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Changes in the expressions of annexin A1, annexin A5, inhibin/activin subunits, and vitamin D receptor mRNAs in pituitary glands of female rats during the estrous cycle: correlation analyses among these factors

Takuya MURATA[1)]*, Shuichi CHIBA[1)], Mitsumori KAWAMINAMI[1)]

[1)]Laboratory of Veterinary Physiology, Faculty of Veterinary Medicine, Okayama University of Science, Ehime, Japan

ABSTRACT. Pituitary gonadotropin secretion is regulated by several pituitary factors as well as GnRH and ovarian hormones. To elucidate the regulatory mechanisms of pituitary gonadotropin secretions, we observed changes in the mRNA levels of pituitary factors, namely annexin A1 (*Anxa1*) and *Anxa5*, inhibin/activin subunits, follistatin (*Fst*), and vitamin D receptor (*Vdr*), in female rat pituitary glands during the estrous cycle. Additionally, levels of LHβ subunit (*Lhb*), FSHβ subunit (*Fshb*), and GnRH receptor (*Gnrh-r*) mRNA were examined. During proestrus, *Anxa1, Anxa5, Vdr,* and inhibin α-subunit (*Inha*) exhibited the lowest levels, while during estrus, activin βB-subunit (*Actbb*), *Lhb,* and *Gnrh-r* were the lowest. Moreover, *Fshb* exhibited the highest value during metestrus, whereas *Fst* did not differ significantly. Correlation analyses revealed 16 statistically significant gene combinations. In particular, four combinations, namely *Anxa5* and *Inha, Anxa5* and *Actbb, Inha* and *Vdr, and Inha* and *Actbb,* were highly significant ($P<0.0001$), while four combinations, *Anxa1* and *Anxa5, Anxa1* and *Vdr*, *Anxa5* and *Vdr*, and *Lhb* and *Gnrh-r,* were moderately significant ($P<0.001$). The remaining eight combinations that exhibited statistical significance were *Anxa1* and *Inha, Anxa1* and *Actbb*, *Vdr* and *Actbb*, *Anxa1* and *Fshb, Inha* and *Lhb*, *Actbb* and *Fshb*, *Actbb* and *Lhb*, and *Fst* and *Fshb* ($P<0.05$). These results highlight strong correlations among *Anxa1, Anxa5, Vdr, Inha,* and *Actbb,* thereby suggesting that an interaction among ANXA1, ANXA5, and VDR may lead to further communications with inhibin and/or activin in the pituitary gland.

KEYWORDS: annexin, estrous cycle, gonadotropins, inhibin/activin, vitamin D receptor

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The hypothalamic-pituitary-gonadal axis regulates the reproductive functions in vertebrates [21]. The secretion of gonadotropins from the pituitary gland is regulated by the gonadotropin-releasing hormone (GnRH) from the hypothalamus, steroid hormones and inhibin from the ovaries, as well as certain factors produced by the pituitary gland [40]. These factors exhibit varying concentrations and functions during the different phases of the estrous cycle in female mammals. To elucidate the regulatory mechanisms of pituitary gonadotropins, it is necessary to understand the changes in and interactions among these factors during the estrous cycle.

Annexins (ANX) constitute a family of structurally related proteins that possess a calcium-dependent phospholipid binding property [9, 16]. In vertebrates, there are 12 annexins, namely ANXA1–ANXA11 and ANXA13 [34]. These proteins consist of a conserved C-terminal core domain, four (eight in ANXA6) approximately 60 amino acid sequence repeats, and a variable N-terminus [9]. Moreover, the ANXAs perform various functions, including membrane repair, signaling, hormone secretion, inhibition of blood coagulation, and regulation of inflammation; additionally, they serve as biomarkers for various pathophysiological changes [9, 16, 46]. Reportedly, ANXA1 and ANXA5 are produced in the pituitary gland [15, 24, 25, 27, 50]. In fact, we have previously demonstrated that a GnRH agonist (GnRHa) can stimulate *Anxa1* and *Anxa5* expressions via the GnRH receptor (GnRH-R)-mitogen-activated protein kinase (MAPK) cascade in the mouse gonadotrope-derived cell line, LβT2 [36].

Identification of inhibin, activins, and follistatin (FST) is based on their abilities to regulate follicle stimulating hormone (FSH) secretion from the pituitary gland; inhibin and FST inhibit, while activins stimulate FSH secretion [30, 31, 33, 41, 43, 51]. Inhibins

*Correspondence to: Murata T: t-murata@ous.ac.jp, Laboratory of Veterinary Physiology, Faculty of Veterinary Medicine, Okayama University of Science, 1-3 Ikoinooka, Imabari, Ehime 794-8555, Japan

are hetero-dimers of α- and β-subunits; the latter has two different forms, namely βA and βB. The two isoforms of inhibin are inhibin A (composed of αβA) and inhibin B (αβB). Activins are the homo- or hetero-dimers of the βA- and βB-subunits. The three isoforms of activin are activin A (βAβA), activin AB (βAβB), and activin B (βBβB) [54]. Incidentally, activins have diverse effects on several tissues; for instance, they promote cell growth, differentiation, and death [5–7, 11, 45, 53]. Activins bind to the activin type II receptor, which in turn forms a complex with the type I receptor, leading to its phosphorylation, ultimately triggering the phosphorylation of SMAD2 and SMAD3 [1, 18]. Inhibin and FST suppress the action of activins by binding to activin type II receptor [29] and activin [38], respectively. The predominant activin produced in the pituitary is activin B, which reportedly functions in a paracrine/autocrine manner [3].

