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ORIGINAL ARTICLE

# Gonadotrophin-Inhibitory Hormone Receptor Expression in the Chicken Pituitary Gland: Potential Influence of Sexual Maturation and Ovarian Steroids

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Gonadotrophin-inhibitory hormone (GnIH), a hypothalamic RFamide, has been found to inhibit gonadotrophin secretion from the anterior pituitary gland originally in birds and, subsequently, in mammalian species. The gene encoding a transmembrane receptor for GnIH (GnIHR) was recently identified in the brain, pituitary gland and gonads of song bird, chicken and Japanese quail. The objectives of the present study are to characterise the expression of GnIHR mRNA and protein in the chicken pituitary gland, and to determine whether sexual maturation and gonadal steroids influence pituitary GnIHR mRNA abundance. GnIHR mRNA quantity was found to be significantly higher in diencephalon compared to either anterior pituitary gland or ovaries. GnIHR mRNA quantity was significantly higher in the pituitaries of sexually immature chickens relative to sexually mature chickens. Oestradiol or a combination of oestradiol and progesterone treatment caused a significant decrease in pituitary GnIHR mRNA quantity relative to vehicle controls. GnIHR-immunoreactive (ir) cells were identified in the chicken pituitary gland cephalic and caudal lobes. Furthermore, GnIHR-ir cells were found to be colocalised with luteinising hormone (LH)$\beta$ mRNA-, or follicle-stimulating hormone (FSH)$\beta$ mRNA-containing cells. GnIH treatment significantly decreased LH release from anterior pituitary gland slices collected from sexually immature, but not from sexually mature chickens. Taken together, GnIHR gene expression is possibly down regulated in response to a surge in circulating oestradiol and progesterone levels as the chicken undergoes sexual maturation to allow gonadotrophin secretion. Furthermore, GnIHR protein expressed in FSH$\beta$ or LH$\beta$ mRNA-containing cells is likely to mediate the inhibitory effect of GnIH on LH and FSH secretion.

**Key words:** oestradiol, progesterone, LH, FSH, sexual maturation.

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In the past 10 years, it has been shown that the vertebrate brain produces a number of RFamide peptides that regulate the secretion of anterior pituitary hormones (1–11). The RFamide family of peptides is a group of neuropeptides that share a common caroboxyl-terminal motif comprised of RF-$NH_2$ (R = arginine, F = phenylalanine). Gonadotrophin-inhibitory hormone (GnIH), a newly discovered hypothalamic peptide, is a dodecapeptide belonging to this family of RFamide peptides with a C-terminal Leu-Pro-Xaa-Arg-Phe-$NH_2$ motif (LPXRFamide motif). First isolated from quail hypothalamus, GnIH (9), has been shown to inhibit luteinising hormone (LH) secretion from the Japanese quail anterior pituitary gland both *in vitro* and *in vivo* (12). The gene encoding a precursor protein for GnIH in Japanese quail, chickens and song birds has been cloned and found to share a high degree of homology (13–15). GnIH-immunoreactive (ir) neurones (9) have been exclusively found in the hypothalamic paraventricular nucleus, whereas GnIH-ir fibres are found throughout the brain, including the median eminence in Japanese quail (11) and song birds (15). In addition to avian species, GnIH immunoreactivity have been identified in hamsters, rats, and mice (16), wherein GnIH-ir cell bodies were found to be clustered in the mediobasal hypothalamus with pronounced projections and terminals throughout the central nervous system. This extensive distribution of GnIH-ir fibres in multiple brain areas is consistent with multiple functions of GnIH, such as feeding behaviour in chickens

(17), and sexual behaviour in white-crowned sparrows (18). GnIH-ir neurones were found to have close appositions with GnRH-ir cells in the preoptic area of the sparrow and starling brain as well as midbrain of the starling brain, thereby suggesting a possible interaction of GnIH and GnRH neurones (15, 19). In addition, GnIH expression in the Japanese quail brain was found to be photoperiodically regulated, with the greatest expression observed under short-day photoperiod or when melatonin was administered to pinealectomised and orbital enucleated birds (20).

