

## Parallel expression patterns of NR4A nuclear receptor family genes in the pituitary gland of proestrus rats

Ryota TERASHIMA<sup>1</sup>, Daiki NAGAO<sup>1</sup>, Masato IKEO<sup>1</sup>, Keisuke MORIOKA<sup>1</sup>, Titaree LAOHARATCHATATHANIN<sup>2</sup>, Shiro KURUSU<sup>1</sup> and Mitsumori KAWAMINAMI<sup>3</sup>

<sup>1</sup>Laboratory of Veterinary Physiology, School of Veterinary Medicine, Kitasato University, Aomori 034-8628, Japan

<sup>2</sup>Clinic for Small Domestic Animals and Radiology, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok 10530, Thailand

<sup>3</sup>Laboratory of Veterinary Physiology, School of Veterinary Medicine, Okayama University of Science, Ehime 794-8555, Japan

**Abstract.** The NR4A nuclear receptor family (NR4As), encompassing NR4A1, NR4A2, and NR4A3, exerts pivotal roles in cellular processes through intricate expression patterns and interactions. Despite the influence of some NR4As on anterior pituitary functions regulated by the hypothalamus, their physiological expression patterns remain unclear. In our prior work, we demonstrated the specific upregulation of NR4A3 in the rat anterior pituitary gland during the proestrus afternoon, coinciding with a gonadotropin surge. In this study, we investigated changes in pituitary *Nr4a* gene expression throughout the estrous cycle in rats and a gonadotropin surge-induced model. *Nr4a1* and *Nr4a2* gene expression significantly increased during proestrus, aligning with previous observations for *Nr4a3*. Furthermore, *prolactin* gene expression increased sequentially with rising *Nr4a* gene expression, while *thyroid-stimulating hormone beta* gene expression remained stable. Immunohistochemistry revealed a widespread and differential distribution of NR4A proteins in the anterior pituitary, with NR4A1 and NR4A3 being particularly abundant in thyrotrophs, and NR4A2 in gonadotrophs. In estrogen-treated ovariectomized rats, elevated luteinizing hormone secretion corresponded to markedly upregulated expression of *Nr4a1*, *Nr4a2*, and *Nr4a3*. In gonadotroph and somatomammotroph cell lines, gonadotropin- and thyrotropin-releasing hormones transiently and dose-dependently increased the expression of *Nr4a* genes. These findings suggest that hypothalamic hormone secretion during proestrus may induce the parallel expression of pituitary *Nr4a* genes, potentially influencing the pituitary gene expression program related to endocrine functions before and after ovulation.

**Key words:** Anterior pituitary gland, Estrous cycle, NR4A1, NR4A2, NR4A3

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The NR4A nuclear receptor family (NR4As), comprising NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor1), consists of orphan receptors with unknown physiological ligands. *Nr4as*, as immediate early response genes, respond to various stimuli and exhibit both overlapping and distinct functions [1, 2]. They are frequently co-regulated in parallel across different cell types, including hepatocytes, skeletal muscle cells, thymocytes, macrophages, ovarian granulosa cells, and pituitary corticotroph cells [3–8].

NR4As play crucial roles in diverse cellular processes [9]. Their activity primarily relies on changes in gene expression, post-translational modifications, and interactions with co-regulatory proteins, rather than ligand binding. NR4A1, for instance, is implicated in endocrine signaling, as it can interact with activated estrogen receptor alpha, androgen receptor, and glucocorticoid receptor, inhibiting their DNA binding and activity [10–13]. This interaction suggests that NR4As are involved in endocrine feedback regulation at the hypophyseal and pituitary levels.

Comprising three domains, NR4As exhibit an exceptionally high

degree of sequence homology within their DNA binding domain and approximately 60% homology within their ligand-binding domain [9]. NR4As function as monomers when binding to the NGFI-B response element sequence [14]. They also bind to palindromic Nur77 response element (NurRE) sequences as homodimers or heterodimers of NR4As [9]. The first functional NurRE was identified in the promoter of the *pro-opiomelanocortin (Pomc)* gene in pituitary-derived AtT-20 cells [15]. Notably, the heterodimer activated *Pomc* transcription more potently than the NR4A1 homodimer, indicating an interdependence among NR4As in activating their target genes [16]. Furthermore, NR4As can potentially regulate transcriptional activity by interacting with other transcription factors, either by directly enhancing or inhibiting the activities of these transcription factors [9]. Therefore, exploring the intricate relationship between NR4As and their interactions with other factors is crucial to elucidate their multifaceted roles.

The hypothalamus-hypophyseal system critically regulates endocrine-controlled biological functions, such as reproduction, metabolism, growth, and stress responses, by integrating internal and external signals. Hypothalamic hormones, such as corticotropin-releasing hormone and thyrotropin-releasing hormone (TRH), influence the expression of NR4As in anterior pituitary endocrine cells [17–19]. Pituitary NR4As may also participate in regulating *Pomc* gene expression and providing feedback control for the hypothalamic-pituitary-adrenal axis during stress responses [13, 16–18, 20]. However, limited data are available on changes in

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Correspondence: R Terashima (e-mail: rterashi@vmas.kitasato-u.ac.jp)

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pituitary NR4A expression under different physiological conditions. In a recent study, we observed a significant transient upregulation of *Nr4a3* expression in the rat anterior pituitary during the afternoon of proestrus [21]. We further demonstrated that this increase in *Nr4a3* expression was induced by gonadotropin-releasing hormone (GnRH) and TRH [21, 22]. During this period, elevated levels of 17 $\beta$ -estradiol ( $E_2$ ) secreted from developing follicles triggered positive feedback effects on the hypothalamus, activating hypothalamic hormone secretion and resulting in gonadotropin surges and the activated secretion of pituitary hormones [23, 24]. These observations strongly suggest that the secretion of hypothalamic hormones activated by  $E_2$  induces pituitary NR4As, including NR4A3.

