

Effect of prolonged progesterone treatment on the proenkephalin mRNA gene expression and enkephalins concentration in the sheep brain

*Krystyna Pierzchała-Koziec¹, Joanna Zubel, Janusz Rząsa
Department of Animal Physiology, Agricultural University,
Cracow, Poland*

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SUMMARY

The opioids modulate reproduction in sheep mostly by inhibiting the activity of the hypothalamo-pituitary-gonadal axis. However, the mechanism by which the negative feedback control systems regulate opioid synthesis and secretion in sheep is still not recognized. As a part of a research dealing with interaction between opioids and steroids, the effect of prolonged administration of progesterone (P_4) and opioid receptor agonist or antagonist on the Met-enkephalin synthesis and concentration was examined in sheep brain. Long term P_4 treatment significantly decreased the synthesis and the concentration of the opioid peptide in the hypothalamus and pituitary, however, the effect was more pronounced in the hypothalamus. Injections of Met-enkephalin completely or partially reversed the effect of P_4 . Naltrexone given together with opioid peptide modulated the response to the opioid agonist. The results show that there is an interaction between P_4 and endogenous opioids in the

¹Corresponding author: Department of Animal Physiology, University of Agriculture, Al. Mickiewicza 24/28, 30-059 Cracow, Poland; e-mail: rzkoziec@cyf-kr.edu.pl

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INTRODUCTION

Although there was much interest during the last decade in the opioid system's role in the control of reproductive processes [10, 14], more scientific attention was directed to the involvement of a particular opioid receptor type [3, 8, 13] and interaction with other cell systems [9, 15]. It is known that the opioid, methionine-enkephalin (Met-enkephalin) is involved in the regulation of the estrous cycle, pregnancy and development in many species [2, 16]. Two known active forms of Met-enkephalin are: a small, native pentapeptide and a large molecule called cryptic enkephalin. Under special conditions, the native form is released from the cryptic form by enzymatic hydrolysis [12]. The ratio of native/cryptic form strongly depends on the synthesis of proenkephalin and is affected by stress [1, 12]. The opioid action on the hypothalamo-pituitary-gonadal (HPG) axis is mediated by specific G protein-coupled receptors: μ , delta and kappa, and depends on the synthesis and concentration of endorphins, enkephalins and dynorphins [3].

The opioids modulate male and female reproduction of many mammalian and avian species mostly by inhibition of the activity of the HPG axis [6, 17]. The effect of opioids on the concentration of steroid hormones was documented in several experiments [8, 14,] but the data concerning the effects of progesterone (P_4) and estrogens on the synthesis and concentration of endogenous opioids are very scarce [2, 4, 7]. Thus, as a part of a study dealing with the interaction of opioids and gonadal steroids, the experiment was carried out to assess the effects of prolonged administration of P_4 as well as opioid receptor antagonist and agonist on the Met-enkephalin synthesis and concentration in sheep brain.

MATERIALS AND METHODS

The experiment was carried out on 2-year-old Polish Mountain Sheep (n=20) kept in standard conditions with free access to food and water. Animals were divided into control (C, with natural estrous cycles) and three experimental groups with synchronized estrous cycles. Synchronized ewes received P₄ (intravaginal sponges for 12 days) and were injected with saline (P), Met-enkephalin (P+MET, 1 mg/kg bw, iv) and Met-enkephalin plus naltrexone (opioid receptor antagonist; P+MET+NAL, 3 mg/kg bw, iv) on Days 1, 2, 5, 12 and 15 after intravaginal sponge placing [13]. In order to synchronize the estrous cycle, sheep from all experimental groups received an injection of 600 I.U. of PMSG after removing sponges. Two days later, hypothalamus and pituitary were dissected, immediately placed on dry ice and kept at -80°C until further processing. Chemicals were provided by Sigma (USA).

Proenkephalin mRNA gene expression

The frozen fragments of tissues were sliced (14 µm sections) using a cryostat microtome (-22°C). The sections were thaw-mounted on gelatin-covered microscopic slides, and stored for 1-4 days at -20°C before the assay. Tissue sections were thawed and fixed for 10 min in 4% formaldehyde in phosphate buffered saline (PBS; pH 7.4). Afterwards, the sections were acylated in triethanolamine/acetyl anhydride (0.25%) for 10 min, dehydrated through graded ethanol (70%, 80%, 95%, 100%) and allowed to air-dry [18].

