Augmentation of Nr4a3 and suppression of Fshb expression in the pituitary gland of female Annexin A5 null mouse

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Abstract

GnRH enhances the expression of annexin A5 (ANXA5) in pituitary gonadotropes, and ANXA5 enhances gonadotropin secretion. However, the impact of ANXA5 regulation on the expression of pituitary hormone genes remains unclear. Here, using quantitative PCR, we demonstrated that ANXA5 deficiency in female mice reduced the expression of Fshb and Gh in their pituitary glands. Transcriptome analysis confirmed a specific increase in Nr4a3 mRNA expression in addition to lower levels of Fshb expression in ANXA5-deficient female pituitary glands. This gene was then found to be a GnRH-inducible immediate early gene, and its increased expression caused protein to accumulate in the nucleus after administration of a GnRH agonist in LβT2 cells, which are an in vitro pituitary gonadotrope model. The increase in ANXA5 protein levels in LβT2 cells clearly suppressed Nr4a3 expression. siRNA-mediated inhibition of Nr4a3 expression increased Fshb expression. The results revealed that GnRH stimulates Nr4a3 and Anxa5 sequentially. NR4A3 suppression of Fshb may be necessary for later massive secretion of FSH by GnRH in gonadotropes, and Nr4a3 would be negatively regulated by ANXA5 to increase FSH secretion.
Gonadotropin-releasing hormone (GnRH) of the hypothalamus promotes the secretion of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (1-3). FSH and LH consist of the specific beta subunits FSHβ and LHβ, respectively, and the common glycoprotein alpha subunit (CGA). GnRH stimulates the release of gonadotropin and facilitates the expression of gonadotropin subunit genes.

A transcriptome analysis of a GnRH-stimulated mouse clonal gonadotrope cell line identified hundreds of regulated genes (4), and several were proposed as required for gonadotropin secretion. For example, in vitro study but not yet in vivo showed the transcriptional complex AP-1, composed of two GnRH-inducible factors, c-Fos and c-Jun (5), promotes the transcriptional activity of the Fshb promoter (6), which is needed for the GnRH-stimulated induction of Fshb. In addition, GnRH stimulation of high-frequency pulse induces the expression of SKIL and TGIF1, which prevent the Fshb promoter activation by AP-1 (7). Therefore, GnRH signaling may also induce the expression of negative regulators that may affect the expression of gonadotropin genes and the responsiveness of cells towards GnRH stimulation.

Annexin A5 (ANXA5), a calcium-dependent phosphatidylserine-binding protein (8), is encoded by a GnRH-inducible gene (9). We previously demonstrated that ANXA5 is induced by GnRH signaling via PKC activation of the mitogen-activated protein kinase (MAPK) pathway (10). Moreover, an antisense oligonucleotide-mediated decrease in Anxa5 suppresses the secretion of LH by GnRH signaling in primary cultures of female rat pituitary cells (9). A recent study also revealed that the administration of the GnRH agonist stimulates the expression of ANXA5 and LHβ in the pituitary glands of female hypogonadal mice (hpg) that lack a functional GnRH-encoding gene (11). These results strongly suggest that ANXA5 is involved in physiological gonadotropin secretion under the influence of GnRH, but knowledge of the role of ANXA5 is limited.

Our previous study showed that ANXA5 is expressed in some pituitary endocrine cells of female rat, not only in gonadotropes (12). Here, we examined the regulation of pituitary hormone genes in ANXA5-deficient mice using transcriptome analysis and qPCR and identified a link between GnRH, NR4A3, ANXA5 and Fshb expression in pituitary gonadotrope. Sequential changes in NR4A3 and ANXA5
expression after GnRH stimulation is suggested to be beneficial for FSH secretion by GnRH.

Materials and Methods

Animals

All animal experiment protocols were approved by the President of Kitasato University through the judgment rendered by the Animal Care and Use Committee of Kitasato University (approval no. 15-032). The establishment of ANXA5-deficient mice (Anxa5<sup>−/−</sup>, Anxa5<sup>tm1Epo</sup>/Anxa5<sup>tm1Epo</sup>) has been described previously (13). C57BL/6J wild-type and Anxa5<sup>−/−</sup> mice were maintained under controlled temperature and lighting: 23 ± 3 °C and 14-hour light/10-hour dark cycle (lights on at 0500 h). They were allowed free access to laboratory chow and tap water. Eight-week-old female mice were administered either 5 ng/50 µl of GnRH agonist (GnRHa, Des-Gly10 (Pro9)-GnRH ethylamide; Intervet K.K., Tokyo, Japan) or 50 µl of saline (control) through repeated intraperitoneal injection (10 times in 30 min intervals). Pituitary samples were collected 30 min after the final administration. The mice were sacrificed by cervical dislocation. Pituitary tissues were immediately collected and frozen in liquid nitrogen.