In the present study, we investigated the kinetics of *Nr4a* gene expression during the estrous cycle and the inducibility of *Nr4a* genes in an  $E_2$ -induced gonadotropin surge model and cell lines stimulated with GnRH and TRH. We compared the expression patterns of *Nr4a* genes and pituitary hormone genes to examine the involvement of NR4As in pituitary hormone synthesis.

## Materials and Methods

### Animals

Female Wistar–Imamichi rats (200–300 g) were obtained from SLC Japan (Shizuoka, Japan) and were housed under controlled conditions (temperature: 23  $\pm$  3°C; light: 14 h; dark: 10 h; lights on at 0500 h). Rats had ad libitum access to laboratory chow and tap water. All animal protocols were approved by the Animal Care and Use Committee of Kitasato University School of Veterinary Medicine (approval no. 19-173).

### Determination of the estrous cycle

The estrous cycle stages (proestrus, estrus, and diestrus) were determined based on specific vaginal cytological characteristics. Vaginal smears were monitored daily for at least 2 weeks before the experiments. Female rats with regular 4-day estrous cycles were selected. Animals were euthanized by decapitation, and anterior pituitary tissues were promptly collected and frozen in liquid nitrogen. For immunohistochemistry, rats under isoflurane anesthesia were perfused with 4% paraformaldehyde in 50 mM phosphate buffer using a peristaltic pump, and pituitary tissues were collected and fixed overnight at 4°C in 4% paraformaldehyde in 50 mM phosphate buffer.

### Estrogen-primed gonadotropin surge-induced rat model

Ovariectomized rats were continuously treated with  $E_2$  (Sigma–

Aldrich, St. Louis, MO, USA) to induce an evening gonadotropin surge. Bilateral ovariectomy was performed under isoflurane anesthesia. One week post-surgery, ovariectomized rats were subcutaneously implanted with a 3 cm long Silastic capsule (100-2N; Kaneka Medix Co., Osaka, Japan) containing either 0.1  $\mu$ g/100  $\mu$ l or 10  $\mu$ g/100  $\mu$ l  $E_2$  in sesame oil. Simultaneously, ongoing cytological evaluation of vaginal smears monitored estrus status. Three days post-implantation, anterior pituitary and trunk blood samples were collected at 1100 h, 1400 h, and 1700 h.

### Cell culture

The L $\beta$ T2 mouse gonadotroph cell line [25] was cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco Fetal Bovine Serum, Thermo Fisher Scientific) at 37°C in a 5% CO<sub>2</sub> environment. The GH3 rat somatomammotroph cell line [26] was cultured in DMEM/F12 (Thermo Fisher Scientific) with 15% normal horse serum (Gibco Horse Serum, Thermo Fisher Scientific) and 2.5% fetal bovine serum under the same conditions. L $\beta$ T2 and GH3 cells underwent treatment with GnRH (Peptide Institute Inc., Osaka, Japan) or TRH (Sigma–Aldrich) at a concentration of 100 nM for various durations (0, 0.5, 1, 2, 4, and 8 h) or various concentrations (0, 0.1, 1, 10, and 100 nM) for 1 h. Subsequently, post-culture, the cells were harvested using ISOGEN (Nippon Gene, Tokyo, Japan) and stored at –80°C.

### Reverse transcription-quantitative PCR

Total RNA was extracted from pituitary tissues and cells using ISOGEN and reverse transcribed to cDNA using a High-Capacity cDNA Synthesis Kit (Thermo Fisher Scientific). Primer sequences for all reverse transcription-quantitative PCR (RT-qPCR) assays are listed in Table 1. qPCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. The cycle-threshold (Ct) values were set at 0.5 delta Rn value, and relative gene expression levels were calculated using the  $\Delta\Delta$ CT method with ribosomal protein L19 (*Rpl19*) as the internal control for normalization.

### Determination of luteinizing hormone levels

Plasma luteinizing hormone (LH) concentrations were measured using a time-resolved fluorometric immunoassay (DELFIASystem; PerkinElmer, Branchburg, NJ, USA). Purified rat LH (LH-I-9, National Institute of Diabetes and Digestive and Kidney Diseases, NIDDK,

**Table 1.** Sequences of the primers used in this study

Species	Gene	Forward primer (5'–3')	Reverse primer (5'–3')
Rat	<i>Nr4a1</i>	ATTTGGACTCCGGGCTAAC	GGAGCCAGAGAGCAAGTCAT
Rat	<i>Nr4a2</i>	TGTCAGCATTACGGTGTTTCG	ACCGACAGTACTGACAACGAT
Rat	<i>Nr4a3</i>	AAAGACGGAACTCCACAGAA	GTCGGGATAGGCGAAGCA
Rat	<i>Prl</i>	AAGGTGTCGAAGGTTTATAAAGTCA	ATCATCAGCAGGAGGAGTGT
Rat	<i>Tshb</i>	CAGCATTAACCTGCCAGTGC	GATGACACTTGCCCCACAAGC
Rat	<i>Rpl19</i>	GGAAGCCTGTGACTGTCCAT	ATCCTTCGCATCCAGGTCAC
Mouse	<i>Nr4a1</i>	GTGGTGACAATGCTTCGTGT	GCAGATGTACTTGGCGCTTT
Mouse	<i>Nr4a2</i>	TGTCGGTTTCAGAAGTGCCCT	CTTCGGCTTCGAGGGTAAAC
Mouse	<i>Nr4a3</i>	CCGAGCTTTAACAGATGCAA	AGCTTCTGGACACGTCAATG
Mouse	<i>Lhb</i>	GTCTGCATCACCTTACCAC	GTAGGTGCACACTGGCTGAG
Mouse	<i>Rpl19</i>	AGCCTGTGACTGTCCATTCC	GCATTGGCAGTACCCTTCT

Torrance, CA, USA) was labeled with europium (Eu) using a DELFIA Eu-Labeling Kit (PerkinElmer, Waltham, MA, USA). Anti-rabbit gamma globulin goat serum was prepared in our laboratory and purified via ammonium sulfate precipitation.