Administration of GnIH either by intracerebroventricular or peripheral route to ovariectomised Syrian hamsters resulted in reduced plasma LH concentrations, providing compelling evidence for the role of GnIH as a gonadotrophin inhibitory factor (16). Treatment of male chicken pituitary slices *in vitro* with GnIH resulted in decreased follicle-stimulating hormone (FSH) and LH synthesis and release (21). Furthermore, GnIH was found to decrease plasma testosterone concentrations, induce testicular apoptosis, and decrease testicular spermatogenic activity when GnIH was continuously administrated to Japanese quail over a 2-week period (12). Based on these findings, GnIH appears to be an important neuropeptide involved in the control of gonadotrophin secretion in both avian and mammalian species.

The chicken and Japanese quail GnIH receptor (GnIHR) cDNA have been cloned and found to be 97% similar to each other (13, 22). The deduced protein sequences of Japanese quail and chicken GnIHR are predicted to belong to a family of G protein-coupled receptors that have a unique seven transmembrane domain (22). The membrane fraction of COS-7 cells wherein putative quail GnIHR was overexpressed showed a dose-dependent high affinity binding to GnIH (13, 22). Furthermore, GnIH treatment of COS-7 cells overexpressing chicken GnIHR resulted in a dose-dependent decrease in the accumulation of $G_{\alpha i2}$ mRNA (13). Using the reverse transcription-polymerase chain reaction (RT-PCR), GnIHR mRNA was found to be expressed in the diencephalon and anterior pituitary gland in chickens and Japanese quail (13, 22). GnIHR mRNA-expressing neurones have been documented using *in situ* hybridisation histochemistry in the songbird hypothalamus (15) as well as in the gonadal tissues of Japanese quail and songbird gonadal tissues (23). Furthermore, we have recently reported that GnIHR mRNA is expressed in the ovarian follicles in addition to the diencephalon and anterior pituitary gland in the chicken (24). To determine the inhibitory effect of GnIH on LH secretion, it is important to characterise the expression of GnIHR protein in the anterior pituitary gland. However, cellular localisation of GnIHR protein or regulation of GnIHR mRNA expression in the pituitary gland has not been studied in any species. Considering the inhibitory effect of GnIH on LH secretion, we hypothesised that GnIHR expression would be higher in the pituitary gland compared to the diencephalon or ovary. The main objectives of the present study were: (i) to characterise the expression of GnIHR mRNA and protein in the chicken pituitary gland; (ii) to determine whether sexual maturation and gonadal steroids would influence pituitary GnIHR mRNA abundance; and (iii) to determine whether GnIH treatment would affect pituitary FSH and LH secretion *in vitro*.

## Materials and methods

### Animals

Female white Leghorn chickens (Hyline W36 strain) were housed in cages under a 16 : 8 h light/dark cycle. The chickens were provided with feed and water *ad libitum*. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee approved protocol.

### Reagents

Trizol and RNeasy kits used to isolate RNA were obtained from Invitrogen (Carlsbad, CA, USA) and Qiagen (Valencia, CA, USA), respectively. Moloney murine leukemia virus (M-MuLV) reverse transcriptase and Taq polymerase used for the RT-PCR were purchased from New England Biolabs (Beverly, MA, USA). Quantitative real-time PCR master mixture (Platinum SYBR Green qPCR Super Mix-UDG), RNAseOut, Alexa Flour 488 goat anti-rabbit immunoglobulin (Ig)G, Alexa Flour 546 anti-mouse IgG, Streptavidin Alexa Flour 488, ProLong Gold antifade reagent, and cell culture reagents were purchased from Invitrogen. Oestradiol 17-$\beta$ and progesterone were purchased from Sigma-Aldrich (St Louis, MO, USA). Putative chicken GnIH peptide (SIRPSAYLPLRF-$NH_2$) was custom synthesised by Sigma Genosys (The Woodlands, TX, USA).