Microarray analysis

Five pituitary glands from either C57BL/6J or Anxa5<sup>−/−</sup> adult female mice were collected and combined. RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. Microarray analysis was performed once each for C57BL/6J and Anxa5<sup>−/−</sup> mice (n = 1). Gene chip analysis and cDNA microarray data was carried out at GeneticLab Co., Ltd. (Sapporo, Japan). Briefly, the quantity and the quality of RNA samples digested with DNase was verified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Rockford, IL) and Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA) (C57BL/6J: 3.12 µg, 2.11 OD, RIN score 8.3; Anxa5<sup>−/−</sup>: 3.77 µg, 2.09 OD, RIN score 8.0). The synthesis of cDNA, cRNA and second cycle cDNA was performed using Ambion WT Expression Kit (Life Technologies, Tokyo, Japan). cDNA was
fragmented and labeled GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). Gene chip data was analyzed by means of GeneChip® Scanner 3000 7G with GeneChip Mouse Gene 1.0 ST (Affymetrix) and GeneChip Command Console software (Affymetrix) and GeneSpring GX Version 11.5.1 software (Agilent Technologies). When differences in the detected gene expression levels were greater than two-fold, the RNA expression was further measured and statistically evaluated by quantitative real-time PCR, as described below.

**Real-time PCR**

Total RNA was extracted from tissues of adult female mice or cells using TRIzol reagent (Life Technologies, Tokyo, Japan) and was reverse-transcribed to generate cDNA using a High Capacity cDNA synthesis kit (Life Technologies). Primer sequences for real-time PCR are given in Table 1. Real-time PCR was performed using Power SYBR® Green PCR Master Mix (Life Technologies) according to the manufacturer’s protocol using the following amplification conditions: 95 °C for 10 min and 50 cycles of 95 °C for 15 sec, 60 °C for 1 min. Relative gene expression levels were calculated by the delta-delta CT method using ribosomal protein L19 (RPL19) as an internal control for normalization. Melting curve analysis revealed no amplification of nonspecific products. Expression levels are given as the relative levels by comparing experimental levels to those of the relevant control sample.

**Mouse gonadotrope LβT2 cell line**

The LβT2 cell line was a kind gift of Prof. P. L. Mellon of the University of California, San Diego. The cells were maintained in Dulbecco’s modified Eagle’s medium with high levels of glucose (DMEM; Life Technologies), 10% fetal bovine serum (Life Technologies) and antibiotic-antimycotic supplements (Life Technologies), and they were maintained at 37 °C with 5% CO2. GnRHa (10^-8 M) was added 24 hours after the cells were plated in multiwell plates, and they were further incubated for 0.5, 1, 2, 4 and 8 hours. In addition, the effect of protein synthesis inhibitors was tested by preincubating the cells with cycloheximide (CHX, 50 µM; Sigma-Aldrich, St. Loise, MO) for two hours before GnRHa stimulation. The
cells were also treated with recombinant rat ANXA5 protein (10), which was added either 30 min before or at the same time as GnRHa administration. For immunoblotting, the cells were cultured in 35 mm dishes. The cells were harvested in Laemmli sample buffer after incubation with GnRHa (10^{-8} \text{ M}) or following transfection of the expression vector.

**Western blotting**

LβT2 cell lysates were loaded on a 12\% SDS-PAGE gel and then were transferred to PVDF membranes (Amersham™ Hybond™ P 0.45, GE Healthcare UK Ltd, Buckinghamshire, UK). After blocking with 5\% nonfat milk in TBST, the membranes were probed with primary antibodies against NR4A3 (1:1,000 dilution; anti-human NGFI-B gamma mouse monoclonal antibody, Perseus Proteomics Inc., Tokyo, Japan) (14), ANXA5 (1:10,000 dilution; polyclonal rabbit sera against rat ANXA5) (15) and β-actin (1:2,000 dilution; mouse monoclonal sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA) (16) at 4 °C overnight. Then, the membranes were incubated with an ECL™ peroxidase-labeled anti-mouse antibody or anti-rabbit antibody (GE Healthcare UK Ltd) (17,18), which function as a secondary antibody. Immunoreactivity was detected by chemiluminescence with ECL Western blotting detection reagents (GE Healthcare UK Ltd), and blots were scanned using an ImageQuant LAS 4000 system (GE Healthcare UK Ltd).

