

Alterations in Prolactin Messenger Ribonucleic Acid Level During the Rat Estrous Cycle: Effect of Naloxone

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The present study examines the physiological alterations in prolactin (PRL) messenger ribonucleic acid (mRNA) and serum PRL levels during the rat estrous cycle and the effect of naloxone, an endogenous opioid peptide receptor antagonist, on PRL gene expression during the rat estrous cycle. Adult female rats exhibiting at least two consecutive 4-day estrous cycles were used in this study. A single injection of naloxone (2mg/kg b.w.) or saline was given sc 30 min prior to decapitation. Animals were sacrificed at 10:00 h of each stage of the estrous cycle, and at 2-h intervals from 10:00 h to 20:00 h during the proestrus. PRL mRNA and serum PRL levels were determined by a RNA-blot hybridization with the rat PRL cDNA probe and by a PRL radioimmunoassay, respectively. PRL mRNA and serum PRL levels were not dramatically altered in the morning of each stage of diestrus I, II and proestrus, and naloxone failed to modify the two parameters. During estrus naloxone clearly suppressed serum PRL levels, but it was unable to modify PRL mRNA levels. A more detailed examination of the proestrus stage revealed that PRL mRNA and serum PRL levels were fluctuated as a function of time: PRL mRNA levels reached a maximum level at 12:00 h and gradually decreased until 18:00 h. PRL mRNA levels then rose at 20:00 h. No difference of PRL mRNA levels between the control and naloxone-treated groups was observed. Changes in serum PRL levels during proestrus were conversely related to changes in PRL mRNA: serum PRL levels were low from 10:00 h to 14:00 h, then increased and reached a maximum level at 16:00-18:00 h. Following then, serum PRL levels were decreased. Naloxone was effective in suppressing the characteristic afternoon surge of PRL from 16:00 h to 20:00 h.

These data clearly showed that alterations in PRL mRNA levels were conversely correlated with changes in serum PRL levels on proestrus, indicating a differential regulation of PRL gene expression and secretion.

KEY WORDS: Prolactin gene expression, Naloxone, Rat estrous cycle

It is well documented that prolactin (PRL) secreted by lactotropes of the anterior pituitary is involved in a variety of physiological processes. In

particular, PRL is essential for the normal development and differentiation of female breast and milk production. PRL also exerts complex yet undefined actions on gonadotropin secretion and growth hormone-like metabolic functions (Frantz, 1978; McNeilly, 1987). It is known that serum PRL levels fluctuates as a function of the rat

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estrous cycle, and a major surge of PRL occurs in the late afternoon of proestrus (Butcher *et al.*, 1974; Neill and Smith, 1974). Endogenous opioid peptides (EOP) are shown to be involved in the regulation of PRL secretion (Rivier *et al.*, 1977; Enjalbert *et al.*, 1979; Ragavan and Frantz, 1981a; Bedran de Castro *et al.*, 1987). It is evident that naloxone (NAL), a specific EOP receptor antagonist, blocks proestrous PRL surge during the rat estrous cycle, and PRL secretion induced by lactation or stressful stimuli (Ieri *et al.*, 1980; Ragavan and Frantz, 1981b; Sirinathsinghji and Martini, 1984).

Following the elucidation of gene sequences and genomic organization of the rat PRL (Chien and Thompson, 1980; Gubbins *et al.*, 1980), it has been shown that gene expression of PRL is regulated by a number of different factors, such as dopamine (Maurer, 1980), TRH (Murdoch *et al.*, 1983; Laverrier *et al.*, 1988), estrogen (Maurer, 1982; Yamamoto *et al.*, 1986; Shull and Gorski, 1989), and VIP (Carrillo *et al.*, 1985). However, few data are only available regarding the physiological changes in PRL mRNA levels during the estrogen-induced PRL surge and the rat estrous cycle (Carrillo *et al.*, 1987; Haisenleder *et al.*, 1989). As far as the EOP action on PRL mRNA levels during the rat estrous cycle is concerned, no evidence is yet available.

