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Estrogen promotes gonadotropin-releasing hormone expression by regulating tachykinin 3 and prodynorphin systems in chicken

Xing Wu,[*,1] Zihao Zhang,[*,1] Yijie Li,[*] Yudian Zhao,[*] Yangguang Ren,[*] Yixiang Tian,[‡] Meng Hou,[*] Yulong Guo,[*] Qi Li,[§] Weihua Tian,[*,†] Ruirui Jiang,[*,†] Yanhua Zhang,[*,†] Yujie Gong,[*,†] Hong Li,[*,†] Guoxi Li,[*,†] Xiaojun Liu,[*,†] Xiangtao Kang,[*,†] Donghua Li,[*,†] and Yadong Tian[*,†,2]

[*]*College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450046, China;* [†]*Henan Key Laboratory for Innovation and Utilization of Chicken Germplasm Resources, Zhengzhou 450046, China;* [‡]*College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, China; and* [§]*Henan zhumadian agricultural school, zhumadian, 463000, China.*

ABSTRACT The "KNDy neurons" located in the hypothalamic arcuate nucleus (**ARC**) of mammals are known to co-express kisspeptin, neurokinin B (**NKB**), and dynorphin (**DYN**), and have been identified as key mediators of the feedback regulation of steroid hormones on gonadotropin-releasing hormone (**GnRH**). However, in birds, the genes encoding *kisspeptin* and its receptor *GPR54* are genomic lost, leaving unclear mechanisms for feedback regulation of GnRH by steroid hormones. Here, the genes tachykinin 3 (***TAC3***) and prodynorphin (***PDYN***) encoding chicken NKB and DYN neuropeptides were successfully cloned. Temporal expression profiling indicated that *TAC3, PDYN* and their receptor genes (*TACR3, OPRK1*) were mainly expressed in the hypothalamus, with significantly higher expression at 30W than at 15W. Furthermore, overexpression or interference of *TAC3* and *PDYN* can regulate the *GnRH* mRNA expression. In addition, in vivo and in vitro assays showed that estrogen (**E2**) could promote the mRNA expression of *TAC3, PDYN*, and *GnRH*, as well as the secretion of GnRH/LH. Mechanistically, E2 could dimerize the nuclear estrogen receptor 1 (**ESR1**) to regulate the expression of *TAC3* and *PDYN*, which promoted the mRNA and protein expression of *GnRH* gene as well as the secretion of GnRH. In conclusion, these results revealed that E2 could regulate the GnRH expression through TAC3 and PDYN systems, providing novel insights for reproductive regulation in chickens.

Keywords: estrogen, *tachykinin 3*, *prodynorphin*, GnRH, chicken

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INTRODUCTION

It is widely acknowledged that gonadotropin-releasing hormone (**GnRH**) plays a pivotal role in regulating reproductive activities through the hypothalamus-pituitary-gonadal (**HPG**) axis (Millar, 2005). GnRH secretion can be autonomous or hormone regulated, such as by estrogen (**E2**) and progesterone (**P4**) (Maeda et al., 2010). Interestingly, physiological doses of P4 and E2 alter GnRH pulse release frequency or genomic expression, while pharmacological doses inhibit GnRH expression (Inserra et al., 2020). Studies have shown that E2-medaited regulation of reproduction is achieved through ESR1 (**ERα**) (Wersinger, et al., 1999; Dorling et al., 2003; Novaira, et al., 2018; Arao et al., 2019; Xiao and Chen, 2022). However, GnRH neurons only express small amounts of ESR2 (**ERβ**) (Skinner and Dufourny, 2005; Ciofi et al., 2006). Thus, the intermediate mediators responsible for hormonal signaling mediated GnRH release has attracted widespread interest.

Increasing evidence has revealed the role of neuropeptides in regulating reproduction, namely kisspeptin, Neurokinin B (**NKB**), and dynorphin (**DYN**), as well as their corresponding receptors GPR54, NK3R, and KOR. (Goodman, et al., 2004; Izzi-Engbeaya, et al., 2013). Kisspeptin were found to be expressed by over 90% of kisspeptin neurons in the hypothalamic arcuate nucleus (**ARC**) that express ESR1 (Smith, et al., 2005). It was reported that kisspeptin also exerted a vital role in controlling the pulsatile secretion of GnRH and gonadotropins in mammals, such as rabbits and goats, thus

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[1]These authors contributed equally to this work.

[2]Corresponding author: tianyadong@henau.edu.cn

mediating the feedback regulation of GnRH and LH by endogenous gonadal steroid hormones (Smith et al., 2007; Li et al., 2009;). NKB has a regulatory effect on the mammalian HPG axis and is expressed in the vicinity of the ARC of GnRH neurons, and it is hypothesised that NKB may directly regulate the secretion of GnRH (Guran, et al., 2009; Krajewski, et al., 2005; Sahu and Kalra, 1992). Furthermore, co-localization of NKB-NK3R neurons with ESR1-expressing neurons suggests a link between changes in estradiol levels and GnRH and LH levels mediated by NKB (Burke et al., 2006; Grachev et al., 2014). Interestingly, NKB-NK3R signalling regulates gonadotropin secretion in steroid (Wakabayashi et al., 2010; Navarro et al., 2011; Yamamura et al., 2015), seasonal (Sakamoto et al., 2012) and prepubertal environments (Nestor et al., 2012; Ramaswamy et al., 2010). Studies have shown that the majority of DYN neurons co-express ESR1 in the hypothalamic ARC of rats (Kanaya et al., 2017). Kinsey-Jones et al. also showed that KOR agonists could induce LH secretion in rats in an E2 dependent manner (Kinsey-Jones et al., 2012). More interestingly, the ARC was a unique subregion of the mammalian hypothalamus where neurons co-express NKB, DYN, and kisspeptin, referred to as the "KNDy neurons" (Wakabayashi et al., 2010; Skrapits et al., 2015;). "KNDy neurons" are the primary target cells of steroidal sex hormones and mediate the feedback regulation of GnRH neurons by gonadal steroid hormones, forming an interaction network of positive and negative regulation (Mittelman-Smith et al., 2012; Overgaard et al., 2014). In addition, GnRH neurons in the preoptic area also receive signal inputs from Kiss neurons located in the periventricular nucleus of the anterior ventral region, which is thought to be the target site within the hypothalamus responsible for the positive E2 feedback observed during the preovulatory period (Nestor et al., 2012).

While the role of TAC3 and PDYN systems in mammalian reproductive regulation is well-established, their role in birds remains poorly understood. Birds possess the GnRH system and exhibit pulsatile GnRH secretion akin to mammals, but they lack the *kiss1* and *GPR54* genes (Senthilkumaran et al., 2006; Pasquier et al., 2014; Zhang et al., 2014). The estrogenic feedback regulation of GnRH expression mediated by the TAC3/TACR3 and PDYN/OPRK1 systems in birds remains unexplored, particularly in chickens (Joseph et al., 2013). We hypothesized that estrogen can regulate the expression of GnRH through *TAC3* and *PDYN* in chickens, but the underlying mechanism may differ from that of mammals.

In this study, we utilized a range of in vivo and in vitro experiments, including gene cloning, enzyme-linked immunosorbent assay (**ELISA**), western blotting (**WB**), quantitative Real-Time PCR (**qRT-PCR**), ovariectomy, cell counting kit-8 (**CCK8**) and gene overexpression and interference techniques, to elucidate the molecular mechanism involved in how E2 regulates GnRH expression through feedback from *TAC3* and *PDYN* in chicken. The aim of our research was to enhance our understanding of avian reproductive regulation and contribute to its theoretical advancement.