A 96-well immunoplate (Nunc, Tokyo, Japan) was coated with anti-rabbit gamma globulin and overlaid with a 1:4,000 dilution of NIDDK anti-rat LH-S-11 (NIDDK). Diluted plasma samples and LH standards (rat LH-RP-3, NIDDK) were incubated at 4°C overnight, and optimally diluted Eu-labeled hormones were added for 2 h at 20–25°C. After washing, the plates were treated with DELFIA Enhancement Solution (PerkinElmer), and specific fluorescence (excitation and emission wavelengths of 340 and 613 nm, respectively) was measured using a Multilabel Reader 2030 ARVO X4 (PerkinElmer). The intra- and inter-assay coefficients of variation for the LH levels were 6.5% and 9.9%, respectively.

### Immunohistochemistry

Each NR4A protein was detected using double-fluorescence immunohistochemistry with a monoclonal antibody and the tyramide signal amplification technique. The anti-NR4A2 mouse monoclonal antibody (#66878-1-Ig; Proteintech Japan, Tokyo, Japan) and anti-NR4A3 mouse monoclonal antibody (#PP-H7833-00; Perseus Proteomics, Tokyo, Japan) were biotinylated using biotin-LC-LC-NHS (Tokyo Chemical Industry, Tokyo, Japan) and purified in 10 kDa MWCO Amicon Ultra-0.5 Centrifugal Filter Units (Merck Millipore, Dublin, Ireland).

The fixed pituitary tissues underwent dehydration using a graded ethanol series, were cleared in xylene, embedded in paraffin, and sectioned at 4 µm with a microtome. Sections were dewaxed in xylene and rehydrated using a graded ethanol series. Heat-induced antigen retrieval was performed using a KOS Histostation Microwave Multifunctional Tissue Processor (Milestone Medical, Sorisole, Italy) with a 10 mM citrate buffer solution (pH 6.0) at 98°C for 25 min.

Endogenous peroxidase was quenched for 30 min with 1% H<sub>2</sub>O<sub>2</sub> in methanol, followed by blocking of endogenous avidin and biotin using an Avidin/Biotin Blocking Kit (Abcam, Tokyo, Japan), as per the manufacturer's instructions. Sections were incubated with 5% normal goat serum in phosphate-buffered saline (PBS) with 0.1% Triton X-100 for 30 min and then with biotinylated NR4A2 antibody (0.1 ng/ml), biotinylated NR4A3 antibody (1 ng/ml), or anti-NR4A1 rabbit monoclonal antibody (1:1,000; #MA5-32647; Thermo Fisher Scientific) overnight at 4°C.

Sections were incubated with HRP-Conjugated Streptavidin (1:4,000; Thermo Fisher Scientific) or goat anti-rabbit IgG HRP conjugate (0.02 µg/ml; #G-21234; Thermo Fisher Scientific) in PBS with 0.5% bovine serum albumin (Sigma–Aldrich) and 0.1% Tween 20 for 30 min. They were then incubated with 50 µM Biotinyl-tyramide (Sigma–Aldrich) in PBS with 0.0015% H<sub>2</sub>O<sub>2</sub> and 0.1% Tween 20 for 15 min. The sections were labeled with fluorescent-labeled streptavidin (1:1,000; Streptavidin, Alexa Fluor 488 Conjugate, ThermoFisher Scientific) for 30 min.

All antibodies (guinea pig antiserum) against the anterior pituitary hormones were purchased from NIDDK. Sections were incubated with primary antibody (LH beta, AFP344191, 1:2,000,000; prolactin (PRL), AFP450191, 1:1,000; thyroid-stimulating hormone (TSH) beta, AFP967793, 1:100,000; growth hormone (GH), AFP12121390, 1:4,000; adrenocorticotrophic hormone (ACTH), AFP71111591, 1:1,000) for 60 min and secondary antibody (1:1,000; goat anti-guinea pig IgG secondary antibody, Alexa Fluor 568, #A110175; Thermo Fisher Scientific) for 60 min. Finally, slides were mounted with Vectashield H1200 mounting medium containing DAPI (Vector

Laboratories, Burlingame, CA, USA), and images were captured using a confocal laser scanning microscope LSM710 (Carl Zeiss, Oberkochen, Germany) with a 63× lens.

### Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Differences were analyzed using a one-way analysis of variance, followed by the Tukey–Kramer test. All statistical analyses were performed using JMP Pro 17.0.0 (SAS Institute, Cary, NC, USA).

## Results

### *Proestrus-specific expression of Nr4a genes in the anterior pituitary gland of rats*

In alignment with our previous findings indicating specific upregulation of pituitary Nr4a3 mRNA between 1400 and 1700 h during proestrus [21], the current study explored the expression kinetics of *Nr4a1* (Fig. 1A) and *Nr4a2* (Fig. 1B) throughout the rat pituitary gland across the estrous cycle. Both genes exhibited a significant increase in mRNA expression between 1400 and 1700 h during proestrus. This pattern was contrasted with the expression of the *prolactin (Prl)* gene in lactotrophs and the *thyroid-stimulating hormone beta (Tshb)* gene in thyrotrophs (both stimulated by TRH). *Prl* mRNA expression gradually increased from 1400 h in proestrus, with a significant rise observed from 2000 h in proestrus to 1100 h in estrus (Fig. 1C). Conversely, no significant change was noted in *Tshb* mRNA expression throughout the cell cycle (Fig. 1D).