Vitamin D_3 is converted to 25-hydroxyvitamin D_3 [25-(OH) D_3] in the liver and to 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], which is the most active form of vitamin D_3, in the kidney [32]. The effect of 1,25-$(OH)_2D_3$ is mediated by the intracellular vitamin D_3 receptor (VDR) [37]. This VDR is involved in calcium and phosphorus homeostasis as well as in cell proliferation, differentiation, immunomodulation, and reproduction [32, 37]. Incidentally, VDR null female mice display hypergonadotropic hypogonadism and reduced ovarian aromatase expression, as well as gonadotropin-resistant and atrophic ovaries [28, 55]. Additionally, 1α-hydroxylase [Cyp27b1; the rate-limiting enzyme that converts 25-(OH) D_3 to 1,25-$(OH)_2D_3$] null mice exhibit peripubertal 1,25-$(OH)_2D_3$ deficiency, leading to a significant delay in vaginal opening [12]. Furthermore, young adult females maintained on a vitamin D_3-deficient diet after puberty exhibit arrested follicular development and undergo prolonged estrous cycles characterized by extended periods of diestrus [12]. Although previous studies have reported the involvement of vitamin D_3 in reproduction, its physiological role with respect to gonadotrope function has not yet been elucidated.

Recently, we found that activin suppresses *Anxa5* mRNA expression and enhances GnRH-mediated *Anxa1* mRNA expression in LβT2 cells [35]. Wöeckel *et al*. [52] reported that 1,25-$(OH)_2D_3$ increases the secretion of activin A, while decreasing that of FST in the human pre-osteoblast cell line SV-HFO. If ANXA1 and ANXA5, activins, and VDR interact *in vivo*, under physiological conditions, the expression of these factors, in the pituitary gland of female rats during the estrous cycle, might be correlated. In fact, examining these relationships during the estrous cycle in the pituitary may provide novel insights in this field. Therefore, in this study, we observed the changes in *Anxa1*, *Anxa5*, inhibin/activin subunits, and *Vdr* mRNA levels in the pituitary glands of female rats during the estrous cycle and analyzed their correlations to determine the nature of the relationship among these factors.

MATERIALS AND METHODS

Animals

Adult female Wistar-Imamichi rats (body weight: 180–220 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were housed in an environmentally controlled room (temperature: 23 ± 3°C; lights on: 0700–1900 hr) with free access to tap water and pelleted rat food. Their estrous cycles were monitored using daily vaginal smears. As genes examined herein included GnRH-inducible genes, such as *Anxa1* and *Anxa5* [36], samples were collected in the morning (1000–1200 hr) to minimize the effect of GnRH surge. The rats were euthanized by decapitation between 1000 and 1200 hr on metestrus (D1), diestrus (D2, the day following metestrus), proestrus (P), and estrus (E). The anterior pituitaries were collected from all rats, immersed overnight in RNA*later* solution (Invitrogen, Carlsbad, CA, USA), and stored at −20°C until RNA extraction. All procedures for animal care, maintenance, and surgery were approved (Exp2021-121) by the Animal Care and Use Committee of Okayama University of Science. The entire study was conducted in accordance with the Guidelines for Animal Experiment, Okayama University of Science.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from the anterior pituitaries of rats using the acid guanidinium thiocyanate-phenol-chloroform extraction method with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. First, 500 µL of TRIzol reagent was added after removal of the RNA*later* solution. Then the anterior pituitary tissue was homogenized with TRIzol reagent, followed by centrifugation at 12,000 × *g* for 10 min. Subsequently, the supernatant was transferred to a 1.5 mL plastic centrifuge tube, to which 100 µL of chloroform was added. The mixture was centrifuged at 12,000 × *g* for 15 min. Thereafter, the aqueous phase was transferred to a new tube, and the RNA was precipitated using isopropanol. Finally, 1 µg of the total RNA was treated with RNase-free DNase I (Invitrogen) to exclude any genomic DNA, followed by reverse-transcription using 200 U ReverTra Ace (TOYOBO, Osaka, Japan) and 10 pmol of random primers (Invitrogen), according to the manufacturer's protocol, to obtain cDNA.

Real-time polymerase chain reaction (PCR)

Real-time PCR analyses of the cDNA samples were performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) on QuantStudio software (Applied Biosystems). The primers for the genes encoding ANXA1, ANXA5, VDR, inhibin/activin βB-subunit (ACTβB), inhibin α-subunit (INHα), FST, luteinizing hormone (LH) β subunit (LHβ), FSHβ subunit (FSHβ), GnRH receptor (GnRH-R), and ribosomal protein L19 (RPL19) [15, 36] were used for each PCR assay. The primer sequences are listed in Table 1. Each mRNA abundance was standardized by dividing its value by the expression of *Rpl19* mRNA in the same sample.

