

Expression of Gonadotropin releasing hormone (GnRH), metastin
and related peptides in the ovary: dynamic changes and their
regulation during estrous cycle of rats

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卵巣における性腺刺激ホルモン放出ホルモン（GnRH）と
メタスチン及びその関連ペプチドの発現：ラット性周期中の発現変
動とその調節機序

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**Expression of Gonadotropin releasing hormone (GnRH), metastin
and related peptides in the ovary: dynamic changes and their
regulation during estrous cycle of rats**

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Chapter 1 Introduction

The reproductive cycle of mammalian females consists of recurring physiological changes induced by reproductive hormones. Among the various hormones, gonadotropin-releasing hormone (GnRH, luteinizing hormone releasing hormone) is sometimes called the master hormone of reproduction and plays a role of the interface between the central nervous system and the endocrine system. GnRH was originally identified as a hypothalamic decapeptide, which stimulates pituitary gonadotropes to synthesize and release of gonadotropins (luteinizing hormone, LH and follicle stimulating hormone, FSH) at pituitary gonadotropes [11]. These gonadotropins in turn, initiate folliculogenesis, steroidogenesis and ovulation. It has been reported that gonadotropin releasing hormone receptor (GnRHR) is expressed in ovarian cells [2, 33]. Beside the long range control by gonadotropins, local regulation factors in the ovary are also involved in the recurrence of the cycle. Interestingly, GnRH is also synthesized in peripheral tissues, including the ovary [40]. It was shown to cause corpus luteum regression and a relationship with follicular atresia was suggested [19, 21].

Mast cells are tissue dwelling immune cells known as the effector of anaphylaxis and inflammation [14, 32]. It has been reported that mast cells accumulate at local tissues in different pathophysiological conditions, including both acute and chronic diseases [4, 7, 14, 34]. Our laboratory recently observed that mast cells in the mammary tissues contain GnRH immunoreactivity and that GnRH facilitates mammary involution after lactation [44]. These results suggest that mast cells are a common producer of GnRH in various tissues. Although GnRH is expressed in the ovary and GnRH is thought to contribute to ovarian cycle within the ovary, the nature of GnRH in the ovary is not known yet. GnRH, as a local regulating factor in the ovary, was examined for its expression rate, producing cells, and relation to mast cells in the ovary.

Recently, metastin also called kisspeptin has been reported to be the product of KISS-1 gene acting via G protein-coupled receptor 54 (GPR54) [37]. KISS-1 is the metastasis suppressor gene of melanoma and other tumors [47, 50]. Beside these functions, it has been demonstrated that metastin plays a critical role in reproduction. Metastin neurons were limitedly localized in the anteroventral periventricular nucleus (AVPV) and hypothalamic arcuate nucleus (ARC) [1, 37], those are candidate regions of the centers for GnRH/LH surges, respectively. Metastin stimulates GnRH release in the hypothalamus [31]. Metastin is also expressed in other peripheral tissues, including the ovaries [30, 47]. So, it is interesting to know whether metastin is involved in GnRH secretion in the ovary. While metastin is synthesized in the ovary, its physiological implication is unknown. Although ovarian expression of metastin mRNA is known to be increase on proestrus, its producing cells and regulating mechanisms are still unknown.

Metastin neurons coexpress also neurokinin B (NKB) and dynorphin at the arcuate nucleus [16]. They are thus called kisspeptin/neurokinin B/dynorphin (KNDY) neurons and are found in sheep, rats, mice, and goats [6, 36, 39, 49]. NKB has been reported to be involved in GnRH secretion also [6, 39]. NKB and dynorphin, however, have not been known for their existence and functions in the ovary. It is interesting to know whether the relationship among these hypothalamic factors exists also in the ovary or not.

In the present study, it was intensively studied that ovarian production of GnRH in relation to possible producing cells, mast cells, and a possible regulator, metastin, with its related peptides, NKB and dynorphin.

It was clarified that GnRH is produced in the ovary and the synthesis of GnRH varies during estrous cycle with two peaks during the estrous cycle of rats. GnRH production probably occurs in various cell species of the ovary. Mast cells were shown to migrate into the ovary by the chemoattraction of GnRH and mast cell itself also synthesizes GnRH. Although metastin,

NKB and dynorphin were all demonstrated to be synthesized in the ovary, there was no evidence to indicate a relationship to GnRH production. The regulating mechanism of GnRH production in the ovary would be different from that in the hypothalamus. Metastin was shown to be synthesized in granulosa cells under the control of the LH surge. The expression of dynorphin was also stimulated by LH surge. The massive synthesis of NKB and dynorphin occur in the interstitial tissues. It is suggested that metastin, dynorphin and NKB are involved in the ovulation and luteinization processes. Metastin was demonstrated to be involved in the augmentation of progesterone production of luteal cells.

In this thesis, changes in the GnRH expression, mast cells, metastin, and related peptides were examined in rat estrous cycle and a local regulating mechanism of ovarian periodicity will be discussed.

Chapter 2 Variation in Ovarian GnRH expression

Introduction

Estrous cycle is primarily controlled by a hypothalamic neuropeptide hormone, gonadotropin-releasing hormone (GnRH). GnRH has been found in all vertebrates examined as a very conserved decapeptides and sometimes called a master hormone of reproduction. GnRH is synthesized by GnRH neurons in the hypothalamus and secreted into portal vessels at the median eminence. It is transported to the pituitary gland and stimulates the synthesis and release of gonadotropins, luteinizing hormone and follicle stimulating hormone via specific GnRH receptor [11]. These gonadotropins regulate, in turn, the gonadal function.

GnRH has been detected outside the hypothalamus [40]. GnRH immunoreactivity was first identified in the rat testis in 1981 [38]. Since then, GnRH is also synthesized in various peripheral tissues with its specific receptor (e.g. ovary, testis, prostate, endometrium, oviduct, placenta and mammary gland) [40]. So, GnRH is presumed to play a role in those tissues. GnRH was shown to cause corpus luteum regression [21] and a relationship with follicular atresia was suggested [19]. Beside pituitary, the GnRH receptor gene is also expressed in extrapituitary sites including ovarian granulosa cells of rats [2]. Although GnRH receptor and its function have been repeatedly described, how the ligand is delivered in the ovary is still obscure. This thesis was investigated a variation of GnRH mRNA expression in the ovary during estrous cycle of rats and changes in the distribution of mast cells, a candidate for a source of ovarian GnRH. The GnRH mRNA expression in the ovary was examined and found that it increased in the evening of diestrus 2 and late afternoon of proestrus. The source of GnRH was examined by Laser Microdissection (LMD) and immunohistochemistry. Mast cell production of GnRH mRNA was confirmed by peritoneal mast cells and Mouse mast cell clone

P 815. GnRH stimulates both GnRH production of mast cells and the migration of mast cells into the ovary. Ovarian mast cells were immuno-positive for GnRH and the number in the ovary, existing profoundly in the interstitial tissues, varied during the estrous cycle with two distinct peaks in the evening of diestrus 2 and late afternoon of proestrus. In this chapter, it is suggested that a new function of GnRH as a chemo-attractant for mast cells. Mast cell migration into the ovary varies according to GnRH expression. Mast cells are thought to have a specific role on the regression of the corpus luteum and follicles. The observed changes in the number of ovarian mast cells and GnRH mRNA suggest their functional relationship and the involvement of mast cells in GnRH production in the ovary.

Materials and methods

1. Animals

Adult female rats of Wistar Imamichi strain were kept in the atmosphere of temperature at $23\pm 3^{\circ}\text{C}$ and light-dark cycle of 14L: 10D (lights on 05:00-19:00 h). Food (laboratory chow, CE-2, Oriental Co., Tokyo, Japan) and tap water were supplied *ad libitum*. Vaginal smears were examined daily and only rats showing at least two consecutive regular four-day estrous cycles were used. At about 17:00 h on the day of proestrus, each female was transferred to the cage of single intact male rat and left overnight. The occurrence of mating was confirmed in the next morning by the presence of spermatozoa in the vaginal smear. The estrous day following mating was designated as day 0 of pregnancy. The day of parturition was designated as day 0 of lactation. For lactating rats, pups number was adjusted to 8 on day 2 of lactation. C57BL/6J mice (control) and C57BL/6- $\text{W}^{\text{sh}}/\text{W}^{\text{sh}}$ mice were used. C57BL/6- $\text{W}^{\text{sh}}/\text{W}^{\text{sh}}$ mice is a mutant with deletion of c-kit and known to be mast cell deficiency. It was obtained from RIKEN Bio Resource Center (Tsukuba, Ibaragi, Japan) and bred in our laboratory. C57BL/6J mice were obtained from Charles River laboratories (Yokohama, Japan). All experiments were performed according to the guideline for animal experiments of Kitasato University that followed the guideline of NIH, and the experimental protocol was approved by the local committee at School of Veterinary Medicine, Towada.

2. RNA extraction and transcription to cDNA

a. Reagents, solutions and equipments

Trizol Reagent (Invitrogen, Carlsbad, CA)

RNeasy Mini Kit (QIAGEN, Valencia, CA)

UltraPure™ DNase/Rnase-Free Distilled Water (Invitrogen, Grand Island, NY)

High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA)

Veriti™ 96 well Thermal cycler (Applied Biosystems)

b. Protocol

Estrous cycling rats were sacrificed by decapitation. RNA was extracted from whole ovary and hypothalamus by means of Trizol Reagent or RNeasy Mini Kit. The hypothalamus was cut out by a horizontal cut about 2 mm in depth with limits of anterior at the chiasm to posterior at the mammillary body, and laterally at the edge of hypothalamus. Tissue samples were snap frozen in liquid nitrogen after dissection. RNA samples were dissolved in UltraPure™ DNase/Rnase-Free Distilled Water to 500 ng/μl and subjected to reverse-transcription with High-Capacity cDNA Reverse Transcription kits using Veriti™ 96 well Thermal cycler according to the protocol supplied by the manufacturer. Briefly, 5 μl of RNA (500 ng/μl) was mixed with 2.5 μl of 10x RT Random Primers, 2.5 μl of 10x RT Buffer, 1.0 μl of 25x dNTP Mix (100 mM), 1.25 μl of MultiScribe™ Reverse Transcriptase (50 U/μl) and 12.75 μl of distilled water. Final volume was 25 μl. Reverse transcription was performed at 25°C for 10 min, 37 °C for 120 min, 85 °C for 5 sec and then cooled immediately. Samples were either used directly for PCR or stored at -30°C until the assay.

3. Quantitative Real-time PCR

a. Reagents, solutions and equipments

Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK)

Sequences for forward and reverse primers used were presented in Table 1 and 2.

StepOnePlus Real-time PCR system (Applied Biosystems)

b. Protocol

The quantitative real-time PCR was performed by StepOnePlus Real-time PCR system. The reaction mixture contained 1 µl of cDNA sample solution obtained as above, 0.06 µl (50 µM) of forward and reverse primers, 5 µl of Power SYBR Green PCR Master Mix and 3.88 µl of distilled water. The final volume was 10 µl. The quantitative real-time PCR was performed with 50 cycles of 95°C for 15 sec, 60°C for 1 min and then one cycle at 95°C for 10 min for getting a melting curve. Each CT measure was divided by that of ribosomal protein L19 (RPL 19) control and revealed as expression rate using $\Delta\Delta CT$ method.

4. PCR

a. Reagents, solutions and equipments

Premix Taq (Ex Taq Version 2.0, TAKARA BIO INC, Otsu, Shiga, Japan)

Sequences for forward and reverse primers used were presented in Table 3 and 4.

20X TAE buffer

- 1,000 ml Milli Q
- 96.8 g Tris
- 28 ml of Acetic acid
- 15.77 g Na₃EDTA

Certified™ Molecular Biology Agarose gel 125 g (Bio-Rad Laboratories, Inc., Hercules, CA)

100 bp DNA Ladder (TAKARA BIO INC, Otsu, Shiga, Japan)

Veriti™ 96 well Thermal cycler (Applied Biosystems)

b. Protocol

cDNA samples were amplified by PCR using Veriti™ 96 well Thermal cycler. Each 20 µl amplification reaction mixture contained 1 µl of cDNA solution obtained as above, 0.2 µl (50 µM) of forward and reverse primers, 10 µl of Premix Taq and 8.6 µl of distilled water. PCR was performed with 35 cycles of 98°C for 3 min, 98°C for 10 sec, 55°C for 30 sec, 72°C for 1 min, 72°C for 2 min and then cooled immediately. The PCR products were separated by gel electrophoresis on 2 % agarose, stained with ethidium bromide and visualized and photographed under ultraviolet illumination. The bands were scanned by a video documentation system and the image analysis software Intelligent Quantifier. Amplicon sizes were verified by comparison with 100 bp DNA Ladder.

5. Laser Microdissection (LMD)

Laser Microdissection was used for collecting follicles, corpora lutea and interstitial tissues separately from ovary sections. Three compartments were identified under a microscope included in the LMD system.

a. Reagents, solutions and equipments

O.C.T. compound (Sakura, Tokyo, Japan)

PEN-membrane 2.0 µm covered with PEN foil (polyethylene naphthalate) (Leica Microsystems, Wetzlar, Germany)

5% Acetic acid

- Acetic acid
- 100% ethanol

0.05% Toluidine blue solution

- Toluidine blue (Sigma-Aldrich, Inc., St. Louis, MO)

RNeasy Micro Kit (QIAGEN)

RNase-Free DNase Set (QIAGEN)

Sequences for forward and reverse primers used were presented in Table 1.

LMD system (Leica Microsystems CMS GmbH, Wetzlar, Germany)

b. Protocol

Adult Wistar-Immamichi rats were sacrificed by decapitation and their ovaries were embedded in O.C.T. compound, frozen in liquid nitrogen, and stored at -80°C until making sections for LMD. Frozen sections of 10 µm thickness were made from ovaries using a cryostat and mounted on Membrane Slides. The sections were fixed with 5% acetic acid for 3 min and then washed with DNase/RNase-Free Distilled Water. Then the sections were stained with 0.05% toluidine blue solution for 1 min and washed with distilled water. The stained sections were washed with 70% ethanol for 5-10 sec and then dried.

The dissected follicles, corpora lutea and interstitial tissues from ovary were collected into three separate tube caps filled with an appropriate volume of Buffer RLT (RNeasy Micro Kit). The tube caps were applied to their tubes and the dissected tissues were collected into the tubes by brief centrifugation and transfer into Eppendorf tubes containing 350 µl buffer RLT. All three compartments were collected by LMD. Total RNA was extracted with RNeasy Micro Kit and treated with RNase-Free DNase Set according to the protocol supplied by the manufacturer. RNA was reverse-transcribed with High-Capacity cDNA Reverse Transcription kits. Finally, total RNA was dissolved in 14 µl.

13 µl of RNA was mixed with 5 µl of 10x RT Random Primers, 5 µl of 10x RT Buffer, 2.0 µl of 25x dNTP Mix (100 µM), 2.5 µl of MultiScribe™ Reverse Transcriptase (50 U/µl) and 22.5 µl of distilled water. Final volume was 50 µl. Reverse transcription was performed at 25°C

for 10 min, 37 °C for 120 min, 85 °C for 5 sec and then cooled immediately. Samples were either used directly for real time PCR or stored at -30°C until the assay as previously described.

6. Histological analysis

Paraffin block preparation

Ovaries were harvested and subjected to histological analysis. Immunohistochemistry for GnRH was performed. Some tissue sections were also stained with Toluidine blue or Hematoxylin-Eosin. Tissue was obtained from rats after perfusion with fixative solution. Outline of the protocol is as follows.

a. Reagents, solutions and equipment

0.1 M Phosphate buffered saline (PBS, pH 7.4)

4% Paraformaldehyde solution (4% PFA)

- Paraformaldehyde (Merck Schuchardt OHG, Hohenbrunn, Germany)
- 0.1 M Phosphate buffered saline (PBS, pH 7.4)

Paraffin (Merck Schuchardt OHG)

Microtome (Microm HM355, Microedge, Tokyo, Japan)

b. Protocol

Rats were deeply anaesthetized with ether and PBS (50-100 ml) was infused through its left ventricle after cutting off the right atrium auricle. Then it was changed to 50-100 ml of 4% PFA. The tissue was then soaked in PBS for another night at 4°C. Dehydration and paraffin embedding were performed per standard procedures. Ovary sections were cut to 2.5 µm thickness and dried in an incubator at 37°C overnight.

7. Immunohistochemistry

The distribution of GnRH in the ovarian tissue was determined by immunohistochemistry with specific antiserum to GnRH. The procedure for immunohistochemistry is outlined below.

a. Reagents and solutions

Antibody-binding buffer (ABB, pH 7.4)

- 150 mM NaCl
- 5 mM EDTA
- 0.25% Gelatin
- 0.05% Nonidet P-40
- 50 mM Tris

1% Hydrogen peroxide (H₂O₂)

- Hydrogen peroxide
- Methanol

Blocking buffer

- 2.5% normal horse serum (ImmPRESS REAGENT KIT PEROXIDASE; Vector Laboratories, Burlingame, CA)

Primary Antibody

- Anti-GnRH rabbit serum (1:10,000 dilutions)
- Normal rabbit serum (1:10,000 dilutions) for negative stain.

Secondary Antibody

- Anti-rabbit IgG POD (ImmPRESS REAGENT KIT PEROXIDASE; Vector Laboratories, Burlingame, CA)

DAB Substrate

Hematoxylin

Mounting medium (Vectashield®; Vector Laboratories, CA)

b. Protocol

Paraffin was removed from the sections by a series of xylene and ethanol rinses and then sections were immersed in 1% H₂O₂ for 20 minutes to exhaust endogenous peroxidase activity. After this treatment, sections were washed three times for 30 minutes in ABB. ABB was also used in the subsequent washing and incubation. After washing, the sections were soaked with the blocking buffer for 1 hour at room temperature to inhibit non-specific binding. Sections were then incubated with primary antibody for overnight at 4°C in a humidified chamber. The antiserum was used at dilution of 1:10,000. After incubation with the primary antibody, sections were washed for three times in ABB and incubated for 2 hours with the anti-rabbit IgG POD at room temperature. Sections were again washed three times in ABB. The sections were counterstained with hematoxylin, dehydrated and mounted, respectively. Negative control was processed with rabbit serum instead of primary antibody.

8. Staining with Toluidine blue

a. Reagents and solutions

Toluidine blue stock solution

- Toluidine blue (Sigma-Aldrich)
- 70% ethanol

Toluidine blue working solution, pH 2.0-2.5 with HCl

- 0.1% Toluidine blue stock solution
- 1% sodium chloride

b. Protocol

Paraffin was removed from the sections by a series of xylene and ethanol rinses. Tissue sections were stained with Toluidine blue working solution for 3 min, rinsed in distilled water for three times and dehydrated through 95, 99 and 100 % of ethanol and xylene. Mast cells were detected with metachromasy of the granules.

9. Staining with Hematoxylin-Eosin

a. Reagents and solutions

Mayer's Hematoxylin (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan)

Eosin Solution

- 20% Pure Eosin Solution (MUTO PURE CHEMICALS CO.,LTD., Tokyo, Japan)
- 80% of 95% ethanol

b. Protocol

Paraffin was removed from the sections by a series of xylene and ethanol rinses. Tissue sections were rinsed in distilled water for 5 min and stained with Hematoxylin solution for 3 min, rinsed in distilled water for 5 min and stained with Eosin solution for 3 min, rinsed in distilled water for three times and dehydrated through a series of ethanol and xylene.

10. Counting mast cells

To examine the variation in number of ovarian mast cells, ovaries were obtained from rats in various physiological statuses. Ovaries were harvested and subjected to Toluidine blue staining from estrous cycling, pregnant, lactating, weaned and pseudopregnant rats.

Pseudopregnancy was induced by mechanical stimulation of the cervix by tapping the uterine cervix with a glass rod about one hundred times in the evening of proestrus. The estrus day following the day of the stimulation was designated as day 0 of pseudopregnancy. Ovaries were harvested at 10:00 h on day 3, 7, 12, 13 and 14 of pseudopregnancy.

When using pregnant rats, pregnancy was confirmed by the presence of spermatozoa in the vaginal smear upon separation from a male. The estrous day following mating was designated as day 0 of pregnancy. Ovaries were harvested at 10:00 h on day 2, 12, 14, 18, 20 and 21 of pregnancy. The day of parturition was designated as day 0 of lactation. Weaning was performed on day 21 of lactation and the day 21 of lactation was designated as day 1 of post-weaning. Ovaries were harvested at 10:00 h of lactation day 1, 3, 21 and post-weaning day 2.

11. Administration of reagents

The effects of systemic and local administration of various reagents on the number of ovarian mast cell were studied.

- 1) The effects of prolactin administration on ovarian mast cell number were examined. Ovine prolactin (10 IU/0.2 ml, Sigma-Aldrich) was administered intraperitoneally at 10:00 h of diestrus 2 or proestrus. Control was treated with saline. Ovaries were harvested at 20:00 h of diestrus 2 or at 17:00 h of proestrus.
- 2) Prolactin secretion was suppressed by dopamine agonist (CB-154, Ergocryptine, Sigma-Aldrich). CB-154 (300 µg/0.2 ml) was administered intraperitoneally at 10:00 h from pseudopregnant day 5. CB-154 was dissolved in 3% tartaric acid. Control was treated with tartaric acid. Ovaries were harvested at 10:00 h on pseudopregnant day 7.
- 3) The direct effect of GnRH agonist (GnRHa: Des-Gly10 [Pro]-GnRH ethylamide, Takeda Pharm.) on ovarian mast cell number was examined. GnRHa (200 ng/50 µl) was given into the hemi-lateral ovarian bursa at 11:00 h of diestrus 1. Saline was given into

the contra-lateral ovary for a control. Ovaries of both sides were harvested five hours later.

