



Gonadotropin-releasing hormone stimulation of annexin A5 expression in the thymus of male rats

Mitsumori KAWAMINAMI^{1,2)*}, Ryota TERASHIMA¹⁾, Takuya MURATA²⁾,
Shuichi CHIBA²⁾ and Shiro KURUSU¹⁾

¹⁾Laboratory of Veterinary Physiology, School of Veterinary Medicine, Kitasato University, Aomori, Japan

²⁾Laboratory of Veterinary Physiology, School of Veterinary Medicine, Okayama University of Science, Ehime, Japan

ABSTRACT. As gonadotropin-releasing hormone (GnRH) is expressed in the thymus, its direct action on thymic cells, including thymic involution, has been suggested. Annexin A5 (ANXA5), a biomarker of GnRH, was used to determine whether GnRH affects the thymus of male rats. Immunohistochemistry showed positive reactions for ANXA5 in large medullary epithelial cells at 30 days of age, and the expression continued until 180 days of age. Organ culture of thymus pieces was performed to examine the direct action of a GnRH agonist (GnRH_a) on the expression of *Anxa5* and *Gnrh* mRNA. Thymus tissues obtained from male rats (40–60 days old) were cut into small pieces (2–3 mm³) and incubated for 3 hr with the GnRH_a. The expression levels of *Anxa5* and *Gnrh* mRNA were augmented by the GnRH_a. Immunohistochemistry of these tissue fragments showed that ANXA5 expression was enhanced, especially in medullary epithelial cells. These results revealed that GnRH synthesis in the thymus could affect thymic epithelial cells after puberty.

KEYWORDS: annexin A5 (ANXA5), gonadotropin-releasing hormone (GnRH), thymus

J. Vet. Med. Sci.

84(5): 638–643, 2022

doi: 10.1292/jvms.22-0052

Received: 1 February 2022

Accepted: 2 March 2022

Advanced Epub:

29 March 2022

Gonadotropin-releasing hormone (GnRH) is a decapeptide neurohormone primarily synthesized in GnRH neurons in the preoptic area and released at the median eminence of the hypothalamus [11, 17]. GnRH is transported to the anterior pituitary gland by the pituitary portal vessel and affects gonadotropin secretion by gonadotropes. Although accumulating evidence on GnRH suggests the significance of GnRH in reproduction, the non-reproductive functions of GnRH expressed in various peripheral tissues are not known [6, 7, 14, 15, 21, 24, 25].

We previously found that the annexin family proteins annexin A5 and A1 (ANXA5 and ANXA1) are expressed in pituitary gonadotropes, and their expression could be augmented by GnRH in rats [8, 10, 13]. Furthermore, ANXA5 augments gonadotropin secretion by enhancing the stimulatory action of GnRH on gonadotropin secretion [10]. These findings suggest that ANXA5 mediates GnRH activity in gonadotropes. In addition, we identified a relationship between GnRH and ANXA5 in the corpus luteum, Leydig cells, and mammary epithelial cells of rats [7, 15, 25]. Locally synthesized GnRH enhances ANXA5 expression in these tissues.

There have been reports of GnRH action on the thymus of mice [22, 23]. Specifically, GnRH modulates cell numbers and cytokine synthesis in the thymus [22]. Although these reports suggest the physiological role of GnRH in the thymus, it is still unclear whether thymic GnRH has physiological effects on thymic cells even though it is synthesized in the thymus [2, 6]. The thymus is the site of T-cell maturation and involution, and tissue remodeling occurs after puberty [4, 16]. In the present study, we examined the expression of ANXA5 in the rat thymus and the effect of GnRH on thymic cells after puberty.

MATERIALS AND METHODS

Experimental animals

Male Wistar-Imamichi rats (0–180 days old) bred in our laboratory were used in this study. The rats were maintained in light- and dark-controlled rooms (room temperature: 22 ± 3°C, 14L:10D, lights on at 5:00 AM). Laboratory chow (CE-2; CLEA Japan, Tokyo, Japan) and water were provided *ad libitum*. All animal experiments in this study were conducted in accordance with the Guidelines for Animal Experiments and the Animal Management Manual of the School of Veterinary Medicine, Kitasato University.