MATERIALS AND METHODS

Ethical Approval

The experimental procedures were conducted in strict compliance with the Animal Experiment Management Regulations of 2004 and were approved by the Animal Care and Use Committee of Henan Agricultural University, China (Permit Number: 19-0068).

Sample Collection and Animal Treatment

Twelve Hyland Brown laying hens at 15 and 30 wk old (6 chickens each stage) were intramuscularly injected with Sumianxin (0.25 mL/kg xylazine hydrochloride, Shengda Animal Drug Company, Dunhua, China). After euthanasia and jugular vein bloodletting, hypothalamus, pituitary, ovary, heart, liver, spleen, lung, and kidney were expeditiously collected.

In addition, 24 Hyland Brown laying hens at 14 wk old were randomly divided into 3 groups (8 chickens each group): 1) a sham-operated group, 2) an ovariectomy group (**OVX**), and 3) an ovariectomy and E2 treatment group with 5 mg/kg (OVX+E2 [5 mg/kg]). Prior to the commencement of the experiment, the chickens were provided with a 7-d acclimatization period with free access to water and food. All animals received an intramuscular injection of antibiotics (10 mg/kg, Enrofloxacin, Baytril, Bayer, Leverkusen, Germany) at 1 d before surgery, along with 12 h of fasting and 2 h of water deprivation. To mitigate bleeding, 200 μL Etamsylate Injection (Sichuan Jishan Home Pharmaceutical Co., Ltd., Sichuan, China) was intramuscularly administered 30 min before the operation. After administering intramuscular injection of Sumianxin II, the chickens were anesthetized, and ovariectomy was performed in groups (2) and (3) following the previous method reported by Song et al (Song and Silversides, 2006). In group (1), an incision was made at the same site with no further surgical intervention. To alleviate pain, Carprofen (2 mg/kg, Rimadyl injectable, Pfizer, New York) was intramuscularly injected 6 h after the operation, and antibiotics were intramuscularly administered. Furthermore, vitamin K3 powder (Hebei weierli animal pharmacy group co., ltd, Hebei, China) was added to the drinking water for 7 consecutive days to promote blood clotting. Before collection of blood and hypothalamic tissue samples, group (3) received 5 mg/kg of E2 (E2758, Sigma, St Louis, MO), while groups (1) and (2) were administered with equal amounts of anhydrous ethanol for a duration of 4 wk. And 500 μL venous blood samples per chicken were collected at 2 h intervals for 6 consecutive times using a negative pressure blood collection needle. Following a 30-min standing period, serum was collected by centrifugation at 3,000 rpm for 3 min and stored at -80°C for further detection of LH

and E2 levels. After the final blood collection, the chickens were humanly euthanized as described above to observe the removal of ovaries and collect hypothalamic tissue for RNA extraction.

Hypothalamic Primary Neuronal Cell Isolation and Culture

Hypothalamic primary neuronal cells were isolated and cultured according to the method as previously described (Wu et al., 2023). Briefly, the hypothalamus of 200 Hyland Brown eggs (embryonic d 16) were extracted and rinsed with a sequential application of ice-cold D-hanks buffer (H1045, Solarbio, Beijing, China) containing 2% penicillin-streptomycin (P1400, Solarbio, Beijing, China) and ice-cold DMEM medium (06-1055-57-1ACS, BI, Kibbutz, Beit Haemek, Israel) containing 2% penicillin-streptomycin (P1400, Solarbio, Beijing, China). Hypothalamic tissue was cut into pieces, digested with 0.25% trypsin (25200056, Gibco, Carlsbad, CA) at 37 °C for 10 min. The dissociated cells were filtered using 40 μm and 70 μm filters and then centrifuged at 1,200 rpm for 7 min. The precipitate was resuspended and subjected to centrifugation at 1,000 rpm for 6 min for 3 times. The resuspended cells were incubated in a serum-free neuronal medium (Neurobasal, 21103049, Gibco, Carlsbad, CA) supplemented with 2% B27 (17504044, Gibco, Carlsbad, CA), 1% penicillin-streptomycin (P1400, Solarbio, Beijing, China), and 1% L-glutamine (G0200, Solarbio, Beijing, China). The cell suspensions were then cultured in 12-well plates coated with 0.1 mg/mL poly-L-lysine (P1399, Sigma, St Louis, MO) at 37 °C in a 5% CO_2 incubator. After 24 h, the growth of non-neuronal cells was arrested by treating the cells with 0.001% Cytosine β-D-arabinofuranoside (C8040, Solarbio, Beijing, China).

RNA Extraction, cDNA Ssynthesis and qRT-PCR

Total RNA extraction was carried out using TriZol (Vazyme, Nanjing, China) in accordance with the manufacturer's protocol, and the total RNA quality and concentration were estimated using a Nanodrop 2000 microspectrophotometer (Thermo Fisher Scientific, Waltham, MA). Then, cDNA was synthesized from 1 μg total RNA using the HiScrip®III First Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China). The qRT-PCR were performed in a 10 μL reaction volume, including 5 μL 2 × Taq PCR Master Mix, 0.5 μL forward primer, 0.5 μL reverse primer, 3 μL RNase-free ddH_2O, and 1 μL cDNA (about 300 ng). The *GAPDH* gene was used as an internal control, and qRT-PCR data was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequence information is listed in Table 1.

PCR Amplification and Sequencing

According to the predicted chicken *TAC3* sequences (XM_040694044.1) in the NCBI databank, specific primers were designed to clone the full-length coding DNA sequence (**CDS**). The PCR was performed in a 50 μL reaction volume using the KOD FX Neo buffer system (TOYOBO, Osaka, Japan), consisting of 1 μL cDNA, 1 μL KOD FX Neo, 25 μL 2 × PCR buffer for KOD FX Neo, 10 μL dNTP, 1.5 μL each of upstream and downstream primers and 10 μL RNase-free ddH_2O. A two-step reaction procedure was employed for PCR amplification, consisting of an initial step at 94 °C for 2 min, followed by 30 cycles of 98 °C for 10 s and 68 °C for 15 s.

Primers were designed according to the *PDYN* sequence (XM_040650978.1) predicted by NCBI database (XM_040650978.1) to amplify the CDS of *PDYN* by KOD One TM PCR Master Mix -Blue- (TOYOBO, Osaka, Japan). The reaction system consisted of 5 μL of KOD OneTM PCR Master Mix -Blue-, 1 μL cDNA, 0.5 μL each of the upstream and downstream primers and 3 μL RNase-free ddH_2O. The reaction procedure was set as follows: 98 °C for 3 min, followed by 30 cycles of 98 °C for 15 s, 65 °C for 10 s, and 68 °C for 10 s, and a final extension at 68 °C for 5 min. The PCR products were sequenced by Shangya Biotechnology

Table 1. The primer sequences of qRT-PCR.

Genes	GenBank number	Primer	Sequences (5′-3′)	Product length (bp)
TAC3	XM_040694044.1	F	CCGTCGCCGCTCTTCTG	82
		R	TGCATATCCCGCTTCTGAGG	
PDYN	XM_040650978.1	F	ACAATCAGAAGCGGTACGGG	93
		R	AGACCTCTCCTGAGTAGGCG	
TACR3	NM_001318454.1	F	CCACTCGGCAAAGCAGTCTA	210
		R	CATCTCCCGCTGTGTTCAGT	
OPRK1	NM_001318772.1	F	TCCGTACTCCTCTCAAGGCA	140
		R	TGGGAACTGCAAGGAGCATT	
ESR1	NM_205183.2	F	ATGATCGGCTTAGTCTGGCG	137
		R	GCAGCAGTAGCCAGTAGCAT	
GnRH	NM_001080877.1	F	TGCTTGGCTCAACACTGGTC	194
		R	CCTTCGATCAGGCTTGCCAT	
GAPDH	NM_204305.1	F	GCACGCCATCACTATCTT	82
		R	GGACTCCACAACATACTCAG	

TAC3, tachykinin 3; *PDYN*, prodynorphin; *TACR3*, tachykinin receptor 3; *OPRK1*, opioid receptor kappa 1; *ESR1*, estrogen receptor 1; *GnRH*, gonadotropin-releasing hormone; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

Table 2. The primer sequences of PCR.