Statistical analysis

Data were obtained by dividing the value of each sample by the mean value of the P phase to obtain the relative expressions. Data are expressed as mean ± standard error of the mean (SEM). The data were statistically evaluated using Tukey's multiple comparison test. Statistical analyses were performed using KaleidaGraph version 4.5 software (Synergy Software, Reading, PA, USA). The data

Table 1. List of primers

Primers	Forward (5′-3′)	Reverse (5′-3′)
Anxa1	GCCCCTACCCTTCCTTCAAT	GAGTGTCTTCATCTGTTCCA
Anxa5	ATGGCTCTCAGAGGCACCGT	CGTGTTTCAGCTCGTAGGCG
Vdr	GACGGAAGTACAGGGAGCTA	CATGTCTAGTGAGGTGGTG
Inha	GTGGGGAGGTCCTAGACAGA	GTTGGGATGGCCGGAATACA
Actbb	TCTTCATCGACTTTCGGCTCAT	TGTCAGGCGCAGCCACACTCCT
Fst	CCCCAACTGCATCCCTTGTA	GGTCCACAGTCCACATTCT
Lhb	GTCTGCATCACCTTCACCAC	GTAGGTGCACACTGGCTGAG
Fshb	CTGCTGCCATAGCTGTGAAC	TAGCTGGGTCCTTATACACCA
Gnrh-r	AATCATCTTCGCCCTCACAC	AGCACGGGTTTAGAAAAGCA
Rpl19	CAGGAGATACCGGGAATCTAAG	TGCCTTCAGTTTGTGGATGTG

Anxa1, annexin A1; *Anxa5*, annexin A5; *Vdr*, vitamin D receptor; *Inha*, inhibin α-subunit; *Actbb*, inhibin/activin βB-subunit; *Fst*, follistatin; *Lhb*, LHβ subunit; *Fshb,* FSHβ subunit; *Gnrh-r*, GnRH-receptor; *Rpl19*, ribosomal protein L19.

were also subjected to Pearson's product-moment correlation analysis. The correlation analysis was performed using R software (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at $P<0.05$.

RESULTS

Altered gene expression in anterior pituitary glands of rats during the estrous cycle

Analysis of the relative mRNA expression in the anterior pituitary glands of female rats during different phases of the estrous cycle revealed that *Anxa1, Anxa5*, and *Vdr* expression was significantly lower during the P phase than in D1 and D2 (Fig. 1A–C).

Additionally, *Inha* expression in the D2 phase was significantly higher than that in the P phase (Fig. 2A). Similarly, the *Actbb* level was higher in D1 and D2, and gradually decreased as the rats approached the E phase of the cycle (Fig. 2B). The *Fst* level tended to decline in the P phase; however, this result was not statistically significant (Fig. 2C).

Furthermore, *Lhb* expression was the highest in D2, and gradually declined as the cycle progressed to the E phase (Fig. 3A). In contrast, *Fshb* expression was significantly higher in D1 than in the other phases (Fig. 3B). Similar to *Lhb, Gnrh-r* expression was the highest in D2, and gradually decreased as the cycle progressed to E (Fig. 3C).

Correlation analyses among different genes in the anterior pituitary of female rats

Correlations among the expressions of different genes within the anterior pituitary of female rats were assessed using Pearson's analysis. Sixteen combinations were statistically significant (Table 2). Four combinations, namely *Anxa5* and *Inha, Anxa5* and *Actbb, Inha* and *Vdr, and Inha* and *Actbb,* were strongly and positively correlated ($0.76<r<0.86$, $P<0.0001$; Table 2A, Fig. 4). Additionally, the correlations among four pairs of genes, namely *Anxa1* and *Anxa5, Anxa1* and *Vdr*, *Anxa5* and *Vdr*, and *Lhb* and *Gnrh-r,* were moderately significant ($0.71<r<0.76$, $P<0.001$; Table 2B, Fig. 5). In fact, significant correlations were noted among eight other gene pairs, particularly *Anxa1* and *Inha, Anxa1* and *Actbb*, and *Vdr* and *Actbb* ($0.60<r<0.66$, $P<0.01$; Table 2C), as well as *Anxa1* and *Fshb, Inha* and *Lhb*, *Actbb* and *Fshb*, *Actbb* and *Lhb*, and *Fst* and *Fshb* ($0.45<r<0.53$, $P<0.05$; Table 2D).

DISCUSSION

In this study, we analyzed changes in the expression levels of *Anxa1, Anxa5, Vdr, Inha, Actbb, Fst, Lhb, Fshb,* and *Gnrh-r* in the anterior pituitary of female rats during the different phases of the estrous cycle. Incidentally, *Anxa1, Anxa5, Vdr,* and *Inha* mRNAs were expressed at the lowest levels during the P phase, while *Actbb, Lhb,* and *Gnrh-r* levels were the lowest in the E phase. Particularly, *Anxa1, Anxa5, Vdr, Inha,* and *Actbb* exhibited a tendency to decrease in both P and E, suggesting a positive correlation among these factors. Since ovarian-derived factors, especially estrogen, are the most likely to cause fluctuations and affect gene expressions in the P and E phases, it is plausible that the abovementioned factors are directly or indirectly affected by estrogen. Additionally, the *Fshb* levels were highest in D1, as compared to those in the other phases, whereas *Fst* expression decreased in the P phase; however, the difference was not statistically significant.