*Correspondence to: Kawaminami, M.: m-kawaminami@vet.ous.ac.jp, Laboratory of Veterinary Physiology, School of Veterinary Medicine, Okayama University of Science, Ikoinooka 1-3, Imabari, Ehime 794-8555, Japan

©2022 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Histological observation

Five male rats aged 0, 30, 60, 90, and 180 days were euthanized by cervical dislocation under anesthesia. The body and thymus weights were measured. The thymus was quickly removed and immediately immersed in 4% paraformaldehyde. Thymus samples were shaken overnight at 4°C and rinsed with phosphate-buffered saline overnight. Fixed tissues were dehydrated by immersion in a series of ethanol and xylene solutions and embedded in paraffin. Sections of 4 µm thickness were prepared. Anti-annexin A5 (MS-2, ×5,000) was used as the primary antibody (PRID: AB_2827744; https://antibodyregistry.org/AB_2827744). We obtained the antibody from rabbit serum and used it for immunohistochemistry and western blotting [15, 19]. ImPRESS REAGENT (anti-rabbit IgG) (Vector Laboratories, Funakoshi, Tokyo, Japan) was used for signal detection. The diaminobenzidine substrate solution (Roche, Basel, Switzerland) was added dropwise to the tissue sections. The reaction was carried out at room temperature, and the degree of coloration was observed under a microscope. The color reaction was stopped with distilled water, followed by immersion in hematoxylin solution for approximately 1 min and rinsing with tap water. The specimens were dehydrated and mounted with a sealant (M-X) (Matsunami Glass, Osaka, Japan).

Quantitative reverse transcription PCR (qRT-PCR)

Thymus samples were immersed in 0.8 ml of TRIzol (Invitrogen, Carlsbad, CA, USA), homogenized with an Eppendorf plastic pestle, and stored at -80°C until RNA extraction. Total RNA for qRT-PCR was extracted according to the manufacturer's protocol. RNA samples were reverse-transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).

The expression levels of *Anxa5* and *Gnrh* mRNA were measured by qRT-PCR. rRNA was measured as an internal standard using TaqMan Ribosomal RNA Control Reagents (VIC-TM Prob, Applied Biosystems). To obtain the RNA calibration curve, *Anxa5* and *Gnrh* cDNA samples were used. *Gnrh* mRNA was measured with SYBR Green (Applied Biosystems). cDNA samples were prepared at 10 ng/µl for *Anxa5* and *Gnrh* mRNA quantification and 20 pg/µl for rRNA quantification. All samples were run in duplicate.

For *Anxa5* mRNA quantification, primers specific to the *Anxa5* sequence (sense primer 5'-AAGTTCTTCGGAAGGCCATG-3', antisense primer 5'-CTCAGCAATCTGCTGGCG-3') and TaqMan probe (5'-FAM CGACGAGGACAGCATCCTGAACCTGTT TAMRA-3') were used. For rRNA quantification, the primers and probes included in TaqMan Ribosomal RNA Control Reagents (Applied Biosystems) were added to TaqMan Universal PCR Master Mix Reagent. Primers specific to the *Gnrh* sequence (sense primer 5'-GGCAAGGAGGAGGATCAAA-3' and antisense primer 5'-CCAGTGCATTCATCTTCTTCTG-3') were added to SYBR Green Master Mix Reagent.

As an internal standard, the rRNA expression level of each sample was measured and standardized by dividing with the measured values of *Anxa5* and *Gnrh* mRNA. The experimental results are expressed as mRNA levels relative to the measured values of the control group.

Organ culture of thymus tissue

Two to three male rats (40–60 days old) were used for tissue collection. The thymus was aseptically removed from decapitated rats and immediately immersed in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen). Then, the thymus was cut into blocks of approximately 2–3 mm³. A few pieces were randomly selected, transferred to 35 mm dishes with DMEM supplemented with FCS alone or 10⁻⁹ M GnRH_a (Des-Gly10 [Pro9]-GnRH ethylamide; Intervet K.K., Tokyo, Japan), and cultured for 3 hr at 37°C with 5% CO₂ and 40% O₂. The tissue pieces were subjected to RNA extraction and immunohistochemistry.

Statistical analysis

Multiple comparisons of the means were performed using the Bonferroni test, and the statistical significance was set at $P < 0.05$. Student's *t*-test was used for the comparison of two groups, and the significance level was $P < 0.05$.