12. Mast Cell Counting

Ovarian tissues were subjected to Toluidine blue staining. Mast cell was counted under a microscope at magnification of x10. Five fields each from ovaries were counted and number was averaged. The number of mast cell was revealed per unit area (mm²). At least three rats per point were used.

13. Mast cell collection and isolation

Purification of rat peritoneal mast cells was performed with Percoll density gradient centrifugation.

a. Reagents and solutions

1% Bovine serum albumin solution (BSA)

- 10 mM *Phosphate Buffered Salts* (TAKARA BIO INC., Shiga, Japan)
- 1% Albumin, from Bovine Serum, Cohn Fraction V, pH7.0 (Wako Pure Chemical Industries, Ltd., Japan)

Culture medium

- α -MEM (Invitrogen, Grand Island, NY)
- 10% Fetal calf serum (FCS, Gibco, BRL)
- 1% Antibiotic-Antimycotic (100x) (Gibco® by Life Technologies, Grand Island, NY)

Isotonic Percoll solution

- 90% Percoll Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)
- 10% 10X Hanks' Balanced Salt Solution (HBSS, Invitrogen, Grand Island, NY)

75% Percoll solution

- 75% Isotonic Percoll solution
- 25% 1X Hanks' Balanced Salt Solution

b. Protocol

Adult Wistar-Kyoto rats were killed by decapitation. Mast cells were collected by washing the peritoneal cavity with 1% BSA solution. The peritoneal cell suspension was centrifuged at 100 x g for 10 min. An aliquot of 10 ml of the cell suspension in culture medium was layered on top of 75% Percoll solution (15 ml). The sample was centrifuged at 600 x g for 20 min. The bottom of 2 ml portion that contained mast cells was collected. Mast cells were washed with culture medium by centrifuged at 100 x g for 10 min.

14. Primary culture of peritoneal mast cells

Peritoneal mast cells were measured for GnRH mRNA expression rates. RNA was extracted by means of Trizol. RNA was incubated in heparinase (Sigma-Aldrich), RNase Inhibitor (TAKARA BIO INC., Shiga, Japan) and 100 mM CaCl₂ - 50 mM Tris (pH 7.5) for 2 h at room temperature. RNA samples were subjected to PCR and real-time PCR.

a. Reagents and solutions

Culture medium

- α -MEM (Invitrogen, Grand Island, NY)
- 10% Fetal calf serum (FCS, Gibco, BRL)
- 1% Antibiotic-Antimycotic (100x)

GnRHa

IWAKI brand 24-well plates (ASAHI GLASS CO., LTD., Tokyo, Japan)

b. Protocol

Peritoneal mast cells at a concentration of 5×10^5 cells/1000 μ l/well in growth medium were incubated in the absence or presence of GnRH α (10^{-9} and 10^{-7} M) for 3 h at 37°C, 5% CO₂ in a humidified atmosphere. After incubation, peritoneal mast cells were subjected to real-time PCR to measure GnRH mRNA expression rate. In addition, peritoneal mast cells were also incubated without GnRH α to measure GnRH by PCR.

15. Mouse mast cell clone P-815

P-815 is a clonal cell line of mouse mast cells. It was obtained from RIKEN Bio Resource Center CELL BANK (Tsukuba, Ibaragi, Japan).

a. Reagents, solutions and equipments

72 cm² flask (Corning Incorporated, NY)

Culture medium

- RPMI (Invitrogen, Grand Island, NY)
- 10% Fetal calf serum (FCS, Gibco, BRL)
- 1% Penicillin-Streptomycin (100 U/ml-100 μ g/ml, Gibco, BRL)

b. Protocol

P-815 mast cells were grown in 72 cm² flask with 20 ml of culture medium at 37°C, in 5% CO₂ in a humidified atmosphere. The medium was changed alternately and the cells were detached from flask by knocking the flask. P-815 mast cells were extracted by means of RNAeasy Mini Kit. RNA samples were subjected to PCR to measure GnRH gene expression.

16. Statistics

Multiple comparisons of differences between mean values were analyzed by Tukey test after ANOVA, and single comparisons were analyzed by Student's *t* test. $p < 0.05$ or less was considered to be significant.

Results

1. Changes in the expression rate of ovarian GnRH mRNA during estrous cycle

The expression of GnRH mRNA was examined in the ovary of cycling female rats. Ovaries were collected at 11:00 and 17:00 h of diestrus 1, 11:00, 17:00, 20:00, and 23:00 h of diestrus 2, 05:00, 11:00, 14:00, 17:00, 20:00 and 23:00 of proestrus, and 02:00 h, 11:00 and 17:00 h of estrus. There were two peaks in the variation of GnRH mRNA expression in the ovary, the first peak was at 20:00 h of diestrus 2 and the second peak was at 20:00 h of proestrus (Fig. 2-1A). These changes were thought to be associated with the estrous cycle, because the expression rate of GnRH was very low at 20:00 h on pregnancy day 3, the day equivalent to proestrus if mating did not take place (Fig. 2-1B).

2. Ovarian components expressing GnRH mRNA

Ovaries were harvested at 20:00 h of proestrus. Corpora lutea, follicles and interstitial tissues were collected with Laser Microdissection (LMD) (Fig. 2-1 C). The expression rate of GnRH mRNA was similar among three compartments (Fig. 2-1D).

3. Immunohistochemical demonstration of GnRH in the ovary

Ovaries were harvested at 20:00 h of diestrus 2. GnRH was demonstrated by immunohistochemistry in almost all compartments of the ovary. The granulosa layer (Fig. 2-2E) and corpus luteum (Fig. 2-2B) were positive. GnRH positive cells were restricted to only a portion of the tissues (Fig. 2-2C, I). Some corpus luteum showed low intensity (Fig. 2-2A). Although interstitial tissues were stained diffusely (Fig. 2-2F), mast cells were very strongly positive (Fig. 2-2G). Normal rabbit serum was used instead of the first antibody for negative control (Fig. 2-2D, H, J).

4. Changes in ovarian mast cell number during estrous cycle of rats

Mast cells in the ovary were counted during estrous cycle. Ovary was harvested from estrous cycling rats at 10:00 and 17:00 h of diestrous 1, 10:00, 17:00, 20:00 and 23:00 h of diestrous 2, 05:00, 10:00, 17:00, 20:00 and 23:00 h of proestrus, and 10:00 and 17:00 h of estrus. Tissue section was made at two different planes to observe whether there was maldistribution in the ovary (Fig. 2-4B). Tissue sections were made at 2.5 μm thickness. Mast cells were demonstrated with toluidine blue metachromasy mainly in the interstitial tissues especially at hilum (Fig. 2-3A-C). There were more mast cells in the interstitial tissues and the number was always more in plane 1 (Fig. 2-4A). Average of the data from two planes were shown in Fig. 2-4C. The number of mast cell was dramatically increased in the evening of diestrous 2 and late afternoon of proestrus. Although there are two peaks in both planes, peaks of two planes were shifted a little (Fig. 2-4A). This variation in the number of mast cells synchronized with the changes in GnRH mRNA content (Fig. 2-4D).

5. Changes in ovarian mast cell number during luteal phase

Ovary was harvested at 10:00 h on day 3, 7, 12, 13 and 14 of pseudopregnancy, and pregnancy day 2, 12, 14, 18, 20 and 21. Ovaries were also harvested at 10:00 h of lactation day 1, 3, 21 and post-weaning day 2. The increase of mast cells was suppressed during pseudopregnancy (Fig. 2-5A), pregnancy and lactation (Fig. 2-5B). When comparing mast cell number among ovaries of diestrus 2, day 2 of pregnancy, proestrus and day 3 of pregnancy, the increase of mast cell was negated (Fig. 2-5C).

6. Prolactin suppresses the accumulation of ovarian mast cells

Ovine prolactin was administered i.p. at 10:00 h of diestrus 2 or proestrus. Ovaries were harvested at 20:00 h of diestrous 2 or at 17:00 h of proestrus. Prolactin inhibited the

accumulation of ovarian mast cells both on diestrus 2 and proestrus, respectively (Fig. 2-6A, B). On the other hand, dopamine agonist, CB-154 i.p. that would decrease prolactin secretion increased ovarian mast cells during pseudopregnancy (Fig. 2-6C).

7. Number of Toluidine blue stained and GnRH immunoreactive mast cells in the ovary

As mast cells were shown to contain GnRH, it was next examined whether number of ovarian mast cells identified with Toluidine blue is always same as mast cells demonstrated with the immunohistochemistry of GnRH during estrous cycle (Fig. 2-7A). Ovaries were harvested at 10:00, 17:00 and 20:00 h of diestrus 2. Toluidine blue stained mast cells were significantly increased but GnRH immuno-positive cells were not (Fig. 2-7B), suggesting a release of GnRH from mast cells in the afternoon of diestrus 2.

8. Estrous cycle of C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice

Ovarian histology was examined in both C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} female mice. Toluidine blue staining showed that the ovary of C57BL/6J mice but C57BL/6-W^{sh}/W^{sh} contained abundant mast cells (Fig. 2-8A). Vaginal smears of C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice were examined daily. Duration between estrus (days) was measured. The length of estrous cycle of C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice was not different (Fig. 2-8B).

9. Changes in ovarian weight of C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice

Whole ovaries were harvested from C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice on day 30, 60, 90, 120 and 170 of age. Ovary was lighter in C57BL/6-W^{sh}/W^{sh} mice (Fig. 2-8C). Ovarian tissues were subjected to Hematoxylin-Eosin staining. Ovarian tissues were compared and found no profound difference between two strains (Fig. 2-9).

10. GnRH mRNA expression in the ovary of C57BL/6-W^{sh}/W^{sh} mice

Ovarian GnRH mRNA expression at 10:00 h of proestrus was not different between C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice (Fig. 2-10).

11. GnRH expression in mast cells

Mast cell production of GnRH mRNA was confirmed by peritoneal mast cells and clonal cell line of mast cell, P 815. Ovaries were harvested from adult female rats at 20:00 h of diestrous 2. Hypothalamus was prepared from adult Wistar-Immamichi rats and C57BL/6J mice. Expression of GnRH was examined by PCR. GnRH mRNA was expressed in peritoneal mast cells, ovary and hypothalamus (Fig. 2-11A). Mouse mast cell clone P 815 was also demonstrated to synthesize GnRH (Fig. 2-11B).

In addition, GnRH and annexin A5 mRNA expression rate in peritoneal mast cells, ovary and hypothalamus from adult Wistar-Immamichi rats were examined by real-time PCR. Peritoneal mast cells were demonstrated to synthesize GnRH and annexin A5 mRNA (Fig. 2-12 A, B) and they were also expressed in the ovary and the hypothalamus.

GnRH and annexin A5 mRNA expression rate in L β T2 cells, MA-10 Leydig cells and mouse mast cell clone P 815 were examined by real-time PCR. GnRH and annexin A5 mRNA were shown to be synthesized in P 815 (Fig. 2-13 A, B) and also expressed in L β T2 cells and MA-10 Leydig cells.

12. The effect of GnRH agonist (GnRHa) on mast cells

GnRHa (200 ng/50 μ l) given into the hemi-lateral ovarian bursa increased the number of ovarian mast cells, suggesting that GnRH would be a chemo-attractant for mast cell migration to the ovary (Fig. 2-14).

The direct effect of GnRH on peritoneal mast cells were examined. When GnRHa was added to the culture medium at 10^{-9} and 10^{-7} M for 3 h, the expression of GnRH mRNA was augmented (Fig. 2-15).

Discussion

Present results clearly demonstrate the changes in the expression of GnRH mRNA during the estrous cycle of rats. There were two peaks in the variation of GnRH mRNA expression rate. The first peak was at 20:00 h of diestrus 2 and the second peak was at 20:00 h of proestrus. These changes were demonstrated to be specific to the estrous cycle. Although the mechanism by which GnRH expression is regulated is not known, the first peak would be related to the regression of corpus luteum and the second peak would be involved in the process of follicular maturation and ovulation.

Corpus luteum is formed after the ovulation and secretes progesterone until the morning of diestrus 2. When there is no luteotropic hormone, prolactin, until the morning of diestrus 2, the corpus luteum stops to produce progesterone and luteal cells results in apoptosis. It has been already demonstrated that the corpus luteum of pseudopregnancy falls into apoptosis when prolactin secretion is inhibited [21]. The induction of apoptosis of luteal cells by the withdrawal of prolactin was shown to be mediated by locally supplied GnRH in the ovary. So, it is quite possible that the first peak of GnRH would be related to the regression of corpus luteum during the estrous cycle.

On the other hand, it has been reported that GnRH would be involved in follicular atresia before the final maturation of follicles during estrous cycle [19]. During normal estrous cycle, a half of growing follicles do not reach the ovulation. The process of degenerating follicles are called atresia. Granulosa cells of atretic follicles become apoptotic and GnRH is postulated to be involved in this process. So, the second peak of GnRH mRNA expression would be regulated so as to meet these physiological changes.

The source of GnRH in the ovary was examined by means of LMD. Corpora lutea, follicles and interstitial tissues were collected with LMD system at 20:00 h of proestrus. The

expression rate of GnRH mRNA was not significantly different among these compartments. GnRH immunoreactivity was detected in all compartments of the ovary, but mast cells were very strongly positive. As the sampling time for LMD is one of the two peaks of GnRH mRNA expression, it was expected that one of these three compartments would contribute significantly to the expression rate of whole ovary. However, there was no obvious difference among compartments. So, it is suggested at least that the augmentation of GnRH expression at 20:00 h of proestrus similarly occurs in the ovary. As strong GnRH immunoreaction of mast cells was noteworthy, the variation of mast cells in the ovary was next examined during the estrous cycle.

Mast cell was demonstrated with toluidine blue metachromasy mainly in the interstitial tissues of the ovary. Tissue section was made at two different planes to observe whether there was maldistribution in the ovary. There were two peaks in both planes and peaks of two planes were shifted a little. This may reflect the movement of mast cells in the ovary. Mast cells are suggested to move from outside to the ovary at the peaks of the number. It is not known whether ovarian mast cells are blood born or from interstitial fluid. As the majority of mast cells were demonstrated at the hilum of the ovary and peritoneal lavage contains abundant mast cells, they would be moved into the ovary through interstitial fluids. Average of the mast cell number from two planes were shown that the number of mast cells varied during the estrous cycle with two distinct peaks in the evening of diestrus 2 and late afternoon of proestrus. This variation matches quite well with the changes in GnRH mRNA content. This result is suggestive that ovarian mast cells are related to the variation of ovarian GnRH function. It has been demonstrated that mast cells migrate into the mammary tissues after weaning and secrete GnRH to induce apoptosis of mammary epithelial cells [44]. So, it was speculated that mast cells would play a role at the corpus luteum regression in the afternoon of diestrus 2. It was, then, examined whether number of ovarian mast cells identified with toluidine blue stained were the same as GnRH immuno-positive cells. Surprisingly, the number of GnRH positive mast cells

was less than that stained with toluidine blue at the peaks of GnRH expression, suggesting a release of GnRH from mast cells in the afternoon of diestrous 2. So, it is suggested, at least, that GnRH from mast cells would be involved in the regulation of corpus luteum regression during estrous cycle.

Both peaks of ovarian mast cell number were suppressed in pregnant rats and also by prolactin. It is thought that the decrease of mast cell is attained at least partly through prolactin receptor during luteal phases. The number of mast cells in the ovary was also very low during pregnancy and lactation. Dopamine agonist, that would decrease prolactin secretion, increased ovarian mast cells during pseudopregnancy. The inverse relationship between prolactin and ovarian mast cells suggest a direct action of prolactin on mast cell movement.

Mast cell is blood-born tissue dwelling cells [13, 32]. There are two types of mast cells. They are mucosal type mast cells and connective tissue type mast cells. Mast cell of the skin is known as typical connective tissue type cell and that of lung is designated as a typical mucosal type cell [27]. GnRH suggested to be a common secretion of two types of mast cells, since mast cells in other tissues were also GnRH positive (data not shown). GnRH mRNA was confirmed in peritoneal mast cells and clonal cell line of mast cell, P 815.

When peritoneal mast cells were incubated with GnRH agonist for three hours, the expression of GnRH mRNA was augmented. Further, as GnRH agonist given into the hemilateral ovarian bursa increased the number of ovarian mast cells, GnRH would be a chemo-attractant for mast cell migration to the ovary. These data suggest that GnRH stimulates both the GnRH production of mast cells and the migration of mast cells into the ovary. Mast cells also expressed annexin A5 (anxa5) [22, 46]. Anxa5 is a gene that expression is stimulated by GnRH in various cell types including the gonadotropes, Leydig cells, mammary epithelial cells and luteal cells [20, 21, 51]. In the present study, MA-10 clonal cell line of Leydig cells was used and shown to express GnRH and anxa5. It has been already shown that GnRH stimulates the

expression of *anxa5* in Leydig cells [51]. So, *anxa5* could be used as a biomarker of GnRH action. Actually, GnRH has been shown to stimulate *anxa5* expression of peritoneal mast cells [43]. Ovarian GnRH would attract mast cells and GnRH produced by mast cells also would facilitate the migration of mast cells.

C57BL/6- W^{sh}/W^{sh} is a mutant allele at the mouse *W* (*c-kit*) locus. Mice carrying this allele lack mast cells. The lengths of estrous cycle of C57BL/6J mice and C57BL/6- W^{sh}/W^{sh} mice were same. Ovary weight was smaller in C57BL/6- W^{sh}/W^{sh} mice. Although this difference seems to reflect the absence of mast cells and GnRH, ovarian GnRH mRNA expression was not different between two strains. These data suggest that GnRH of mast cells does not contribute to a variation of GnRH mRNA expression during the estrous cycle. Rather ovarian variation of GnRH would cause the changes in ovarian mast cells by chemoattraction of mast cells. If it is so, a role of mast cells other than production of GnRH would be there. As mast cells are well known to secrete various bioactive substances, it is of great interest to identify the factor that is involved in ovarian function.

Although the regulating mechanism for phasic GnRH synthesis is not known, the present study suggests a new function of GnRH as a chemo-attractant for mast cells. Mast cell migration into the ovary varies according to the changes in GnRH expression. Mast cells are thought to have a specific role in the ovary probably related to the regression of the corpus luteum and follicles. It is suggested that mast cells are also responsible, at least partly, for the phasic increase of GnRH in the ovary.

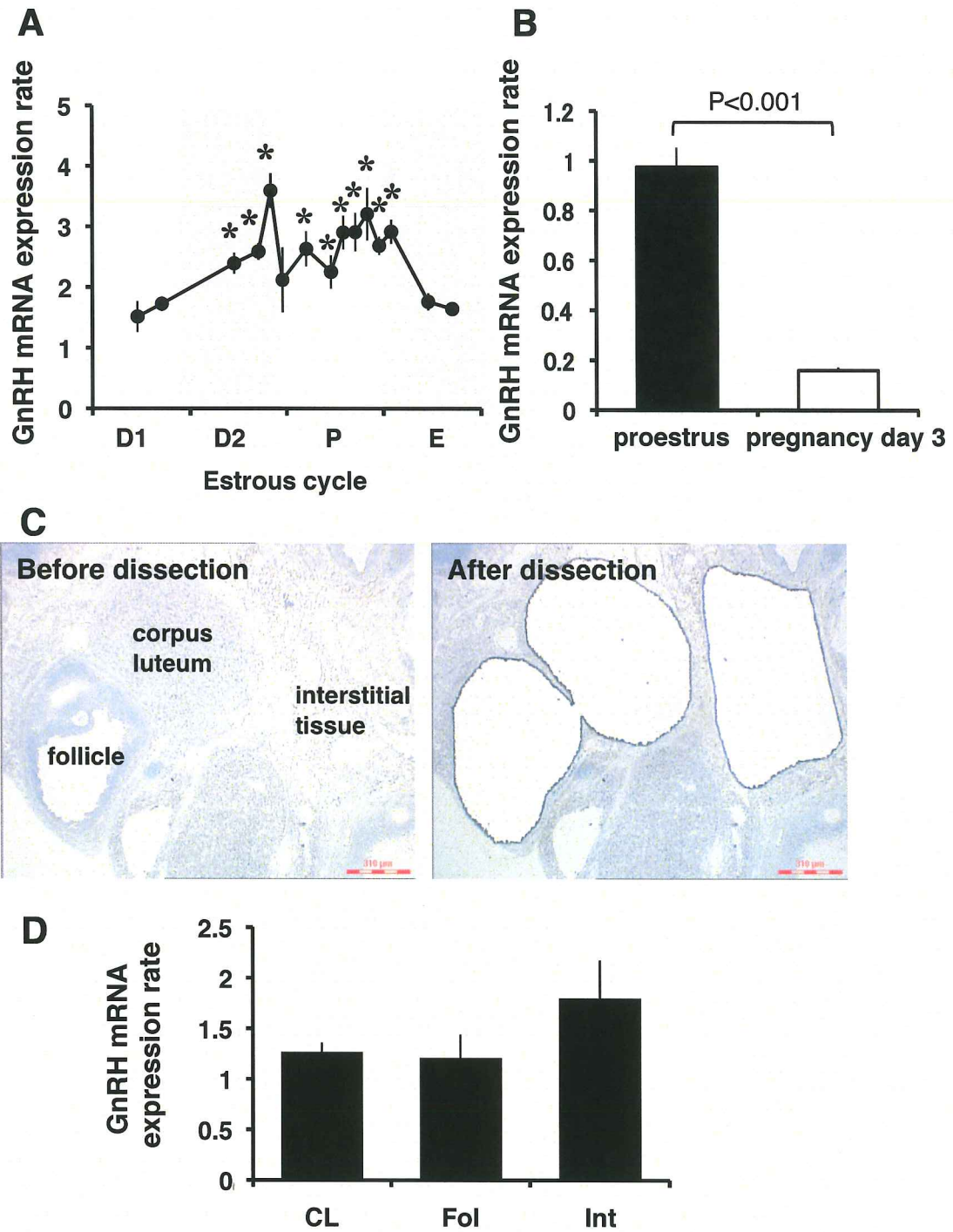


Fig. 2-1. Changes in GnRH mRNA expression during estrous cycle. A; Sampling points: Diestrus 1 (D1): 11:00, 17:00 h, Diestrus 2 (D2): 11:00, 17:00, 20:00, 23:00 h, Proestrus (P): 05:00, 11:00, 14:00, 17:00, 20:00, 23:00 h and Estrus (E): 02:00, 11:00, 17:00 h. Asterisks reveal the peak value and values not significantly different from the peak value (peak values are significantly different from values without asterisk, $p < 0.05$). B; GnRH mRNA expression in pregnant rats. Ovarian samples were harvested at 20:00 h on day 3 of pregnancy. Data are means \pm SEM. P values were obtained with Student's *t* test. C; Ovaries photographs of the sections of rat ovary at 20:00 h of proestrus before and after LMD. Three compartments were dissected out and obtained into a tube cap filled with reagents for RNA extraction. D; Relative expression of GnRH mRNA in corpus luteum (CL), follicle (Fol) and interstitial tissues (Int). Those samples were made from 5 rats.