ANXA1 has been identified as a glucocorticoid-inducible protein in rat macrophages [4]. Reportedly, it is also involved in the secretion of pituitary hormones, such as adrenocorticotropic hormone, prolactin, thyroid stimulating hormone, and LH [19, 20]. According to a previous study, although ANXA1 protein and mRNA levels decrease in the pituitary glands of ovariectomized rats, successive 17β-estradiol treatment again increases the pituitary ANXA1 protein and mRNA levels in these rats [10]. The same study also reported that the pituitary ANXA1 protein levels are the lowest in the P phase [10]. The present study has demonstrated consistent results with respect to the ANXA1 expression at the mRNA level. Collectively, these results suggest that the decreased expression of pituitary ANXA1 protein and mRNA during the P phase of the estrous cycle in rats is likely influenced by the action of 17β-estradiol. Furthermore, ANXA1 is present in abundance in the folliculo-stellate cells of the rat pituitary [50], and the action of 17β-estradiol in the regulation of ANXA1 might be mediated by corticosterone [10]. Additionally, *Anxa1* expression was strongly stimulated by a

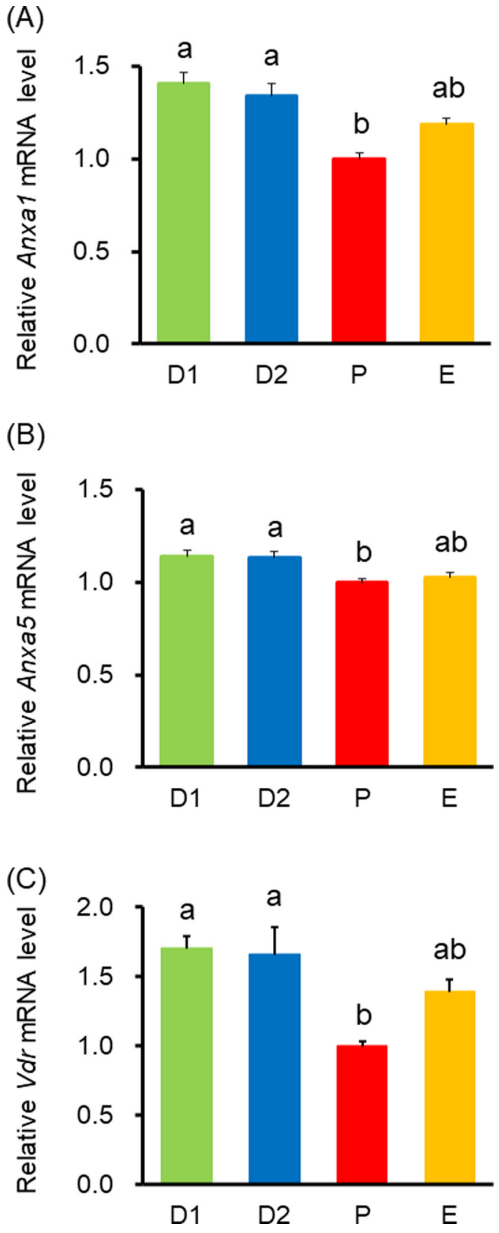

Fig. 1. Changes in the mRNA levels of annexin A1 (*Anxa1*; **A**), annexin A5 (*Anxa5*; **B**), and vitamin D receptor (*Vdr*; **C**) in the pituitary glands of female rats during the estrous cycle. The anterior pituitaries were collected between 1000 and 1200 hr on metestrus (D1), diestrus (D2), proestrus (P), and estrus (E). Values are represented as the mean ± SEM (n=5). Each value is presented as a ratio to the value observed in proestrus. Data labeled with different letters differ significantly from each other (P<0.05, Tukey's multiple comparison test).