Gene	GenBank number	Primer	Sequence of nucleotide (5′-3′)	Product length (bp)
TAC3	XM_040694044.1	F	ATGCGGAGCCGCGCGCTG	399
		R	CGGCGCGGGGCGGGGGG	
PDYN	XM_040650978.1	F	TGTCTCGGCACTGTTCTTCC	1,349
		R	TGTGATACCATCCCCTGGGT	
ESR1	NM_205183.2	F	ATGACCATGACCCTTCACA	1,770
		R	TATTGTATTCTGCATACTCTCC	

TAC3, tachykinin 3; *PDYN*, prodynorphin; *ESR1*, estrogen receptor 1; F, forward; R, reverse.

Company (Zhengzhou, China). Multiple sequence alignment was performed using BioXM2.6 software (Nanjing Agricultural University, Nanjing, China). The primer sequence information used in PCR was presented in Table 2.

Sequence Retrieval and Bioinformatics Analysis

In addition to the chicken TAC3, PDYN and its receptor TACR3, OPRK1, we also obtained the amino acid sequences of TAC3/TACR3 and PDYN/OPRK1, from the NCBI database for 10 species, including four mammals (human, mouse, pig, and rabbit), 3 birds (duck, zebra finch, and willow flycatcher), one fish (zebrafish), one amphibian (xenopus tropicalis), and one reptile (green sea turtle). To construct the phylogenetic trees, we employed the neighbour-joining approach using MEGA7 (http://www.megasoftware.net/). Additionally, we analyzed the exon-intron structures of the chicken *TAC3*/*TACR3* and *PDYN*/*OPRK1* genes using the online software GSDS (http://gsds.cbi.pku.edu.cn/).

Plasmids Construction and Cell Transfection

The CDS sequences of *TAC3* and *PDYN* genes were synthesized and subsequently inserted into the expression vector pcDNA3.1(+) to construct overexpression plasmids for chicken *TAC3* and *PDYN* (pcDNA3.1-TAC3 and pcDNA3.1-PDYN) by Tsingke Biotechnology Co., Ltd. In addition, the CDS sequences of chicken *ESR1* was cloned into the corresponding restriction endonuclease site of the pcDNA3.1(+) vector by double digestion with EcoRI-HindIII, resulting in the production of plasmids pcDNA3.1-ESR1. The interfering fragments of *TAC3, PDYN*, and *ESR1*, as well as the negative control NCR, were synthesized by RiboBio (Guangzhou, China).

When reaching 70% confluence, the hypothalamic neuronal cells were transfected with pcDNA3.1-TAC3, pcDNA3.1-PDYN and pcDNA3.1-ESR1 using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA). For the interference of *TAC3, PDYN*, and *ESR1*, siRNA oligos were synthesized and transfected into hypothalamic neuronal cells using RiboFECT CP Transfection kit (RiboBio, Guangzhou, China). All operations were followed the manufacturer's instructions.

Predicted Regulatory Elements in the Promoter Regions of TAC3 and PDYN

The promoter region sequences for *TAC3* and *PDYN* within 5.0 kb upstream of the transcription start site (**TSS**) were extracted from the NCBI online database (https://www.ncbi.nlm.nih.gov/). The ERE matrixes for vertebrates (ERα: MA0112.2) were obtained from the JASPAR online software (http://jaspar.genereg.net/search?q=&collection). Then, the promoter sequences of *TAC3* and *PDYN* gene were submitted to MEME FIMO (http://meme-suite.org/tools/fimo) to identify their putative ERα binding sites.

ELISA Analysis

Intracellular GnRH levels as well as serum LH and E2 levels were quantified using an ELISA kit (Jiangsu Meimian Industrial Co., Ltd, Yancheng, China) in accordance with the manufacturer's instructions.

WB Assay

WB experimental procedures were conducted in accordance with the method we previously reported (Wu, et al., 2023). The primary antibodies were rabbit anti-GnRH (polyclonal, 20075, ImmunoStar, Hudson, WI, USA, 1:1,000) and anti-GAPDH (polyclonal, (60004-1-IG, Proteintech, Wuhan, China, 1:20,000). The second antibody was HRP-conjugated goat anti-rabbit (E-AB-1003, Elabscience, Wuhan, China, 1:3,000).

Statistical Analysis

All data were reported as the mean ± standard error of the mean (**SEM**) and were statistically analyzed using IBM SPSS statistics V22.0 software (IBM SPSS, Armonk, NY) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). Two independent samples t-tests were used to compare 2 groups, and 1-way ANOVA was used for comparisons between multiple groups. A P-value < 0.05 was considered statistically significant difference.

RESULTS

Cloning and Sequence Analysis of Chicken TAC3 and PDYN Genes

The CDS of *TAC3* gene (accession number XM_040694044.1) was successfully cloned with 399 bp

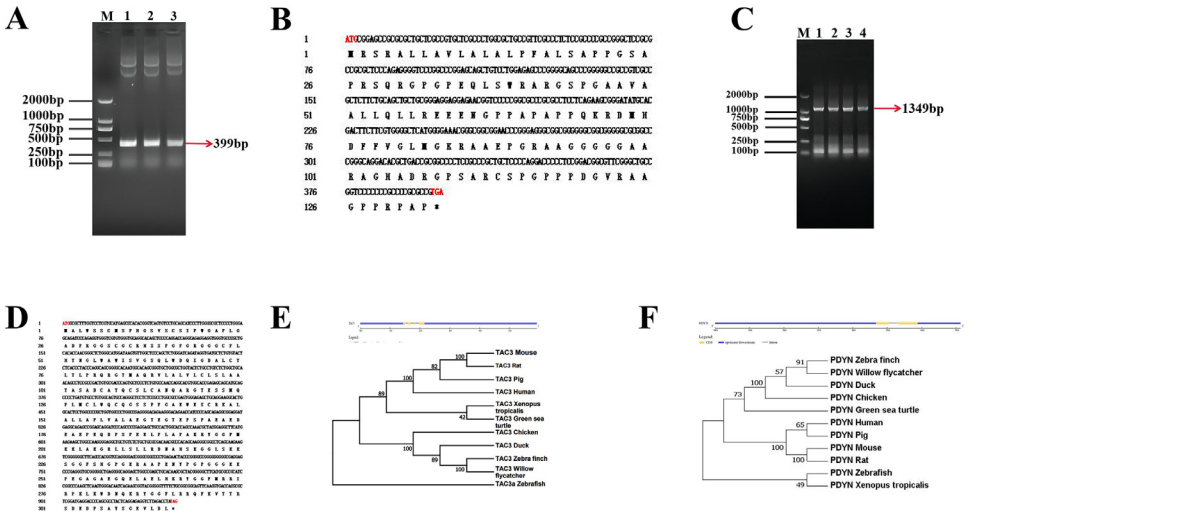

Figure 1. Cloning and sequence analysis of *TAC3* and *PDYN* genes in chickens. (A) Cloning results of *TAC3*, M: DL2000 DNA Marker, 1-3: Biological repetition; (B) Nucleotide and amino acid sequences of *TAC3* (NKB); (C) Cloning results of *PDYN*, M: DL2000 DNA Marker, 1-4: Biological repetition; (D) Nucleotide and amino acid sequences of *PDYN* (DYN); (E) Gene structure and phylogenetic tree of *TAC3*; (F) Gene structure and phylogenetic tree of *PDYN*.