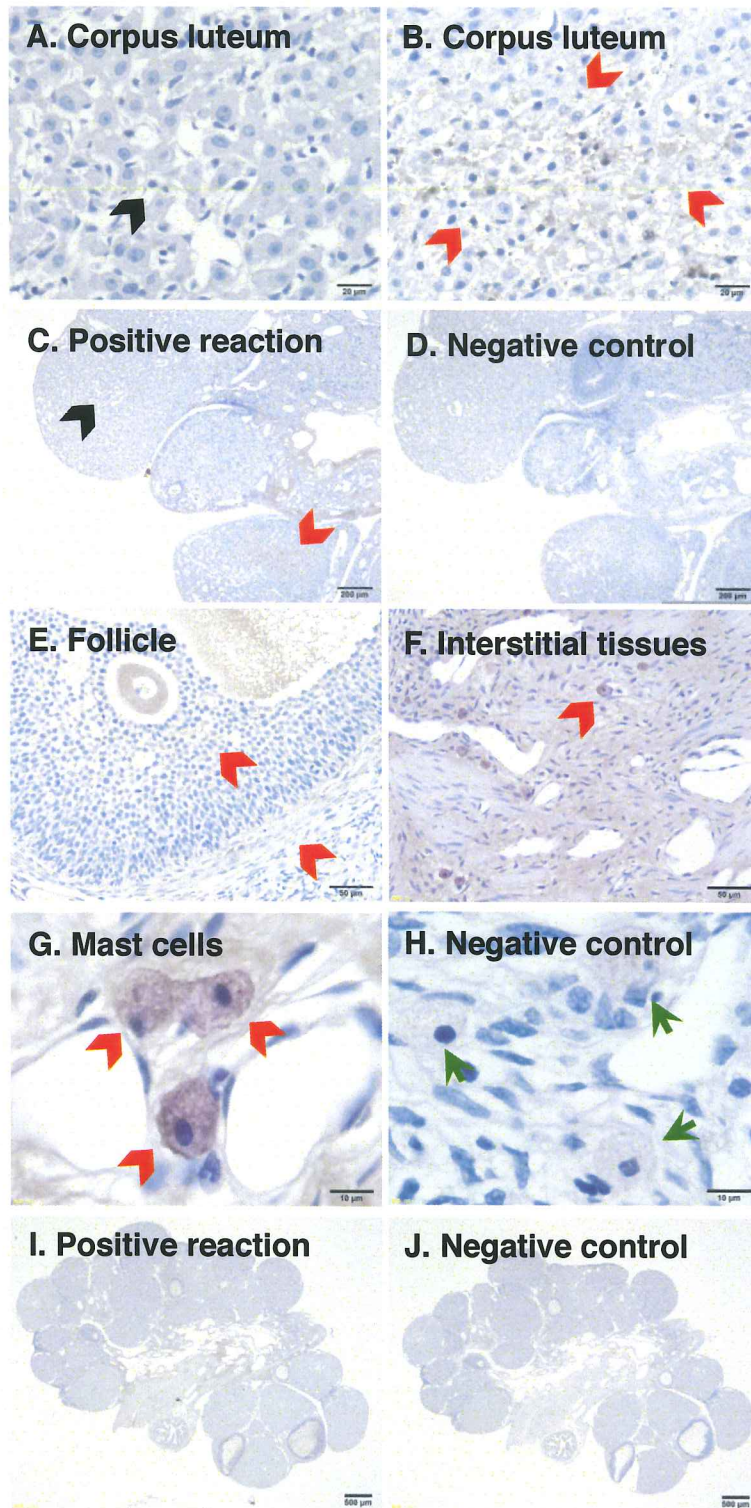


Fig. 2-2. Immunohistochemical demonstration of GnRH in the ovary. Ovaries were harvested at 20:00 h of diestrus 2. Ovarian tissues were subjected to the immunohistochemistry for GnRH. Ovarian tissues were stained with anti-GnRH serum. Normal rabbit serum was used for negative control instead of the first antibody. Arrow heads indicate GnRH positive cells in the ovarian tissues. Red arrow heads were strongly positive cells. Black arrow heads indicate corpus luteum with very low intensity of positive reaction. Green arrow indicate negative control for mast cells.

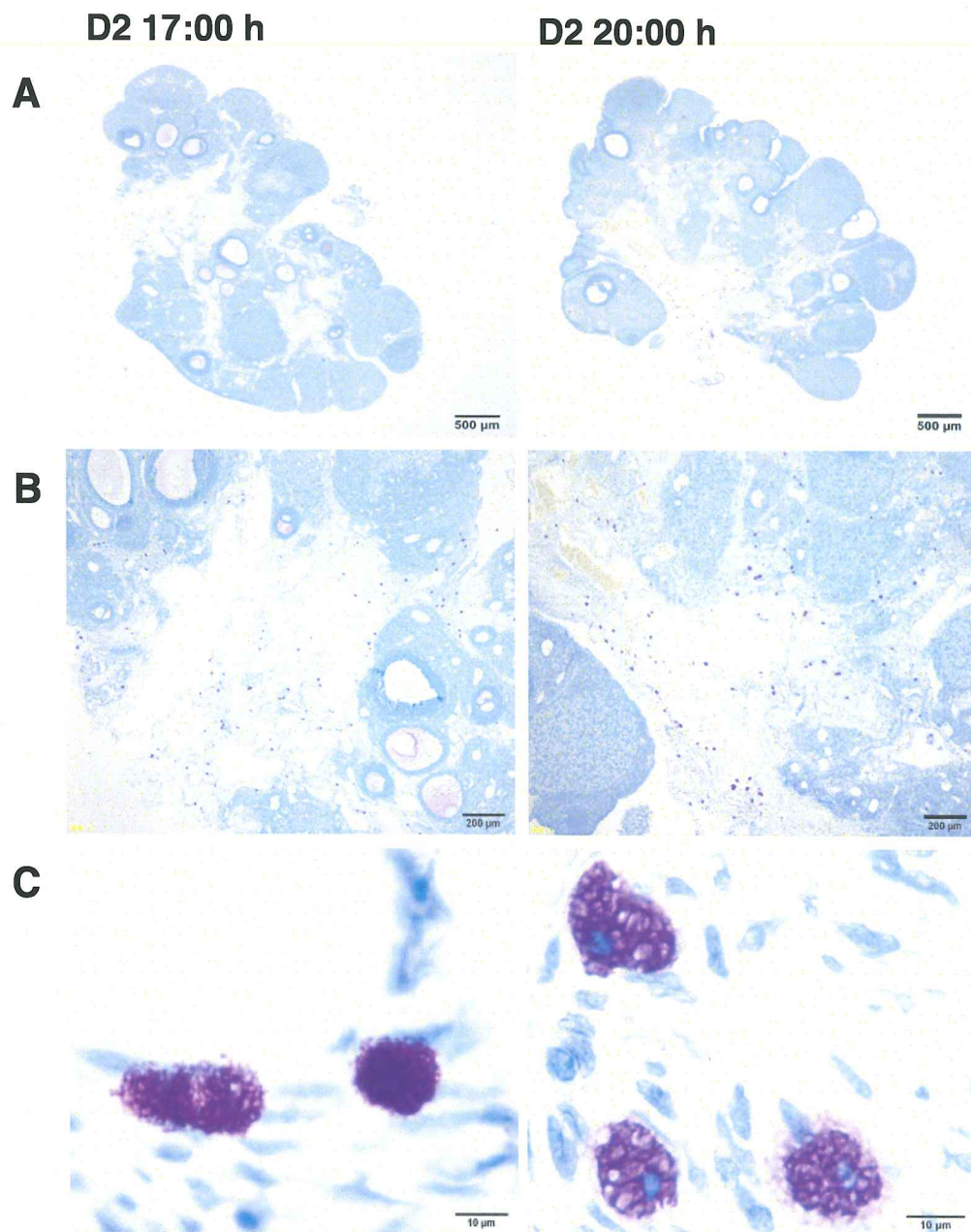


Fig. 2-3. A; Toluidine blue staining of ovarian tissues. Ovarian tissues were harvested at 17:00 h and 20:00 h of diestrous 2. C; Mast cells was demonstrated with toluidine blue metachromasy. B; They distribute to mainly interstitial tissues.

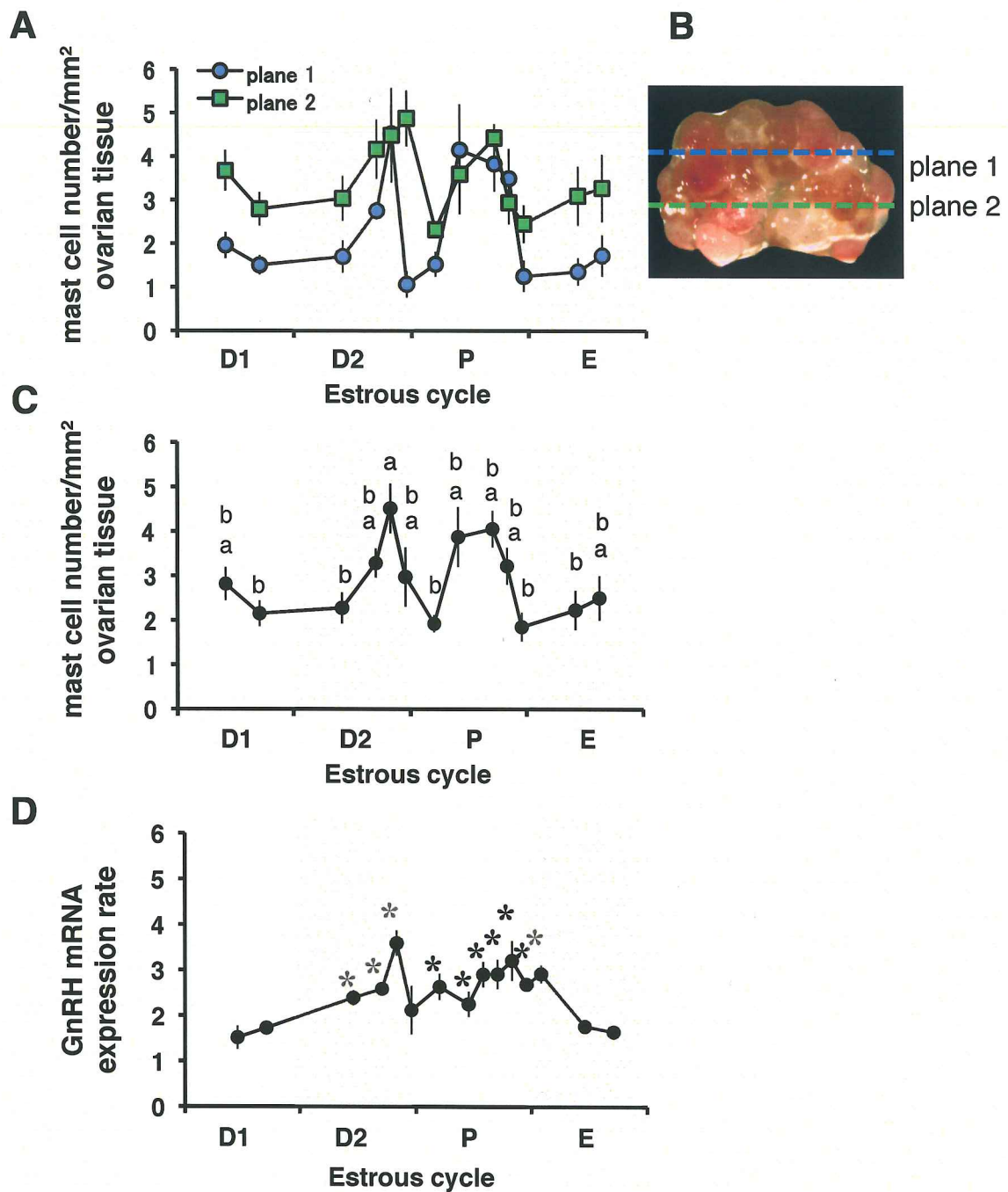


Fig. 2-4. Changes in ovarian mast cell number during estrous cycle of rats. A; Sampling points: Diestrus 1 (D1): 10:00, 17:00 h, Diestrus 2 (D2): 10:00, 17:00, 20:00, 23:00 h, Proestrus (P): 5:00, 10:00, 17:00, 20:00, 23:00 h, Estrus (E): 10:00, 17:00 h. B; Tissue section was made at two different planes. Mast cell was counted under a microscope at magnification of $\times 10$. Five fields each from ovaries were counted and the numbers were averaged. The number of mast cell was revealed per unit area (mm²). C; Changes in ovarian mast cell number (Data are means \pm SEM. Values with different letters are significantly different, $P < 0.05$.) and D; GnRH mRNA expression during estrous cycle (Asterisks reveal the peak value and values not significantly different from the peak value (peak values are significantly different from values without asterisk, $p < 0.05$)), mast cell number is revealed as a combined data of A. Each data are the average of 5-12 rats.

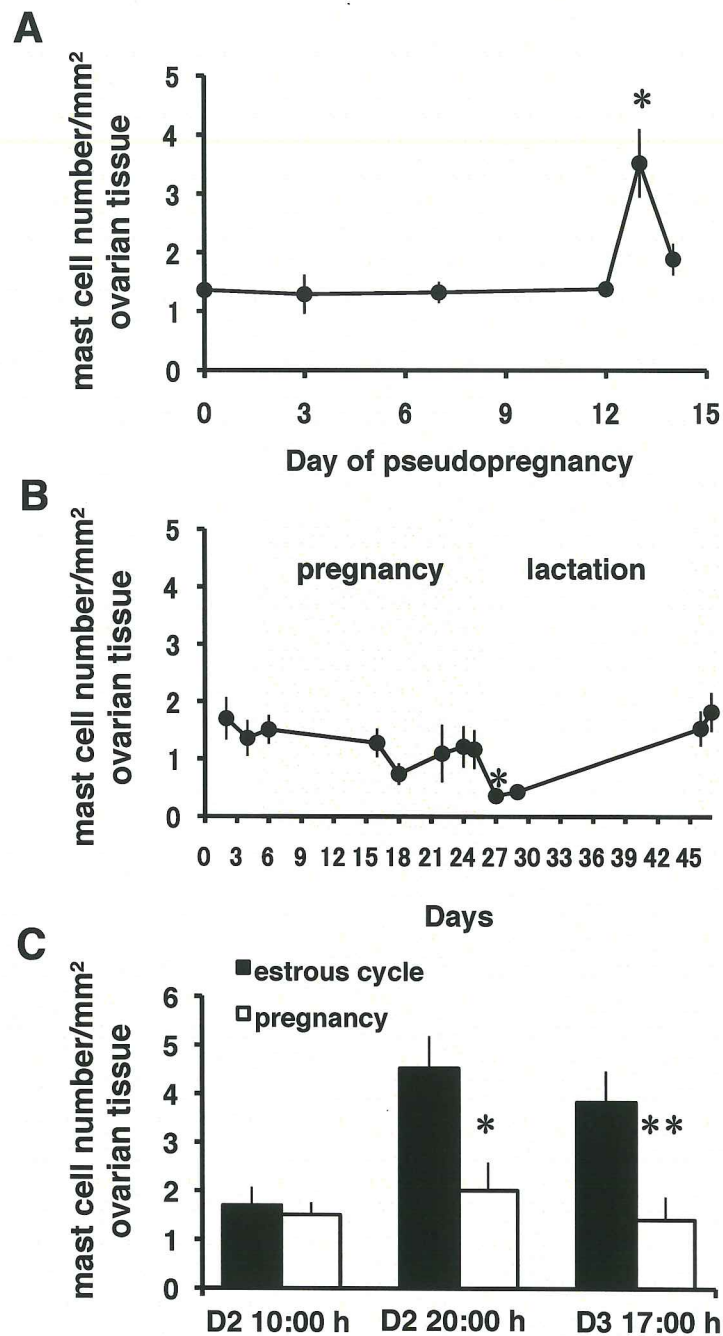


Fig. 2-5. Changes in ovarian mast cell number during luteal phase. A; Ovarian mast cells during pseudopregnancy. Ovaries were harvested at 10:00 h on day 3, 7, 12, 13 and 14 of pseudopregnancy. * $P < 0.05$ vs other days, $n = 3-5$. B; Ovarian mast cells during pregnancy and lactation. Ovaries were harvested at 10:00 h of pregnancy day 2, 12, 14, 18, 20 and 21, lactation day 1, 3, 21 and post-weaning day 2. * $P < 0.05$ vs post-weaning day 2, $n = 4$. P values were obtained with Tukey's test. C; Decrease in ovarian mast cell number in pregnant rats. Ovaries are harvested at each time point shown under the abscissa. D2 is diestrus 2 or day 2 of pregnancy. D3 is proestrus or pregnancy day 3. Data are means \pm SEM. P values were obtained with Student's t test, * $P < 0.05$ and ** $P < 0.01$ vs estrous cycle, $n = 4-9$.