Therefore, the present study attempts to elucidate 1) the changes in PRL mRNA levels during the rat estrous cycle, and 2) the effect of naloxone on PRL gene expression. Serum PRL levels are determined to establish the causal relationship between PRL gene expression and PRL secretion.

Materials and Methods

Animals

Adult female Sprague Dawley rats (Seoul National University Animal Breeding Center) weighing 200-250 g were used. Animals were housed under controlled conditions (lights 07:00 h-21:00 h) with food and water freely available. Vaginal smears were taken daily and rats showing three consecutive 4-day cycles were used in the present study.

Experiments

In order to validate the tissue-specific gene expression of PRL and the size of PRL mRNA, Northern-blot analysis was performed. RNA was extracted from the kidneys, cerebral cortexes, and anterior pituitaries and fractionated by gel electrophoresis as described below.

To investigate the alteration of PRL mRNA levels during the rat estrous cycle, rats were decapitated at 10:00 h of each stage of the estrous cycle. During proestrus, anterior pituitary and serum were obtained at 2-h intervals from 10:00 h to 20:00 h. In addition, to determine the action of EOP on PRL mRNA levels and serum PRL levels, naloxone (2mg/kg b.w.) or saline were injected sc 30 min prior to decapitation.

RNA-cDNA Hybridization

(1) RNA preparation

Total RNA was prepared either by nonidet P-40 method (Papavasiliou *et al.*, 1986) or by CsCl method (Maniatis *et al.*, 1982). The nonidet P-40 method was conventionally used for RNA-blot hybridization. Briefly, pituitaries were homogenized by a glass homogenizer containing 220 μ l homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.5% (v/v) nonidet P-40, pH 7.5). The homogenates were centrifuged at 13,000 \times g at 4°C for 30 min. The supernatant was transferred to a sterile tube and adjusted to a final concentration of 100 mM NaCl, 100 mM Tris-HCl (pH 8.5) and 1% (w/v) SDS and then extracted with phenol/chloroform. RNA was precipitated overnight at -20°C with 3 M sodium acetate (0.1 vol.) and absolute ethanol (2.5-3 vol.). Total RNA was then recovered by centrifugation at 13,000 \times g at 4°C for 1 h. RNA was dissolved in distilled water and quantitated by U.V. spectrophotometry (LKB) at 260 nm. Ratio of O.D. at 260 nm to at 280 nm was about 1.8-2.0.

For Northern-blot analysis, total RNA was extracted by using guanidium thiocyanate/CsCl gradient centrifugation procedure (Maniatis *et al.*, 1982). Briefly, tissues were homogenized by a glass homogenizer containing lysis buffer [4 M guanidium thiocyanate (BRL), 0.1 M Tris-HCl (pH 7.5), 1% 2-mercaptoethanol and 0.5% sodium-lauryl sarcosinate]. Cesium chloride (1 g/2.5 ml)

was added to the homogenate and layered onto a 1.1 ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) in a Beckman SW 60 polyallomer tube. Following ultracentrifugation at 41,000 rpm at 20°C for 12 hr, the supernatants were discarded and the walls of tube were dried thoroughly. The pellets of total RNA were dissolved in solubilization buffer [10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1% SDS] and extracted with 4:1 mixture of chloroform and 1-butanol. The aqueous phase was transferred to a fresh tube and RNA was precipitated and quantitated as described above.

(2) Slot-blot and Northern-blot hybridization

In slot-blot hybridization, total RNA aliquots adjusted to 50 μ l with sterile water were transferred to a sterile tube (1.5 ml) containing 30 μ l of 20X SSC plus 20 μ l of 37% formaldehyde. The mixture was then incubated at 65°C for 10 min, and chilled on ice. RNA was applied onto nitrocellulose filter (BA 85-SB, 0.45 μ m pore size) by using Minifold II apparatus (Schleicher & Schuell, New Hampshire). Wells were rinsed with 150 μ l of 15 \times SSC and the nitrocellulose filters were then baked at 80°C for 2 hr (Papavasiliou *et al.*, 1986).