### *Differential distribution of NR4As in pituitary endocrine cells of rats*

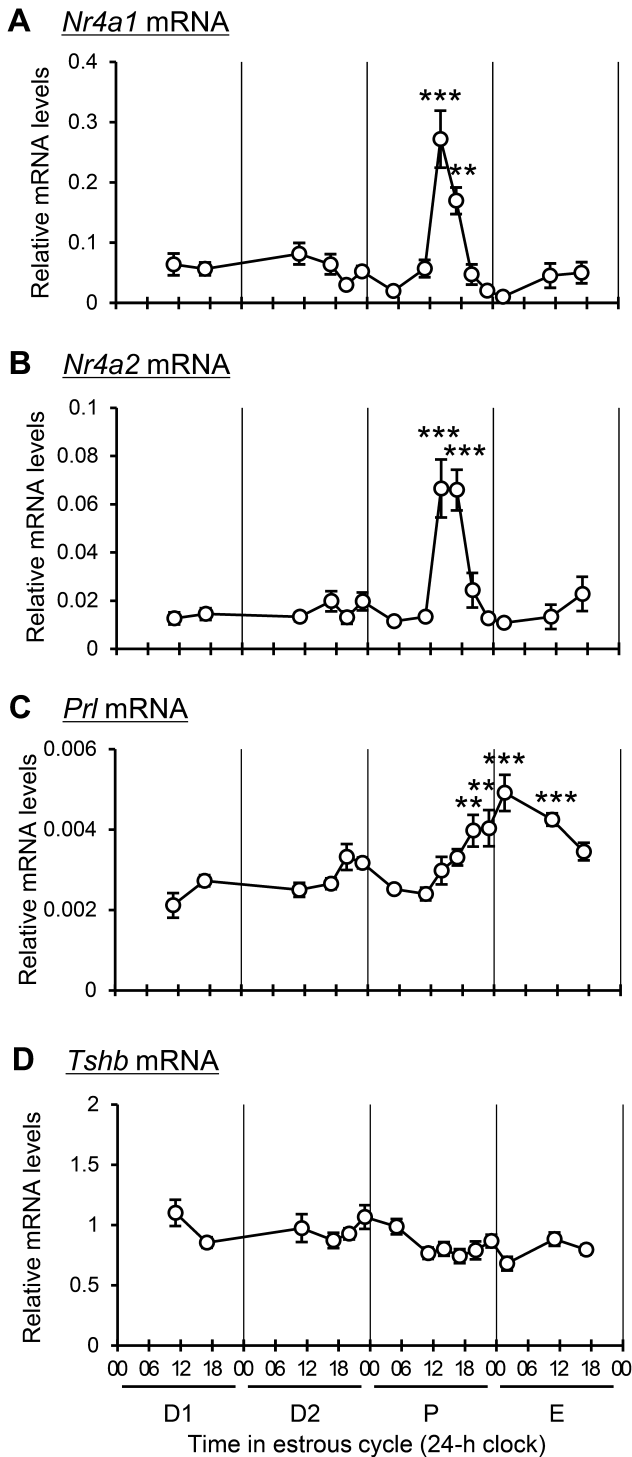
To characterize NR4A-expressing cells with high expression levels in the pituitary, double-fluorescence immunostaining for NR4As and anterior pituitary hormones was conducted on the rat anterior pituitary obtained at 1400 h in proestrus rats (Fig. 2). NR4A1 exhibited abundance in the cytoplasm of TSH-producing cells, with additional expression detected in the nuclei of LH- and PRL-producing cells, and comparatively lower expression in GH- and ACTH-producing cells. NR4A2, in contrast, displayed widespread expression patterns across all cell types, with strong cytoplasmic distribution in LH-producing cells and some TSH-producing cells. In other cell types, NR4A2 primarily localized to the nucleus and showed high expression in GH- and ACTH-producing cells. NR4A3 predominantly localized in the nucleus, exhibiting high expression in TSH-producing cells. Additionally, some PRL-, LH-, and GH-producing cells displayed high expression levels of NR4A3.

### *Time-dependent induction of Nr4a genes in an estrogen-primed, gonadotropin surge-induced rat model*

In investigating the potential correlation between pituitary *Nr4a* expression and hypothalamic activation, ovariectomized rats were continuously exposed to E<sub>2</sub>. Rats exposed to high E<sub>2</sub> concentrations displayed surge-like increases in LH secretion at both 1400 and 1700 h (Fig. 3A). Low E<sub>2</sub> concentrations failed to elicit this response. Similarly, the mRNA expression of *Nr4a1* (Fig. 3B), *Nr4a2* (Fig. 3C), and *Nr4a3* (Fig. 3D) in the pituitary glands of rats exposed to high E<sub>2</sub> concentrations increased at 1400 h and subsequently declined at 1700 h.