in length (Figure 1A) that corresponds to a putative protein of 132 amino acid residues for NKB (Figure 1B). The physicochemical properties of the NKB protein were presented in Table S1. For *PDYN*, we devised primers targeting its 5′ and 3′ untranslated regions (**UTRs**), resulting in a PCR product of 1,349 bp (as per the NCBI GenBank database, accession number XM_040650978.1) (Figure 1C). The *PDYN* CDS region has a length of 948 bp, corresponding to 315 amino acid residues (Figure 1D). The physical and chemical properties of the DYN protein were summarized in Table S2. The *TAC3* and *PDYN* CDS sequence obtained in this study have been deposited on the NCBI website under registration numbers Banklt2644718 and Banklt2644649, respectively. Gene structure analysis revealed that the *TAC3* gene contains four exons and 3 introns (Figure 1E). Exon 1 encodes 29 amino acids, exon 2 encodes 32 amino acids, exon 3 encodes 10 amino acids, and exon 4 encodes 61 amino acids. *PDYN* gene was composed of 2 exons and one intron (Figure 1F). Exon 1 encodes 127 amino acids, and exon 2 encodes 188 amino acids. The phylogenetic analysis has revealed that both *TAC3* and *PDYN* genes were clustered in the avian branch, with a close affinity to ducks, followed by zebra finch and willow flycatcher. The mammalian *TAC3* and *PDYN* genes sequences were grouped in another branch, as depicted in Figures 1E and 1F.

Dynamic Expression Profiles of Chicken TAC3, PDYN and its Receptor Genes in Different Tissues and Stages

To determine the expression pattern of *TAC3*/*TACR3* and *PDYN*/*OPKR1* genes in chickens, qRT-PCR was performed using cDNA synthesized from RNA isolated from 8 different tissues at 15 and 30 wk of age. The results revealed that the *TAC3* exhibited high expression levels in the hypothalamus, pituitary, ovary, spleen and lung. However, *TAC3* gene showed a relatively low mRNA expression level in the heart, liver, and kidney. Additionally, the *TAC3* mRNA expression level was significantly higher in the hypothalamus, pituitary, and ovary in chicken at 30 wk old than that at 15 wk old ($P < 0.05$) (Figure 2A). Similarly, the *TACR3* was highly expressed in the hypothalamus, pituitary, and lungs, but was expressed at low levels in the heart, liver, spleen, kidney and ovary. (Figure 2B). The *PDYN* was highly expressed in the hypothalamus, pituitary, and lungs. Conversely, *PDYN* displayed low expression levels in the heart, liver, spleen, kidney, and ovary (Figure 2C). The *OPRK1* was highly expressed in the hypothalamus, spleen, and lung, while exhibiting low levels of expression in the heart, liver, kidney, pituitary, and ovary. Furthermore, the expression level of *OPRK1* in the hypothalamus was significantly higher in 30-wk-old chicken than 15-wk-old chicken (P <0.05). These results indicated that *TAC3*/*TACR3* and *PDYN*/*OPKR1* were commonly expressed in various tissues, and the expression level of hypothalamus at 30 wk old was significantly higher than that at 15 wk old ($P <$ 0.05).

TAC3 or PDYN Promotes GnRH Expression

Next, we explored the effects of *TAC3* and *PDYN* on *GnRH* expression. The relative mRNA expression of *GnRH* was detected after *TAC3* and *PDYN* overexpression or knockdown. The results showed that *TAC3* overexpression could significantly promote the expression of *GnRH* in hypothalamic neurons cells ($P < 0.05$) (Figures 3A−3B). After successful interference with *TAC3*, *GnRH* expression was significantly inhibited (P <0.05) (Figures 3C−3D). The expression of *GnRH* was

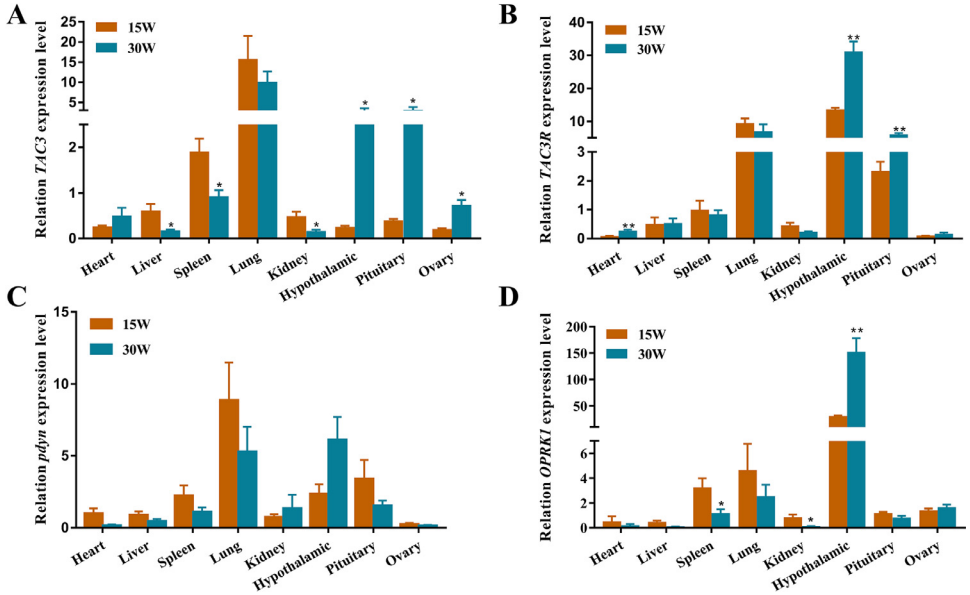

Figure 2. Expression patterns of chicken *TAC3/TACR3, PDYN/OPRK1.* (A) qRT-PCR analysis of TAC3 gene expression in different tissues of 15 W and 30 W chickens; (B) qRT-PCR analysis of PDYN gene expression in different tissues of 15 W and 30 W chickens; (C) qRT-PCR analysis of TACR3 gene expression in different tissues of 15 W and 30 W chickens; (D) qRT-PCR analysis of OPRK1 gene expression in different tissues of 15 W and 30 W chickens. ** indicated extremely significant differences ($P < 0.01$), and * indicated significant differences ($P < 0.05$).

significantly up-regulated after overexpression of *PDYN,* while the effect of interfering with *PDYN* was opposite (Figures 3E−3H).