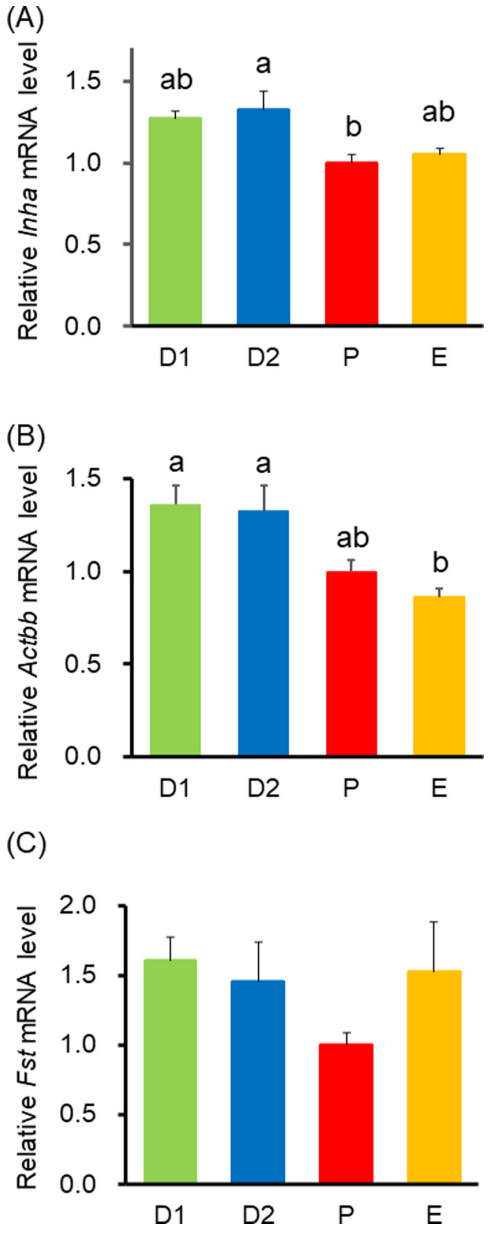

Fig. 2. Changes in the mRNA levels of inhibin α-subunit (*Inha*; **A**), inhibin/activin βB-subunit (*Actbb*; **B**), and follistatin (*Fst*; **C**) in the pituitary glands of female rats during the estrous cycle. The anterior pituitaries were collected between 1000 and 1200 hr on metestrus (D1), diestrus (D2), proestrus (P), and estrus (E). Values are represented as the mean ± SEM (n=5). Each value is presented as a ratio to the value observed in proestrus. Data labeled with different letters differ significantly from each other (P<0.05, Tukey's multiple comparison test).

GnRH analog in LβT2 cells [15, 36] and was stimulated by 17β-estradiol in the folliculo-stellate-like cell line, TtT/GF [10]. Hence, further studies need to investigate the regulation of ANXA1 and the involvement of estrogen with respect to its direct action on gonadotropes *in vivo*.

Of the four strongest positive correlations observed in this study, three combinations comprised *Anxa5*, *Inha*, and *Actbb* expressions. This relationship can be represented as a triangle (Fig. 6). Reportedly, ANXA5 stimulates FSH secretion in primary cultures of the rat anterior pituitary [26]. In fact, *Fshb* expression is lower in ANXA5-deficient mice than in wild-type mice [49]. Thus, ANXA5 has a stimulatory effect on FSH secretion from the pituitary. The correlation between *Inha* and *Actbb* expression appears to be associated with the production of inhibin B. However, estimating the production of activin B based on the expression of the inhibin/activin subunits

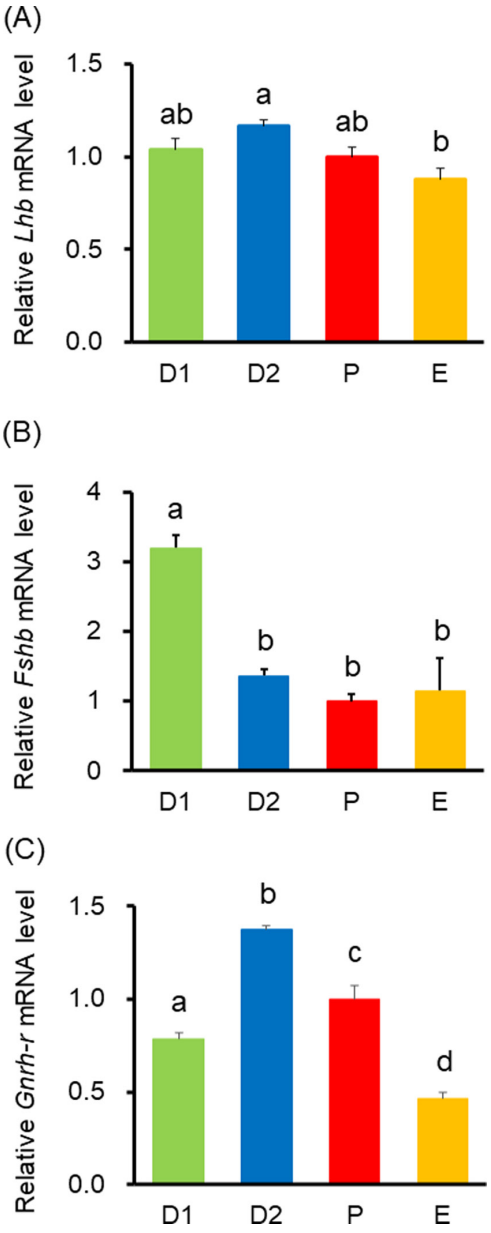

Fig. 3. Changes in the mRNA levels of luteinizing hormone β subunit (*Lhb*; **A**), follicle stimulating hormone β subunit (*Fshb*; **B**), and gonadotropin-releasing hormone receptor (*Gnrh-r*; **C**) in the pituitary glands of female rats during the estrous cycle. The anterior pituitaries were collected between 1000 and 1200 hr on metestrus (D1), diestrus (D2), proestrus (P), and estrus (E). Values are represented as the mean ± SEM (n=5). Each value is presented as a ratio to the value observed in proestrus. Data labeled with different letters differ significantly from each other ($P<0.05$, Tukey's multiple comparison test).

Table 2. The following gene combinations exhibit a statistically significant correlation in the pituitary glands of female rats during the estrous cycle

(A) $0.76<r<0.86$, $P<0.0001$

	Anxa5	*Inha*
Vdr		0.7607[a] 0.000099[b]
Inha	0.7773 0.000055	
Actbb	0.8177 0.000011	0.8520 0.0000019

(B) $0.71<r<0.76$. $P<0.001$

	Anxa5	*Vdr*
Anxa1	0.7323 0.00024	0.7273 0.00028
Anxa5		0.7198 0.00035
	Gnrh-r	
Lhb	0.7521 0.00013	