**Immunocytochemistry**

LβT2 cells were seeded on poly-L-lysine-coated coverslips and were grown for two days. After incubation with GnRHa (10^{-8} \text{ M}), the cells were fixed with 4\% PFA-PBS at room temperature for 10 min and then were blocked by incubating with 10\% normal goat serum for 30 min. Immunocytochemistry was performed using an indirect immunofluorescence technique with an anti-NR4A3 primary antibody (1:2,000) and an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:200; Life Technologies) (19). To visualize the actin cytoskeleton, F-actin was stained with Alexa Fluor 568 phalloidin (Life Technologies) for 30 min. The specimens were mounted with VECTASHIELD mounting medium with DAPI (Vector...
Laboratories, Burlingame, CA), and they were visualized using a confocal laser microscope (LSM710, Carl Zeiss Japan, Tokyo, Japan).

**Transfection of the vector and siRNA**

Rat *Anxa5* cDNA was previously cloned into BamHI site of plasmid pUC119 (20), subcloned into BamHI site of plasmide pcDNA3.1(−) (Invitrogen, Carlsbad, CA) and sequenced (LC533519). The transfection of the expression vectors for ANXA5 (pcANXA5) was performed by means of electroporation using an NEPA21 electroporator and electroporation cuvettes (Nepa Gene Co. Ltd., Chiba, Japan). A suspension of LβT2 cells (10^6 cells/100 µl) was prepared by detaching the cells with trypsin, and the cells were then mixed with 10 µg plasmid vector and electroporated by applying two pulses of 175 V for 5 ms at 50 ms intervals.

Nr4a3 Silencer® Select siRNA (ID: s70687) and Silencer® Select Negative Control #1 siRNA were obtained from Ambion (Austin, TX). LβT2 cells were transfected with each type of siRNA (66 pmol/ml final concentration) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

After transfection with the expression vectors or siRNA, the cells were incubated for 48 hours, and then they were treated with GnRHa (10^{-8} M).

**Statistics**

The results are presented as the mean ± SEM. Significant differences were analyzed with Student’s t-tests or one- or two-way ANOVA followed by a Tukey-Kramer test. A P-value less than 0.05 was considered statistically significant.
Results

Expression changes in the anterior pituitary glands of Anxa5−/− mice

To determine the effect of ANXA5 deficiency on anterior pituitary gland hormone production, we analyzed the relative expression levels in RNA extracts from the pituitary glands of C57BL/6J mouse controls and Anxa5−/− mutants using real-time PCR. Among the seven pituitary hormone genes analyzed, we observed that the FSH beta subunit (Fshb) and growth hormone (Gh) expression levels were significantly reduced in the Anxa5−/− pituitary glands (Fig. 1). We also studied global expression changes and performed a transcriptome analysis of the anterior pituitary glands of C57BL/6J and Anxa5−/− mice. The results from the microarray analysis showed a 1.4-fold decrease in Fshb mRNA in the Anxa5−/− pituitary glands compared to the level of the control, whereas the expression of Gh and other pituitary hormone genes remained unchanged compared to that of the control (Table 2). Fourteen of 23,304 genes were differentially expressed, as defined by having a two-fold or greater difference. To confirm the results, quantitative PCR analysis was performed. Here, we confirmed that Slitrk3, C10orf11, Nr4a3 and Mme were upregulated 2.9-, 7.0-, 6.8- and 4.3-fold, respectively, and Mid1 was downregulated 6.7-fold in the pituitary glands of Anxa5−/− mice (Fig. 2).

ANXA5-controlled genes responding to GnRH stimulus in the pituitary glands

To identify the GnRH-responsive genes within the cluster of differentially expressed genes, C57BL/6J mice were received multiple administrations of GnRHa or saline. Then, RNA from their pituitary glands was isolated, and relative gene expression was determined using quantitative PCR. Here, we observed that the administration of GnRHa significantly increased Nr4a3 expression two-fold and decreased Akr1c18 expression three-fold in the pituitary glands compared to the levels determined for the saline control (Fig. 3). AKR1C18 is a 20α-hydroxysteroid dehydrogenase (20α-HSD) that converts progesterone to the nonactive metabolite 20α-hydroxyprogesterone in ovaries (21). Akr1c18 was shown to be induced by PPARalpha in the pituitary gland (22). In addition, we confirmed a previously described increase in the
expression of Anxa5.