E2 Regulates TAC3, PDYN and GnRH Expression and LH Secretion in Vivo

To corroborate the regulatory effect of E2 on the mRNA expression of *TAC3, PDYN*, and *GnRH* and the secretion of LH in vivo, we developed a chicken model with removal of the ovary and assessed the levels of *TAC3, PDYN*, and *GnRH* mRNA expression, as well as serum E2 and LH levels, following E2 treatment. The NC group exhibited normal ovarian development, with distinct prehierarchical and preovulatory follicles, while the 2 groups that underwent oophorectomy had no developing follicles and ovarian stroma (Figure 4A). The qRT-PCR results indicated that the mRNA expression of *TAC3, PDYN*, and *GnRH* were significantly higher in the OVX group than the NC group ($P < 0.05$). The mRNA expression of *TAC3, PDYN*, and *GnRH* were significantly lower in the OVX+E2 (5 mg/kg) group than the OVX group ($P < 0.05$) (Figures 4B −4D). The results of ELISA showed that the serum E2 level was the lowest in OVX group and the highest in OVX+E2 (5 mg/kg) group (Figure 4E). Furthermore, the serum LH levels were increased in the OVX group in

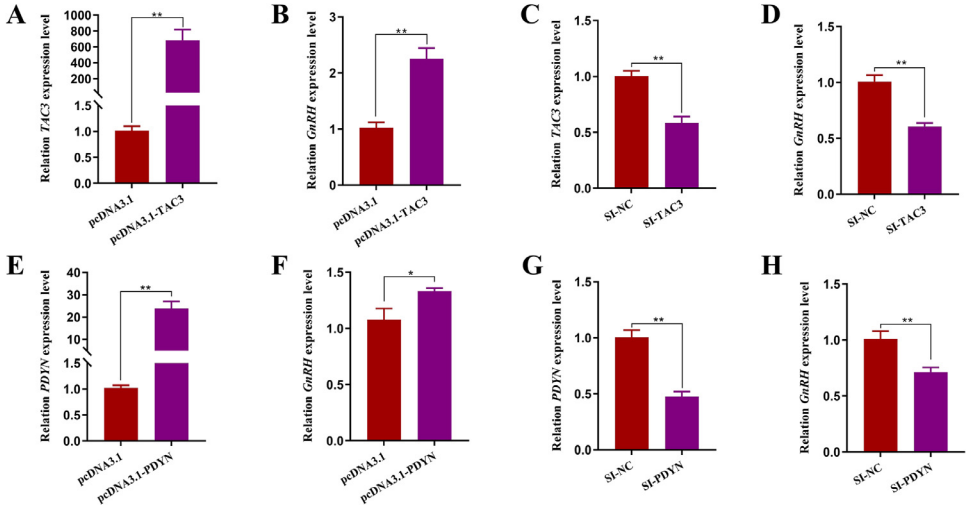

Figure 3. Overexpression or interference with *TAC3* and *PDYN* regulates the expression of *GnRH.* (A) Efficiency assay of TAC3 overexpression vector in primary hypothalamic neuronal cells; (B) Effect of overexpression of TAC3 on GnRH mRNA expression; (C) Efficiency assay of TAC3 interference in primary hypothalamic neuronal cells; (D) Effect of interference with TAC3 on GnRH mRNA expression; (E) Efficiency assay of PDYN overexpression vector in primary hypothalamic neuronal cells; (F) Effect of overexpression of PDYN on GnRH mRNA expression; (G) Efficiency assay of PDYN interference in primary hypothalamic neuronal cells; (H) Effect of interference with PDYN on GnRH mRNA expression. ** indicated extremely significant differences ($P < 0.01$), and * indicated significant differences ($P < 0.05$).

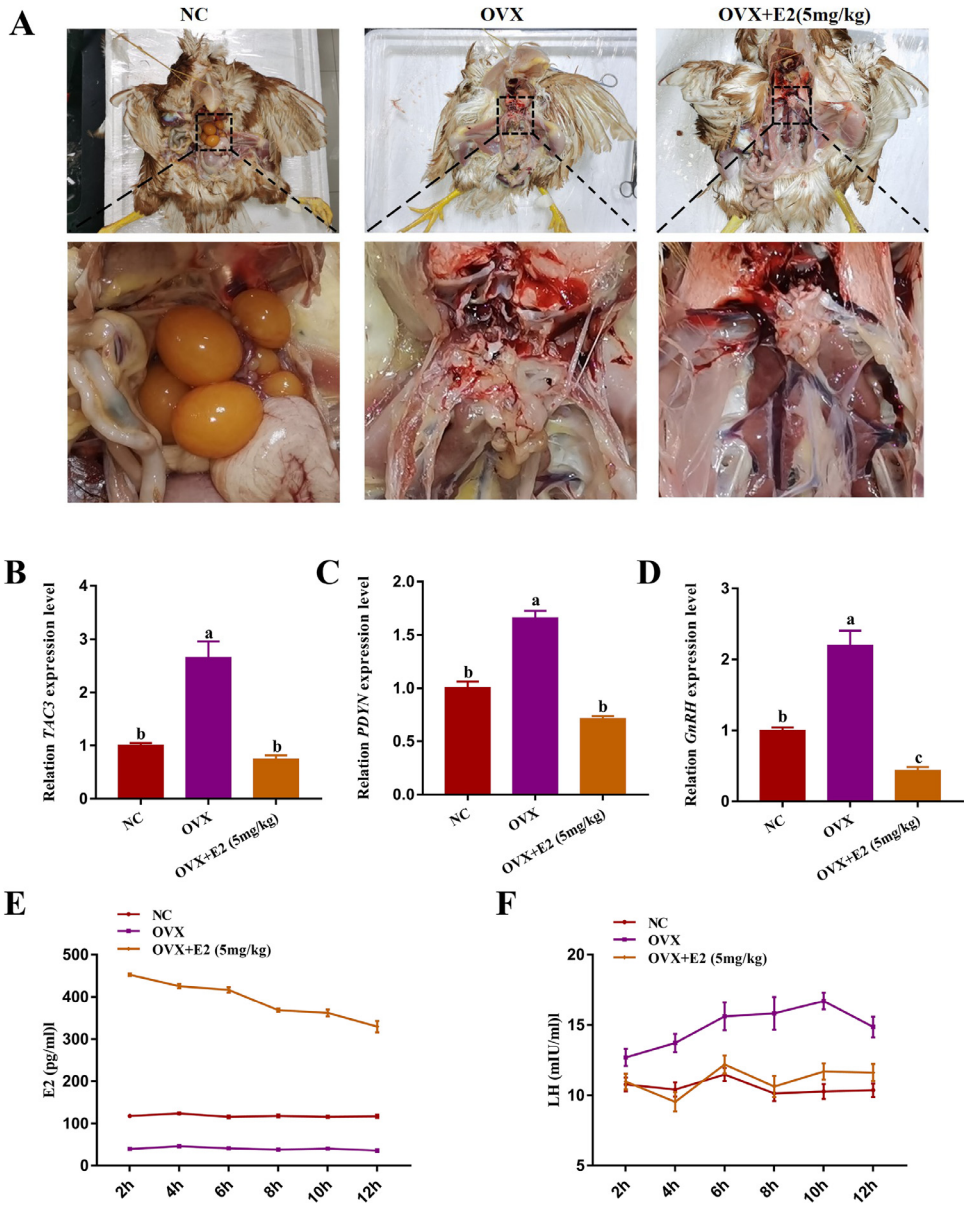

Figure 4. Regulatory effects of ovarian removal and E2 supplementation on the expression of *TAC3, PDYN*, GnRH, and LH secretion in chickens. (A) Morphological observations on the removal of chicken ovaries; (B) Effect of ovarian removal and E2 supplementation on the expression of TAC3; (C) Effect of ovarian removal and E2 supplementation on the expression of PDYN; (D) Effect of ovarian removal and E2 supplementation on the expression of GnRH; (E) Changes in E2 secretion after removal of the ovaries and supplementation with E2; (F) Changes in LH secretion after removal of the ovaries and supplementation with E2. Different letters represent significant and the same letters represent no significant.

comparison to the NC group, and the serum LH level in the OVX+E2 (5 mg/kg) group was returned to a similar level to that of the NC group (Figure 4F). In summary, the findings suggested that changes in E2 levels may have an impact on the expression levels of *TAC3, PDYN*, and *GnRH* mRNA, as well as the secretion levels of LH.