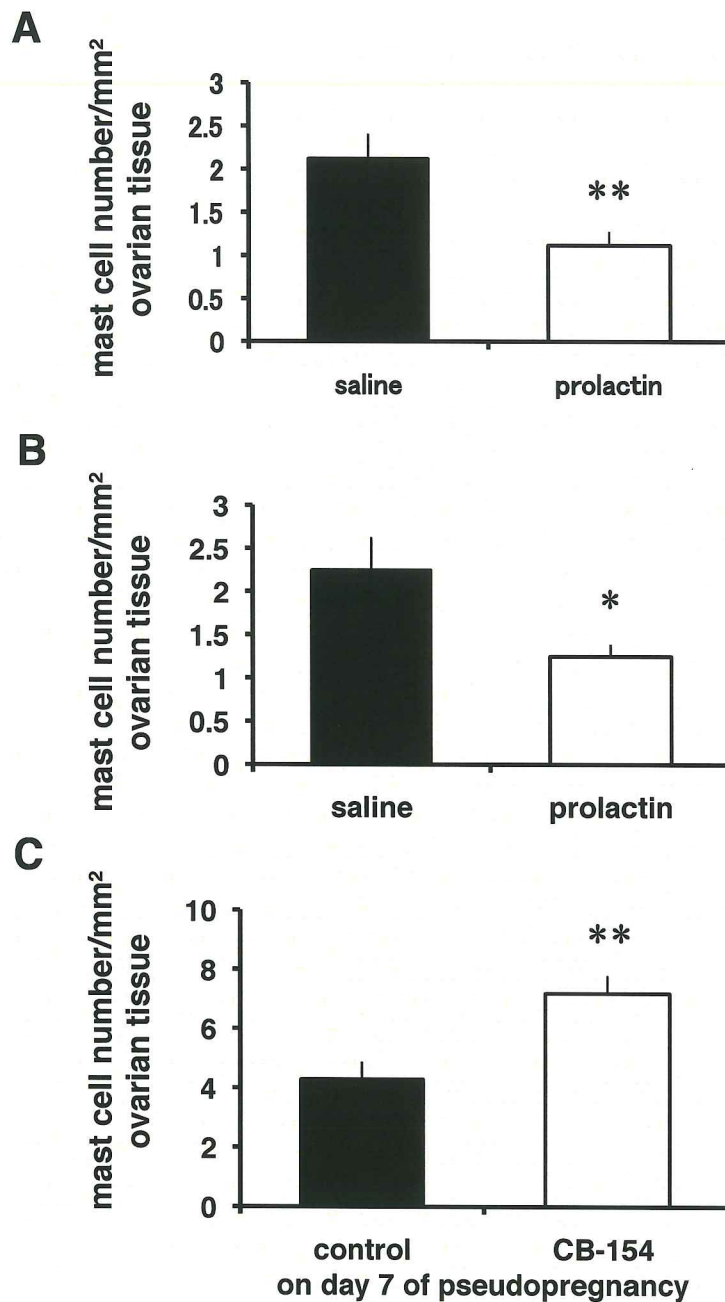
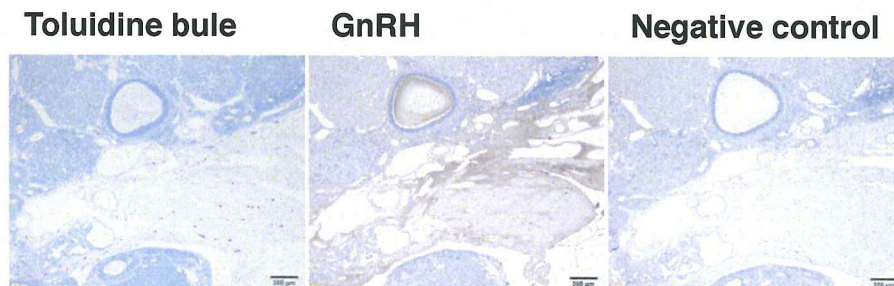


Fig. 2-6. The effect of prolactin on ovarian mast cell number. A; Ovine prolactin (10 IU) or saline was given at 10:00 h of diestrus 2 and harvested at 20:00 h of the same day. B; Ovine prolactin (10 IU) or saline was given at 10:00 h of proestrus and harvested at 17:00 h of the same day. C; The effect of dopamine agonist (CB-154) on the number of ovarian mast cell. CB-154 (300 μ g) or tartaric acid (vehicle) was given at 10:00 h of pseudopregnant day 5. Ovary was harvested at 10:00 h on pseudopregnant day 7. Those samples were made from 5-10 rats each. Data are means \pm SEM. P values were obtained with Student's *t* test, * $P < 0.05$ and ** $P < 0.01$.

A



B

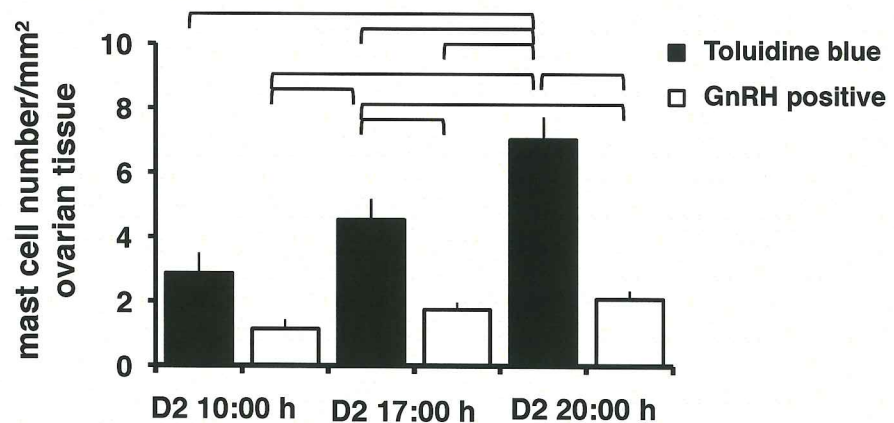


Fig. 2-7. A; Adjacent sections of ovary were made and subjected to immunohistochemistry of GnRH and Toluidine blue staining. B; Changes in the mast cell and GnRH positive cell numbers at 10:00 h, 17:00 h and 20:00 h of diestrus 2. Mast cell was counted under a microscope at magnification of x10. Five fields each from ovaries were counted and the numbers were averaged. The number of mast cell was revealed per unit area (mm²). Those samples were made from 3 rats each. The measures were standardized to each area of tissue. Values connecting with bars indicate significant differences, P<0.05.

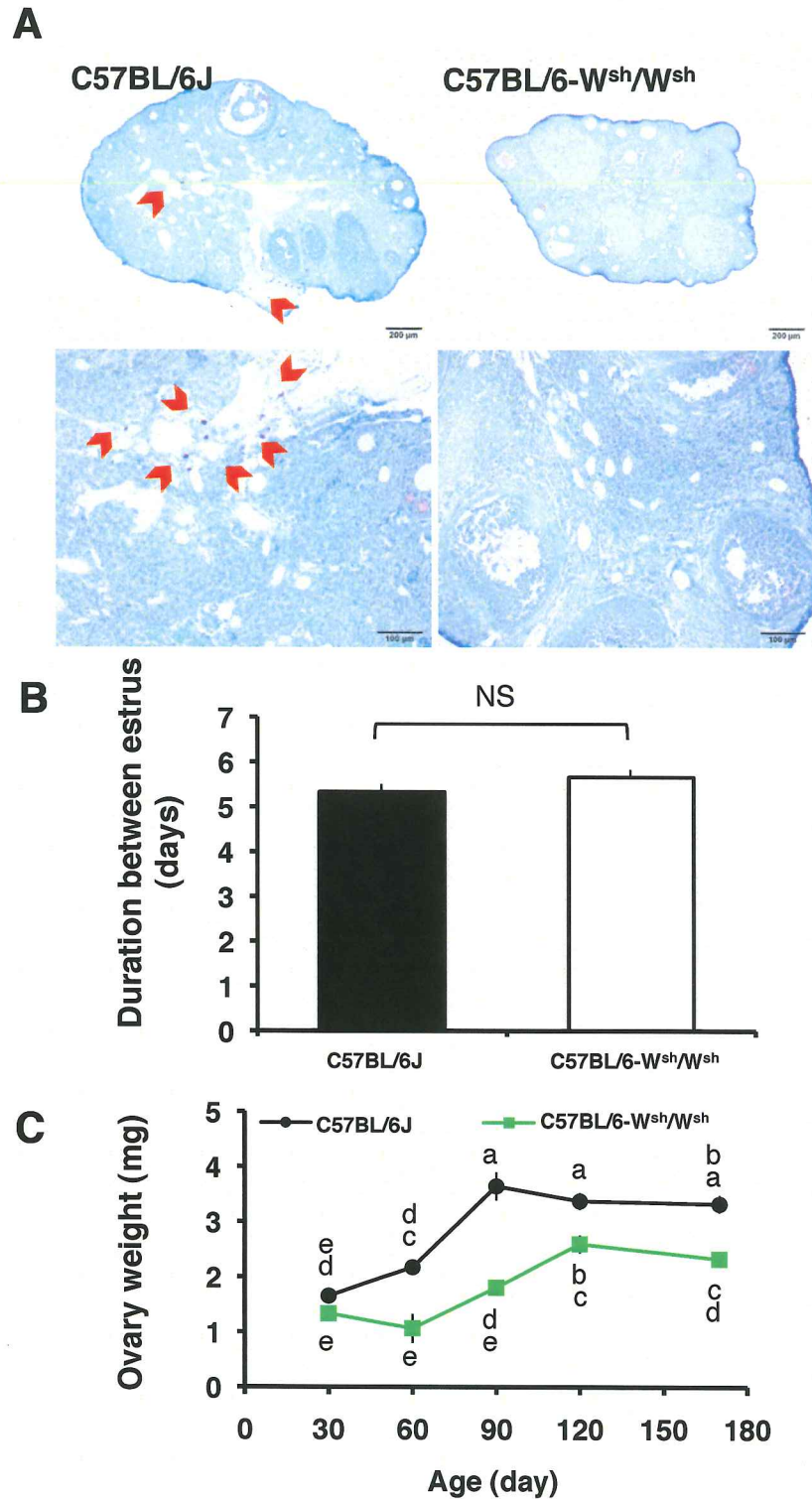


Fig. 2-8. Changes in the ovary of C57BL/6- W^{sh}/W^{sh} mice. A; Toluidine blue staining of the ovary. Arrow heads indicate mast cells. B; The length of estrous cycle of C57BL/6J mice and C57BL/6- W^{sh}/W^{sh} mice. Vaginal smears were examined daily, $n = 19-22$ per group. C; Changes in ovary weight of C57BL/6J mice and C57BL/6- W^{sh}/W^{sh} mice on day 30, 60, 90, 120 and 170 of age, $n = 3-5$ per each group. Data are presented as mean \pm SEM. Values with different letters are significantly different, $P < 0.05$. NS: non significant.

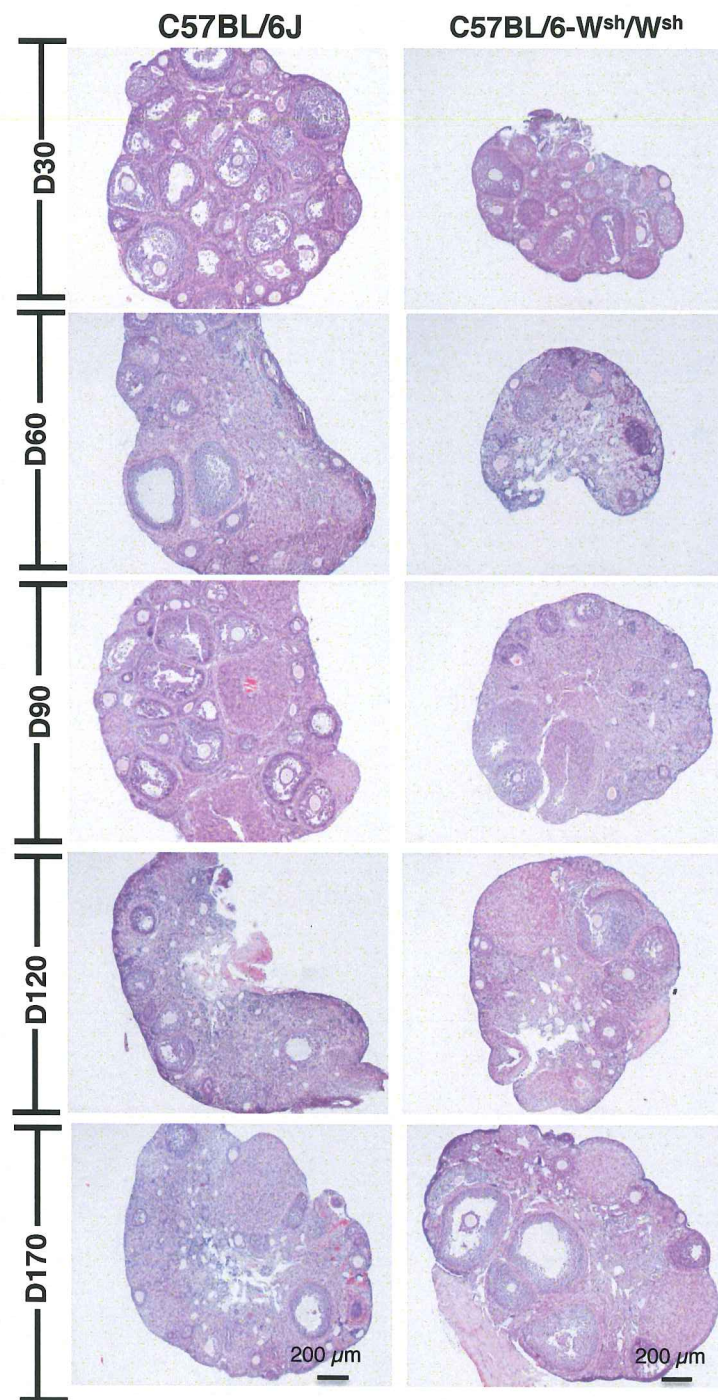


Fig. 2-9. Hematoxylin-Eosin staining of ovarian tissues. Ovarian tissues were harvested on day 30, 60, 90, 120 and 170 of age from C57BL/6J and C57BL/6-W^{sh}/W^{sh} mice.

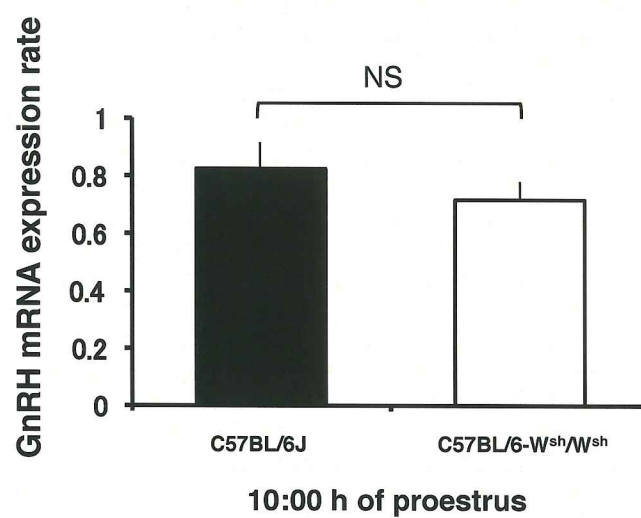


Fig. 2-10. Expression rate of GnRH mRNA of ovary in C57BL/6J mice and C57BL/6-W^{sh}/W^{sh} mice. Ovaries were harvested at 10:00 h of proestrus and subjected to real-time RT-PCR. NS: non significant, n = 4-5 per each group.

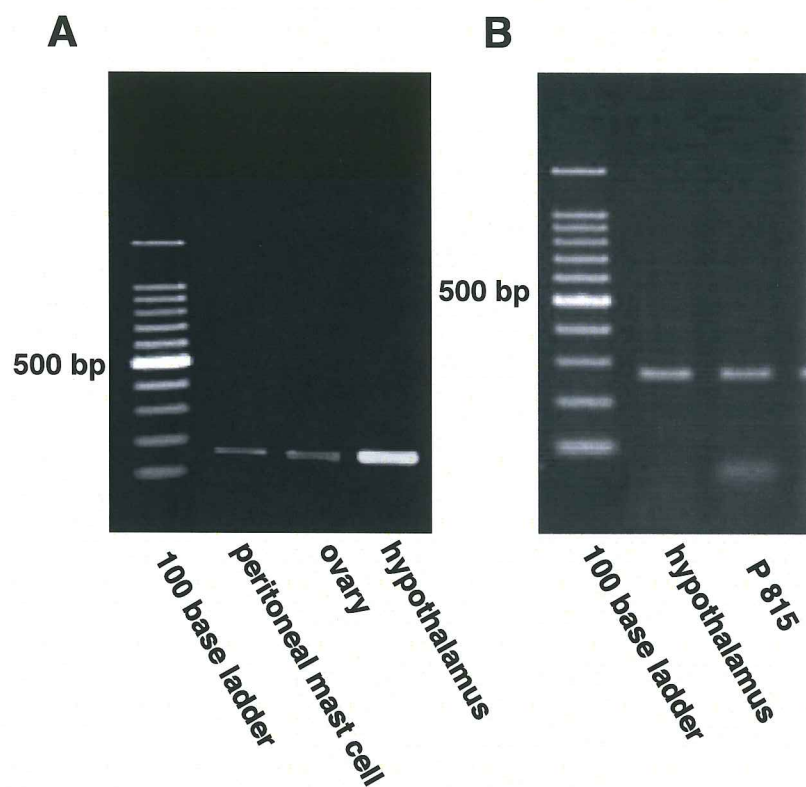


Fig. 2-11. Detection of GnRH gene expression in mast cells. A; Mast cells were prepared from peritoneal lavage of adult female rats and RNA was extracted. Expression of GnRH was examined by PCR. B; PCR demonstration of GnRH in Mouse mast cell clone P 815.

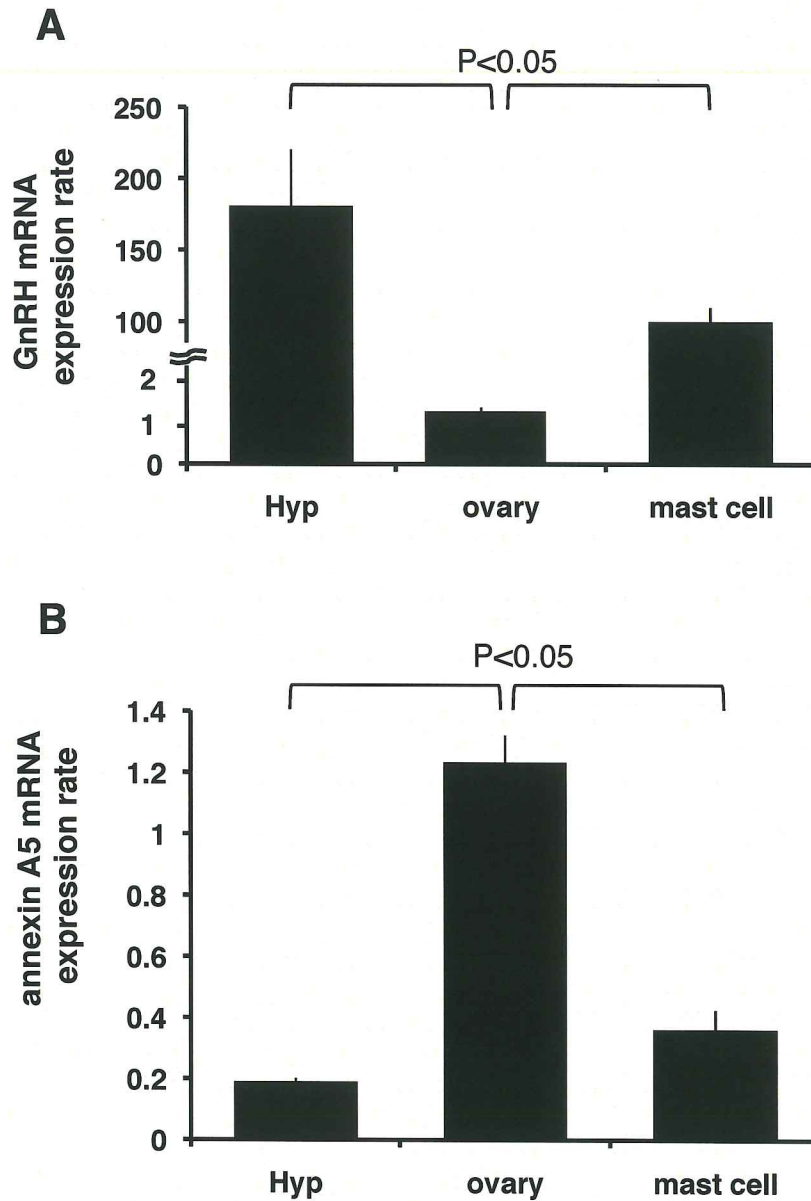


Fig. 2-12. The expression rate of GnRH and annexin A5 of hypothalamus (Hyp), ovary and peritoneal mast cells from female rats. Ovaries were taken at 20:00 h of diestrus 2. RNA was extracted from cells and subjected to each real-time RT-PCR. The expression rate of A; GnRH and B; annexin A5 mRNA were measured. Those samples were made from 4 rats each. P values were obtained with Tukey's test.

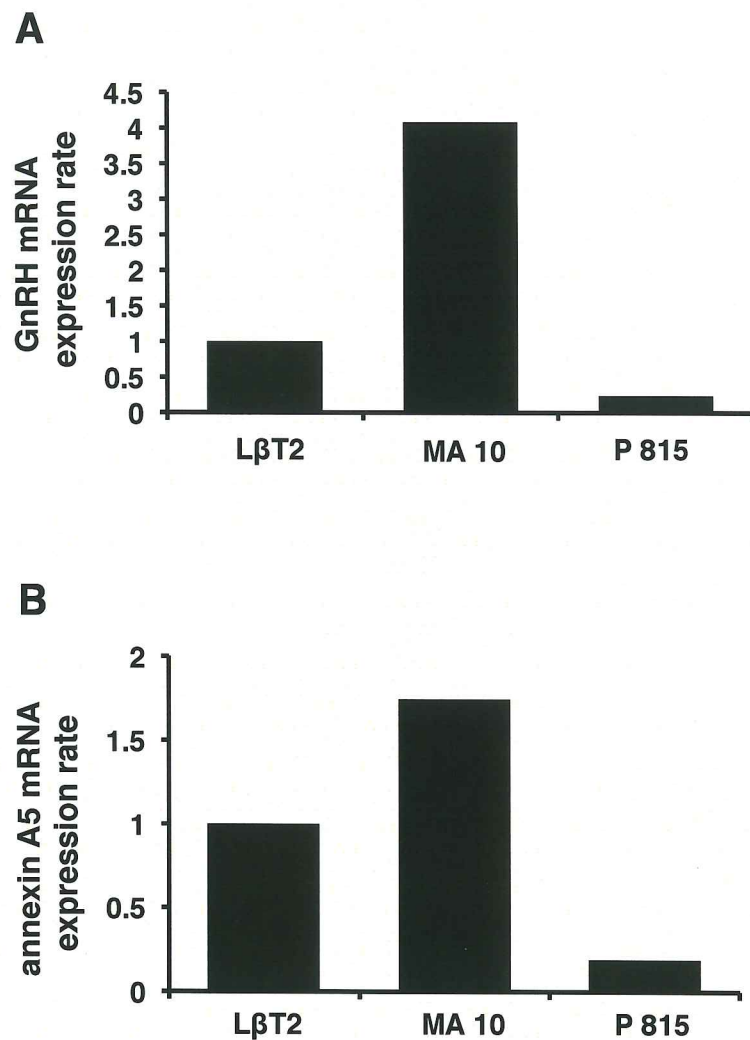


Fig. 2-13. The expression rate of GnRH and annexin A5 of LβT2, MA10 and P 815. RNA was extracted from cells and subjected to each real-time RT-PCR. The expression rate of A; GnRH and B; annexin A5 mRNA were measured.