### *Induction of Nr4a genes by GnRH and TRH in lβT2 and GH3 cells*

To validate the impact of GnRH on *Nr4a* gene expression, lβT2



**Fig. 1.** Changes in the expression levels of *Nr4a1*, *Nr4a2*, *Prl*, and *Tshb* mRNA during the estrous cycle. Relative mRNA expression levels of *Nr4a1* (A), *Nr4a2* (B), *Prl* (C), and *Tshb* (D) in the anterior pituitary gland were measured during diestrus 1 (D1; 1100 and 1700 h), diestrus 2 (D2; 1100, 1700, 2000, and 2300 h), proestrus (P; 0500, 1100, 1400, 1700, 2000, and 2300 h), and estrus (E; 0200, 1100, and 1700 h) by RT-qPCR. Data are presented as mean  $\pm$  SEM (n = 5). Statistical analyses were performed using the Tukey–Kramer test; \*\* P < 0.01, \*\*\* P < 0.001 versus proestrus at 1100 h.

gonadotroph cells were exposed to GnRH, resulting in a significant increase in *luteinizing hormone beta* (*Lhb*) mRNA expression (Fig. 4A). Simultaneously, transient peaks in *Nr4a1* (Fig. 4B), *Nr4a2* (Fig.

4C), and *Nr4a3* (Fig. 4D) mRNA expression were observed within 1–2 h. Gene expression also exhibited a dose-dependent increase 1 h after GnRH administration, especially at concentrations of 10 nM or higher.

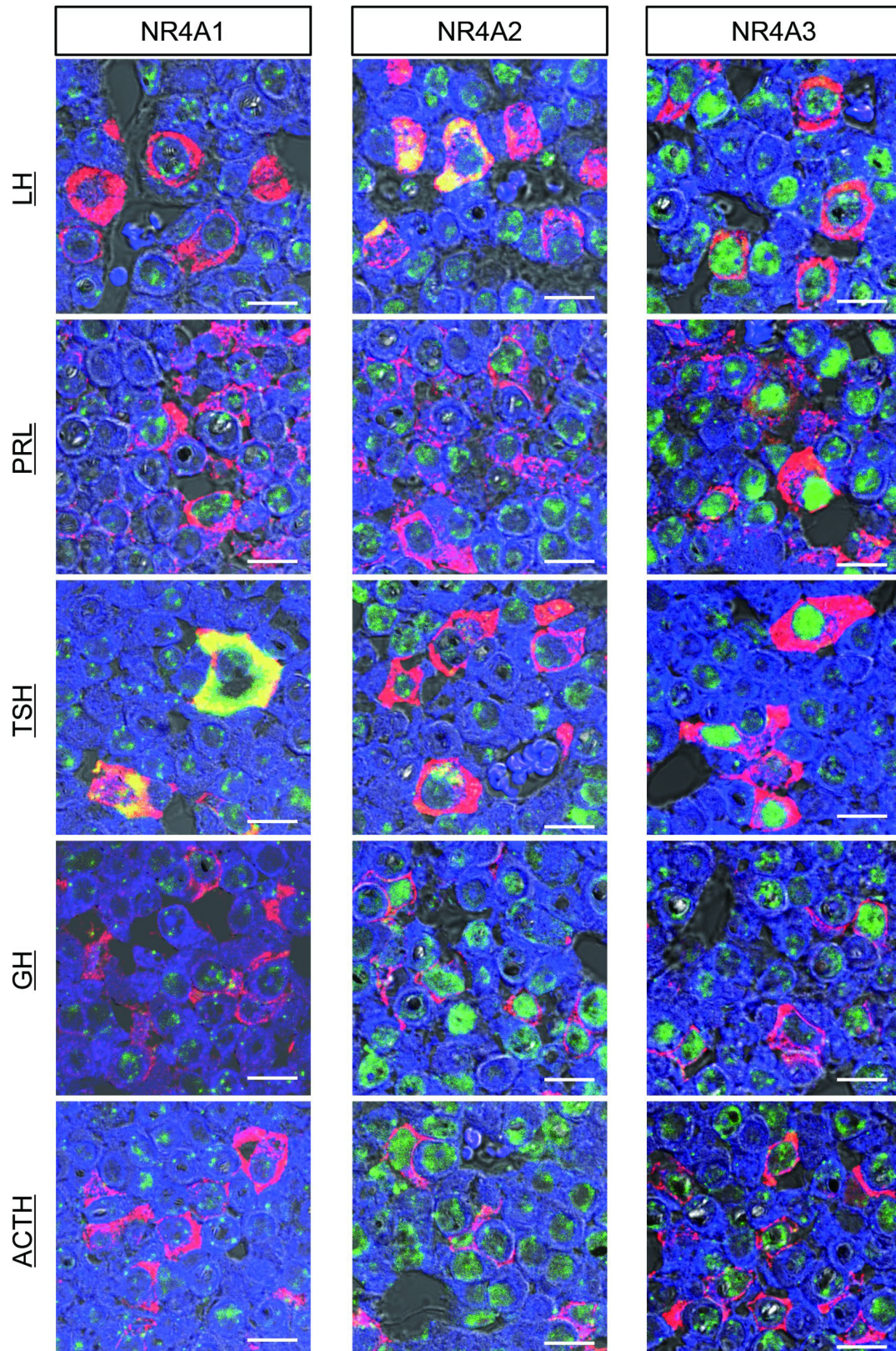
Given the impact of hypothalamic TRH on *Nr4a3* expression during proestrus afternoons, the effect of TRH on *Nr4a* genes was examined in TRH-sensitive GH3 somatomammotrophic cells. TRH treatment significantly increased *Prl* mRNA expression at 8 h (Fig. 5A). TRH transiently induced *Nr4a1* (Fig. 5B), *Nr4a2* (Fig. 5C), and *Nr4a3* (Fig. 5D) mRNA expression, peaking after 1 h. Furthermore, the expression of these genes 1 h after treatment exhibited a dose-dependent augmentation at TRH concentrations exceeding 1 nM.