RESULTS

Immunohistochemistry of ANXA5 in rat thymus tissue

The expression of ANXA5 in the thymus of rats aged 0–180 days was examined by immunohistochemistry using an anti-ANXA5 antibody (Fig. 1A and 1B). Positive reactions were noted in the cortex on day 0. From 30 days of age, both the medulla (red arrows) and cortex (black arrows) showed positive reactions. Quantitative analysis was not performed; nevertheless, the expression seemed to increase with age, especially in the medulla. A high magnification of thymic medullary tissues from 30 to 180 days of age showed positive reactions in large medullary epithelial cells and structures that may be Hassal corpuscles (Fig. 1B, blue arrowheads). Similarly, the staining of positive cells appeared to increase with age.

Changes in body and thymus weights with age

The body and thymus weights of male rats were measured daily. The body weight increased over time during the observation period until 180 days of age (Fig. 2A). The thymus weight peaked at 60 days of age and decreased at 90 days of age (Fig. 2B). Both the body and thymus weights were considerably low on day 0.

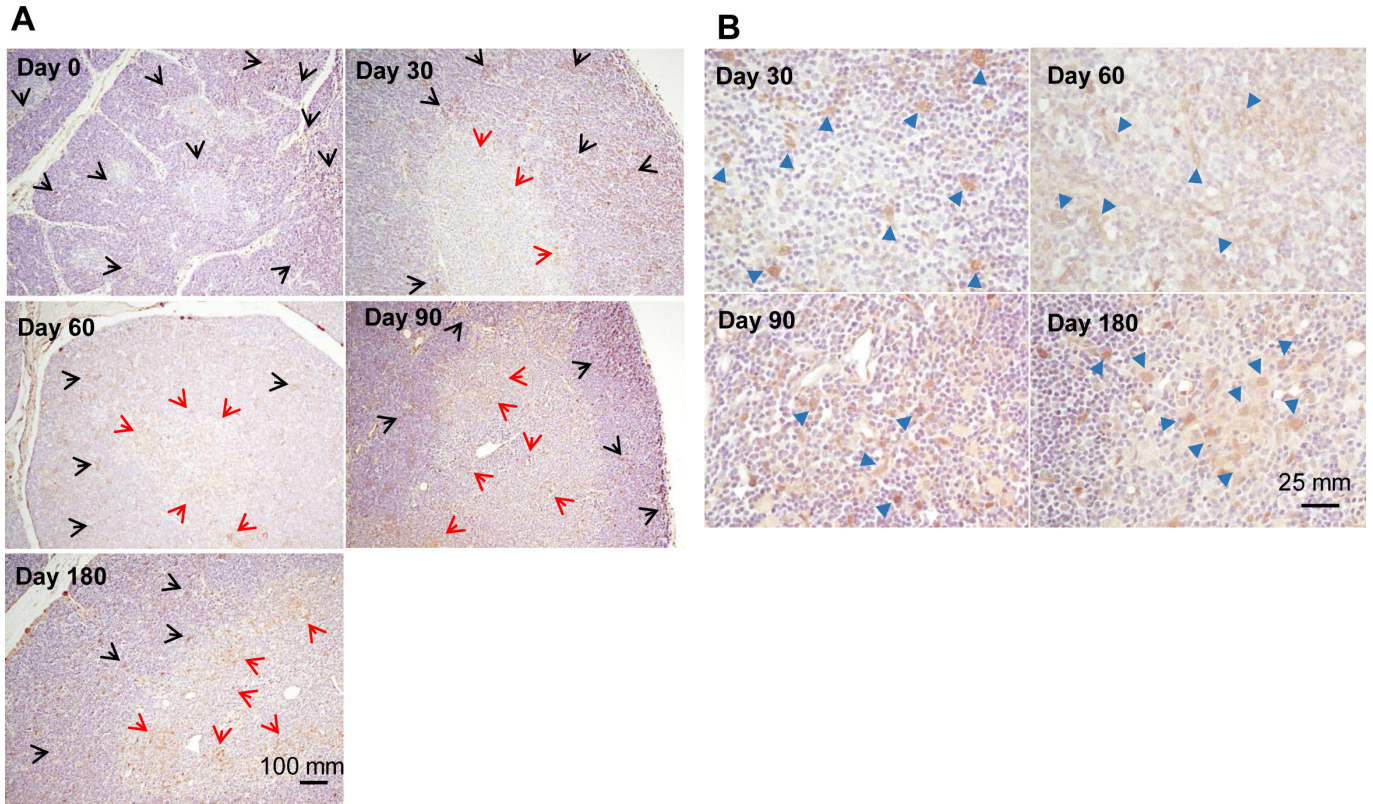


Fig. 1. Immunohistochemistry of annexin A5 (ANXA5) in rat thymus tissue. The expression of ANXA5 in the thymus of rats aged 0–180 days was examined by immunohistochemistry using an anti-ANXA5 antibody. (A) Thymus tissues showing the cortex and medulla. Intense immunoreactions in the cortex and medulla are indicated by the red and black arrows, respectively. (B) Enlarged medulla. Blue arrowheads indicate epithelial cells stained for ANXA5. Blue arrowheads indicate Hassall corpuscle-like structures.