**Induction and intranuclear accumulation of NR4A3 by GnRH**

Interestingly, *Nr4a3* expression was increased in *Anxa5*−/− pituitary gland tissue, and GnRH could induce *Nr4a3* and *Anxa5* expression. Therefore, we compared the levels of *Anxa5* and *Nr4a3* expression in the mouse gonadotrope cell line LβT2. GnRHa stimulation caused a single peak of transiently increased *Nr4a3* mRNA expression one h after stimulation (Fig. 4A). In contrast, *Anxa5* mRNA expression showed a constant increase eight h after the LβT2 cells were treated (Fig. 4B). To examine whether the synthesis of *Nr4a3* mRNA was dependent on protein synthesis, LβT2 cells were incubated with the protein synthesis inhibitor cycloheximide (CHX) before GnRHa treatment. The kinetics of *Nr4a3* mRNA expression were not affected by the addition of the inhibitor. Hence, *Nr4a3* is a direct immediate early gene of GnRH, and stimulation of *Nr4a3* mRNA expression is independent of protein synthesis processes (Fig. 4C). The results from the immunoblot analysis showed that the increase in mRNA expression was accompanied by a peak of increased NR4A3 protein (~75 kD band) expression two h after GnRHa stimulation (Fig. 4D). Immunocytochemistry clearly showed that NR4A3 protein levels were increased, and NR4A3 was detected within the nuclei of the LβT2 cells after GnRHa treatment (Fig. 4E).

**Inhibitory effect of de novo synthesized and exogenous ANXA5 on *Nr4a3* expression**

Because *Nr4a3* expression is enhanced in the pituitary glands of ANXA5-deficient mice, we hypothesized that *Nr4a3* expression is inhibited in the presence of ANXA5. We therefore studied the effect of increasing ANXA5 levels on *Nr4a3* mRNA expression in LβT2 cells using transient transfection protocols (Fig. 5A). ANXA5 expression was successfully increased two days after transfection, and the *Nr4a3* mRNA levels were significantly reduced compared to the levels in the control cells after GnRH stimulation (Fig. 5B). We previously showed a stimulatory effect of externally added recombinant rat ANXA5 (rANXA5) on gonadotropin release from primary pituitary cells (9), which indicated that exogenous ANXA5 can act on gonadotropes to cause release the hormone. Therefore, the LβT2 cells were
simultaneously incubated with GnRHa and rANXA5 to study the combined effect on *Nr4a3* expression. The administration of rANXA5 in addition to GnRHa significantly inhibited *Nr4a3* mRNA expression after one h in a dose-dependent manner (Fig. 5C). The preincubation with rANXA5 for 30 min (Fig. 5D) or the preincubation and simultaneous incubation with GnRHa (Fig. 5E) showed a similar inhibitory effect on *Nr4a3* mRNA expression.

**Effect of Nr4a3 on gonadotropin gene expression**

Next, we used siRNA to knock down *Nr4a3* in LβT2 cells, so that we could determine whether NR4A3 could regulate gonadotropin gene expression. The *Nr4a3* mRNA levels were significantly reduced between one and two h after GnRHa administration (expression ratio of NR4A3 siRNA transfected cells to Control siRNA transfected cells: zero h, 55 %; one h, 66 %; two h, 47 %; 4 h, 56 %; 8 h, 62 %) (Fig. 6A); further, *Fshb* mRNA expression, which is known to be increased after stimulation, was increased in the siRNA for NR4A3 transfected cells between one and eight h after GnRHa administration compared to the expression levels observed in the control cells (Fig. 6B). *Lhb* mRNA expression levels showed a 40% decrease within the first two h of GnRHa administration, and thereafter, normal levels were reestablished (Fig. 6C); however, the expression of the common alpha subunit (*Cga*) mRNA was not significantly altered in the NR4A3 siRNA transfected LβT2 cells (Fig. 6D). This finding suggests that the induction of NR4A3 by GnRH can suppress *Fshb* gene expression.
Discussion

Here, we demonstrated a unique system for regulating Fshb expression among gonadotropin subunits through GnRH and ANXA5 in pituitary gonadotropes. While GnRH stimulates both LH and FSH secretion, the secretion patterns of these hormones are not always identical. Therefore, specific regulation for each subunit has been presumed.