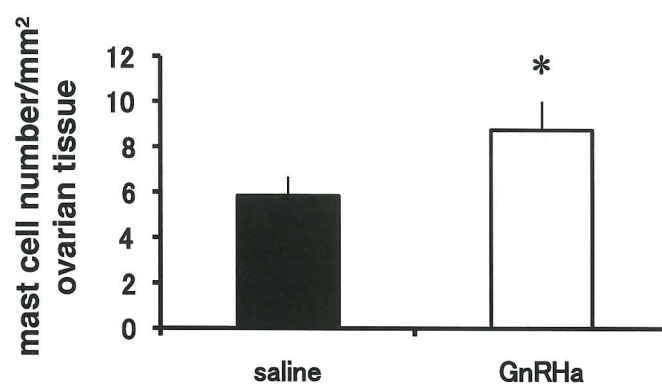


Fig. 2-14. The effect of GnRH agonist (GnRHa) on ovarian mast cell number. GnRHa (200 ng/50 μ l) was given into the hemi-lateral ovarian bursa at 11:00 h of diestrus 1. Saline was given into the contra-lateral ovary for a control. Ovaries of both sides were harvested five hours later. Data were made from 5 rats each and mean \pm SEM. P values were obtained with Student's *t* test, **P*<0.05.

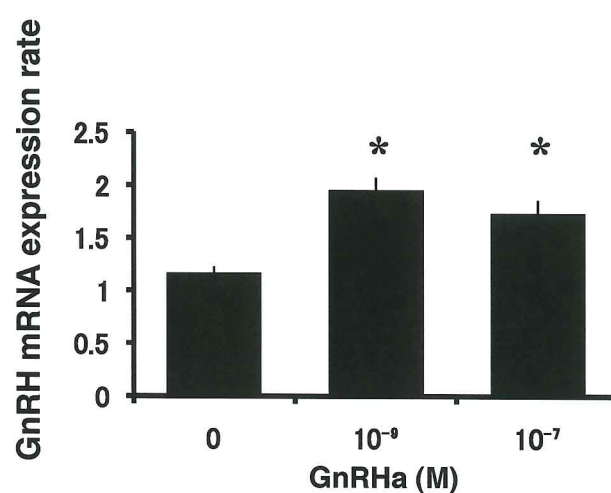


Fig. 2-15. The effect of GnRH agonist (GnRHa) on GnRH mRNA expression in peritoneal mast cells. Peritoneal mast cells were incubated with or without GnRHa at 10^{-9} and 10^{-7} M for three hours. Cells were harvested and subjected to quantitative real-time RT-PCR. P values were obtained with Tukey's test, * $P < 0.05$ vs 0.

Table 1. Sequences of Real time PCR primers (Rat)

Target	Forward primer 5' to 3'	Reverse primer 5' to 3'
GnRH	GGCAAGGAGGAGGATCAAA	CCAGTGCATTACATCTTCTTCTG
Metastin	ATGATCTCGCTGGCTTCTTG	AGGCTTGCTCTCTGCATAACC
Dynorphin	ACAGGCTTTGAGATCTGCGT	GGCAGTCTGCTGTAACCTCA
NKB	GTGAGGAACCTCAGGAGCAG	GCTAGCCTTGCTCAGCACTT
GPR54	GTCGGGAACTCACTGGTCAT	ACGCAGCACAGAAGGAAAGT
Annexin A5	GGAAACCATTGACCGAGAGA	TCTCTGCAAGGTAGGCAGGT
RPL19	CAGGAGATACCGGGAATCTAAG	TGCCTTCAGTTTGTGGATGTG

Table 2. Sequences of Real time PCR primers (Mouse)

Target	Forward primer 5' to 3'	Reverse primer 5' to 3'
GnRH	GGGATCTGCGAGGAGCTCTGGAAA	ACAGGTCACAAGCCTCAGGGTCA
Annexin A5	GGTACCGATGAGGACAGCAT	TCCCTGCCAAACAGAGTCTT
Metastin	ACTGTAGACCTGCCCCCTTCC	CTTTTCCCAGGCATTAACGA
GPR54	TCCCCTGTTTTTCGCTACAC	GTAGAGGAGTGCGGTGAAGG
RPL19	CAGGAGATACCGGGAATCTAAG	TGCCTTCAGTTTGTGGATGTG

Table 3. Sequences of PCR primers (Rat)

Target	Forward primer 5' to 3'	Reverse primer 5' to 3'
GnRH	CAGCACTGGTCCTATGGGTT	CAGACGTTCCAGAGCTCCTC

Table 4. Sequences of PCR primers (Mouse)

Target	Forward primer 5' to 3'	Reverse primer 5' to 3'
GnRH	CTCAACCTACCAACGGAAGC	GGGCCAGTGCATCTACATCT

Chapter 3 Expression of Metastin and Related Peptides in the Ovary

Introduction

Metastin is a product of KISS-1 anti-metastatic gene [37]. It was first identified as a metastasis suppressor gene in breast cancer and melanoma cells [47, 50]. The KISS-1 gene encodes a 145 amino acid protein that is cleaved into a 54-amino acid peptide known as kisspeptin-54 (Kp-54) as well as short peptides of 14, 13 or 10 amino acids. The product of KISS-1 gene acting via G protein-coupled receptor 54 (GPR54) [25, 37] has been found to stimulate the preovulatory luteinizing hormone (LH) surge through the augmentation of gonadotropin-releasing hormone (GnRH) neuron secretion at the median eminence [23]. Metastin neurons of the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus are thought to be responsible for LH surges and pulses, respectively [30]. Metastin is found in both the peripheral and the central nervous system (CNS). In peripheral tissues, metastin has been identified in the testis, ovary, anterior pituitary gonadotrophs, pancreas and small intestine [15, 37, 42]. The suppressive influence of metastin on metastasis has also been shown in lung [28], stomach [10], and other cancers [27, 30]. Metastin is involved in insulin secretion [18], aldosterone production [35] and implantation [48]. The most impactful finding has been the principal role of GPR54 in reproduction. Loss-of-function mutations of GPR54 in humans have been shown to accompany hypogonadotrophic hypogonadism [8]. Under these circumstances, metastin is preferentially called kisspeptin, probably due to its function in reproduction. In this thesis, it is called metastin according to the original nomenclature.

Changes in the expression of metastin and GPR54 in the ovary have been demonstrated to occur with physiological changes in the ovaries of humans, hamsters, and rats [3, 15, 45]. Immunohistochemical studies have shown that metastin is distributed to theca, granulosa, luteal,

and interstitial cells of human, marmoset, hamster, and rat ovaries [3, 15, 45]. It has been revealed by semi-quantitative RT-PCR that metastin mRNA is increased in the afternoon of proestrus, and that human chorionic gonadotropin (hCG) stimulates the expression of metastin mRNA in rats [3]. These results suggest a role for local metastin in ovulation. As polycystic ovary syndrome has been reported to accompany high plasma levels of metastin [5], it may have a relationship to pathological conditions. Nevertheless, the regulating mechanism of synthesis, the source cells, and the function of ovarian metastin are still not known.

Metastin neurons also contain neurokinin B (NKB) and dynorphin at the arcuate nucleus of the hypothalamus, and are called KNDY neurons there [16]. NKB has been shown to play a crucial role in the feed back control of GnRH release. Similarly to metastin, loss-of-function mutation results in reproductive failure [17]. Tachykinins, including NKB, are also expressed in the ovary [39]. It is therefore of interest whether cells expressing metastin also synthesize NKB and dynorphin in the ovary.

In this chapter, detailed variations of the expression rate, source and physiological relevance of metastin and related peptides, neurokinin B (NKB), dynorphin and GPR54 were examined in the ovary during the estrous cycle of rats. In addition, a possibility that metastin is involved in GnRH control in the ovary was also examined.

Materials and methods

1. Animals

All experiments were performed according to the guideline for animal experiments of Kitasato University that followed the guideline of NIH, and the experimental protocol was approved by the committee. Female Wistar Imamichi rats bred in our vivarium were used. They were kept in the atmosphere of temperature at 23 ± 3 °C and light-dark cycle of 14L:10D (lights on 05:00-19:00 h). Food (laboratory chow, CE-2, Oriental Co., Tokyo, Japan) and tap water were supplied *ad libitum*.

2. Quantitative Real-time PCR

Expression rate of metastin, GPR54, NKB, dynorphin and GnRH mRNA in ovarian tissues and granulosa cells and were determined by real-time PCR. Sequences of forward and reverse primers were shown in Table 1. Procedure for Quantitative Real-time PCR is described in Chapter 2.

3. Administration of reagents

The effect of systemic and local administration of various reagents on metastin, GnRH, NKB and dynorphin mRNA expression of the ovary and ovarian morphology were examined as listed below.

- 1) The effects of GnRH antagonist and pentobarbital sodium on metastin expression in the ovary were examined. GnRH antagonist (Cetrorelix, 1 µg/ 100 g) or pentobarbital sodium (4.0 µg/ 100 g) was administered intraperitoneally at 12:00 h of proestrus. Control group was given saline. Ovaries were harvested at 20:00 h of proestrus.

- 2) The effects of prolactin administration on metastin and GnRH expression in the ovary were examined. Ovine prolactin (10 IU/0.2 ml, Sigma-Aldrich) was administered intraperitoneally at 10:00 h or 17:00 h of proestrus. Control was treated with saline. Ovaries were harvested at 20:00 h of proestrus.
- 3) The effects of dopamine agonist (CB-154, Ergocryptine, Sigma-Aldrich) were studied. CB-154 (300 µg/ 0.2 ml/ day) was administered intraperitoneally at 10:00 h from pseudopregnancy day 5. CB-154 was dissolved in 3% tartaric acid. Control was treated with the vehicle. Ovary was harvested at 10:00 h on pseudopregnancy day 7. The expression rate of GnRH mRNA was measured.
- 4) The effects of estradiol on metastin expression in the ovary was examined. Estradiol (0, 0.1, 1, 10 µg/0.1 ml) was administered subcutaneously at 14:00 h of diestrus 2. Estradiol was dissolved in sesame oil. Ovaries were harvested at 17:00 h of diestrus 2.
- 5) To see the effect of human chorionic gonadotropin (hCG) on metastin, dynorphin, NKB and GnRH expression, hCG (0, 0.1, 1, 10 IU/0.1 ml) was administered intravenously at 14:00 h of diestrus 2. Ovaries were harvested 3 h later at 17:00 h of diestrus 2.
- 6) To examine the direct effect of biologically active metastin peptide on GnRH expression. Kisspeptin-10 (Rat)/Metastin (Rat, 43-52) (10 µg/50 µl, PEPTIDE INSTITUTE, INC., Osaka, Japan) or Kisspeptin-10 (Human)/Metastin (Human, 45-54) (6.45 µg/50 µl, PEPTIDE INSTITUTE, INC., Osaka, Japan) was given into the hemi-lateral ovarian bursa at 10:00 h of diestrus 2. Saline was given into the contra-lateral ovary for a control. Ovaries of both sides were harvested at 16:00 h of diestrus 2.
- 7) To study the effect of local metastin antagonist p234 on ovarian morphology, metastin antagonist p234 (Phoenix Pharmaceuticals INC., Burlingame, CA) dissolved in 0.9% NaCl (1 nmol/24 µl) was continuously infused into the hemi-lateral ovarian bursa by an osmotic minipump (1.0 µl/h delivery rate, DURECT Corporation, Cupertino, CA) with

polyethylene tubing for 3 days from the morning (10:00 h) of proestrus. The contralateral ovary was infused with an equal volume of 0.9% NaCl as a control. Ovaries of both sides were collected three days later (10:00 h). Ovarian tissues were subjected to morphological examination with Hematoxylin-Eosin staining.

4. Laser Microdissection (LMD)

Laser Microdissection is used for collecting follicle, corpora lutea and interstitial tissues separately from ovary sections. Three compartments were identified under a microscope included in the LMD system. Procedure for LMD is described in Chapter 2.

5. Histological analysis

Histological examination was also performed in this chapter. General methods of experiments are the same as described in Chapter 2.

6. Immunohistochemistry

The distribution of metastin in the ovarian tissues was determined by immunohistochemistry with rabbit anti-Kisspeptin 10 polyclonal antibody (Chemicon, Millipore Corporation). The antiserum used at dilution of 1:1,000. The procedure for immunohistochemistry is described in Chapter 2.

7. Hematoxylin-Eosin Staining

The procedure for Hematoxylin-Eosin staining is described in Chapter 2.

8. Primary culture of granulosa cells

a. Reagents, solutions and equipments

Pregnant mare serum gonadotropin (PMSG) was supplied from National Hormone & Peptide Program, HARBOR-UCLA MEDICAL CENTER (Torrance, CA).

Growth medium

- Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) (Gibco® by Life Technologies, Grand Island, NY)
- 10 mM HEPES (1x) (Gibco® by Life Technologies, Grand Island, NY)
- Antibiotic-Antimycotic (100x) (Gibco® by Life Technologies, Grand Island, NY)
- 10% fetal bovine serum

24-well plates (IWAKI brand, ASAHI GLASS CO., LTD., Tokyo, Japan)

hCG

metastin antagonist, p234 (Phoenix Pharmaceuticals INC., Burlingame, CA)

b. Protocol

Granulosa cells were prepared by reported protocols. Briefly, PMSG (15 IU/150 µl) was given subcutaneously on 25 days of age. Rats were killed 48 h later by cervical dislocation under ether anesthesia. Ovaries were excised under aseptic condition and placed in sterile growth medium. Medium and large sized antral follicles were punctured with a 26-gauge needle to release granulosa cells. Granulosa cells were pelleted and resuspended in growth medium and plated in 24-well plates at approximately 5×10^5 viable cells per well and were incubated at 37°C, 5% CO₂-95% air for an additional 24 h before the experiments.

Cells were treated for 3 h with doses of hCG (0, 0.001, 0.003, 0.01, 0.03 and 0.1 IU/ml). At the end of incubation, granulosa cells were collected and stored at -80°C until assay

with real time PCR. Expression rate of metastin, dynorphin and NKB mRNA in granulosa cells were studied. RNA samples were subjected to real time PCR. Total RNA was extracted with Trizol as previously described. Sequences of forward and reverse primers were shown in Table 1.

Cells were treated for 3 h with various doses of hCG (0, 0.001, 0.01 and 0.1 IU/ml) with/without metastin antagonist p234 (0, 0.1 and 1 μ M). At the end of incubation, conditioned medium was collected and stored at -80°C until assay for progesterone content.

9. Time resolved immunofluorescent assay of progesterone

a. Reagents and buffers

Hexane

Eu-labeling reagent

- 0.2 mg (300 nM) Eu-DTTA

Assay buffer (pH7.8)

- 50 mM Tris-HCl buffered saline
- 0.5% Albumin, from bovine serum (BSA, Sigma-Aldrich, Inc., St. Louis, Mo)
- 0.05% Bovine gamma globulin, serum (EMD Chemicals, Inc, San Diego, CA)
- 0.01% Tween 20
- 20 mM DTPA
- Phenol red 15 mg/ ℓ
- 0.05% NaN₃

Coating buffer

- 50 mM K₂HPO₄ buffered saline
- 0.05% NaN₃

Blocking buffer

- 50 mM Na₂HPO₄
- 0.1% BSA

Washing buffer

- 50 mM K₂HPO₄ buffered saline (pH 7.8)
- 0.05% Tween 20

BSA conjugated progesterone (4-pregnen-3, 20-dione 3-O-calboxymethyloxime: BSA, Steraroid, Newport, RI)

Eu labeling

- Eu-labeling reagent
- Anti progesterone IgG solution
- Sodium carbonate buffer (pH 9.8)

Protein G HP Spin Trap (GE Healthcare Life Sciences, Pittsburgh, PA)

DELFI[®]A Enhancement Solution (PerkinElmer[™], Wallac Oy, Turku, Finland)

96-well assay plate (Thermo Scientific, Roskilde, Denmark)

b. Protocol

Progesterone levels in the medium were measured by the Time resolved immunofluorescent assay. Progesterone was extracted from medium with hexane, dried and dissolved into appropriate volume of assay buffer. BSA conjugated progesterone was diluted with coating buffer to 3 µg/ml and added 100 µl of the solution to each well of 96-well assay plate. The plate was shaken at room temperature for overnight and washed for three times. Then 200 µl of blocking buffer was added to each well and the plate was shaken for overnight at 4°C. They were washed for three times. Anti-progesterone IgG was prepared from home made anti-progesterone rabbit serum with Protein G HP Spin Trap. IgG was labeled with Eu by means of

DELFLIA® Eu-Labeling Kit. Progesterone standards and medium samples were incubated in anti-progesterone antibody-coated 96-well plates. The plate was shaken for overnight at 4°C and washed for six times. The plate was then rinsed off, and fluorescence was measured after suspension with DELFLIA® Enhancement Solution.

10. Statistics

Multiple comparisons of differences between mean values were analyzed by Tukey test after ANOVA, and single comparisons were analyzed by Student's *t* test. $p < 0.05$ was considered to be significant.

Results

1. Changes in the expression rate of ovarian metastin, dynorphin, NKB and GPR54 mRNAs during estrous cycle of rats

Ovarian RNA was harvested at 11:00 and 17:00 h of diestrus 1, 11:00, 17:00, 20:00 and 23:00 h of diestrus 2, 05:00, 11:00, 14:00, 17:00, 20:00 and 23:00 h of proestrus, and 02:00, 11:00 and 17:00 h of estrus from estrous cycling rats. There was only one steep peak in ovarian metastin mRNA expression in the afternoon of proestrus. It was dramatically increased from 17:00 h of proestrus and reached a peak at 20:00 h (Fig. 3-1A). Then it quickly declined to a base level until 23:00 h. There was no obvious fluctuation in metastin mRNA expression during estrous cycle other than this proestrous increase. Dynorphin mRNA expression showed a similar pattern to that of metastin (Fig. 3-1B). NKB mRNA expression was also increased, accompanying that of metastin, but its peak was delayed until 02:00 h of estrus (Fig. 3-1C). GPR54 mRNA expression rate started to increase from diestrus 2 and showed a peak at 14:00 h of proestrus (Fig. 3-1D). It decreased inversely to the increase of metastin mRNA expression at 17:00 h.

2. The metastin mRNA expression after suppression of LH surge

The significant increase of metastin mRNA expression rate of proestrus was disappeared in pregnant rats when it was examined at the equivalent time, 20:00 h of pregnancy day 3 (Fig. 3-2A). To see the relevance of proestrous LH surge on the acute increase of metastin mRNA expression, the effects of pentobarbital or GnRH antagonist given at noon during proestrus were examined. Both treatments are expected to suppress the LH surge and extinguished the increase of metastin mRNA expression rate (Fig. 3-2 B).

3. The effect of prolactin on expression of GnRH and metastin mRNAs in the ovary

When ovine prolactin was administered intraperitoneally at 10:00 or 17:00 h of proestrus and ovaries were harvested at 20:00 h of the same day. Prolactin did not inhibit the expression of GnRH and metastin mRNA at 20:00 h (Fig. 3-3A, B).

As prolactin secretion was suppressed by dopamine agonist. CB-154 was administered intraperitoneally at 10:00 h from pseudopregnancy day 5 and ovaries were harvested two day later. It stimulated GnRH mRNA expression a little but not significantly (Fig. 3-3C).

4. The effect of estradiol (E2) on metastin mRNA expression in the ovary

To examine the effect of estradiol on ovarian metastin expression, rat was administered with estradiol at 14:00 h of diestrus 2. Ovaries were harvested at 17:00 h of the same day. Low dose estradiol significantly increased the ovarian expression of metastin (Fig. 3-4).

5. The effect of human chorionic gonadotropin (hCG) on metastin, dynorphin, NKB and GnRH mRNA expression in the ovary

As the increase of metastin mRNA expression was supposed to be stimulated by LH surge, the effect of hCG on the mRNA expression of metastin was examined. It was administered at 14:00 h on diestrus 2 and ovaries were harvested three hours after at 17:00 h. Administration of hCG significantly increased the ovarian expression of metastin (Fig. 3-5A) and dynorphin mRNAs (Fig. 3-5B), but not NKB (Fig. 3-5C) and GnRH (Fig. 3-5D).

6. Ovarian components expressing metastin, dynorphin, NKB and GPR54 mRNAs

Laser Microdissection (LMD) was applied for assessing ovarian components expression of metastin, dynorphin, NKB and GPR54 mRNAs. Corpora lutea, follicles and

interstitial tissues were processed from the ovaries obtained at 20:00 h of proestrus. Metastin mRNA was shown to be synthesized almost solely in the follicles (Fig. 3-6A), while dynorphin and NKB mRNAs were detected mainly in interstitial tissues (Fig. 3-6B, C). There were no significant differences in the expression of GPR54 mRNA among corpora lutea, follicles, and interstitial tissues (Fig. 3-6D).