Effects of E2 Treatment on the Expression of GnRH, TAC3 and PDYN in Vitro

The gradients of different concentrations of E2 were established to further investigate the effects of E2 treatment on *GnRH, TAC3* and *PDYN* in hypothalamic neuron cells. CCK8 results revealed that 400 nmol/L E2 significantly reduced the viability of hypothalamic neuronal cells ($P < 0.05$) (Figure 5A). Meanwhile, qRT-PCR results showed that the mRNA expression levels of *GnRH, TAC3* and *PDYN* genes was the highest in 200 nmol/L group ($P < 0.05$) (Figures 5B–5D). These findings suggested that 200 nmol/L E2 could not affect the viability of hypothalamic neuronal cells, but significantly enhances the expression of *GnRH, TAC3*, and *PDYN*.

ERE Element Analysis of Chicken TAC3 and PDYN Gene Promoters and Correlation Analysis Between Gene Expression Levels

To explore potential associations between *TAC3* or *PDYN* with *ESR1* and *GnRH*, we extracted the promoter regions (5 kb upstream of TSS) of the *TAC3* and

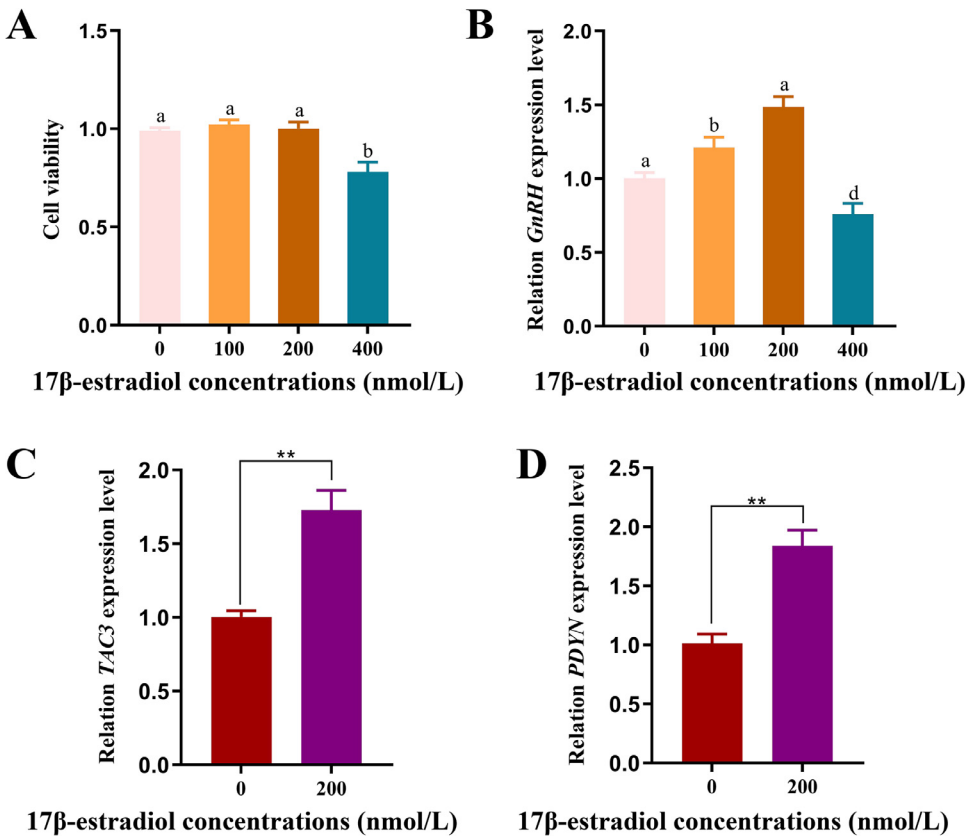

Figure 5. Effect of E2 treatment on the expression of *GnRH*, *TAC3* and *PDYN* in chicken hypothalamic neuronal cells. (A) Effect of different concentrations of E2 on the viability of hypothalamic neuronal cells; (B) Effect of different concentrations of E2 treatment on expression of GnRH; (C) Effect of 200 nmol/L E2 on TAC3 mRNA expression in chicken hypothalamic neuronal cells; (D) Effect of 200 nmol/L E2 on PDYN mRNA expression in chicken hypothalamic neuronal cells. Different letters represent significant and the same letters represent no significant. ** indicated extremely significant differences ($P < 0.01$), and * indicated significant differences ($P < 0.05$).

PDYN to identify putative estrogen-responsive elements (**ERE**). The results showed that the promoter region of the *TAC3* gene harbors 2 putative ESR1 (ERα) sites, and the promoter region of the *PDYN* gene contained one putative ESR1 (ERα) binding site (Figure 6A). We also analyzed the correlation between the expression levels of *ESR1* and *TAC3* or *PDYN*, as well as between *TAC3* or *PDYN* and *GnRH*. The results showed that the expression levels of *ESR1* were significantly and positively correlated with *TAC3* or *PDYN* at 15W, 30W, and cellular levels, respectively ($r^2 = 0.980$, $P < 0.001$ and $r^2 = 0.907$, $P < 0.001$) (Figures 6B−6C). And we observed a significant and positive correlation between the expression levels of *TAC3* or *PDYN* with *GnRH* ($r^2 = 0.931$, $P < 0.001$ and $r^2 = 0.934$, $P < 0.001$), respectively (Figures 6D−6E).

E2 Regulates the Expression of TAC3 and PDYN by Binding to ESR1, thereby Promoting the Expression of GnRH

To elucidate the molecular pathway by which E2 regulates GnRH expression, we evaluated the impact of *ESR1* overexpression on *TAC3* and *PDYN* expression, as well as the influence of *TAC3* and *PDYN* overexpression and interference on GnRH expression. Our results indicated that the mRNA expression of *TAC3* and *PDYN* remained unchanged in hypothalamic neuronal cells upon *ESR1* overexpression in comparison to the control group. However, co-treatment with 200 nmol/L E2 and *ESR1* overexpression significantly enhanced the mRNA expression of *TAC3* and *PDYN* when compared to the *ESR1* overexpression group ($P < 0.05$) (Figures 7A−7C). Our findings also demonstrated that the overexpression and interference of *TAC3* or *PDYN* in hypothalamic neuronal cells led to significant changes in GnRH mRNA, hormone, and protein compared to the control group ($P < 0.05$) (Figure 7D−7O). Moreover, in the presence of 200 nmol/L E2, overexpression and interference with *TAC3* resulted in high expression of GnRH mRNA, protein and hormone levels ($P < 0.05$) (Figures 7D−7I). Similarly, overexpression and interference with *PDYN* significantly promoted GnRH mRNA, hormone and protein expression in the presence of 200 nmol/L E2 ($P < 0.05$) (Figures 7J−7O). In summary, these results suggested that E2 modulated GnRH expression by binding to nuclear receptor ESR1 and regulating the expression of *TAC3* and *PDYN*, with *TAC3* and *PDYN* playing a promoting role in GnRH expression.