Mittag et al. first reported that Fshb mRNA expression levels were specifically lowered among anterior pituitary hormone genes in immature three-week-old male Anxa5−/− mice (23). As ANXA5 was suggested to enhance the expression of Fshb mRNA, we have now identified the genes specifically regulated in ANXA5-deficient pituitary glands of adult female mice. The present study shows a regulatory network involving ANXA5 and the transcription factor NR4A3, and both were enhanced by GnRH in pituitary gonadotrope cells. NR4A3 is proposed to suppress the expression of Fshb mRNA.

The expression of Nr4a3 is enhanced in the ANXA5-deficient pituitary glands, and in contrast, its expression is suppressed by the overexpressed ANXA5 protein such that ANXA5 represents a negative regulator of Nr4a3 expression. The effect of ANXA5 on NR4A3 was highly effective as pre-administration of ANXA5 elicited the same effect as when ANXA5 was given with GnRHa. In turn, NR4A3 was suggested to suppress Fshb mRNA, since overexpression of Nr4a3 in ANXA5-deficient mice accompanied decreased Fshb expression and the suppression of Nr4a3 by siRNA augmented Fshb mRNA expression.

Here, we demonstrated the influence of ANXA5 and NR4A3 on Fshb mRNA expression. Nr4a3 was shown to be an immediate early gene (IEG) induced by the GnRH receptor. NR4A3 (also known as neuron-derived orphan receptor 1, NOR1) is known as a nuclear orphan receptor in the NR4A protein family, which includes NR4A1 and NR4A2. It has been reported that the NR4A protein is induced by various stimuli, including the activation of GPCRs (24-31). In the present study, the NR4A3 protein immediately accumulated in the nucleus upon GnRHa administration. The NR4A protein was also reported to translocate into nuclei in a ligand-independent manner (32). Our data further support the idea that NR4A3 has a primary role in regulating gene expression upon GnRH stimulation. As ANXA5 expression is also stimulated by
GnRH after NR4A3, we assume that the sequential expression of NR4A3 and ANXA5 may define a regulatory network linked with GnRH effects on FSH secretion.

In Nr4a3 knockdown experiments, the increase in Fshb mRNA following GnRHa administration continued for at least 8 hours after administration. This result suggests that the transient increase in NR4A3 after GnRHa stimulation elicits long-lasting effects, such as the following: 1) direct inhibition of Fshb transcription activity, 2) repression of the effector of Fshb expression and 3) suppression of Fshb mRNA stability. Verification of multiple aspects of this mechanism, such as measuring transcription activity by reporter gene assay and analyzing direct DNA binding by ChIP assay, will be helpful for elucidating the effect of NR4A3 on Fshb mRNA expression.

NR4A3 is known as a nuclear receptor that is predicted to interact with a specific sequence. To date, we do not know how NR4A3 affects Fshb mRNA expression, and we need to examine the relationship between NR4A3 and the reported gene products that affected Fshb mRNA. Because NR4A3 was shown to interact with the SIX3 homeodomain transcription factor and SIX3 is expressed in gonadotropes (33,34), we assume the involvement of the interaction between NR4A3 and SIX3 in the functional network of GnRH-ANXA5-NR4A3-Fshb.

Although various molecular functions have been proposed for ANXA5, including the inhibition of protein kinase C (PKC) (35), the formation of calcium channels in phospholipid membranes (36), the binding of calcium ions, and the interaction with actin and collagen (37,38), the physiological significance of these functions is still unknown. Therefore, the functional relationship between ANXA5 and NR4A3 expression is a part of the next subject of investigation into the molecular mechanism for ANXA5 function. Although the present study showed that ANXA5 enhances FSH synthesis, it is not known how it works physiologically. We have already revealed that ANXA5 augments gonadotropin secretion (9), but ANXA5-deficient mice exhibit regular estrous cycles and ovulation numbers (39). Brachvogel et al. also did not observe any apparent phenotype in reproductive function in ANXA5−/− (13). This is probably due to the presence of a redundant mechanism of the other annexin family members, ANXA1~13, 12 does not exist.
Recently, we demonstrated that the expression of ANXA1 is also facilitated by GnRH (40). Further analysis of ANXA5 function as it relates to gonadotropin secretion is needed.