(C) $0.60<r<0.66$, $P<0.01$

	Inha	*Actbb*
Anxa1	0.6208 0.0035	0.6582 0.0016
Vdr		0.6468 0.0021

(D) $0.45<r<0.53$, $P<0.05$

	Fshb	*Lhb*
Anxa1	0.4649 0.039	
Inha		0.5253 0.017
Actbb	0.4817 0.031	0.4540 0.044
Fst	0.5021 0.024	

a: *r* value; b: *P* value. *Anxa1*, annexin A1; *Anxa5*, annexin A5; *Vdr*, vitamin D receptor; *Inha*, inhibin α-subunit; *Actbb*, inhibin/activin βB-subunit; *Lhb*, LHβ subunit; *Fshb*, FSHβ subunit; *Gnrh-r*, GnRH-receptor; *Fst*, follistatin.

is difficult. Considering that inhibin restricts FSH secretion from the pituitary by binding to the activin receptor and suppressing the action of activin [29], the combination of activin and inhibin is likely important for regulating FSH secretion. Therefore, this study suggested that FSH secretion from the pituitary might be modulated by a crosstalk among ANXA5, activin, and inhibin. Indeed, activin A has been found to suppress *Anxa5* expression in LβT2 gonadotrope cells [35]. Although the effect of activin on Anxa5 expression *in vivo* and in pituitary primary cultures warrants further examination, these results indicate an interaction between ANXA and activin.

Another strongly positive correlation was observed between *Vdr* and *Inha* expression. Reportedly, 1,25-$(OH)_2D_3$ treatment increased activin A and decreased FST secretions from the human pre-osteoblast cell line, SV-HFO [52]. Although transforming growth factor-

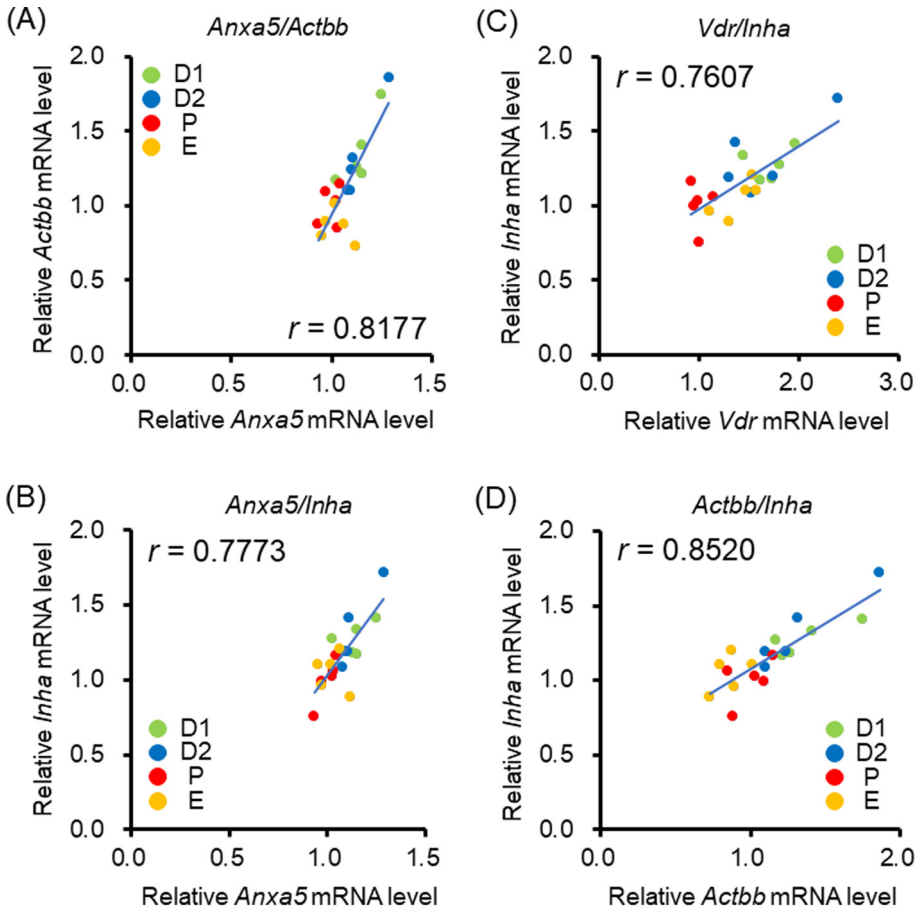

Fig. 4. Scatterplots denoting the correlations between the mRNA levels of annexin A5 (*Anxa5*) and inhibin/activin βB-subunit (*Actbb*; **A**), *Anxa5* and inhibin α-subunit (*Inha*; **B**), vitamin D receptor (*Vdr*) and *Inha* (**C**), and *Actbb* and *Inha* (**D**) in the pituitary glands of female rats during the estrous cycle. The anterior pituitaries were collected between 1000 and 1200 hr on metestrus (D1), diestrus (D2), proestrus (P), and estrus (E). Values are presented as a ratio to the mean value observed in proestrus, and a regression line is displayed. Statistical analyses were performed using Pearson's product-moment correlation.