7. The effect of hCG on metastin mRNA expression in ovarian components

To see the effect of hCG on metastin synthesis in each ovarian component, hCG was administered at 14:00 h of diestrus 2 and ovaries were harvested 3 h after. Corpora lutea, follicles and interstitial tissues were separately collected by means of LMD. hCG was shown to stimulate metastin synthesis in the follicle (Fig. 3-7).

8. Immunohistochemical demonstration of metastin in the ovary

Ovaries were harvested at 20:00 h of proestrus. Immunohistochemistry demonstrated that metastin distributed to almost all compartments of the ovary. In the granulosa cells, theca cells (Fig. 3-8F) and luteal cells were all positive (Fig. 3-8B), but metastin positive cells were restricted to only a portion of each tissues (Fig. 3-8C, E). Some corpora lutea were without positive reaction (Fig. 3-8A) and interstitial tissues were stained diffusely (Fig. 3-8E). Normal rabbit serum was used for the negative control instead of the first antibody showing no reaction (Fig. 3-8D).

9. Comparison of the expression rate of metastin and GnRH mRNAs during the estrous cycle of rats

Metastin and GnRH mRNA expression rates were compared. Metastin mRNA expression (Fig. 3-9A) and GnRH mRNA expression rate (Fig. 3-9B) during the estrous cycle were not synchronized (Fig. 3-9C).

10. Effects of Kisspeptin-10

The effect of biologically active metastin derived peptide was examined. Kisspeptin-10 (Rat)/Metastin (Rat, 43-52) (10 µg/50 µl) or Kisspeptin-10 (Human)/Metastin (Human, 45-54) (6.45 µg/50 µl) was given into the hemi-lateral ovarian bursa at 10:00 h of diestrus 2. Ovaries were harvested at six hours later. There were no significant difference in the expression rate of GnRH mRNA in the ovary (Fig. 3-10A, B). Ovarian metastin is thought not to be involved in local GnRH synthesis.

11. Effects of metastin antagonist p234

To see the effect of metastin on ovarian morphology, P234 was given into the hemi-lateral ovarian bursa for three days from proestrous morning with an osmotic minipump and saline was given into the contra-lateral ovary. Ovarian sections were stained with Hematoxylin Eosin. Hemilateral administration of metastin antagonist p234 induced a histological change in corpus luteum (Fig. 3-11). The borders of the corpus luteum frequently merged with the interstitial tissues or another corpus luteum in ovaries given This change was not seen in the contra-lateral ovary given same amount of saline p234. (Fig. 3-12).

12. Primary cultures of granulosa cells

To see the direct effect of hCG on metastin, dynorphin and NKB expression, the primary culture of granulosa cells was established. PMSG (15 IU) was given on day 25 and granulosa cells were harvested by mechanical dissociation from follicles on day 27. Various

doses of hCG was given for three hours and RNA was extracted from cells. The granulosa cells were shown to synthesize significant amounts of metastin in response to hCG (Fig.3-13A). Metastin and dynorphin mRNA expression were stimulated by hCG in a dose response manner. NKB mRNA expression was also stimulated by hCG, but the extent was less than those of metastin and dynorphin (Fig. 3-13A-C).

Cells were incubated with various concentrations of hCG in combination with p234 for three hours and conditioned medium was assayed for progesterone. Granulosa cells were shown to synthesize progesterone in a dose-response manner to hCG. Simultaneous administration of p234 suppressed the progesterone production (Fig. 3-14).

Discussion

The present study clearly demonstrates that metastin mRNA expression in the ovary occurs in granulosa cells and is well regulated by the proestrous LH surge in rats. Dynorphin, NKB and GPR54 mRNAs were also shown to change in the afternoon of proestrus. Dynorphin and NKB were increased along with metastin and GPR54 was decreased. While an increase in ovarian metastin mRNA in the afternoon of proestrus has been already demonstrated by others [3], the significantly increased number of sampling time points in this study demonstrated that the augmentation of metastin is a well-regulated and time-specific change. Metastin mRNA expression was suppressed in pregnancy and treatments those were expected to suppress proestrous LH surge negated the increase of metastin mRNA. So, as the increase of metastin mRNA expression was inhibited in pregnant rats, the effect of prolactin, that secretion is augmented during the pregnancy, was examined [12]. Administration of prolactin nor dopamine agonist CB154 did not affect the metastin expression. Dopamine is a hypothalamic prolactin inhibiting hormone. So, only LH surge of proestrus is thought to be enough stimulation of metastin synthesis. A small dosage of estradiol stimulated the metastin expression in the present study and this may be the result of positive effect of low dosage of estradiol on LH secretion.

Castellano *et al.* have already reported that hCG stimulates metastin expression in GnRH antagonist-treated proestrous rats [3]. Their observation was confirmed in the present study. Metastin mRNA expression was stimulated by hCG in a dose response manner. Dynorphin expression was also stimulated by hCG, while NKB nor GnRH was not affected by hCG treatment. A mechanism for the increase of NKB expression in the afternoon of proestrus would not be related to LH surge. As the increase of NKB expression it was a time specific change, a putative stimulating factor of NKB synthesis would be involved in pre-ovulatory changes. The

expression of metastin and dynorphin mRNAs were also stimulated by hCG in the primary culture of granulosa cell. These data reveal the direct effect of hCG, namely LH, on the expression of metastin and dynorphin. Hence, this is the first report showing a well-regulated acceleration of metastin and dynorphin mRNA expression by the LH surge in the ovary of cycling rats. The increase of metastin and dynorphin mRNA expression during proestrus is likely to be related to ovulation-related changes in the ovary.

As hCG did not stimulate the expression of GnRH mRNA in diestrous rats, metastin seemed not to augment GnRH production in the ovary. The variation of GnRH mRNA expression was not synchronized with metastin in the ovary. Furthermore, biologically active metastin peptide, Kiss-10, from rat and human given into ovarian bursa for six hours on diestrus 2 did not stimulate GnRH mRNA expression. Although metastin was shown to stimulate GnRH release in the hypothalamus [31], the present results suggest that metastin is not responsible for GnRH mRNA expression in the ovary.

By means of LMD, the source of metastin and also other peptides in the ovary was examined. LMD analysis clearly demonstrated that metastin is synthesized solely in follicles. Furthermore, hCG was shown to increase metastin synthesis exclusively in follicles. As substantial expression of metastin mRNA was shown in the primary culture of granulosa cells and it was also stimulated by hCG, the main source of metastin in the ovary is therefore the granulosa cells. Immunohistochemical studies have shown the distribution of metastin in the theca layer and interstitial tissues [3, 15, 45], suggesting that metastin is supplied by granulosa cells. As the vascular system is not developed in the granulosa layer, metastin may diffuse to outer tissues similar to inhibin [24].

It has been demonstrated at the arcuate nucleus of the hypothalamus that metastin, dynorphin and NKB coexist in the same neurons in rat and sheep [6, 39]. The present study clearly demonstrates that the major source of dynorphin and NKB is the interstitial tissues rather

than follicles in the ovary. Although hCG stimulated the expression of dynorphin mRNA in the primary culture of granulosa cells, the quantity of expression should be lower than other ovarian components. Follicles obtained at 20:00 h of proestrus showed quite less dynorphin mRNA than the interstitial tissues. So, a substantial contribution to total ovarian dynorphin synthesis would be attained by the interstitial tissues. There was no difference among corpus luteum, follicles, and interstitial tissues in regard to the expression of GPR54 mRNA. Metastin thus should work on these tissues simultaneously at least at 20:00 h of proestrus.

Dynorphin is an endogenous opioid peptide, and NKB is a member of the tachykinins [26, 41]. Both peptides work as neurotransmitters or neuromodulators in the hypothalamus. Dynorphin and NKB have been shown to co-exist at a part of the metastin neurons in the arcuate nucleus [29]. NKB has been shown to play a crucial role in the control of GnRH neurons, and loss-of-function mutations of NKB or its receptor in humans have been shown to result in hypogonadism [17]. This reproductive failure has been thought to be due to a distortion of GnRH secretion at the hypothalamus, but the present study also suggests that the ovary could be another site of function for NKB. Even though the expression of these neuropeptides in the ovary has been reported previously [9, 39], this is the first report to reveal significant fluctuations during the estrous cycle with a dramatic increase after the LH surge. Time-courses of this phenomenon suggest a relationship between the increase of the synthesis of these peptides and ovulation. The functions of NKB and dynorphin in the ovary require further study.

Hemilateral administration of metastin antagonist p234 from proestrous in the morning for 3 days induced morphological changes in the corpus luteum. Marginal distortion was sometimes seen between the corpus luteum and interstitial tissues. This change was not seen in the contra-lateral ovary given the same amount of saline. However, the change was not induced consistently. This is probably due to the method for the administration of p234. This experiment used an osmotic minipump with a polyethylene cannula. The cannula was inserted into the

ovarian bursa. The position of the tip in the bursa could therefore affect the efficacy of p234 administration and cause inconsistent results. It appeared that the outer layer of the corpus luteum was not well formed in ovaries treated with p234.

KISS-1 was first discovered as an anti-metastatic gene, and the gene product metastin was found to suppress the metastasis of melanoma and mammary tumor cells [47, 50]. While the mechanism by which metastin suppresses metastasis is not known, the function may be related to the present histological findings. The findings suggest that metastin is involved in tissue remodeling during the luteinization after ovulation. It is not known that the physiological relevance of this observation, but it is hypothesized that metastin is physiologically involved in the formation of the corpus luteum. Follicular development, follicular rupture, and corpus luteum formation are accompanied by extensive tissue remodeling. So, metastin would affect factor(s) involved in these processes.

P234 was also shown to inhibit progesterone production stimulated by hCG in a dose-response manner. Primary cultures of granulosa cells from PMSG-primed immature rats were examined. Given that hCG is thought to mimic the LH surge of proestrus at least in part, this suggests that metastin would work during the luteinization of granulosa cells. During culture with hCG, the granulosa cells produced progesterone in a dose-response manner, and this effect was suppressed in a dose-response manner by p234. Metastin synthesized after the LH surge would therefore precede functional and/or morphological luteinization.

The present data demonstrate that 1) ovarian metastin is synthesized in the granulosa cells under the control of LH. The synthesis was increased dramatically by the preovulatory LH surge, 2) Dynorphin and NKB mRNA expressions were augmented simultaneously with metastin. These closely related peptides, which are found in the arcuate nucleus, reveal a tight relationship also in the ovary, but the source of metastin is the granulosa cells, while NKB and dynorphin are mainly synthesized in the interstitial tissues. Cell species responsible for NKB

and dynorphin synthesis is remained to be clarified. Present results suggest that metastin acts on the differentiation of granulosa cells, namely on luteinization. 3) metastin is not to be responsible for the regulation of GnRH mRNA expression in the ovary.

Metastin, dynorphin and NKB have been known as a neurotransmitter or a neuromodulator in the central nervous system [41]. Present study clearly shows that they are also expressed in the ovary and the synthesis of these substances is well regulated along with physiological events. Surprisingly, the granulosa cells express both metastin and dynorphin under the same regulation by LH, while NKB is synthesized in the interstitial tissues by unknown but not LH. On proestrous day, ovarian production of estrogen and progesterone are increased sequentially. Changes in steroid hormones elicit the surge secretion of LH, FSH and prolactin. Along with these changes, other factors including eicosanoids, cytokines and growth factors would change also. So, NKB could be affected by one or more of these factors in the ovary. Novel intra-ovarian mechanisms related to follicular development, ovulation and luteinization are demonstrated.

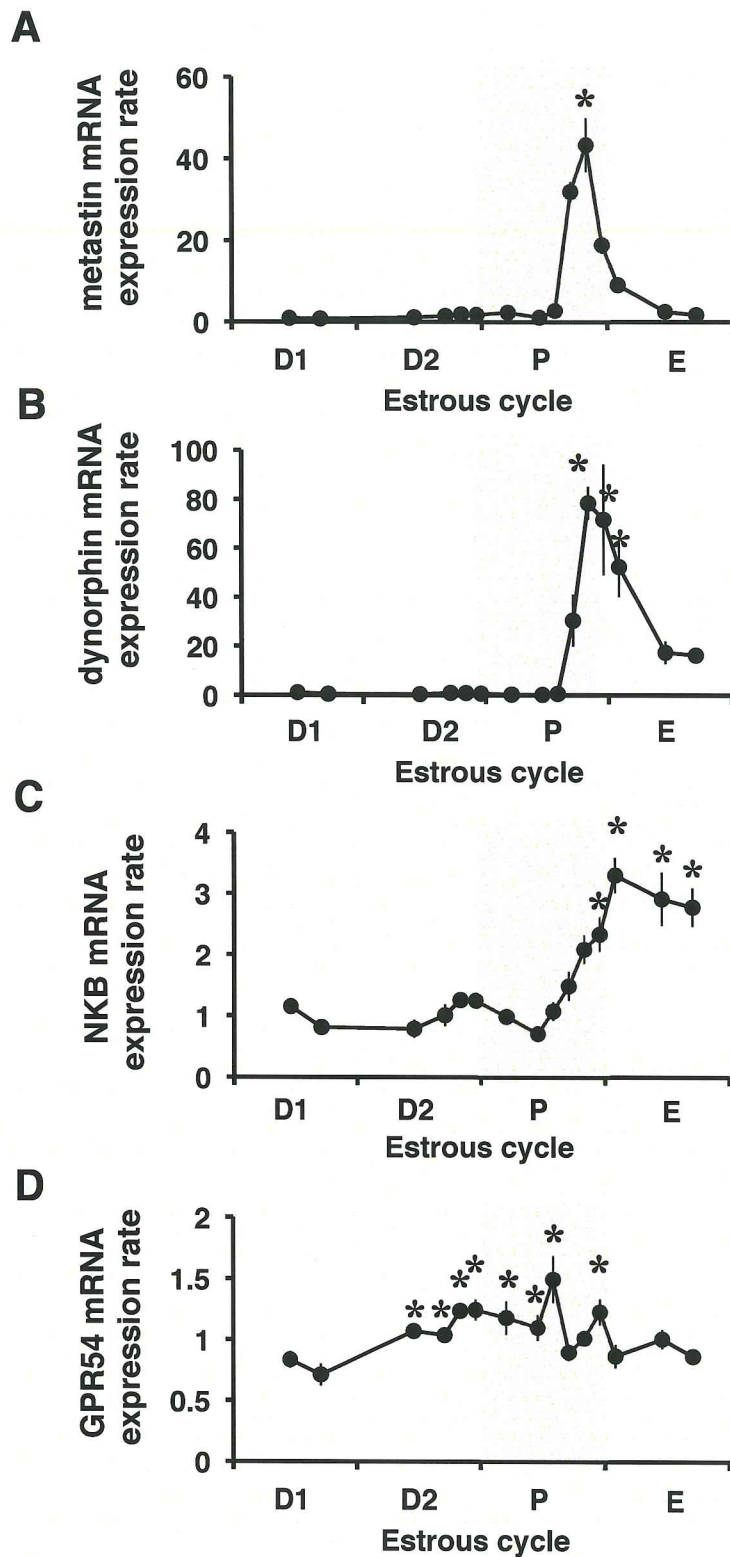


Fig. 3-1. Changes in the expression rate of ovarian metastin, dynorphin, NKB and GPR54 mRNA during estrous cycle of rats. A; metastin, B; dynorphin, C; NKB and D; GPR54 mRNA. Sampling points: Diestrus 1 (D1): 11:00, 17:00 h, Diestrus 2 (D2): 11:00, 17:00, 20:00, 23:00 h, Proestrus (P): 05:00, 11:00, 14:00, 17:00, 20:00, 23:00 h and Estrus (E): 02:00, 11:00, 17:00 h. Those samples were made from 5 rats each. Asterisks reveal the peak value and values not significantly different from the peak value (peak values are significantly different from values without asterisk, $p < 0.05$).

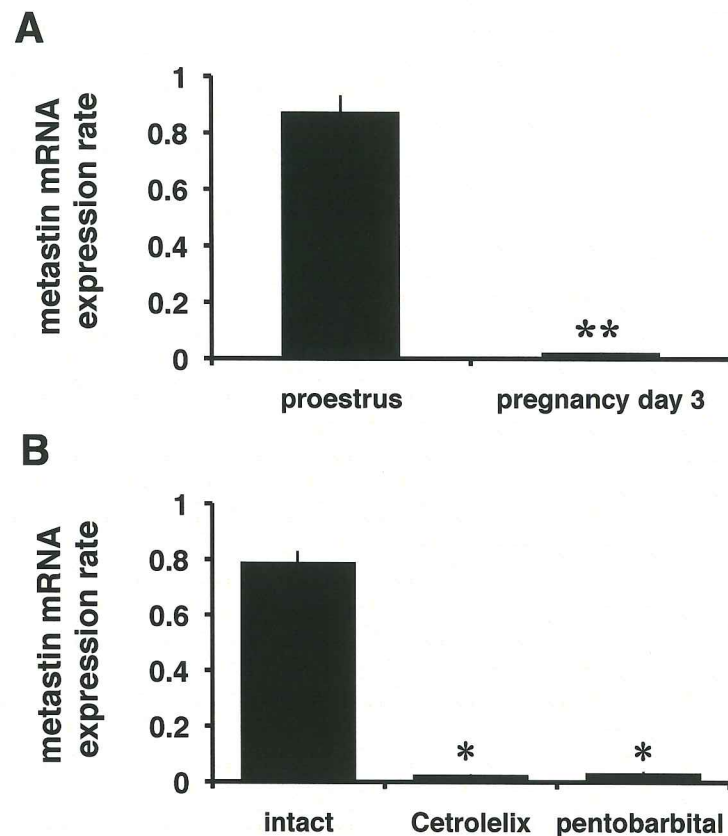


Fig. 3-2. Change in the expression rate of metastin mRNA of ovary. A; Metastin mRNA expression in pregnant rats. Ovarian samples were harvested at 20:00 h on day 3 of pregnancy. Those samples were made from 5 rats each. Data are means \pm SEM. P values were obtained with Student's *t* test, ** $P < 0.001$. B; Effects of GnRH antagonist or pentobarbital on metastin expression in the ovary. GnRH antagonist (Cetorelix, 1 μ g/rat 100 g) or pentobarbital sodium (4.0 μ g/rat 100 g) or vehicle (0.06 ml saline/rat 100 g) was given at 12:00 h of proestrus. Ovarian samples were harvested at 20:00 h of the same day. Those samples were made from 5 rats each. P values were obtained with Tukey's test, * $P < 0.05$ vs intact.

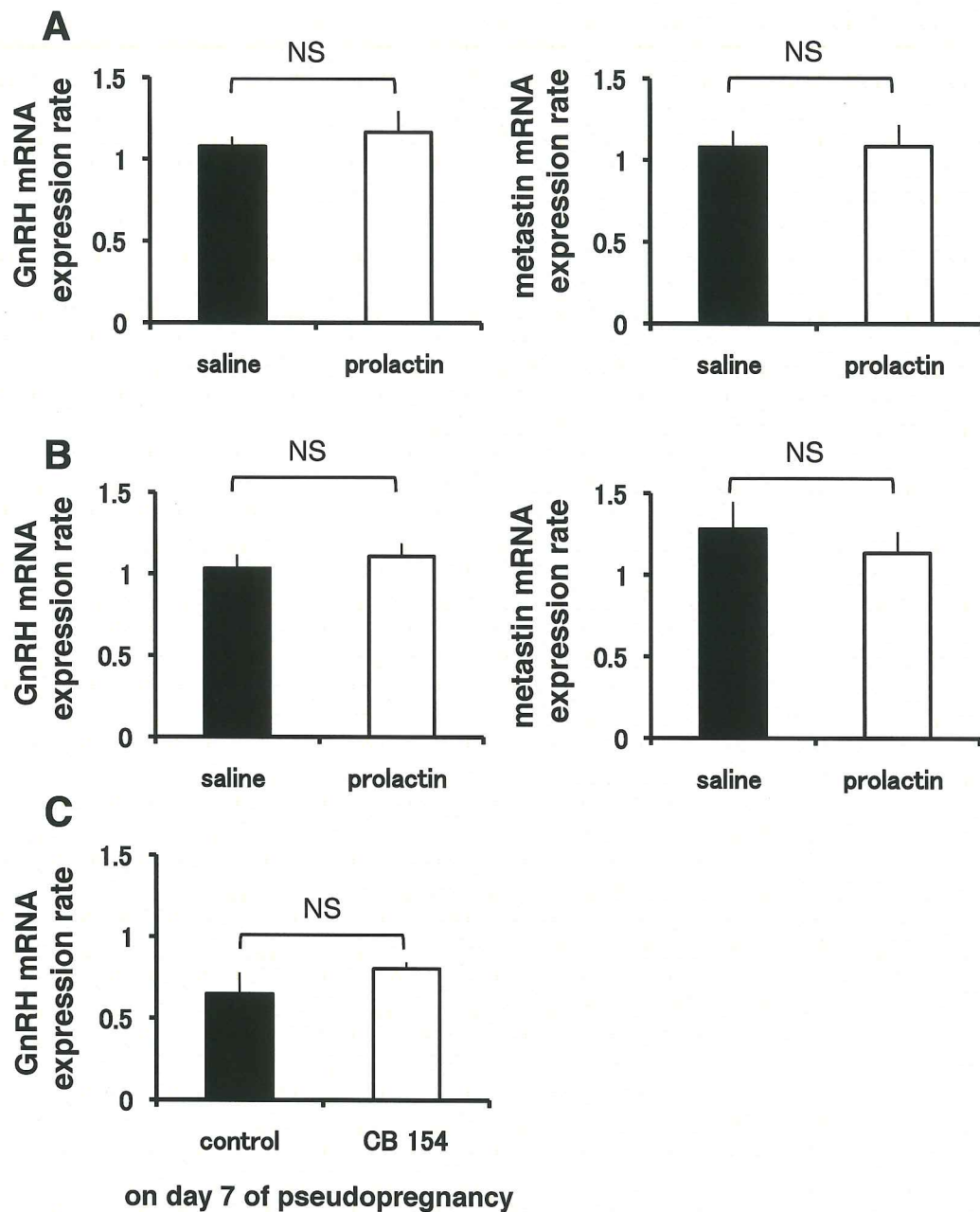


Fig. 3-3. The effect of prolactin or dopamine agonist on ovarian expression rate of GnRH and metastin. A; Ovine prolactin (10 IU) or saline was given at 10:00 h of proestrus and ovaries were harvested at 20:00 h of the same day. B; Ovine prolactin (10 IU) or saline was given at 17:00 h of proestrus and ovaries were harvested at 20:00 h of the same day. C; CB-154 (300 μ g) or tartaric acid (vehicle) was given at 10:00 h of day 5 of pseudopregnancy. Ovary was harvested at 10:00 h on pseudopregnant day 7. Those samples were made from 4-5 rats each. NS: non significant.