In Northern-blot hybridization, RNA was dissolved in D.W. and denatured in 50% formamide, 6.2% formaldehyde, 20 mM MOPS [3-(N-morpholine) propanesulfonic acid], 5 mM sodium acetate and 1 mM EDTA at 60°C for 5 min. RNA was then fractionated by size using electrophoresis on 1.2% agarose gel containing 6.2% formaldehyde and 20 mM MOPS. The RNA ladder (0.16–1.77 kb, BRL 5623 SA) was also denatured and electrophoresed on the gel for size markers. Bromophenol blue and xylene cyanol were used as a tracking dye. Electrophoresis was performed in the form of nonsubmarine gel and carried out at 100 volts for 3 hr. RNA was transferred onto nitrocellulose filters (0.45 μ m) according to the diffusion blotting. After 3hr transfer, the filters were dried in air and baked for 2 hr at 80°C.

(3) Probe preparation

The rat PRL cDNA in plasmid SP65 was a kind gift from Dr. R.A. Maurer (University of Iowa, Iowa city, Iowa). Its size was 0.8 kb and contained the complete prolactin coding sequence

(pPRL-2; Gubbins *et al.*, 1980). The plasmid was transformed to *E. coli* (HB101 strain), amplified in LB medium and the transformants were subsequently lysed by alkali (Maniatis *et al.*, 1982). The PRL cDNA was cut by EcoRI and purified by electroelution.

The random primer labeling reaction was carried out at 37°C for 2 hr by adding of the following reagents (Feinberg and Vogelstein, 1984): Prolactin cDNA (40–50 ng), 10 μ l of oligo-labeling buffer, 2 μ l of BSA(10mg/ml), 5 μ l of alpha- 32 P] dCTP (New England Nuclear, 3000Ci/mmol), and 5 units of large fragments of *E. coli* DNA polymerase I (Klenow fragment)(BRL No. 8012). Oligo-labeling buffer was made from the following components: solution 0; 1.25 M Tris-HCl, 0.125 M MgCl₂, pH 8.0, solution A; 1 ml of solution 0 + 18 μ l of 2-mercaptoethanol + 5 μ l dATP, 5 μ l dTTP, 5 μ l dGTP (each triphosphate, 0.1 M), solution B; 2M Hepes, pH 6.6, solution C; Hexadeoxyribonucleotides (Pharmacia 2166) in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) at 90 OD units/ml. Mix solution A:B:C in a ratio of 100:250:150 to make oligo-labeling buffer. The reaction was stopped by addition of 5 μ l of 0.2 M EDTA and 10 μ g yeast tRNA. The 32 P-labeled probes were separated from unincorporated 32 P] dCTP by gel filtration on a Sephadex G-50 column (1 \times 15 cm) preequilibrated with TE buffer, pH 8.0. The specific activity was 1–2 $\times 10^9$ cpm/ μ g DNA.

(4) Hybridization procedure and Autoradiography

The hybridization procedure was similar to that previously described by Papavasiliou *et al.* (1982). Nitrocellulose filters were prehybridized in 50% deionized formamide, 5 \times SSPE, 5 \times Denhardt's solution (1 \times ; 0.01% polyvinyl pyrrolidone, 0.01% ficoll, 0.01% BSA), 0.1% SDS and 2 mg heat-denatured salmon sperm DNA at 42°C for 3 hr. 32 P-labeled PRL cDNA was added to hybridization buffer and incubated at 42°C for 24 hr. The hybridization solution was the same as prehybridization solution except for 0.1 \times Denhardt's solution. After hybridization, the filters were washed three times (5 min/wash) in 2 \times SSC and 0.1% SDS at room temperature and twice (30 min/wash) in 0.1 \times SSC and 0.1% SDS at 42°C. The filters were then dried and exposed to X-ray film (Kodak X-Omat) at –70°C for 4 hr.