It is well known that FSH and LH have different secretion patterns. This difference is sometimes explained by the difference in the pulsatile pattern of GnRH release. The increase in GnRH pulse frequency leads to downregulation of FSH synthesis and release, which is recognized as one of the switching processes between follicular development and maturation (41-43). This inhibitory mechanism is suggested to be involved in the suppression of Fshb under high-frequency GnRH stimulation, in which negative effectors on Fshb gene transcription, such as ICER, SKIL and TGIF1, are induced (7,44,45). Since Nr4a3 is transiently induced by GnRH as an IEG and is suggested to specifically suppress the Fshb gene expression level, NR4A3 would also be involved in the frequency-dependent mechanism by which GnRH regulates FSH secretion.

The present results clearly demonstrate a specific functional relationship among GnRH, ANXA5 and NR4A3. GnRH stimulation of NR4A3 expression that is suggested to suppress FSH expression is followed by augmentation of ANXA5 that downregulates NR4A3 expression. This proposed relationship would consist of a regulatory network that controls Fshb expression through association with the mechanisms that establish the harmonized secretion of LH and FSH.

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**Table and Figure Legends**

Table 1. Primer list

Table 2. Gene expression in the pituitary gland of *Anxa5*−/− mice.

Genes showing more than a 2-fold change and those encoding pituitary hormones are listed. Gene expression in *Anxa5*−/− mice was compared to that of C57BL/6 mice.

Fig. 1. Pituitary hormone gene expression in *Anxa5*−/− mice

The levels of *Lhb*, *Fshb*, *Cga*, *Tshb*, *Gh*, *Prl* and *Pomc* mRNA expression in wild-type (C57BL/6) and *Anxa5*−/− mice were analyzed by quantitative real-time PCR. Data are depicted as the mean ± SEM (n = 5). Statistical analysis was performed with Student’s *t*-tests (*: *P* < 0.05). (*Lhb*, luteinizing hormone beta subunit; *Fshb*, follicle stimulating hormone beta subunit; *Cga*, common alpha glycoprotein subunit; *Tshb*, thyroid stimulating hormone beta subunit; *Gh*, growth hormone; *Prl*, prolactin; and *Pomc*, pro-opiomelanocortin).

Fig. 2. Expression levels of the thirteen genes affected by ANXA5 deficiency in the pituitary glands listed in Table 2

Relative mRNA levels in the pituitary glands of C57BL/6 and *Anxa5*−/− mice were measured by real-time q-PCR, and the relative expression levels in the control animals were set at 1.0. Data are depicted as the mean ± SEM (n = 5). Statistical analysis was performed with Student’s *t*-tests (**: *P* < 0.01).

Fig. 3. Effects of GnRHa stimulation on genes differentially expressed in ANXA5-deficient pituitary glands

C57BL/6 control mice were repeatedly administered a GnRH agonist (GnRHa, 5 ng/50 µl, 10 times) to obtain a large response, or they were administered saline alone; then, relative mRNA levels in the
pituitary glands were measured by real-time q-PCR. Data are depicted as the mean ± SEM (n = 5). Statistical analysis was performed with Student’s t-tests (*: P < 0.05).

Fig. 4. Induction of Nr4a3 expression and accumulation of NR4A3 protein in the nuclei after GnRH stimulation in gonadotrope cell line LβT2

Nr4a3 (A) and Anxa5 (B) mRNA expression was observed after LβT2 cells were incubated with GnRHa (10⁻⁸ M) for 0-8 h (mean ± SEM; n = 4). Statistical analysis was performed with one-way ANOVA and a Tukey-Kramer test (**: P < 0.01 versus 0 h). (C) LβT2 cells were preincubated with or without cycloheximide (CHX, 50 µM) for 2 h. Nr4a3 mRNA expression was measured after GnRHa (10⁻⁸ M) treatment for 1 h (mean ± SEM; n = 4). Statistical analysis was performed with two-way ANOVA and a Tukey-Kramer test (**: P < 0.01). NR4A3 protein expression was observed by Western blotting (D) and immunocytochemistry (E) of LβT2 cells in the presence of GnRHa (10⁻⁸ M) for 0-8 h and 0-4 h, respectively. (E) Distribution of the NR4A3 protein (green), actin cytoskeleton (red) and nuclei (blue) in cell aggregates was observed by confocal laser scanning microscopy. Scale bars indicate 10 µm (E).