### Quantification of GnIHR mRNA

To determine the relative GnIHR mRNA quantity, sexually immature (16 weeks old; n = 6) female chickens were killed by decapitation to collect diencephalon, anterior pituitary gland and total ovary. Anterior pituitary gland and total ovary were immediately frozen in liquid nitrogen. The brain was removed from the cranium and frozen in powdered dry ice. All the tissues were stored at −80 °C until processed. Using a cryostat (Microm, Walldorf, Germany), 40-$\mu$m thick coronal sections of the chicken brain were made. Diencephalic tissue sections in the coronal plane, starting from the anterior commissure and ending with the mammillary nucleus including the median eminence, were collected following the coordinates of the chick brain stereotaxic atlas (25). Total RNA from diencephalon, anterior pituitary gland, and ovary was extracted using Trizol (Invitrogen) and/or RNeasy kit (Qiagen). After DNase-I (Qiagen) treatment, 1 $\mu$g of total RNA was reverse transcribed using oligo $d(T)_{30}A/G/CA/G/C/T$ primer, and 2 U reverse transcriptase in a 20-$\mu$l reaction. Both GnIHR mRNA and chicken $\beta$-actin mRNA were quantified using 3 $\mu$l of the reverse transcription reaction (equivalent of 150 ng of single-stranded cDNA) as template in the real-time quantitative PCR (qPCR). A 140-bp product for chicken GnIHR cDNA (Gen Bank Accession no. AB193127) and a 123-bp product of chicken $\beta$-actin cDNA (Gen Bank Accession no. L08615) were amplified using the oligonucleotide primers shown in Table 1. The real-time qPCR consisted of Platinum SYBR Green qPCR Super Mix-UDG and 300 nm of forward and reverse primers in a 20 $\mu$l reaction. The reactions were performed in DNA engine Opticon II (MJ Research, Reno, NV, USA) with the settings: 50 °C for 2 min, 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. At the end of amplification, a melting curve analysis was perfomed by heating the PCR products to 65—95 °C for 15 s at increments of 0.2 °C, and the fluorescence was detected to confirm the presence of a single amplification product. Each sample was run in duplicate to obtain average log-linear threshold ($C_T$) values for GnIHR mRNA and $\beta$-actin mRNA. For negative controls, reverse transcription reactions using 1 $\mu$g total RNA without reverse transcriptase (—RT) were used as a template in place of single stranded cDNA. The log-linear threshold values during the exponential phase of the PCR for GnIHR mRNA were subtracted from that of $\beta$-actin mRNA. GnIHR mRNA quantity was expressed as a proportion of $\beta$-actin mRNA

**Table 1.** Oligonucleotide Primer Sequences To Amplify Partial cDNA Encoding Chicken Luteinising Hormone $\beta$ Subunit (LH$\beta$), Follicle-Stimulating Hormone $\beta$ Subunit (FSH$\beta$), Gonadotrophin-Inhibitory Hormone Receptor (GnIHR), or $\beta$-Actin Utilised in *in situ* Hybridisation Histochemistry or Real-Time Quantitative Polymerase Chain Reaction.

| Name | Sequence (5′ to 3′) | Product length (bp) |
|---|---|---|
| LH$\beta$ | | |
| Forward | GTTGGTGCTGATGACCCTTT | 194 |
| Reverse | TGGTGGTCACAGCCATACAT | |
| FSH$\beta$ | | |
| Forward | TTGCTGGAAAGCAATCTGTT | 357 |
| Reverse | TTGCTTCCATTGTGACTGAAG | |
| GnIHR | | |
| Forward | CACTGATGCTGCTGACAGACTA | 140 |
| Reverse | CTCATTGAAGTAGCCGTAGATGAT | |
| $\beta$-Actin | | |
| Forward | CTGGCACCTAGCACAATGAA | 123 |
| Reverse | CTGCTTGCTGATCCACATCT | |

quantity following the $2^{-\Delta\Delta Ct}$ method for converting log-linear $C_T$ values to linear term (26).