After prehybridization, a synthetic deoxyoligonucleotide, complementary to the fragment of rat proenkephalin (PENK), was labeled using ³⁵S-dATP (1200 Ci/nmol) to obtain a specific activity about 4×10⁶ cpm/µl. The probes were diluted in a hybridization buffer (formamide, dextran sulfate, SSC, Denhardt's solution, yeast tRNA, herring sperm DNA). Hybridization occurred overnight (~20 h) in humidified chamber at 37°C. Then, the sections were washed once in SSC for 10 min, then four times for 15 min, each in SSC/ 50% formamide at 40°C, rinsed in SSC and distilled water at room temperature and air-dried. The sections were exposed to Kodak film

for four weeks (-80°C). The photo-stimulated luminescence (PSL) density of the irradiated plates was measured with BAS-1000 readout system. The PSL/mm² at the resultant film images was determined using computer image analysis system.

Met-enkephalin concentration

Native and cryptic Met-enkephalin was estimated by the method of Pierzchała et al. [12]. Small fragments of tissues were homogenized in phosphate buffer, pH 6.5, centrifuged (4000×g, 4°C, 20 min), and supernatants were stored at -80°C. Enkephalin containing peptides (cryptic enkephalin) were hydrolyzed with trypsin and carboxypeptidase B. Optimal conditions for hydrolysis of the cryptic enkephalins included incubation with trypsin (1 mg/ml, 37°C) for 30 min followed by incubation with carboxypeptidase B (5 mg/ml) plus trypsin inhibitor (2.5 mg/ml) for 15 minutes.

Native and cryptic enkephalins were purified on Porapak Q (Waters, 100-120 mesh) in 2 ml of absolute ethanol, than lyophilized and radioimmunoassayed. Met-enkephalin immunoreactivity was quantified using commercial antiserum developed in rabbit, ¹²⁵I-Met-enkephalin and Met-enkephalin standard. Lyophilized samples were reconstituted with 100 µl of 0.06 M phosphate buffer (pH 6.5, 0.2% bovine serum albumin, 0.002% sodium azide). Then, 50 µl antiserum (1:10 000) and 50 µl of ¹²⁵I-Met-enkephalin (~1500 cpm) were added, and the samples were incubated (4°C). After 24 h, 50 µl of rabbit γ-globulin (1%) were added and incubation was maintained for 30 min. Bound and free complexes were separated by adding 250 µl of 25% polyethylene glycol (PEG 8000). After 30 min of incubation samples were centrifuged (2000×g, 4°C, 20 min); the supernatants were discarded and the pellets were counted in a γ-counter (Wizard).

Statistical analysis

Results are presented as means±SEM. The analysis was performed using the Statistica program (StatSoft Inc.,Tulsa, OK, USA). One-way analysis

of variance followed by LSD test was used to determine the effects of P_4 on the proenkephalin mRNA gene expression as well as native and cryptic Met-enkephalin concentrations.

RESULTS

Long-term treatment with P_4 significantly decreased the synthesis of pre-proenkephalin in the hypothalamus from 292.4 ± 8.7 to 150.0 ± 8.7 PSL/mm² ($p < 0.01$) and in the pituitary from 383.0 ± 11.6 to 210.0 ± 11.3 PSL/mm² ($p < 0.01$, fig. 1). Injections of delta opioid receptor agonist, Met-enkephalin significantly, but not completely, reversed the inhibiting effect of P_4 in hypothalamus and pituitary. The antagonistic effect of naltrexone was observed in the hypothalamus where the stimulating effect of Met-enkephalin was abolished.