Fig. 5. Suppression of Nr4a3 expression by ANXA5

(A) LβT2 cells were transfected with an ANXA5 expression vector (pcANXA5) or an empty vector (pcDNA), and the expression of the ANXA5 protein was detected by Western blotting for mouse and rat ANXA5 protein. (B) Nr4a3 mRNA expression in the cells transfected with pcANXA5 or pcDNA was measured after induction with GnRHa (10⁻⁸ M) for 1 h (mean ± SEM; n = 4; *: P < 0.05; and **: P < 0.01). (C, D and E) LβT2 cells were incubated for 1 h in the absence or presence of GnRHa (10⁻⁸ M), and the Nr4a3 mRNA levels were measured (mean ± SEM; n = 4; **: P < 0.01). Recombinant ANXA5 (rANXA5, 0-10 µg/ml) was used as a treatment in the three conditions: simultaneous administration with GnRHa (C, Simultaneous), preadministration 30 min before incubation with GnRHa alone (D, Pre) or preadministration and simultaneous administration (E, Pre+simultaneous). Statistical analysis was performed with two-way ANOVA and a Tukey-Kramer test.
Fig. 6. Effect of RNA interference targeting *Nr4a3* on gonadotropin genes

LβT2 cells were transfected with an *Nr4a3*-specific siRNA (*Nr4a3* siRNA; filled triangle) or a negative control siRNA (Control; open circle), and then the cells were incubated for 48 h. *Nr4a3* (A), *Fshb* (B), *Lhb* (C) and *Cga* (D) mRNA expression in cells treated with GnRHa (10<sup>-8</sup> M) was measured by q-PCR at 0-8 h (mean ± SEM; n = 4). Relative levels of mRNA are indicated compared with levels of the control group at the start (0 h) of treatment. Statistical analysis was performed with two-way ANOVA and a Tukey-Kramer test (***: P < 0.01). *: P < 0.05 and **: P < 0.01 indicate comparison to the levels at 0 h under the same conditions as those of the siRNA transfected cells, and #: P < 0.05 and ###: P < 0.01 indicate comparison to the control group at each time point.
### Table 1.

**Primer list**

| Primers | Forward (5’-3’) | Reverse (5’-3’) |
|---------|----------------|-----------------|
| Lhb     | GTCTGCA TCACCTTCACCAC | GTAGGTGCACACTGGCTGAG |
| Fshb    | CTGCTGCCA TAGCTGTGAAT | GAGCTGGGTCCTTTATAACACCA |
| Cga     | ATCACCTGCCCAGAACACTAT | ACATGGACAGCATGACAGCAGA |
| Tshb    | CCA TCAACACCACCTCTGT | CCTGGTATTTCCACCCTTCT |
| Gh      | GTGGACAGATC ACTGCTTGG | GGAAAAGC TACAGCCTCCTG |
| Prl     | CTCAGGGCATCTGGAGAAG | TCGGAGAGAAGTCTGGCAAG |
| Pomc    | GCCACTGAACATCTTTGTC | CGACTGTA CGAGAATCTCG |
| Slitrk3 | TCTGAGGACTCTGGCTCAATC | AA TGGGACAGGTGTCAG |
| C10orf11 | TACCCCACTTGCACACCTTA | CAAGTTGACAGCTCATTGG |
| Nr4a3   | CCGAGCTTTAAACAGATGCAA | AGCTTCTGGC ACACTCAATG |
| Gm7120  | CGGGATTTTTAGCTTGTCTT | ATGGTGATCAGAATGGACAG |
| Mme     | TTCTGTGGCCAAGACTGTACC | ATGGGTCAATTCGCTTTC |
| Gm5148  | CACGAACGCTGTGATCTTCT | CTCATGGCAAGGAAATGTG |
| Mpz     | TCCTTCTTGGTCCTAGTGAAGT | AAGGTGTCCCTTGGCAATG |
| Mid1    | CACCATATTCCACCCGCAAGA | GTGGTCTCTGGCAGTGGG |
| Fabp6   | ACCATGGGCAAAGGTGATGA | GACCTCCAAGGTTCTGGAT |
| Akr1c18 | GATAGGGCCAGGCCATTCTAA | AATTTTTCAAGCTGGGCTTG |
| Pdk4    | CACCACATGCTTTCGAAACT | CTACTGGGTCCAAGGAAAGG |
| Cetn4   | ACAACTGATCGTGAATCG | CGTAGCATCGCATCAAAT |
| Grp     | TCAGGCTCTCTAGCTTCTG | TCCTCCCTTTCTTGGAGAA |
| Anxa5   | GGTACCCAGATGAGGACAGCAT | TCCCTGCCAAACAGAGTCTTG |
| Rpl19   | AGCCTGTGACTGTCACCCTTC | GCATTGGCAGTACCCTTCT |
Table 2.