DISCUSSION

Our study demonstrates that E2 modulates the expression of *TAC3* and *PDYN* by binding to its

A

Genes	ESR1 (ERα)
Consensus	
TAC3	GGGGTAAAGGTGACCTC
	AAGGGCGACGTGGCCGT
PDYN	GGTCTCACTCTGACCCT

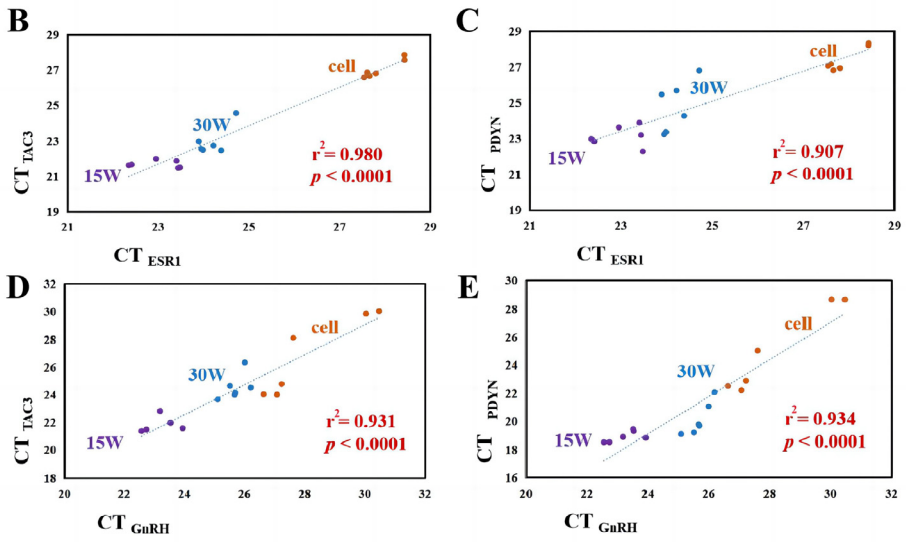

Figure 6. ERE elements in the promoters of chicken *TAC3* and *PDYN* genes and correlation analysis between gene expression levels. (A) Putative binding sites of chicken TAC3 or PDYN to ESR1; (B) Correlation analysis of CT values between ESR1 TAC3; (C) Correlation analysis of CT values between ESR1 and PDYN; (D) Correlation analysis of CT values between TAC3 and GnRH; (E) Correlation analysis of CT values between PDYN and GnRH.

receptors ESR1, thereby regulating GnRH expression (Figure 8). These findings provide a scientific basis for refining the theory of reproductive regulation in chickens, as our results well-verify the competence of chicken *TAC3* and *PDYN* in mediating the feedback regulation of E2 on GnRH expression.

The role of NKB and DYN in regulating reproduction, especially in GnRH secretion and puberty initiation, has been well confirmed in mammals and fish (Topaloglu et al., 2009; Biran et al., 2012; Uenoyama et al., 2022a). However, little was known about their role in reproduction regulation in birds. Therefore, we successfully cloned the CDS region of the genes encoding *TAC3* and *PDYN* using chicken as a model animal. The *TAC3* CDS region was 399 bp, containing 4 exons, whereas zebrafish (Biran et al., 2012), rat (Bonner et al., 1987) and cattle (Kotani et al., 1986) consisted of 7 exons. The *PDYN* CDS region was found to be 946 bp, containing 2 exons, while humans (Yuferov et al., 2018) and mice (Yang et al., 2023) had 4 exons. In addition, we also found that *TAC3, PDYN* and their receptor genes are widely expressed in various tissues, especially in the hypothalamus, which is consistent with previous studies (Biran et al., 2012; Liu et al., 2018). Interestingly, the expression level of hypothalamus at 30W (the peak laying period) was higher than that at 15W (initial ovarian development), suggesting that NKB and DYN may regulate the reproductive function of chickens. However, as a unique branch of vertebrate evolution, the *TAC3* and *PDYN* gene structures of birds differ from those of other vertebrates, potentially leading to different effects of NKB and DYN than in mammals and fish.

GnRH is widely acknowledged as the primary and pivotal regulatory signaling molecule on the HPG axis, which is capable of receiving negative feedback regulation from gonadal steroid hormones, including estrogen (Filicori and Crowley, 1984; Millar, 2005). Since GnRH neurons lack estrogen receptors, it is believed that estrogen indirectly modulates GnRH expression through interneurons. Our study found that *TAC3* and *PDYN* can regulate the expression of GnRH in hypothalamic neuron cells, which is basically consistent with previous studies that NKB and DYN regulated the release and synthesis of GnRH/LH in fish and mammals through direct and indirect actions (Crespo et al., 2022; Glidewell-Kenney et al., 2013; Młotkowska et al., 2020). This tentatively confirms our speculation.

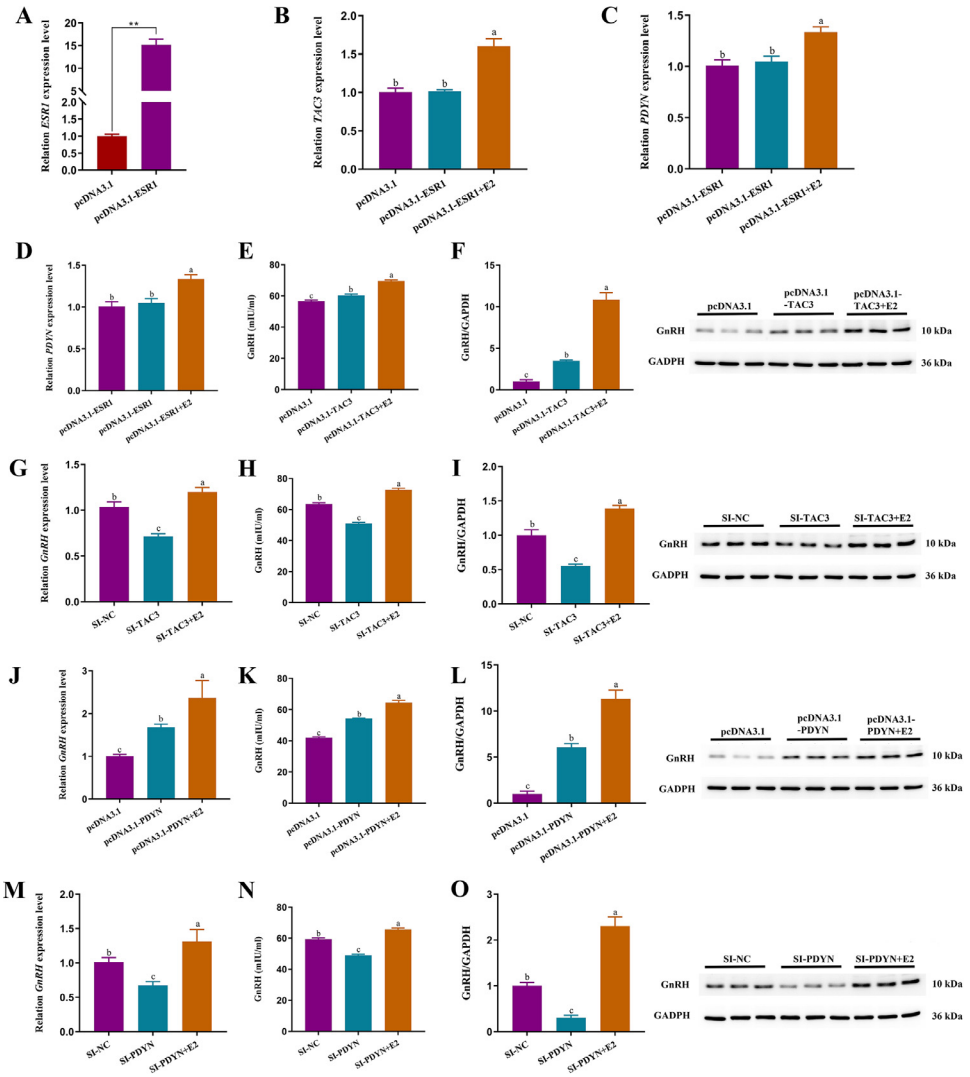

Figure 7. E2 regulates the expression of *TAC3* and *PDYN* by binding to ESR1, thereby promoting GnRH mRNA and protein expression and hormone secretion. (A) Efficiency assay of ESR1 overexpression vector in primary hypothalamic neuronal cells; (B) Effect of overexpression of ESR1 on the expression of TAC3; (C) Effect of overexpression of ESR1 on the expression of PDYN; (D-I) E2 regulates GnRH mRNA, protein expression and hormone secretion through TAC3; (J-O) E2 regulates GnRH mRNA, protein expression and hormone secretion through PDYN. Different letters represent significant and the same letters represent no significant. ** indicated extremely significant differences ($P < 0.01$), and * indicated significant differences ($P < 0.05$).