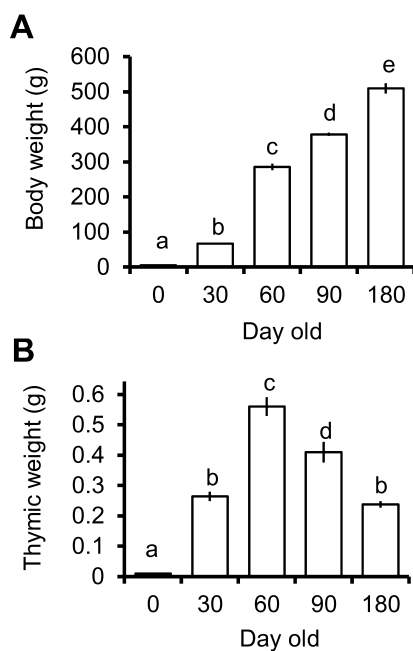


Fig. 2. Changes in the thymus weight. The body and thymus weights of male rats were measured at 0, 30, 60, and 180 days of age. The (A) body weight, and (B) thymus weight. Different letters indicate a statistical difference at $P < 0.05$.

Changes in thymic *Anxa5* and *Gnrh* mRNA in the organ culture of thymus tissue

To investigate the direct action of a GnRHa on the thymus, organ culture of thymus tissue pieces was performed. In addition to *Anxa5* mRNA, we measured *Gnrh* mRNA considering that GnRH could stimulate its own expression [15]. The expression levels of thymic *Anxa5* and *Gnrh* mRNA were measured by qRT-PCR, and both *Anxa5* and *Gnrh* mRNA levels were significantly increased with GnRHa treatment ($P < 0.05$, Fig. 3A, 3B).

The expression of ANXA5 in the thymus was examined by immunohistochemistry after GnRHa treatment. ANXA5 expression in the medulla of the thymus was enhanced after incubation with the GnRHa (Fig. 4). Representative positive reactions are indicated by the arrowheads.

DISCUSSION

The expression and distribution of ANXA5 in the thymus changed with age, and both the cortex and medulla of the thymus showed positive reactions after 30 days of age. The intensity of the staining in the medulla was particularly pronounced after 60 days of age. Positive reactions for ANXA5 were observed in large epithelial-like cells.

ANXA5 is a member of the annexin family of proteins, which is characterized by calcium-dependent phospholipid binding [12]. Annexins consist of four repeats of approximately 60 amino acids (eight repeats for ANXA6) [5]. Twelve annexins have been identified

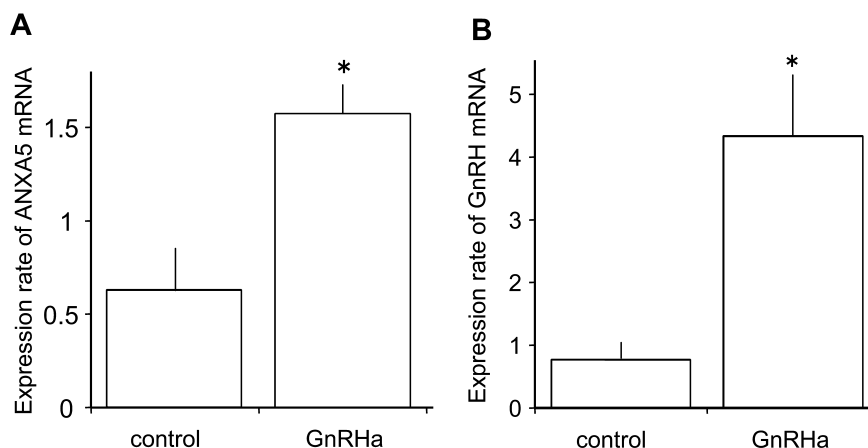


Fig. 3. Changes in thymic *Anxa5* and *Gnrh* mRNA after gonadotropin releasing hormone agonist (GnRH) treatment in the organ culture of thymus tissue. Thymus tissues from male rats (40–60 days old) were cut into 2–3 mm³ blocks and incubated with 10⁻⁹ M GnRH for 3 hr at 37°C with 5% CO₂ and 40% O₂. The expression levels of thymic (A) *Anxa5* and (B) *Gnrh* mRNA were measured by qRT-PCR. Asterisks indicate a significant difference ($P < 0.05$).