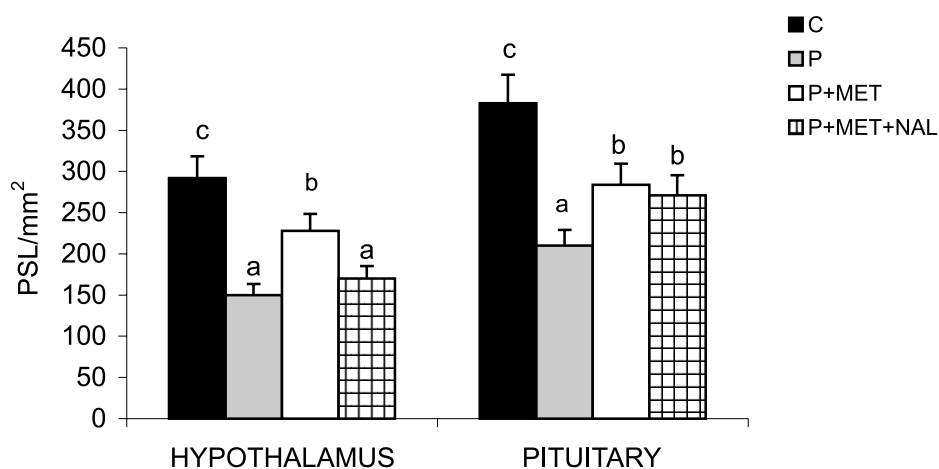


Figure 1. The proenkephalin mRNA gene expression (means \pm SEM) in the hypothalamus and pituitary expressed as PSL/mm² (photo-stimulated luminescence/mm²) of control (C), progesterone (P_4)-treated (P), P_4 -treated and injected with Met-enkephalin (1 mg/kg bw, P+MET), P_4 -treated and injected with Met-enkephalin and naltrexone (3 mg/kg bw, P+MET+NAL) sheep. Bars with different superscripts are significantly different ($p < 0.01$).

The concentration of native Met-enkephalin was decreased by administration of P_4 in the hypothalamus (10.4 ± 0.8 vs. 5.5 ± 0.4 pmol/mg tissue, $p < 0.01$, fig. 2). Injections of exogenous agonist reversed the diminishing effect of the steroid and increased the concentration of endogenous opioid to 8.9 ± 0.6 pmol/mg tissue. Naltrexone, administered together with Met-enkephalin blocked the stimulating effect of opioid which resulted in lowering the level of this peptide's native form. In contrast, the concentration of Met-enkephalin in the pituitary was increased only after injections of exogenous peptide from 9.7 ± 0.7 in control sheep to 19.1 ± 1.9 pmol/mg tissue ($p < 0.01$).

The concentration of cryptic Met-enkephalin was decreased by P_4 in both tissues ($p < 0.01$). The exogenous opioid reversed the effect of P_4 in hypothalamus (entirely) and in the pituitary (partially). Naltrexone injected with Met-enkephalin attenuated the stimulating effect of the opioid peptide in both tested tissues; however this reaction was stronger in the hypothalamus than in the pituitary (fig. 3).

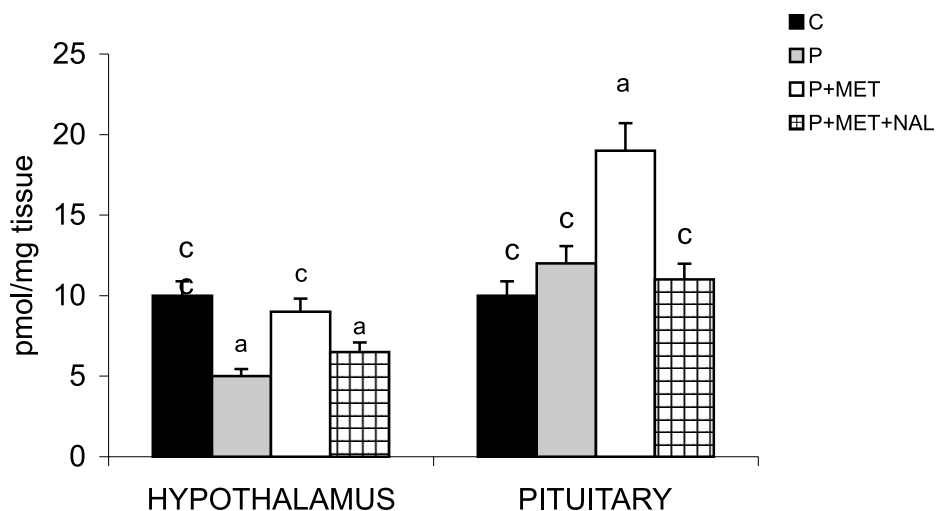


Figure 2. Native Met-enkephalin concentrations (means \pm SEM) in the hypothalamus and pituitary (pmol/mg tissue) of control (C), progesterone (P_4)-treated (P), P_4 -treated and injected with Met-enkephalin (1 mg/kg bw, P+MET), P_4 -treated and injected with Met-enkephalin and naltrexone (3 mg/kg bw, P+MET+NAL) sheep. Bars with different superscripts are significantly different ($p < 0.01$).