Density of each slot band on film was scanned with densitometric scanner (Transidyne General Corp).

PRL Radioimmunoassay (RIA)

The concentrations of PRL in serum were measured in duplicate by a double antibody RIA procedure. PRL RIA kit was supplied by the NIADDK and PRL levels were expressed in terms of rPRL-RP-3 standards. rPRL-I-5 was iodinated by chloramine T method and ^{125}I -labeled PRL (8000-9000cpm/tube) was used. Anti-rPRL-S-9 serum was used at a final dilution of 1: 12,500. The sensitivity was 0.02 ng/ml and inter- and intra-assay coefficients of variation were 6.5% and 3.4%, respectively.

Statistics

Student's *t*-test was employed for analyzing the difference between control and treatment groups. Data were also analyzed by one-way analysis of variance (ANOVA) with $p < 0.05$ required for a statistical significance. Duncan's multiple range test was used for *post-hoc* comparison.

Results

Validation of RNA-blot hybridization

Northern-blot analysis showed the tissue-specific expression of PRL gene (Fig. 1). Hybridization signals were observed in the sample only from the anterior pituitaries, but not from kidneys and cerebral cortices (negative controls). Fig. 1 also depicts that the size of rat PRL mRNA hybridized with PRL cDNA probe was approximately 0.8 kb, which is in a good agreement with the previous report (Maurer, 1982). PRL mRNA was dose-dependently increased as a function of the amounts of RNA per slot (Fig. 2).

Alteration of PRL mRNA and serum levels during the rat estrous cycle and the effect of naloxone

Figs. 3 and 4 represent the effects of NAL on the levels of serum PRL and PRL mRNA at each stage during the estrous cycle. In the saline-treated control groups the serum PRL levels were not highly fluctuated on diestrus I, II, and proestrus (54.4 ± 4.5 , 60.6 ± 7.1 and 43.0 ± 5.5 ng/ml,

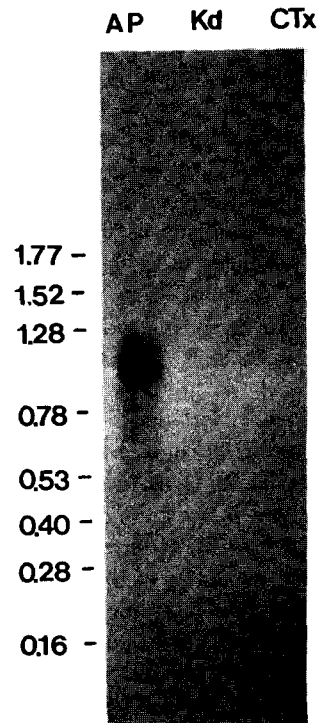


Fig. 1. Northern-blot analysis of total RNA from the rat anterior pituitary (AP), kidney (Kd) and cerebral cortex (CTx). RNA ladder (0.16-1.77 kb, BRL) was used as the size marker.

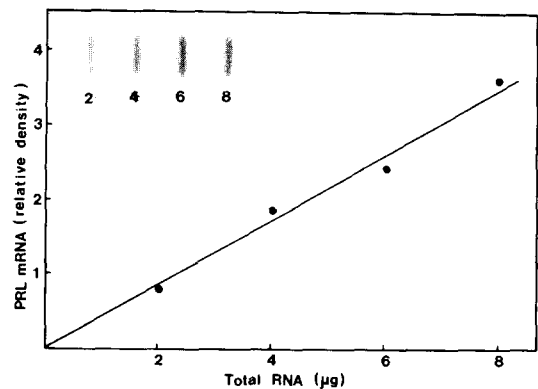


Fig. 2. Linear increase in PRL mRNA as a function of the amounts of total RNA blotted onto nitrocellulose filter. Total RNA derived from the anterior pituitaries were blotted as 2, 4, 6 and 8 μg /slot. The coefficient of regression was 0.985.