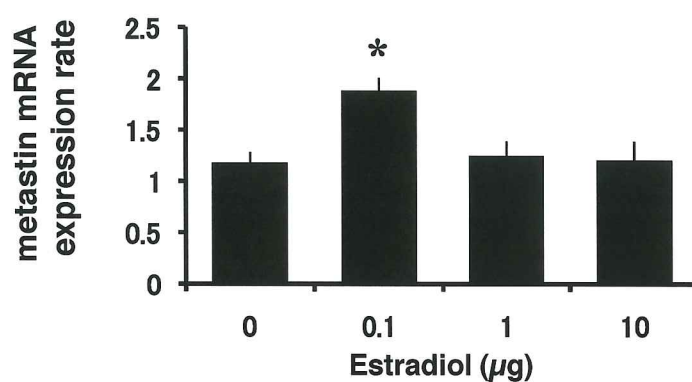


Fig. 3-4. The effect of estradiol on metastin expression in the ovary. Estradiol (0.1, 1, 10 µg/0.1 ml) was given at 14:00 h of diestrus 2. Estradiol was dissolved in Sesame Oil. Ovaries were harvested at 17:00 h of the same day. Those samples were made from 5 rats each. P values were obtained with Tukey's test, *P<0.05 vs 0.

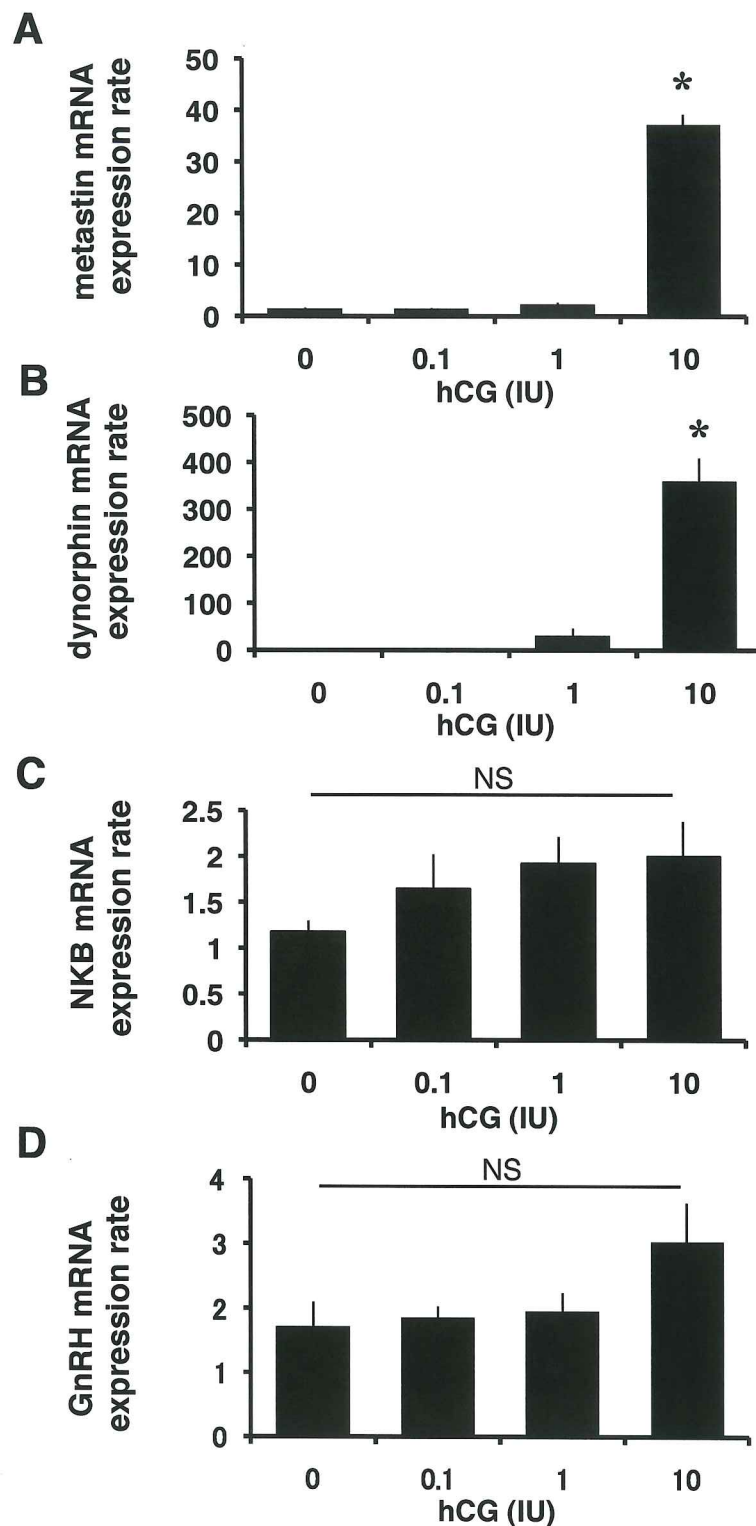


Fig. 3-5. The effect of hCG on metastin, dynorphin, NKB and GnRH expression. The expression rate of A; metastin, B; dynorphin, C; NKB and D; GnRH mRNA were measured. hCG (0.1, 1, 10 IU/rat) was given at 14:00 h of diestrus 2. hCG was dissolved in saline. Ovarian samples were harvested at three hours after hCG or saline injection. Those samples were made from 5 rats each. Asterisk reveals the significant difference from other groups ($P < 0.05$). NS: non significant.

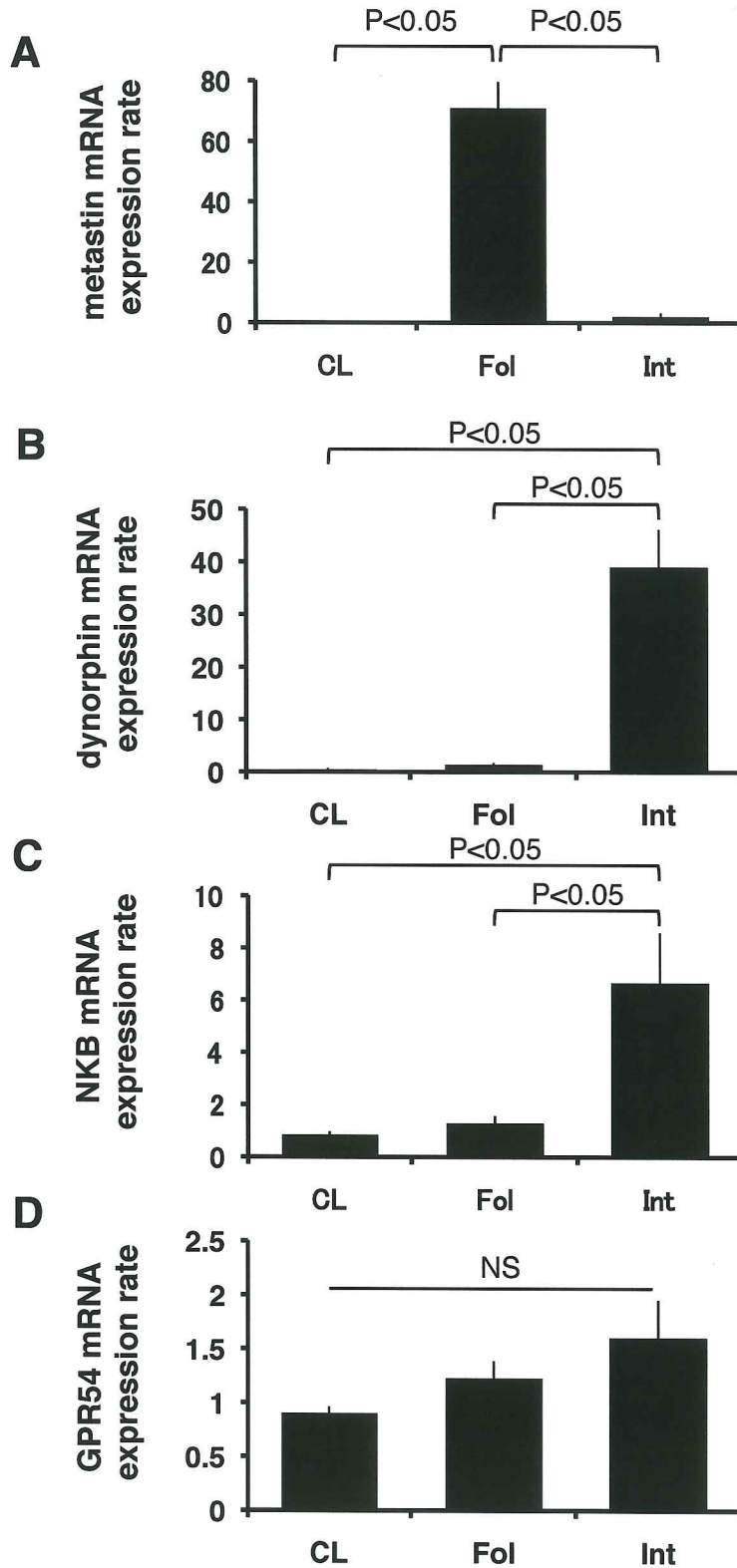


Fig. 3-6. Ovarian components expressing metastin, dynorphin, NKB and GPR54 mRNA. Ovaries were harvested at 20:00 h of proestrus and subjected to LMD to collect sections of corpus luteum (CL), follicle (Fol) and interstitial tissues (Int). RNA was extracted from each tissues and expression rate of A; metastin, B; dynorphin, C; NKB and D; GPR54 were measured by real-time RT-PCR. Those samples were made from 5-6 rats each. P values were obtained with Tukey's test, $P < 0.05$. NS: non significant.

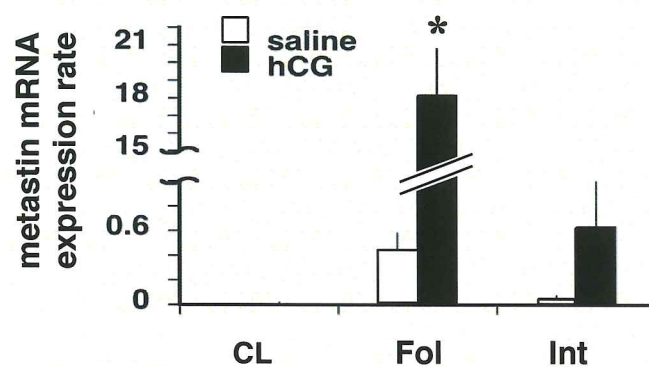


Fig. 3-7. The effect of hCG on metastin mRNA expression in ovarian components. Ovaries treated with hCG 10 IU/rat or vehicle (saline) at 14:00 h of diestrus 2. Ovarian samples were harvested at three hours after hCG or saline injection. Three compartments were subjected to LMD to collect sections of corpus luteum (CL), follicle (Fol) and interstitial tissues (Int). RNA was extracted from each tissues and expression rate of metastin was measured by real-time RT-PCR. Those samples were made from 4 rats each. Data are mean \pm SEM. P values were obtained with Student's *t* test, * $P < 0.05$ vs saline.

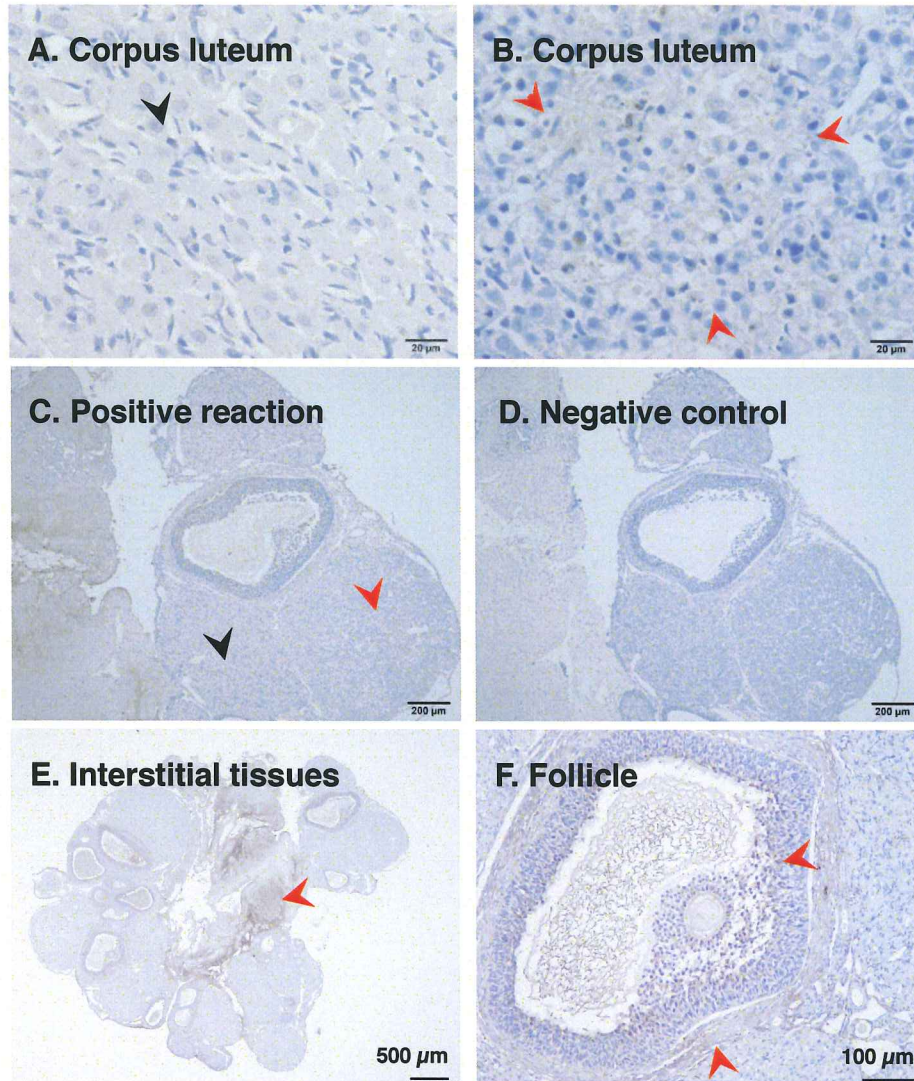


Fig. 3-8. Immunohistochemistry for metastin in the ovary. Ovaries were harvested at 20:00 h of proestrus. Ovarian tissues were subjected to the immunohistochemistry for metastin. Anti-kisspeptin10 (kp10) polyclonal antibody was used. Arrow heads indicate metastin positive cells in the ovarian tissues. Black arrow heads indicate corpus luteum without positive reaction.

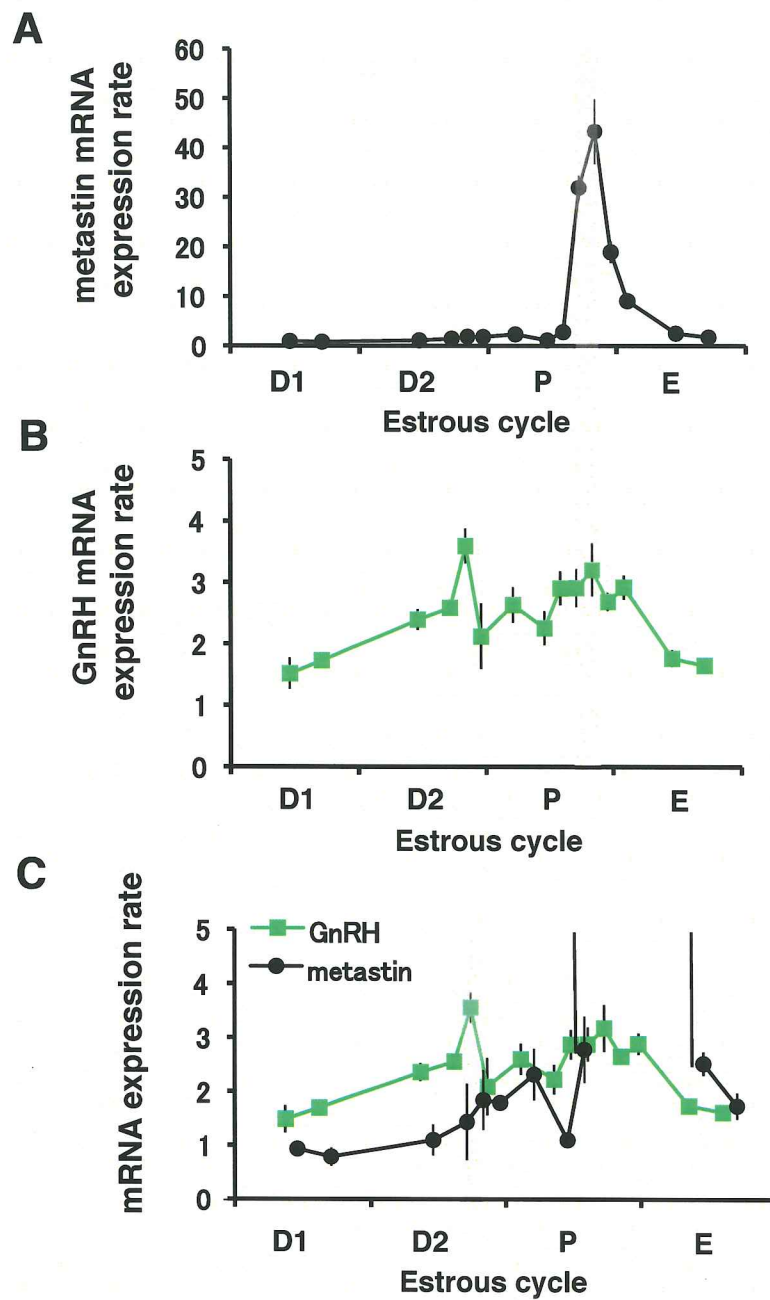


Fig. 3-9. Comparison of the expression rate of metastatin and GnRH mRNA during estrous cycle of rats. A; metastatin, B; GnRH and C; metastatin combine GnRH mRNA. Sampling points: Diestrus 1 (D1): 11:00, 17:00 h, Diestrus 2 (D2): 11:00, 17:00, 20:00, 23:00 h, Proestrus (P): 05:00, 11:00, 14:00, 17:00, 20:00, 23:00 h and Estrus (E): 02:00, 11:00, 17:00 h. Each experimental group consisted of 5 rats.