## Discussion

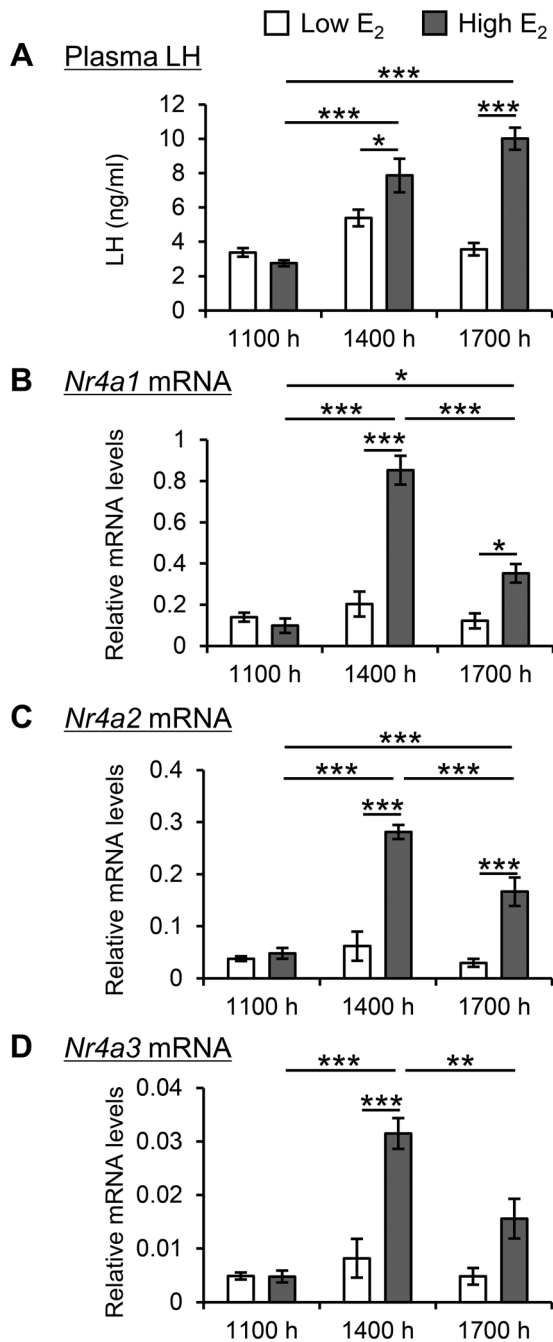
Considering the suggested cooperative or antagonistic interactions between NR4A proteins, this study compared their expression patterns. Previous research confirmed specific upregulation of pituitary *Nr4a3* expression in response to GnRH and TRH during proestrus. The current study extended this assessment to *Nr4a1* and *Nr4a2*, revealing synchronized expression patterns linked to preovulatory hypothalamic activation in proestrus rats. This indicates potential interactions among NR4As in the pituitary gland. Insights from a previous study on double-knockout mice lacking both NR4A1 and NR4A3 emphasized compensatory functions within the NR4A family [27]. These mice displayed heightened acute leukemia severity and reduced lifespan compared to single-gene knockout mice, highlighting the importance of maintaining functional balance within the NR4A network. The existence of mechanisms ensuring the multifaceted functions of NR4As underscores the significance of synchronized pituitary *Nr4a* regulation.

Our results underscore the pivotal role of high-level estrogen-dependent activation of the hypothalamus in regulating pituitary *Nr4a* genes during proestrus. Subsequent experiments with cell lines demonstrated that physiological levels of GnRH and TRH induce synchronized *Nr4a* expression. Established evidence indicates time-dependent and extensive GnRH release in E<sub>2</sub>-primed ovariectomized rats [28]. While increased TRH secretion is noted during proestrus [29], its association with E<sub>2</sub> feedback remains unconfirmed. The heightened activities of GnRH and TRH during proestrus enhance *Nr4a3* expression [21, 22], strongly suggesting that the positive feedback effect of E<sub>2</sub> stimulates the secretion of both GnRH and TRH, subsequently inducing *Nr4a* gene expression.

Our investigation unveiled differentially expressing cells and subcellular distribution patterns of NR4A proteins in the anterior pituitary. This suggests divergent roles and mechanisms of action of NR4As in regulating pituitary endocrine secretion. While tentative comparisons indicate that *Nr4a1* gene expression may be the highest and *Nr4a3* gene expression may be the lowest in the entire anterior pituitary, caution is warranted due to the use of different primer sets. Additionally, variations in gene expression among cell lines were observed, supporting the idea that different cell types have distinct functional NR4A profiles. However, it is crucial to exercise caution as the present data lack a substantial comparison of expression levels, and the cell lines may not fully replicate physiological cellular differences. Considering NR4A3's impact on *Fshb* expression in gonadotroph cell lines [30] and the known interactions and compensatory mechanisms among NR4As [27], dismissing their involvement in functional regulation based solely on lower expression levels in specific cell types would be premature. A more comprehensive assessment is imperative to determine the relative contribution of each NR4A to