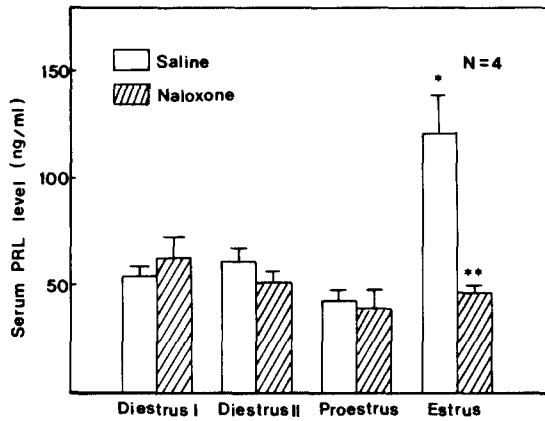


Fig. 3. The effect of naloxone on serum PRL levels at 10:00 h a.m. during the rat estrous cycle. *, significantly different from those of DI, DII, and E ($p < 0.05$), **, significantly different from the saline-treated group ($p < 0.05$).

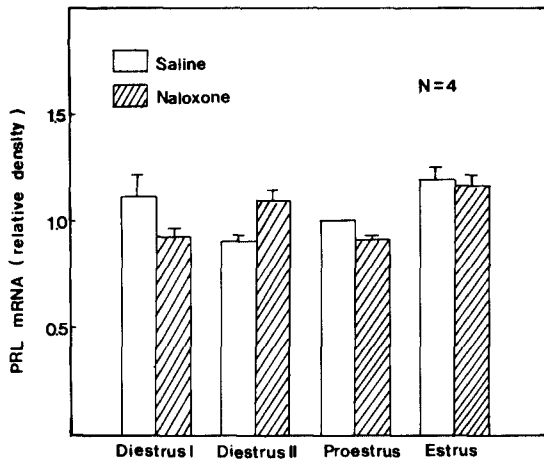


Fig. 4. Changes in PRL mRNA levels in response to naloxone treatment during the rat estrous cycle.

respectively), but were significantly increased (120.9 ± 10.2 ng/ml) on estrus. Administration of NAL did not affect to alter the basal serum PRL levels on diestrus I, II, and proestrus, but decreased serum PRL levels on estrus. No difference of PRL mRNA levels was observed between the control and NAL-treated groups when PRL mRNA levels were compared at 10:00 h during the different stages of the rat estrous cycle. At the day of proestrus, the PRL surge began at 14:00 h (62.5 ± 8.8 ng/ml), reached a maximum at 18:00 h (177.5 ± 14.1 ng/ml). The magnitude of serum PRL levels induced by

NAL was different at the various time points during the proestrus surge. NAL clearly decreased serum PRL level from 14:00 h to 20:00 h, but did not produce any effect at 10:00 h and 12:00 h (Fig. 5). On proestrus stage, PRL mRNA levels reached a maximum at 12:00 h (1.30 unit) and gradually decreased until 18:00 h (0.56 unit). Interestingly, it is evident that PRL mRNA levels then increased at

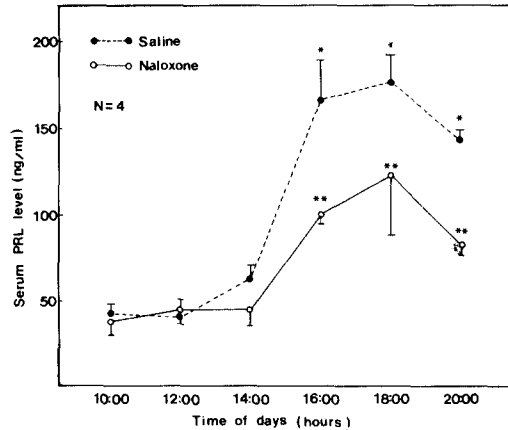


Fig. 5. Alteration of serum PRL levels during the proestrus stage. Saline (●—●) or naloxone (○—○) were treated sc 30 min prior to decapitation at 2-h intervals from 10:00-20:00 h. *, significantly different from the control values at 10:00, 12:00, and 14:00 h ($p <$), **, significantly different from the saline-treated group ($p < 0.05$).