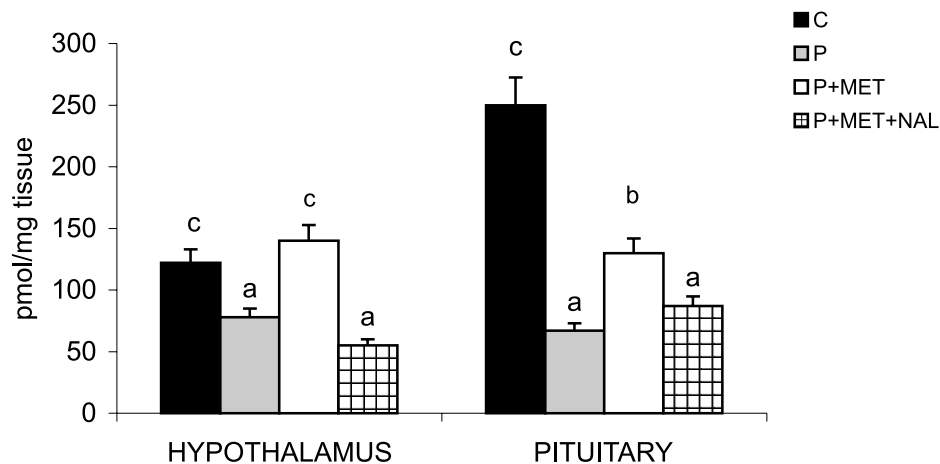


Figure 3. Cryptic Met-enkephalin concentrations (means \pm SEM) in the hypothalamus and pituitary (pmol/mg tissue) of control (C), progesterone (P_4)-treated (P), P_4 -treated and injected with Met-enkephalin (1 mg/kg bw, P+MET), P_4 -treated and injected with Met-enkephalin and naltrexone (3 mg/kg bw, P+MET+NAL) sheep. Bars with different superscripts are significantly different ($p < 0.01$).

DISCUSSION

The regulation of reproduction is a very complex, multihormonal phenomenon which occurs at every level of the HPG axis. The effect of opioids on steroid hormone concentration was documented in several experiments but data concerning the effects of P_4 on the synthesis and concentration of endogenous opioids are very limited. The opioids modulate the reproduction in sheep mostly by inhibition of the HPG axis' activity. However, the mechanisms of negative feedback control systems controlling opioid synthesis and secretion in sheep are not elucidated.

We demonstrated that proenkephalin synthesis in the hypothalamus and pituitary was affected by long-term administration (12 days) of P_4 supporting the hypothesis on the steroid-opioid interaction in the brain. Our previous results showed that prolonged administration of P_4 decreased the proenkephalin mRNA expression and opioid binding to all three types of opioid receptors in the sheep ovary [13]. Changes in

pro-opiomelanocortin and pre-proenkephalin mRNA levels in the ovine brain in response to P_4 and estrogens were found by Broad et al. [2]. These authors showed a decrease of pre-proenkephalin synthesis in three discrete hypothalamic nuclei (the ventromedial, paraventricular and supra-chiasmatic nuclei) in sheep treated with P_4 during pregnancy, parturition and lactation. The decreased level of newly synthesized precursor of Met-enkephalin may be a reason for the low concentration of the cryptic form of enkephalin.

Unexpectedly, the concentration of native Met-enkephalin in the hypothalamus decreased after P_4 treatment. This is in contrast to our previous results showing an increase in the level of the small form of Met-enkephalin as a result of enzymatic hydrolysis of the cryptic form [11]. It is possible that P_4 increased the level of enkephalin-degrading aminopeptidase in the brain which resulted in lowering the native enkephalin concentration. A single dose of P_4 significantly increased the activity of this enzyme in the hypothalamus and pituitary of female mice [5]. In contrast, the level of native Met-enkephalin in the pituitary was not changed by P_4 in spite of very low proenkephalin mRNA expression and cryptic enkephalin level. It cannot be excluded that Met-enkephalin released from hypothalamus was stored in the pituitary. Our results showed that P_4 affected the Met-enkephalin mainly in hypothalamus which suggests that opioid interaction with GnRH may be dominant during the estrous cycle.

Injections of opioid receptor agonist significantly, but not completely, reversed the inhibiting effect of P_4 on the synthesis and concentration of both forms of Met-enkephalin in the hypothalamus and pituitary. Previous experiments showed that Met-enkephalin completely reversed the diminishing effect of P_4 on proenkephalin mRNA expression in the sheep ovary [13]. On the other hand, naltrexone, administered together with Met-enkephalin modulated the effect of opioid indicating the opioidnergic character of the response. In conclusion, these results show that there is an interaction between P_4 and endogenous opioids in the central nervous system of cyclic sheep.

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