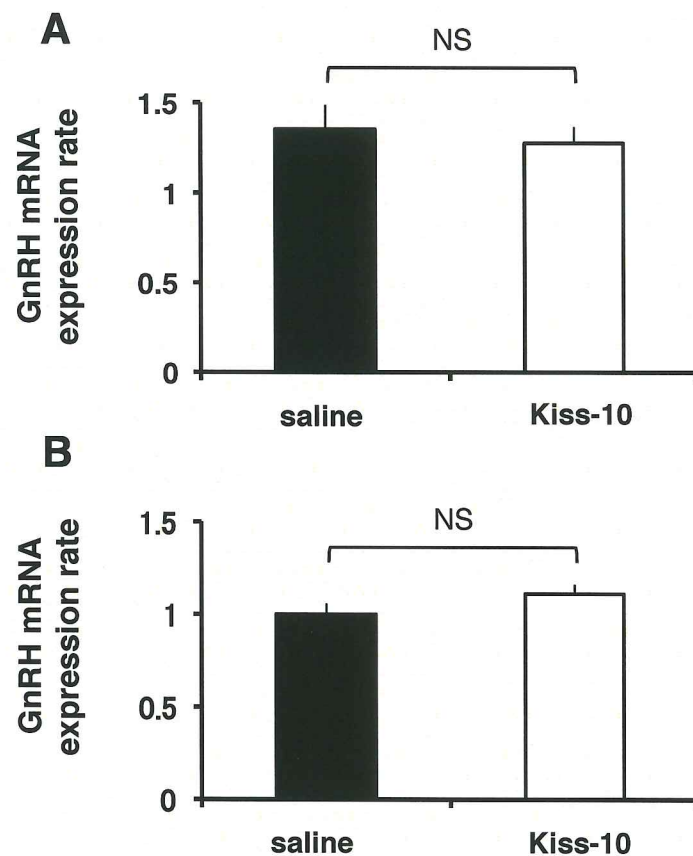


Fig. 3-10. Ovarian GnRH mRNA expression after local treatment of Kiss-10. A; Kisspeptin-10 (Rat)/Metastin (Rat, 43-52) ($10 \mu\text{g}/50 \mu\text{l}$,) or B; Kisspeptin-10 (Human)/Metastin (Human, 45-54) ($6.45 \mu\text{g}/50 \mu\text{l}$) was given into the hemi-lateral ovarian bursa at 10:00 h of diestrus 2. Saline was given into the contra-lateral ovary for a control. Ovaries of both sides were harvested at six hours after. Those samples were prepared from 5 rats each. NS: non significant.

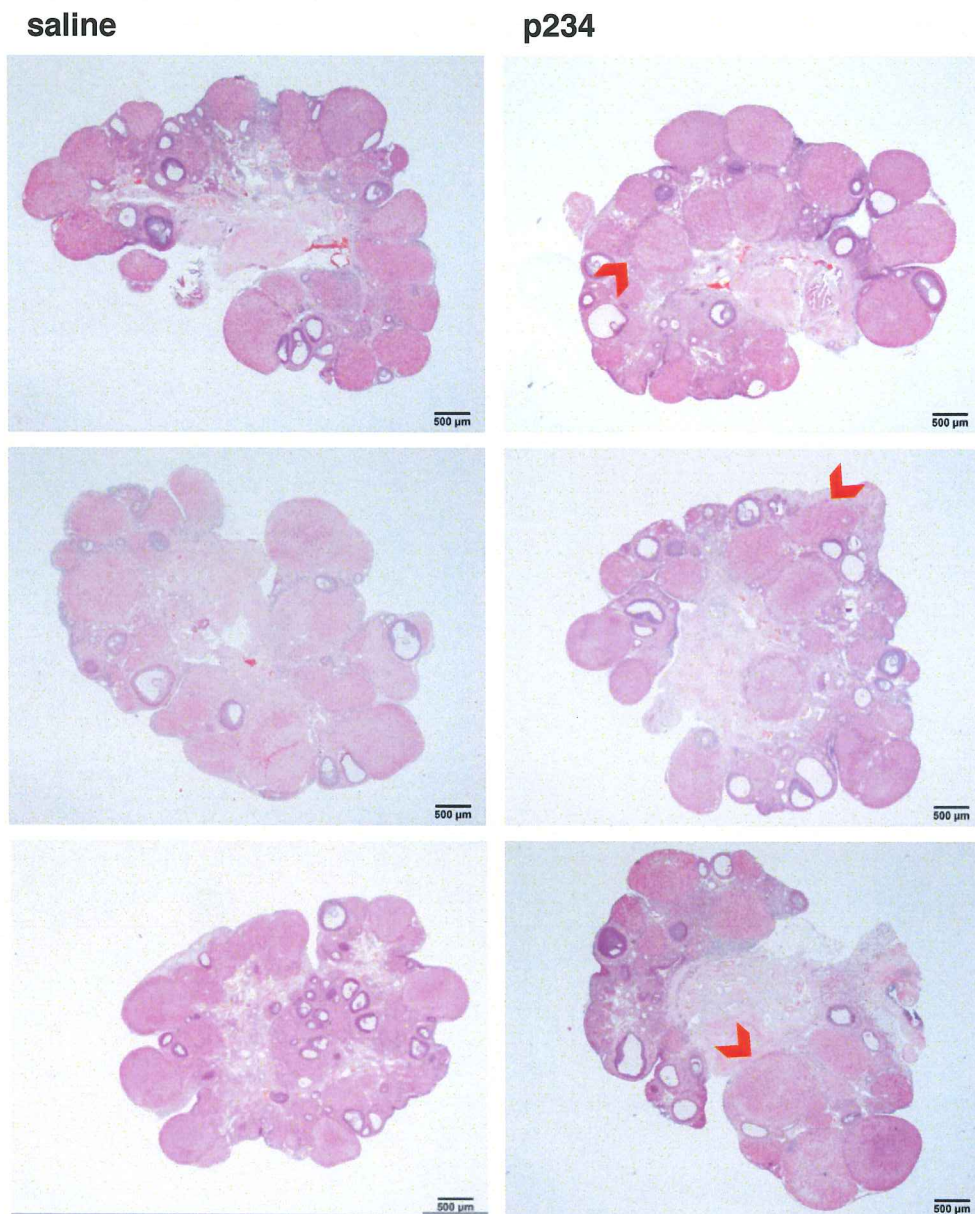


Fig. 3-11. Hematoxylin Eosin staining of ovarian tissues after local administration of metastin antagonist p234. Metastin antagonist p234 (1 nmol/24 μ l) or saline was given by means of osmotic minipumps to each ovaries of a rat from 10:00 h of proestrus for three days. n=3 per each group. Arrow heads indicate p234 induced a histological change.

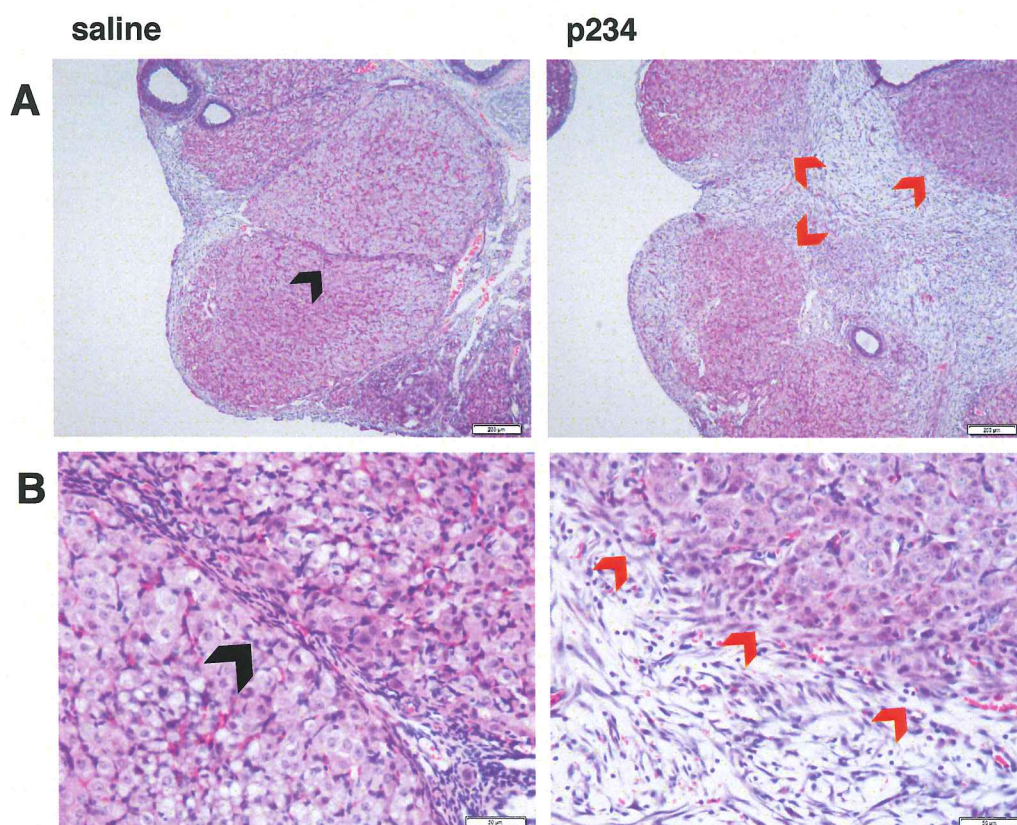


Fig. 3-12. Histology of ovarian tissues after local administration of metastin antagonist p234. Metastin antagonist p234 (1 nmol/24 μ l) or saline was given by means of osmotic minipumps to each ovaries of a rat from 10:00 h of proestrus for three days. Ovaries were collected and subjected to histological examination. Sections were stained with Hematoxylin and Eosin. Black arrow heads indicate well formed outer layer of the corpus luteum in the control ovary and red arrow heads indicate a distortion at borders between the corpus luteum and surrounding tissues by p234 treatment. A; Scale bars are 200 μ m in lower and B; 50 μ m in higher magnification.

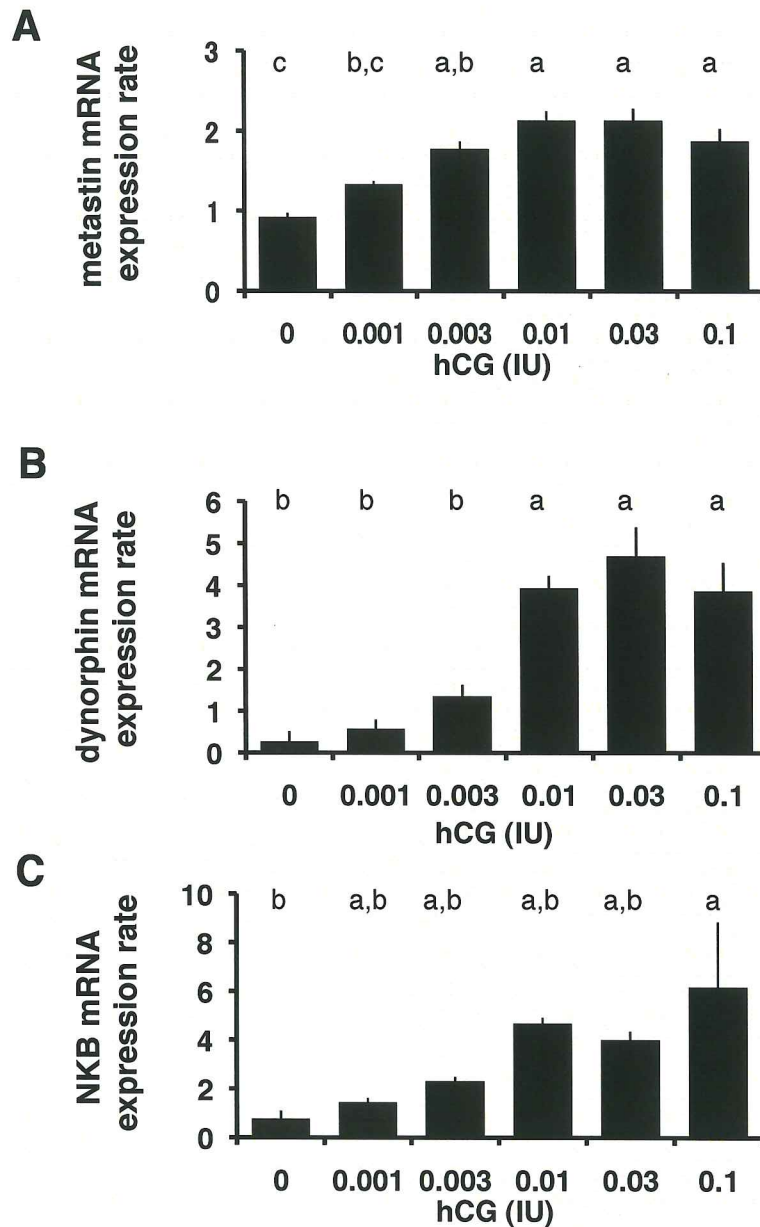


Fig. 3-13. Response to hCG of the expression rates of metastatin, dynorphin and NKB in the primary culture of granulosa cells. PMSG (15 IU) was given on day 25 and granulosa cells were harvested by mechanical dissociation from follicles on day 27. Various doses of hCG was given for three hours and RNA was extracted from cells. Reverse-transcribed cDNA was subjected to each real-time RT-PCR. The expression rate of A; metastatin, B; dynorphin and C; NKB mRNA were measured. Data are presented as mean \pm SEM. Values with different letters are significantly different each other, $P < 0.05$.

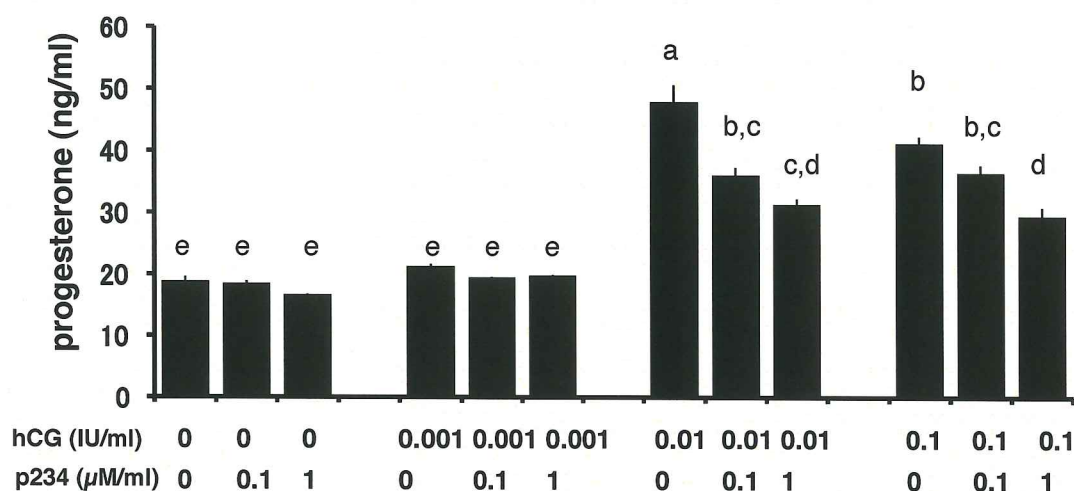


Fig. 3-14. The effect of metastin antagonist p234 on hCG augmented progesterone production in the primary culture of granulosa cells. Primary culture of granulosa cells were established from PMSG treated immature rats. Cells were incubated with various concentration of hCG in combination with p234 for three hours and conditioned medium were assayed for progesterone production. Data are presented as mean \pm SEM. Values with different letters are significantly different, $P < 0.05$.

Chapter 4 Summary

Female reproduction is based on cyclic ovulation. It is performed by the orchestration of factors produced in the hypothalamo-hypophyseal-ovary axis. Metastin or kisspeptin is the product of KISS1 gene and the ligand of G protein coupled receptor 54 (GPR54). Metastin stimulates GnRH release in the hypothalamus. Hypothalamus dictates the gonadotropin secretion by means of GnRH. In the arcuate nucleus, metastin, neurokinin B (NKB), dynorphin are coexpressed in the same neuron, namely KNDY neurons. This thesis investigated GnRH, as a local regulating factor in the ovary, for its expression rate, producing cells, and relation to mast cells, metastin, and related peptides in the ovary. The results of this thesis are summarized in Figure 4-1.

The present study demonstrated that 1) the variation of GnRH mRNA expression rate through the estrous cycle of rats. There were two peaks in the variation of GnRH mRNA expression rate. One was at 20:00 h of diestrous 2 and another was at 20:00 h of proestrus. These changes were demonstrated to be specific to the estrous cycle. The source of GnRH in the ovary was examined by means of Laser Microdissection (LMD). Corpora lutea, follicles and interstitial tissues were collected with LMD at 20:00 h of proestrus. The expression rate of GnRH mRNA was not different among these compartments. Furthermore, GnRH was demonstrated by immunohistochemistry to distribute to almost all compartments of ovary. In the granulosa layer and corpus luteum, GnRH positive cells were restricted to only a portion of the tissues. Interstitial tissues were stained diffusely, but mast cells were very strongly positive.

Mast cells demonstrated with toluidine blue metachromasy distributed mainly in the interstitial tissues of the ovary. When mast cells were observed in different planes of the ovary at one third depth each, peaks of the number were shifted a little. It was thought that this shift would reflect the movement of mast cells from outside into the ovary through hilum. It was

demonstrated that the number of mast cells in the ovary varied during the estrous cycle with two distinct peaks in the evening of diestrus 2 and late afternoon of proestrus. This variation matches with the changes in GnRH mRNA content. Mast cells were shown to contain GnRH and toluidine blue stained mast cells were significantly increased but GnRH immuno-positive cells were not in the afternoon of diestrus 2, suggesting a release of GnRH from mast cells.

It was demonstrated that the increase of mast cells was suppressed during pseudopregnancy, pregnancy and lactation. Ovine prolactin (10 IU/0.2 ml ip) was given at 10:00 h of diestrus 2 or proestrus decreased the number of ovarian mast cell at 20:00 h of diestrus 2 or 17:00 h of proestrus, respectively. In contrast, dopamine agonist, CB-154 that would decrease prolactin secretion, increased ovarian mast cells during pseudopregnancy. So, prolactin is suggested to inhibit the migration of mast cells into the ovary.

C57BL/6- W^{sh}/W^{sh} is a mutant allele at the mouse *W* (*c-kit*) locus. Mice carrying this allele lack mast cells. The estrous cycle length was not different between C57BL/6J mice and C57BL/6- W^{sh}/W^{sh} mice. Ovary weight was smaller in C57BL/6- W^{sh}/W^{sh} mice. Although this difference seems to reflect the absence of mast cells and GnRH, ovarian GnRH mRNA expression was not different between the two strains, indicating that mast cells do not contribute ovarian synthesis of GnRH at least at basal levels of expression.

GnRH and annexin A5 mRNA were confirmed in peritoneal mast cells and clonal cell line of mast cell, P 815. With the primary culture of peritoneal mast cells, GnRH mRNA was shown to be stimulated by GnRH agonist. As GnRH agonist was given into the hemi-lateral ovarian bursa. It increased number of ovarian mast cells. GnRH would be a chemo-attractant for mast cell migration to the ovary. These data suggest that GnRH stimulates both the GnRH production of mast cells and the migration of mast cells into the ovary. It seems that the fluctuation of ovarian GnRH expression takes place in ovarian tissues and GnRH would attract

mast cells into the ovary. The mast cell production of GnRH would contribute to the peaks of GnRH expression of the ovary.

The expression of hypothalamic factors those were already demonstrated to be involved in GnRH neuronal activity was examined in the ovary. The present study demonstrate that 2) changes in metastin and dynorphin mRNA expression in the ovary during estrous cycle. Metastitin and dynorphin mRNAs were dramatically increased at 20:00 h of proestrus. NKB mRNA also increased, but its peak was delayed until 2:00 h of estrus. GPR54 mRNA increased from diestrous 2 and showed a peak at 14:00 h of proestrus then it declined inversely with metastin.

Metastin was demonstrated to be synthesized in the granulosa cells. The expression of metastin and dynorphin was also shown to be directly regulated by LH surge, despite that major site of dynorphin synthesis was in the interstitial tissues. NKB was also revealed to be synthesized in the afternoon of proestrus, but LH was shown not to be involved in this process.

Metastin stimulates GnRH release in the hypothalamus. However the variation of GnRH mRNA expression rate was not synchronized with that of metastin in the ovary. Furthermore, when metastin 45-54, kiss-10 (Human) or metastin 43-52, kiss-10 (Rat) was given into ovarian bursa for 6 hours on diestrus 2, it did not stimulate GnRH mRNA expression in the ovary. So, ovarian metastin would not be a factor regulating GnRH in the ovary.

When an inhibitor of metastin action, p234, was given into the hemi-lateral ovarian bursa with an osmotic-minipump for three days from proestrus, distortion of the corpus luteum and surrounding tissue borders was sometimes seen. On the other hand, p234 suppressed the stimulating effect of hCG on progesterone production in the primary culture of granulosa cells prepared from pregnant mare serum gonadotropin-pretreated immature rats. These data demonstrate the effects of ovarian metastin on granulosa cells. While the physiological meaning of the results is not known yet, metastin would play a role other than stimulating GnRH

secretion in the ovary and it is thought that it may be related to its suppressing effect on metastasis.

The results from this thesis demonstrate that the specific variation of ovarian GnRH expression during the estrous cycle, the first peak of GnRH mRNA expression would be responsible for luteal regression during the estrous cycle. The second peak on proestrus suggests a relationship with follicular atresia. Although the regulating mechanism for phasic GnRH synthesis is not known, the present study shows mast cell migration into the ovary by chemotaxis to GnRH. Mast cells are thought to have a specific role on the regression of the corpus luteum and follicles. It is suggested that mast cells are also responsible, at least partly, for the phasic increase of GnRH in the ovary. The present study clearly demonstrates that metastin is synthesized in granulosa cells under the control of the proestrous LH surge in rats. Although NKB and dynorphin are synthesized in the granulosa cells to a lesser extent, the massive synthesis of NKB and dynorphin occurs in the interstitial tissues. It is suggested that metastin, dynorphin and NKB are involved in the ovulation and luteinization processes. Metastin is not responsible for GnRH mRNA expression in the ovary. The results reveal that metastin, NKB, dynorphin and GnRH shown in the hypothalamus form a different network in the ovary.

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