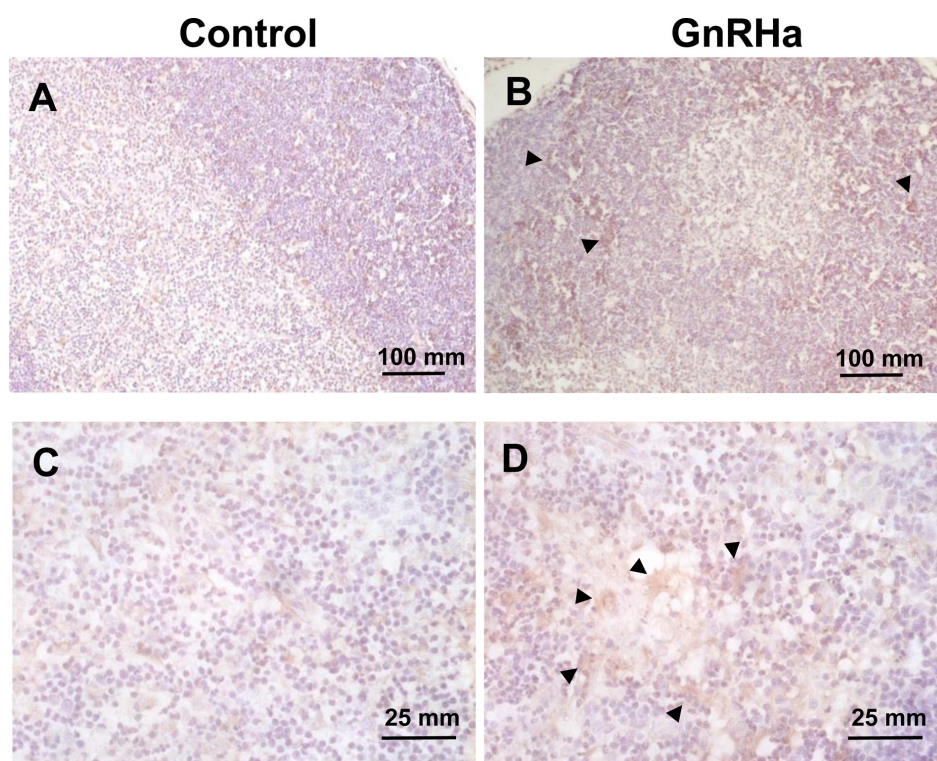


Fig. 4. Immunohistochemistry of annexin A5 (ANXA5) in cultured thymus tissue. (A and C) Control. (B and D) gonadotropin releasing hormone agonist (GnRH)-treated tissues. Black arrowheads indicate intense staining.

in mammals. ANXA5 is a relatively well-studied annexin and is characterized by a high affinity for phosphatidylserine [18, 20]. We previously found that ANXA5 is expressed in pituitary gonadotropes, and GnRH stimulates ANXA5 expression [9, 10]. Furthermore, previous studies revealed that GnRH could augment ANXA5 expression in luteal cells, Leydig cells, and mammary epithelial cells in rats [7, 15, 25]. The studies demonstrated that locally expressed GnRH could affect these cells.

The thymus weight peaked at 60 days of age, after which it rapidly decreased, which is consistent with previous findings [3]. The ratio of thymus weight to body weight reached a maximum at 30 days of age and decreased rapidly thereafter. These results indicated that the increase in ANXA5 staining in the medulla occurred in parallel with the involution of the thymus. The reduction

in the thymus weight appeared to be related to the presence of ANXA5. As the expression of ANXA5 could be affected by locally expressed GnRH, GnRH may have specific functions in the tissue, and the action of GnRH may increase with age. Therefore, GnRH may be involved in thymic involution after puberty. We previously reported that GnRH could stimulate the expression of ANXA5 and apoptosis in regressing luteal cells and involuting mammary epithelial cells [7, 15]. Augmentation of ANXA5 expression in the thymus is hypothesized to be involved in thymic involution.