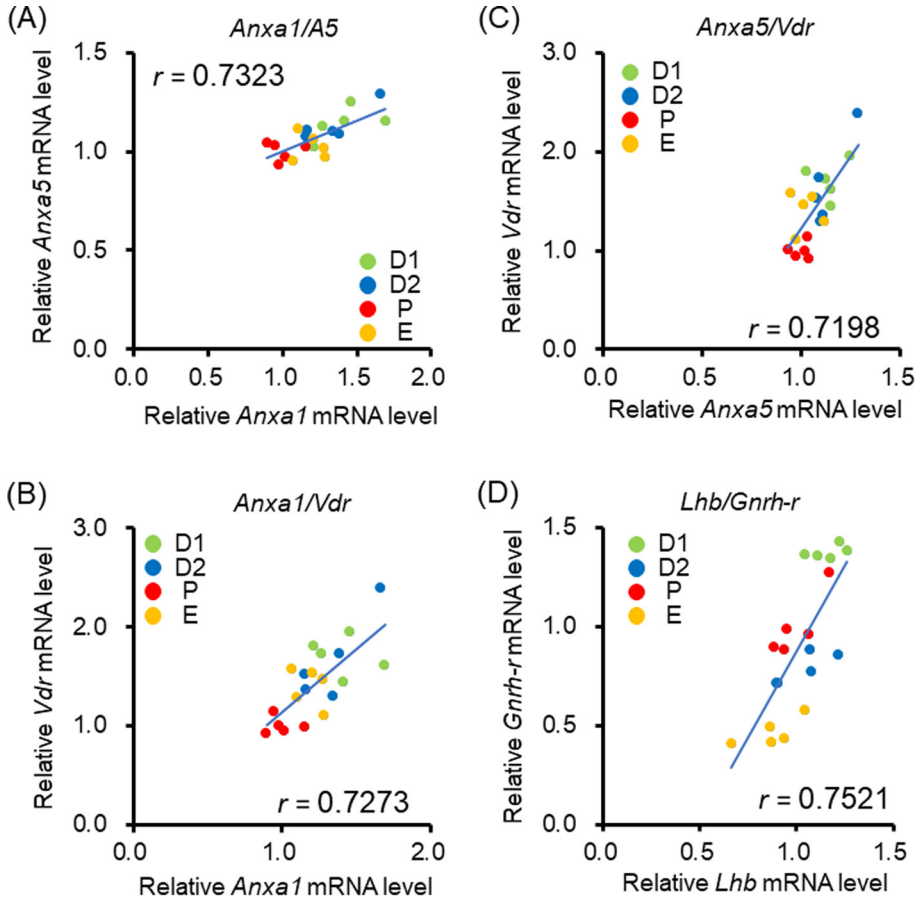

Fig. 5. Scatterplots denoting the correlations between the mRNA levels of annexin A1 (*Anxa1*) and annexin A5 (*Anxa5*; **A**), *Anxa1* and vitamin D receptor (*Vdr*; **B**), *Anxa5* and *Vdr* (**C**), and luteinizing hormone β subunit (*Lhb*) and gonadotropin-releasing hormone receptor (*Gnrh-r*; **D**) in the pituitary glands of female rats during the estrous cycle. The anterior pituitaries were collected between 1000 and 1200 hr on metestrus (D1), diestrus (D2), proestrus (P), and estrus (E). Values are presented as a ratio to the mean value observed in proestrus, and a regression line is displayed. Statistical analyses were performed using Pearson's product-moment correlation.

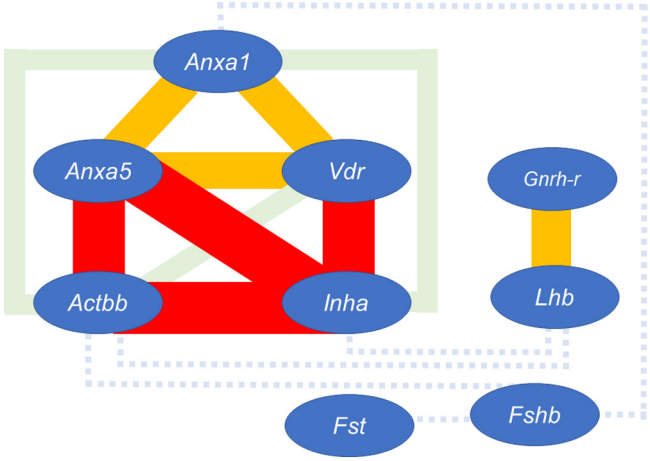

Fig. 6. Diagram summarizing the correlations among different gene expressions, namely annexin A1 (*Anxa1*), annexin A5 (*Anxa5*), vitamin D receptor (*Vdr*), inhibin/activin βB-subunit (*Actbb*), inhibin α-subunit (*Inha*), gonadotropin-releasing hormone receptor (*Gnrh-r*), luteinizing hormone β subunit (*Lhb*), follicle stimulating hormone β subunit (*Fshb*) and follistatin (*Fst*), in the pituitary glands of female rats during the estrous cycle. The lines connecting the different factors indicate the correlations among them. The thickest line (red), second thickest line (yellow), third thickest line (green), and dotted line (blue) represent the correlations among the groups A, B, C, and D presented in Table 2, respectively.

beta (TGFβ) binds to different receptors other than activin, it activates SMAD2 and SMAD3 in a manner similar to that of activin. In fact, it induces the proliferation of primary mouse lung fibroblasts and stimulates the expression and polymerization of α-smooth muscle actin in them [39]. Moreover, TGFβ can induce pro-fibrotic gene expression in primary mouse and rat hepatic stellate cells [13]. However, 1,25-$(OH)_2D_3$ has the ability to suppress these TGFβ-induced phenomena [13, 39]. Ding *et al.* [13] reported that VDR activation does not significantly affect TGFβ1-induced phosphorylation or nuclear translocation of SMAD3; moreover, the antagonism of TGFβ signaling is regulated by co-occupation of the same genomic sites by VDR and SMADs. Based on the similarity between TGFβ and activin, if a comparable genomic crosstalk exists between activin and VDR, inhibin may function as an inhibitor of activins, and the correlation between *Vdr* and *Inha* expression might reflect this mechanism.