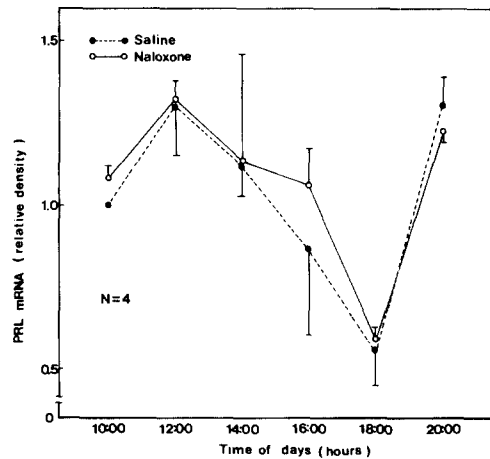


Fig. 6. Alteration of PRL mRNA levels in the anterior pituitaries derived from the proestrus rats at 2-h intervals. Saline (●—●) or naloxone (○—○) was treated 30 min prior to decapitation.

20:00 h (1.31 unit). No effect of NAL on PRL mRNA levels was observed during the proestrus PRL surge (Fig. 6).

Discussion

The present experiment provides information on alterations in PRL mRNA and serum PRL levels during the rat estrous cycle. The overall changes in serum PRL levels were previously well examined (Butcher, 1974; Smith *et al.*, 1975). In the present study, codetermination of serum PRL and PRL mRNA levels was made at a single time point (10:00 h) of each stage, except for the proestrous stage. Major PRL surge occurred in the afternoon of proestrus and maintained at a relatively high level in the morning of estrus, but serum PRL levels were basal in the morning of diestrus I, II, and proestrus (Figs. 3 and 5). Although no significant change in PRL mRNA levels was observed in the morning during the rat estrous cycle, a more detailed examination of proestrus stage with a 2-h intervals showed that PRL mRNA levels were changed as a function of time: PRL mRNA was high in the morning of proestrus, gradually decreased until 18:00 h, and rebounded at 20:00 h (Figs. 4 and 6). Interestingly enough, the overall profile of PRL mRNA levels during the proestrus appears to be very different from that of the recent findings (Carrillo *et al.*, 1987; Haisenleder *et al.*, 1989). Haisenleder *et al.* (1989) found that PRL mRNA levels began to rise at 17:00 h and continued to increase until 23:00 h, although PRL mRNA levels transiently decreased between 11:00 and 12:00 h. At the present, we are unaware of these discrepancies. Anyway, it is of importance to notice that alterations in PRL mRNA were conversely related to changes in serum PRL levels, indicating a differential regulation of PRL gene expression and secretion. It seems likely that the increment of PRL mRNA levels and PRL synthesis (Carrillo *et al.*, 1987; Arbogast and Ben-Jonathan, 1988) in the morning of proestrus may precede a characteristic surge of PRL in the afternoon. The exact mechanism regulating PRL gene expression and secretion remains, however, unknown. Probably, it may due to a complex interaction between stimulatory and inhibitory effects on neurotransmitters,

neuropeptides and ovarian steroids (Maurer, 1980; Murdoch *et al.*, 1983; Carrillo *et al.*, 1985; Yamamoto *et al.*, 1986; Laverriere *et al.*, 1988; Shull and Groski, 1989).