The thymic function of GnRH *in vivo* has been demonstrated in previous studies, in which a GnRHa was administered systemically (intraperitoneally or subcutaneously) [22, 23]. In the present study, we used organ culture to show that a GnRHa could act directly on the thymus, as demonstrated by the increased level of *Anxa5* mRNA following GnRHa administration. Immunohistochemical analysis of the thymus after culture showed that ANXA5 expression was increased in the medulla of the GnRHa-treated thymus, indicating the existence of an auto-amplification mechanism by which GnRH induces GnRH, thereby increasing ANXA5 in the thymus. However, the functions of the thymus regulated by autocrine or paracrine mechanisms remain to be elucidated.

GnRH is expressed in the hypothalamus and various other tissues [6, 7, 14, 15, 21, 24, 25]. Although the function of GnRH in these tissues is of interest, it is not well understood. Considering that GnRH has been reported to affect cell proliferation in some studies [1, 14], GnRH and ANXA5 may be involved in lymphocyte proliferation and differentiation in the thymus. Elucidation of the physiological functions of GnRH receptors in the thymus would be useful for analyzing their functions in other tissues.

In conclusion, the present study demonstrated that ANXA5 expression was enhanced in the thymus after puberty, especially in medullary epithelial cells, and that the thymus weight decreased rapidly at this time. As the expression of GnRH was found to be stimulated by GnRH itself in the thymus, GnRH is proposed as a novel local regulator of the thymus.

CONFLICT OF INTEREST. There is no conflict of interest in this study.

ACKNOWLEDGMENTS. The experiments in this study were performed by Ms. Asaami Kitamura for her graduation thesis under the supervision of Kawaminami. The authors appreciate the help of Ms. M. Nakata in drafting the manuscript.