Among the moderately strong correlations, associations among *Anxa1, Anxa5,* and *Vdr* expressions were notable. This relationship can also be represented as a triangle (Fig. 6), in addition to that among the aforementioned *Anxa5, Inha,* and *Actbb*. Since both ANXA1 and ANXA5 are GnRH-inducible proteins that affect LH secretion from gonadotrope cells [14, 15, 22, 23, 26, 36], the correlation between *Anxa1* and *Anxa5* expression is not unusual. However, to the best of our knowledge, this is the first study to report that the regulation of *Vdr* is possibly linked to the expressions of *Anxa1* and *Anxa5*. Previous studies have demonstrated that VDR null female mice display hypergonadotropic hypogonadism and reduced ovarian aromatase expression, as well as gonadotropin-resistant and atrophic ovaries [28, 55]. Therefore, the effects of vitamin D deficiency have been investigated at the ovarian level. The present study demonstrates that *Vdr* expression fluctuates during the estrous cycle, its expression is highly correlated with *Inha* expression, and it has a certain degree of correlation with both ANXA1 and ANXA5. Hence, there is a strong probability that the regulation of *Vdr* expression in the pituitary, probably the gonadotropes, is also involved in reproductive function.

A previous study has described the *Inha* and *Actbb* levels in the pituitary glands of female rats during the estrous cycle; however, no significant changes were reported [17]. Incidentally, *Fst* [2, 17] and *Fshb* [17, 48] levels exhibit a great increase, and *Gnrh-r* [44] level changes in a characteristic manner in the afternoon of the P phase. Although *Anxa1* and *Anxa5* are GnRH-inducible genes, the samples of this study were collected in the morning (1000–1200 hr) to minimize the effect of GnRH surge. Therefore, it is difficult to compare the results of this study with those of previous studies that included samples collected in the afternoon of the P phase.

Immunoreactive ANXA5 was localized in the majority of rat anterior pituitary cells and colocalized with LH [25] and prolactin [27]. After ovariectomy, the castration cells contained abundant ANXA5 in rat pituitary [25, 26]. Although immunoreactive ANXA1 was colocalized with S100 protein, a specific marker of folliculo-stellate cells [8], with the exception of a few cells [50], *Anxa1* mRNA expression was strongly increased by stimulation with GnRH agonist in LβT2 cells [15, 36]. Immunoreactive inhibin α and inhibin/activin βB subunits were localized in gonadotropes of rat female pituitary, and ovariectomy increased these immunoreactivities [42]. Autoradiograms after the injection of radiolabeled 1,25-$(OH)_2D_3$ showed a strong and extensive radioactivity in thyrotropes and a weak radioactivity in gonadotropes, lactotropes, and somatotropes [47]. The present study might suggest that these factors function via a cross-talk in the pituitary to regulate the secretion of anterior pituitary hormones, including gonadotropins, in an autocrine or paracrine manner, although further precise study is needed.

Correlations between gene expressions suggest that common factors or pathways may be involved. The present study focused on the changes among the stages of the estrous cycle and the correlations among the genes throughout the estrous cycle, with less influence of the GnRH surge. Terashima *et al*. [48] showed that among *Anxa5*, *Fshb*, and the nuclear receptor 4A3 (*Nr4a3*), which are GnRH-inducible genes, there is a negative correlation between *Anxa5* and *Nr4a3*, and *Fshb* and *Nr4a3*, and a positive correlation between *Anxa5* and *Fshb* in the afternoon of the P phase. As no correlation was detected among *Anxa5*, *Fshb* and *Nr4a3* herein (data not shown), the correlations among genes during P around GnRH surge appear to be different from those during the estrous cycle in this study and are necessary to be investigated in the near future. In addition, future analyses of promoter regions and the transcription factors involved may further reveal the complicated network that occurs in the pituitary gland during the estrous cycle.

In summary, we have revealed changes in the mRNA expression of nine genes, namely *Anxa1, Anxa5, Vdr, Inha, Actbb, Fst, Lhb, Fshb,* and *Gnrh-r,* in the pituitary glands of female rats during the estrous cycle and demonstrated significant differences in the expressions of eight genes. Furthermore, the correlation analyses among these mRNA levels confirmed strong associations among *Anxa1, Anxa5, Vdr, Inha,* and *Actbb.* To the best of our knowledge, this is the first study to report the possible involvement of VDR

in pituitary functions with a certain degree of association with ANXA1 and ANXA5, as well as inhibin/activin. Although this study does not elucidate the physiological significance of the observed gene expressions, it suggests a triangular interaction among ANXA1, ANXA5, and VDR, as well as an interaction between this triangle and inhibin/activin. Thus, future studies must focus on each correlation and analyze the interaction *in vivo* and *in vitro* to clarify the regulation of pituitary function.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this research.

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