The presence of elevated concentrations of EOP in the hypothalamus and other brain structures involved in the control of neuroendocrine processes has suggested that EOP might participate in the regulation of anterior pituitary function. Indeed, the systemic or intraventricular administration EOP as well as morphine results in an increase in serum PRL levels (Rivier *et al.*, 1977; Enjalbert *et al.*, 1979; Ragavan and Frantz, 1981b). NAL prevented the β -endorphin-induced stimulation of PRL release (Rivier *et al.*, 1977) and counteracted the effects of morphine and methionine-enkephalin on PRL release. In addition, NAL alone significantly reduced serum levels of PRL (Bruni *et al.*, 1977). It is generally believed that EOP plays an important role in the regulation of PRL secretion. However, in the present study where NAL was administered 30 min prior to decapitation, there was no difference of PRL mRNA levels between the control and NAL-treated groups at any stages during the rat estrous cycle, although NAL was effective in suppressing a characteristic afternoon surge of PRL from 16:00 to 20:00 h. The failure of NAL to produce any effects on PRL mRNA levels remains to be resolved, but it appears difficult to demonstrate the EOP effect in the normal condition. These data again reinforce the hypothesis that PRL gene expression and secretion may be differentially regulated as mentioned above: EOP may be the regulator of PRL release rather than that of PRL gene expression. It is also conceivable that the treatment paradigm of NAL used in this study may not be enough to produce any changes in the PRL mRNA accumulations. Indeed, PRL mRNA have relatively a long half-life (Laverrier *et al.*, 1983; Rosenfeld *et al.*, 1987) and the estrogen-induced PRL mRNA accumulations cannot be observed 6h following treatment, whereas the estrogen-induced PRL transcription can be detected within 20 min (Maurer, 1982).

In summary, it appears that the gene expression of PRL fluctuates during the rat estrous cycle. On proestrus, the increment of PRL mRNA level in the morning appears to precede the proestrus PRL surge in the afternoon. Moreover, alterations in PRL

mRNA was conversely correlated with changes in serum PRL levels on proestrus, indicating a differential regulation of PRL gene expression and secretion.

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흰쥐 성주기간 동안 Prolactin mRNA의 변화 : Naloxone의 효과

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본 연구는 prolactin (PRL) 유전자 발현, 분비의 생리적 변화와 성주기 특정 시기의 PRL mRNA 수준 및 분비에 미치는 내인성 오피오이드의 영향을 조사하였다. 최소한 두번의 연속적인 성주기를 거친 성숙한 흰쥐에서 성주기의 각 시기 (10:00시)에, proestrus 시기에는 10:00-20:00 시동간에는 2시간 간격으로 도살하였고, naloxone (2 mg/kg b.w.)은 도살 30분전에 피하주사 하였다. PRL mRNA의 수준은 흰쥐의 PRL cDNA를 probe로 하여 RNA-blot hybridization 방법에 의해서, 혈중 PRL 농도 변화는 방사면역측정법에 의해 측정하였다. 뇌하수체 PRL mRNA의 수준과 혈중 PRL 수준은 diestrus I, II, proestrus 그리고 estrus 시기의 10:00시에는 급격한 변화를 보이지 않았다. 이때 naloxone처리는 영향을 미치지 못했다. proestrus 시기를 세분하여 조사한 결과 PRL mRNA의 수준은 정오에 최고 수준에 도달하였고, 오후 6:00까지 점차적으로 감소하였다. 그후 8:00시에 다시 증가하였다. estrus 동안 naloxone은 혈중의 PRL 수준을 명백히 억제했으나 PRL mRNA 수준에는 영향을 없었다.

proestrus 시기 동안의 혈중 PRL 변화와 뇌하수체 PRL mRNA 변화는 서로 상이하게 조절되며, PRL mRNA 수준이 흰쥐 성주기 동안 변화하고 있는 사실에서 PRL 유전자 발현이 생리적으로 조절되고 있음을 시사한다.