Gene expression in the pituitary gland of Anxa5<sup>−/−</sup> mice

| Gene ID   | Gene description                                                  | Gene          | Fold |
|-----------|-------------------------------------------------------------------|---------------|------|
| **Genes upregulated in the anterior pituitary gland of Anxa5<sup>−/−</sup>** |                                                                    |               |      |
| NM_198864 | SLIT and NTRK-like family, member 3                               | Slitrk3       | 2.84 |
| NM_028275 | RIKEN cDNA 1700112E06 gene                                        | C10orf11     | 2.64 |
| NM_015743 | nuclear receptor subfamily 4, group A, member 3                   | Nr4a3        | 2.28 |
| NM_001039244 | predicted gene 7120                                           | Gm7120       | 2.09 |
| NM_008604 | membrane metalloendopeptidase (CD10)                             | Mme          | 2.02 |
| **Genes downregulated in the anterior pituitary gland of Anxa5<sup>−/−</sup>** |                                                                    |               |      |
| NM_198657 | predicted gene 5148                                              | Gm5148       | -4.71|
| NM_008623 | myelin protein zero                                              | Mpz          | -4.20|
| NM_010797 | midline 1 (Tripartite motif protein 18)                           | Mid1         | -3.16|
| NM_008375 | fatty acid binding protein 6 (gastrotropin)                      | Fabp6        | -2.73|
| NM_134066 | aldo-ketoreductase family 1, member C18                         | Akr1c18      | -2.46|
| NM_013743 | pyruvate dehydrogenase kinase, isoenzyme 4                       | Pdk4         | -2.46|
| NM_009673 | annexin A5                                                       | Anxa5        | -2.19|
| NM_145825 | centrin 4                                                        | Cetn4        | -2.02|
| NM_175012 | gastrin releasing peptide                                        | Grp          | -2.00|
| **Pituitary hormone genes** |                                                                    |               |      |
| NM_008497 | luteinizing hormone beta                                         | Lhb          | 1.05 |
| NM_008045 | follicle stimulating hormone beta                                | Fshb         | -1.41|
| NM_009889 | glycoprotein hormones, alpha subunit                            | Cga          | 1.02 |
| NM_00943  | thyroid stimulating hormone beta                                 | Tshb         | 1.10 |
| NM_008117 | growth hormone                                                   | Gh           | -1.01|
| NM_011164 | Prolactin                                                        | Prl          | -1.00|
| NM_008895 | pro-opiomelanocortin-alpha                                       | Pomp         | 1.10 |
Figure 1

![Bar Chart]

Bar chart showing relative gene expression of C57BL/6 and Anxa5^{-/-} for various genes:
- Lhb
- Fshb
- Cga
- Tshb
- Gh
- Prl
- Pomc

* indicates a significant difference.
Figure 2

[Bar chart showing gene expression levels relative to C57BL/6 and Anxa5−/− genotypes.]

Bar labels include: S1rk3, C10orf11, Nr4a3, Gm7120, Mme, Gm5148, Mpz, Mid1, Fabp6, Akr1c18, Pdk4, Cen14, Grp.
Figure 3

[Bar graph showing relative gene expression data for various genes after saline and GnRHa administration.]
Figure 5

A

B

C Simultaneous

D Pre

E Pre+Simultaneous

Relative Mr4a3 mRNA expression levels

rANXA5 (µg/ml)

Control  GnRHa (1 h)

Relative Mr4a3 mRNA expression levels

rANXA5 (µg/ml)

Control  GnRHa (1 h)

Relative Mr4a3 mRNA expression levels

rANXA5 (µg/ml)

Control  GnRHa (1 h)
Figure 6

A. Relative NR4a3 mRNA expression levels over time (h) after GnRHa treatment.

B. Relative Fshr mRNA expression levels over time (h) after GnRHa treatment.

C. Relative Lhβ mRNA expression levels over time (h) after GnRHa treatment.

D. Relative Cge mRNA expression levels over time (h) after GnRHa treatment.

- Control
- NR4A3 siRNA