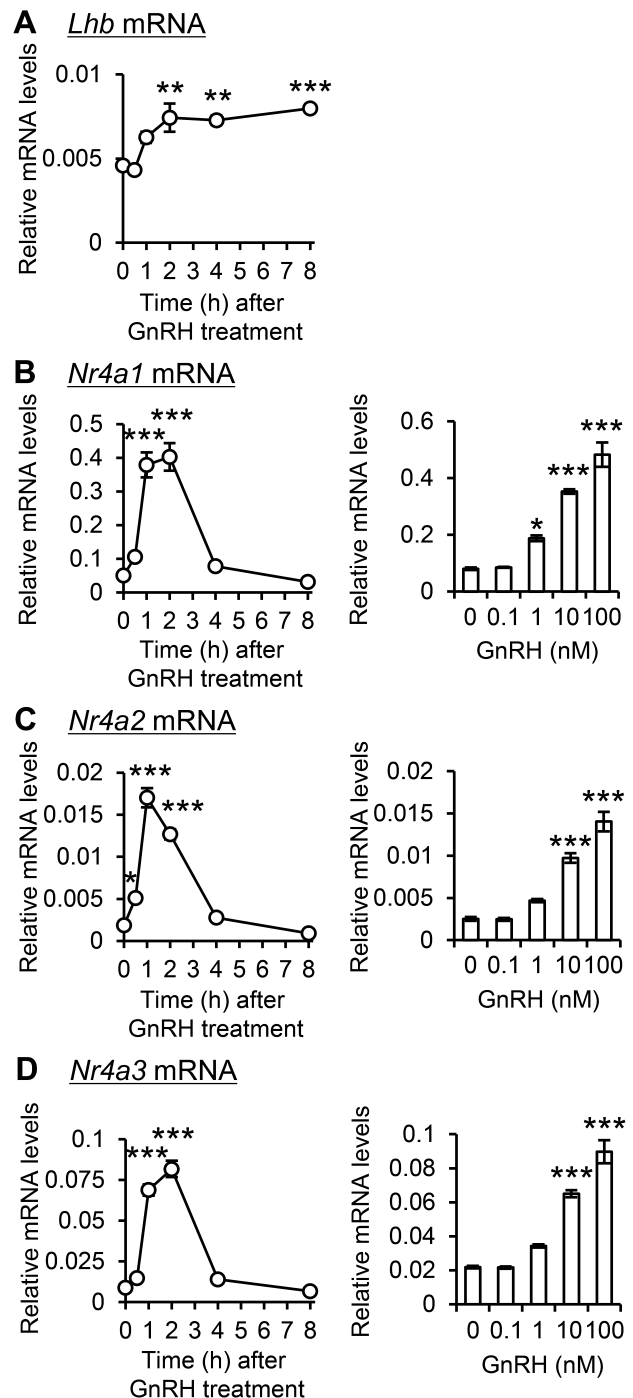
**Fig. 2.** Expression of NR4A proteins in the anterior pituitary gland of proestrus rats. Double-fluorescence immunohistochemistry on rat pituitary gland biopsies obtained at 1400 h during proestrus. NR4As (NR4A1, NR4A2, and NR4A3; green) and pituitary hormones (LH, PRL, TSH, GH, and ACTH; red) were observed by confocal laser scanning microscopy. Colocalized images are shown in yellow, and DAPI (blue) was used as a nuclear counterstain. Scale bars indicate 10  $\mu$ m.



**Fig. 3.** Changes in *Nr4a* expression in estrogen-primed rats. Ovariectomized rats were implanted with silastic capsules containing either 0.1  $\mu\text{g}/100 \mu\text{l}$  (Low) or 10  $\mu\text{g}/100 \mu\text{l}$  (High) E<sub>2</sub> for 3 days. Anterior pituitary tissue and blood samples were collected at 1100, 1400, and 1700 h. Plasma LH concentration (A) and *Nr4a1* (B), *Nr4a2* (C), and *Nr4a3* (D) mRNA expression were measured. Relative mRNA levels were compared to those in the Low E<sub>2</sub> group at 1100 h. Data are presented as mean  $\pm$  SEM (n = 5). Statistical analyses were performed using the Tukey–Kramer test; \* P < 0.05, \*\* P < 0.01, and \*\*\* P < 0.001.

pituitary functionality.

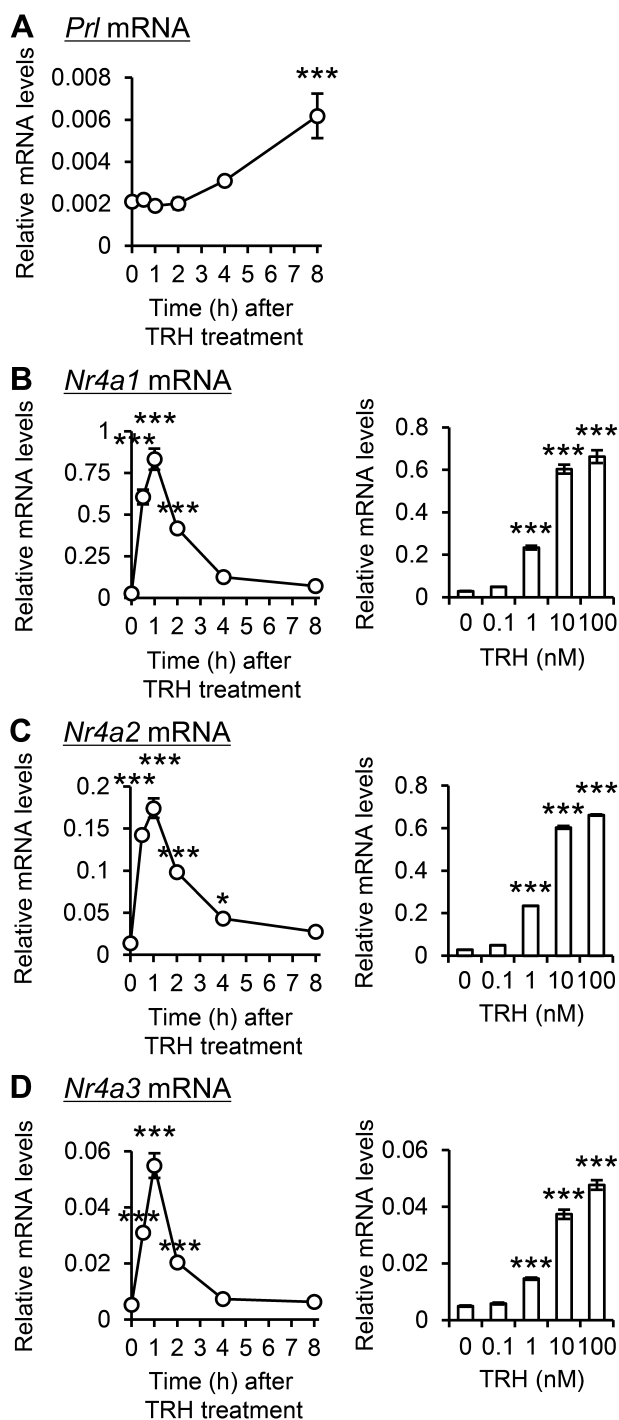
Among anterior pituitary cells, the expression of NR4As, particularly NR4A1 and NR4A3, was prominent in thyrotrophs, indicating a potential inductive effect of TRH. Previous studies have shown reduced NR4A1 protein distribution in the thyrotrophs of TRH-deficient mice [19], supporting TRH-induced upregulation