The ovary serves as the primary source of estrogen production in the body. Previous studies have demonstrated that ovariectomy in mammals, such as monkeys (Sandoval−Guzmán et al., 2004), rats (Rance and Bruce, 1994), and mice (Kauffman et al., 2009; Navarro et al., 2009), has resulted in an increase in NKB expression in the hypothalamus, while E2 replacement therapy has been found to suppress it (Abel et al., 1999; Pillon et al., 2003). Similarly, the removal of ovaries has been shown to reduce DYN expression in the hypothalamus of sheep (Foradori et al., 2005) and rats (Gottsch et al., 2009; Navarro et al., 2009). Our results showed that the mRNA expression of *TAC3, PDYN*, and *GnRH*, as well as the secretion of LH, were significantly elevated following ovariectomy and could be inhibited by E2 treatment. Similarly, our in vitro experiments also showed that 200 nmol/L E2 could promote the mRNA expression of *TAC3, PDYN* and *GnRH.* These results suggest that E2 can regulate the expression of *TAC3* and *PDYN.* However, the differential effects of E2 on DYN expression in birds and mammals may contribute to the significant differences in physiological and gene expression regulation due to their evolutionary independence (Zhang et al., 2014). Notably, our study is the first to investigate the effects of E2 on the mRNA expression of *TAC3, PDYN*, and *GnRH* in chickens.

Extensive evidence in mammals supports the critical role of "KNDy neurons" in the pulsatile secretion of GnRH and LH, as well as the feedback regulation of GnRH neurons by mediating glandular steroids (Ruka, et al., 2013; Overgaard, et al., 2014). The intricate mechanisms involved in this process are kisspeptin and NKB as stimulatory signals and DYN as inhibitory signals mediating the negative feedback of gonadal steroid secretion to circulating GnRH/LH (Herbison, 2018). Putative ERE elements were found in the promoter regions of *TAC3* and *PDYN*, which is similar to previous studies (Page et al., 2001; Rance et al., 2010), suggesting

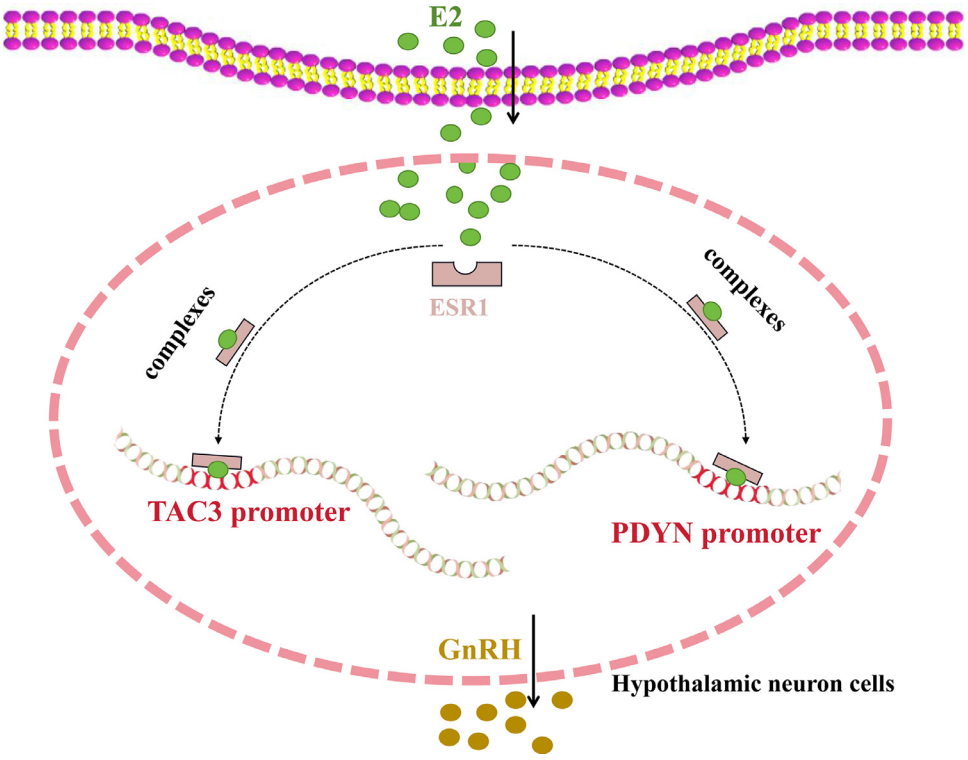

Figure 8. The working model of E2 regulation of GnRH expression through *TAC3* and *PDYN*.

that *TAC3* and *PDYN* were direct targets of E2. Furthermore, *TAC3* was observed to have a significant correlation between the mRNA expression of *ESR1* and *GnRH*, respectively. Similarly, *PDYN* was significantly correlated with *ESR1* and *GnRH*, respectively. These results provide an important basis for our in-depth understanding of the interactions among E2, *TAC3*, *PDYN*, and *GnRH* in the chicken reproductive system. Therefore, we further explored the molecular mechanism by which E2 regulates GnRH expression. The results indicated that E2 can regulate the expression of *TAC3* and *PDYN* by binding to ESR1, thereby promoting the expression of GnRH mRNA and protein as well as hormone secretion. However, in mammals, DYN could inhibit the expression of GnRH (Gallo 1990; Lopez et al., 2016; Uenoyama et al., 2022b). The different effects of DYN on GnRH secretion may be attributed to species-specific differences in the regulation of the HPG axis. While the overall structure and function of the HPG axis in mammals and birds are similar, the neuropeptides and hormones involved are significantly different. For example, kisspeptin, which plays a key role in regulating GnRH secretion in mammals, is absent in birds (Joseph et al., 2013; Pasquier et al., 2014). Likewise, the effect of gonadotropin-inhibitory hormone (**GnIH**), which inhibits GnRH secretion in birds (Bédécarrats et al., 2009; Bentley et al., 2006), exhibits a dual effect on mammalian GnRH secretion (Ubuka and Parhar, 2018). These differences may result from evolutionary differences in the regulation of reproductive function in different species, which needs further study.

CONCLUSIONS

In the present study, we successfully cloned the genes *TAC3* and *PDYN* which encode for NKB and DYN neuropeptides and conducted a comprehensive analysis of their physicochemical properties, gene structure, evolutionary history and tissue expression patterns. In addition, we further investigated the molecular pathway of E2 feedback regulation of GnRH expression, which involves E2 binding to ESR1 and regulating the expression of *TAC3* and *PDYN*, thereby promoting GnRH mRNA and protein expression and hormone secretion. These findings enrich the mechanisms of poultry breeding regulation, aiding in the improvement of conventional selection techniques and facilitating the breeding and conservation of rare birds.

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Author Contributions: Xing Wu, Zihao Zhang, Yijie Li, Yudian Zhao, Yangguang Ren, Yulong Guo, Meng Hou and Qi Li performed the experimental process. Xing Wu and Zihao Zhang wrote the manuscript. Yixiang Tian, Weihua Tian, Yujie Gong, Yanhua Zhang, Donghua Li, and Hong Li analyzed the data and were involved in the study design. Xiangtao Kang, Yadong Tian, Guoxi Li, Xiaojun Liu and Ruirui Jiang critically

revised the manuscript for important intellectual content. All authors approved the final manuscript.

DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103820.

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