EP, \(\alpha\)-MSH, and \(\gamma\)-MSH also fell significantly in the AH of the castrated naltrexone-treated rats \((P < 0.05)\). POMC mRNA levels were higher in the castrated than in the intact rats, \(1.14 \pm 0.06\) vs \(0.85 \pm 0.09 \, \text{pg/µg RNA}\) \((P < 0.05)\), consistent with our previous findings in long-term castrated male rats. However, POMC mRNA increased significantly to \(1.40 \pm 0.09 \, \text{pg/µg RNA}\) in castrated animals treated with naltrexone \((P < 0.05\) compared to castrated untreated animals) (Fig. 4).

DISCUSSION

In these studies we have shown that opioid receptor blockade has significant effects on POMC peptide levels and gene expression in the hypothalamus. We had previously reported that treatment with naltrexone for 1 month caused a fall in the concentration of \(\beta\)-EP in the hypothalamus where POMC is synthesized as well as in...
several other brain regions where POMC neurons project (13). We have now shown that naltrexone causes a fall in the concentrations of α-MSH, CLIP, and γ3-MSH, the other major POMC-derived peptide products in the hypothalamus. A decline in peptide levels was noted as early as 2 days of treatment and persisted for 3 weeks. All 4 POMC-derived peptides appeared to decline in parallel and no consistent significant changes were noted in the molar ratios of these peptides after naltrexone. Although naltrexone caused a consistent decline in POMC peptide levels, POMC mRNA levels were unchanged in the MBH after 2 or 5 days of treatment. By 3 weeks, however, there was a significant increase in POMC mRNA levels. These changes in both POMC peptide and mRNA levels are consistent with feedback regulation of POMC by endogenous opioids probably occurring at multiple sites.

Since gonadal steroids are known to affect POMC gene expression, peptide concentration, and peptide release in the hypothalamus (4, 22–24), it was important to eliminate potential effects of naltrexone on LH and testosterone release (6–10). When naltrexone was administered to orchiectomized animals there was again a decline in POMC peptide levels and an increase in POMC mRNA in the MBH. Thus the effects of naltrexone on POMC are not related to changes in testosterone secretion.

Several studies support acute effects of opiates on POMC peptide release (25, 26). When intracellular recordings were made from hypothalamic arcuate neurons, μ opioid agonists were found to induce membrane hyperpolarization and to decrease the spontaneous firing of the POMC neuron (27). Conversely, opioid antagonism has been shown to increase β-EP release from the perfused hypothalamus in vitro (25). Both basal- and potassium-stimulated β-EP release increased in response to 10^{-6} M naloxone. These studies support the interpretation that the fall in POMC peptide content that we begin to see by 2 days of naltrexone results from increased peptide release.
lease. Chronic exposure to opioid antagonists including naltrexone has been shown to increase brain opiate receptor binding sites (14–17). Opiate receptor upregulation is not seen acutely after 1 day of treatment but is seen after 8 days or longer. An increase in the analgesic potency of opiates was also noted after a period of pretreatment with naltrexone (16, 17). In the rat 8 days of naltrexone produced a 50% increase in the analgesic potency of morphine. Functional supersensitivity therefore parallels the increase in opioid receptor presence after chronic exposure to naltrexone. Thus chronic opioid receptor blockade appears to cause an increase in brain β-EP release as well as an enhanced ability to bind this endogenous opioid peptide.

The increase in POMC mRNA noted after 3 weeks of exposure to naltrexone is consistent with the hypothesis that there is feedback regulation of POMC gene expression by endogenous opioids. Although there are conflicting results, recent studies show that hypothalamic POMC mRNA levels fall with chronic morphine administration (28, 29). The current studies suggest that endogenous opioids tonically have an effect on POMC mRNA levels. The time course of the changes in mRNA levels however is quite different from that of POMC peptide levels. While β-EP release increases immediately in vitro and peptide content in the hypothalamus falls within days, POMC mRNA levels were unchanged with up to 5 days of treatment but did increase when studied after 3 weeks of naltrexone exposure. Clearly increased biosynthesis and turnover of β-EP might be taking place during the first 5 days of treatment without any changes in POMC mRNA levels. However with long-term naltrexone administration additional regulation at the level of gene expression occurs. Whether this takes place by a direct effect on opioid receptors on POMC neurons or by an indirect effect through the modulation of other systems that impinge on POMC neurons is unknown. When taken together, however, our studies support a compensatory increase in brain β-EP release and synthesis in the setting of chronic opioid receptor blockade. These effects will need to be considered when designing treatments that involve antagonism of endogenous opioids.

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REFERENCES