REFERENCES

1. Aguilar-Rojas, A. and Huerta-Reyes, M. 2009. Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells (Review). *Oncol. Rep.* **22**: 981–990. [Medline] [CrossRef]
2. Azad, N., LaPaglia, N., Agrawal, L., Steiner, J., Uddin, S., Williams, D. W., Lawrence, A. M. and Emanuele, N. V. 1998. The role of gonadectomy and testosterone replacement on thymic luteinizing hormone-releasing hormone production. *J. Endocrinol.* **158**: 229–235. [Medline] [CrossRef]
3. Boehm, T. and Swann, J. B. 2013. Thymus involution and regeneration: two sides of the same coin? *Nat. Rev. Immunol.* **13**: 831–838. [Medline] [CrossRef]
4. Chidgey, A., Dudakov, J., Seach, N. and Boyd, R. 2007. Impact of niche aging on thymic regeneration and immune reconstitution. *Semin. Immunol.* **19**: 331–340. [Medline] [CrossRef]
5. Gerke, V. and Moss, S. E. 2002. Annexins: from structure to function. *Physiol. Rev.* **82**: 331–371. [Medline] [CrossRef]
6. Jacobson, J. D., Crofford, L. J., Sun, L. and Wilder, R. L. 1998. Cyclical expression of GnRH and GnRH receptor mRNA in lymphoid organs. *Neuroendocrinology* **67**: 117–125. [Medline] [CrossRef]
7. Kawaminami, M., Shibata, Y., Yaji, A., Kurusu, S. and Hashimoto, I. 2003. Prolactin inhibits annexin 5 expression and apoptosis in the corpus luteum of pseudopregnant rats: involvement of local gonadotropin-releasing hormone. *Endocrinology* **144**: 3625–3631. [Medline] [CrossRef]
8. Kawaminami, M., Tsuchiyama, Y., Saito, S., Katayama, M., Kurusu, S. and Hashimoto, I. 2002. Gonadotropin-releasing hormone stimulates annexin 5 messenger ribonucleic acid expression in the anterior pituitary cells. *Biochem. Biophys. Res. Commun.* **291**: 915–920. [Medline] [CrossRef]
9. Kawaminami, M., Tanaka, K., Asawa, T., Osugi, S., Kawauchi, H., Kurusu, S. and Hashimoto, I. 1992. Association of annexin V with prolactin in the rat anterior pituitary gland. *Biochem. Biophys. Res. Commun.* **186**: 894–898. [Medline] [CrossRef]
10. Kawaminami, M., Etoh, S., Miyaoka, H., Sakai, M., Nishida, M., Kurusu, S. and Hashimoto, I. 2002. Annexin 5 messenger ribonucleic acid expression in pituitary gonadotropes is induced by gonadotropin-releasing hormone (GnRH) and modulates GnRH stimulation of gonadotropin release. *Neuroendocrinology* **75**: 2–11. [Medline] [CrossRef]
11. Millar, R. P. 2005. GnRHs and GnRH receptors. *Anim. Reprod. Sci.* **88**: 5–28. [Medline] [CrossRef]
12. Moss, S. E. 1997. Annexins. *Trends Cell Biol.* **7**: 87–89. [Medline] [CrossRef]
13. Murata, T., Chiba, S. and Kawaminami, M. 2021. The expression of annexin A1 and A5 mRNA by gonadotropin-releasing hormone in LβT2 gonadotrope cells. *Endocr. J.* [Medline]
14. Park, M. K., Kanaho, Y. and Enomoto, M. 2013. Regulation of the cell proliferation and migration as extra-pituitary functions of GnRH. *Gen. Comp. Endocrinol.* **181**: 259–264. [Medline] [CrossRef]
15. Rieanrakwong, D., Laoharatchathathanin, T., Terashima, R., Yonezawa, T., Kurusu, S., Hasegawa, Y. and Kawaminami, M. 2016. Prolactin suppression of gonadotropin-releasing hormone initiation of mammary gland involution in female rats. *Endocrinology* **157**: 2750–2758. [Medline] [CrossRef]
16. Savino, W., Mendes-da-Cruz, D. A., Lepletier, A. and Dardenne, M. 2016. Hormonal control of T-cell development in health and disease. *Nat. Rev. Endocrinol.* **12**: 77–89. [Medline] [CrossRef]
17. Schally, A. V. 1970. Hypothalamic regulation of FSH and LH secretion. *Res. Reprod.* **2**: 2–3. [Medline]
18. Tait, J. F. and Gibson, D. 1992. Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. *Arch. Biochem. Biophys.* **298**: 187–191. [Medline] [CrossRef]
19. Terashima, R., Saigo, T., Laoharatchathathanin, T., Kurusu, S., Brachvogel, B., Poschl, E. and Kawaminami, M. 2020. Augmentation of Nr4a3 and suppression of Fshb expression in the pituitary gland of female annexin A5 null mouse. *J. Endocr. Soc.* **4**: bvaa096.

20. Thiagarajan, P. and Tait, J. F. 1990. Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J. Biol. Chem.* **265**: 17420–17423. [[Medline](#)] [[CrossRef](#)]
21. Tungmahasuk, D., Fungbun, N., Laoharatchathanin, T., Terashima, R., Kurusu, S. and Kawaminami, M. 2018. Effects of gonadotropin-releasing hormone agonist on human chorionic gonadotropin activity in granulosa cells of immature female rats. *J. Reprod. Dev.* **64**: 129–134. [[Medline](#)] [[CrossRef](#)]
22. Ullewar, M. P. and Umathe, S. N. 2014. Gonadotropin-releasing hormone agonist selectively augments thymopoiesis and prevents cell apoptosis in LPS induced thymic atrophy model independent of gonadal steroids. *Int. Immunopharmacol.* **23**: 46–53. [[Medline](#)] [[CrossRef](#)]
23. Ullewar, M. P. and Umathe, S. N. 2016. Gonadotropin-releasing hormone agonist prevents l-arginine induced immune dysfunction independent of gonadal steroids: Relates with a decline in elevated thymus and brain nitric oxide levels. *Nitric Oxide* **57**: 40–47. [[Medline](#)] [[CrossRef](#)]
24. Wang, L., Cao, H., Jiang, N., Zhang, N., Zhang, J., Hou, R., Chen, C., Wang, Y., Li, X., Li, D. and Ji, Q. 2009. Differential expression of gonadotropin-releasing hormone (GnRH) in pancreas during rat pregnancy. *Endocrine* **36**: 538–545. [[Medline](#)] [[CrossRef](#)]
25. Yao, B. and Kawaminami, M. 2008. Stimulation of annexin A5 expression by gonadotropin releasing hormone (GnRH) in the Leydig cells of rats. *J. Reprod. Dev.* **54**: 259–264. [[Medline](#)] [[CrossRef](#)]