**Fig. 4.** Effect of GnRH on *Nr4a* genes expression in L $\beta$ T2 gonadotroph cells. L $\beta$ T2 cells were incubated with 100 nM GnRH for 0 to 8 h or 0 to 100 nM GnRH for 1 h. *Lhb* (A), *Nr4a1* (B), *Nr4a2* (C), and *Nr4a3* (D) mRNA expression was measured. Data are presented as mean  $\pm$  SEM (n = 4). Statistical analysis was performed using the Tukey–Kramer test; \* P < 0.05, \*\*\* P < 0.001 versus 0 h or 0 nM.

of NR4As in these cells. The sensitivity of lactotrophs to TRH [31] suggests its potential involvement in the induction of NR4As in this cell type. Additionally, the clear distribution of NR4A2, along with NR4A3, in gonadotrophs suggests the potential induction of its expression by GnRH.

The prevailing consensus suggests that NR4As are predominantly induced by shared signaling pathways, and our study revealed that



**Fig. 5.** Effect of TRH on expression of *Nr4a* genes in TRH-sensitive GH3 somatomammotroph cells. GH3 cells were incubated with 100 nM TRH for 0–8 h or with 0–100 nM TRH for 1 h. *Prl* (A), *Nr4a1* (B), *Nr4a2* (C), and *Nr4a3* (D) mRNA expression were measured. Relative mRNA levels were compared with those in the 0 h and 0 nM groups. Data are presented as mean ± SEM (n = 4). Statistical analysis was performed using the Tukey–Kramer test; \* P < 0.05, \*\*\* P < 0.001 versus 0 h or 0 nM.

both GnRH and TRH amplify *Nr4a* genes with comparable expression ratios despite different basal levels. This finding suggests the common involvement of intracellular signaling pathways in the induction of *Nr4a* genes by GnRH and TRH.

Notably, there have been multiple reported inductive effects of

GnRH and TRH on *Nr4a1*, accompanied by conflicting evidence. Experiments using the PRL-producing cell line GH4C1 demonstrated that TRH induces *Nr4a1* expression not through calcium (Ca<sup>2+</sup>) or protein kinase A (PKA) but via protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signaling pathways [19]. Conversely, earlier research on LβT2 cells revealed that GnRH induces *Nr4a1* through the PKA and Ca<sup>2+</sup> signaling pathways, excluding the involvement of PKC or MAPK [32]. However, conflicting evidence from other groups, utilizing a gonadotroph-derived cell line (αT3-1) and primary pituitary cultured cells from knockout mice for signaling factors, implicated PKC and MAPK in *Nr4a1* induction [33]. These observations suggest that, at least under physiological conditions, the PKC and MAPK signaling pathways are likely primary regulators. Although information on *Nr4a2* and *Nr4a3* remains limited, it is plausible that the PKC and MAPK signaling pathways are the principal regulators of these members of the NR4A family.

Significant changes in pituitary endocrine secretion, such as notable gonadotropin and PRL surges, have been observed during proestrus in rats [34]. Our previous research demonstrated that NR4A3 expression in gonadotrophs suppresses *Fshb* expression, suggesting a regulatory role for upregulated NR4As, particularly in *Fshb* gene expression [30]. *Fshb* gene expression significantly increases following a gonadotropin surge, leading to a secondary FSH surge [35]. Although the precise significance of this secondary surge remains unclear, it may affect follicular development during the subsequent estrous cycle [36]. Additionally, in this study, we observed a concurrent increase in *Prl* expression following the upregulation of *Nr4a* genes, suggesting the possible involvement of NR4As in *Prl* expression during this period. Increased *Prl* expression persisted for half a day to one day, consistent with previous observations [37]. *Prl* expression is believed to ensure adequate PRL secretion during early pregnancy and the post-ovulation estrous cycle. Therefore, the induction of pituitary NR4As during proestrus may play a role in establishing pregnancy and/or influence the subsequent normal estrous cycle by modulating several pituitary hormone genes. Furthermore, although its specific involvement in *Tshb* expression remains unknown, it is noteworthy that the significant expression of NR4As in thyrotrophs may also contribute to TSH secretory function.

In summary, this is the first demonstration of the synchronized upregulation of *Nr4a1*, *Nr4a2*, and *Nr4a3* in the anterior pituitary gland during the afternoon of proestrus. This coordinated response is orchestrated by the estrogen-dependent activation of the hypothalamic system, with strong implications for GnRH and TRH in this regulatory process. The increased expression of these NR4As in the anterior pituitary gland during proestrus may affect the sexual cycle and pregnancy by modulating the secretory capacity of pituitary hormones, including FSH, PRL, and TSH. Further investigations into the diverse actions and intricate interaction mechanisms of NR4As will provide potential insights into fertility and reproductive health.

**Conflicts of interests:** None of the authors have any potential conflicts of interest associated with this research.

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