## Development of GnIHR antibody

Rabbit polyclonal antisera were custom generated (Sigma Genosys, Austin, TX, USA) against a keyhole limpet haemocyanin (KLH) conjugated synthetic peptide (FRAPFCLPPRRQHRG) corresponding to the predicted GnIHR protein sequence in the C-terminal extracellular domain (amino acids 347–361; Gen Bank Accession no. BAE17050). Two New Zealand White rabbits were given six immunisations each with a total of 700 $\mu$g of the peptide-KLH mixed in either complete (first dose) or incomplete (booster doses) Freund's adjuvant. Blood samples were obtained at various intervals until 110 days after immunisation. Serum antibody titre, as determined by enzyme-linked immunosorbent assay using the peptide antigen as the standard, was found to be 1 : 50 000 after 70 days of immunisation. A part of the antisera was affinity purified using the peptide and utilised for localisation of GnIHR-immunoreactive (ir) cells in the pituitary gland.

GnIHR antiserum was validated by western blotting using chicken pituitary gland and testis protein extracts prepared using a protocol as described previously (27). Briefly, entire pituitary gland or approximately 0.5 g of chicken testis was homogenised in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich). The homogenate was then passed through a 22-gauge needle and shaken in a thermomixer (Eppendorf, Westbury, NY, USA) at 1000 rpm for 30 min at 4 °C. The lysate was centrifuged at 14 000 *g* for 15 min at 4 °C and the supernatant was collected. Total protein concentration was estimated by a protein dye binding assay using a commercial kit (Bio-Rad, Hercules, CA, USA), with chicken ovalbumin as the standard. One-dimensional gel electrophoresis was performed with the NuPAGE Novex minigel system (XCell SureLock Mini Cell; Invitrogen) according to manufacturer's recommendations. Samples were prepared by combining approximately 10 $\mu$g of total protein extract with 4 × NuPAGE LDS sample buffer and 10 × NuPAGE reducing agent, and heated for 10 min at 70 °C prior to electrophoresis. Proteins were separated on a 10% Bis-Tris polyacrylamide gel (Invitrogen) and electrotransferred onto Immun-Blot PVDF membranes (0.20 $\mu$m; Bio-Rad). Membranes were incubated in blocking buffer (Pierce Biotechnology, Rockford, IL, USA) for 2 h at room temperature before incubating overnight in rabbit anti-chicken GnIHR antiserum (1 : 4000) at 4 °C with gentle agitation. The membranes were washed once for 15 min in Tris-buffered saline-Tween (TBS-T; 10 mM Tris, 150 mM NaCl, 0.1% Tween-20), followed by three washes for 5 min each in TBS-T. Immunoreactive GnIHR band was detected by incubation in horseradish peroxidase conjugated to goat anti-rabbit IgG (1 : 10 000; Pierce) for 1 h at room temperature. The membrane was washed as previously described, and treated with ECL Plus Chemiluminescence Detection Reagent (Amersham Biosciences Piscataway, NJ, USA). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences).

## Immunohistochemical detection of GnIHR in the pituitary gland

Anterior pituitary glands collected from sexually mature Leghorn chickens (32 weeks old; n = 6) were used for localising GnIHR-ir cells as described previously (28). Briefly, chickens were killed by decapitation and the anterior pituitary gland was quickly removed from the cranium. The anterior pituitary gland was fixed in Bouin's solution for 24 h followed by storage in phosphate-buffered saline buffer (pH 7.2) at 4 °C until processing. Pituitaries were dehydrated, cleared and embedded in paraffin using an automated tissue processor. Pituitary gland tissue sections in the sagittal plane were cut at 4 $\mu$m thickness using a rotary microtome (Microm, Walldorf, Germany) and serial sections were mounted on Superfrost Plus glass slides (VWR, West Chester, PA, USA). For immunostaining, pituitary tissue sections were deparaffinised in Histoclear (National Diagnostics, Atlanta, GA, USA) and hydrated in descending concentrations of ethyl alcohol in water and rinsed in TBS (0.01 M Tris-HCl and 0.15 M sodium chloride solution, pH 7.4). Following several washes in TBS containing 0.50% Triton X-100 (TBSX; Sigma), slides were treated in a blocking solution (2.5% normal goat serum in TBSX) for 1 h. Tissue sections were incubated with affinity purified anti-chicken GnIHR IgG (1 : 100) at 4 °C for approximately 18 h. Following washes in TBS, the slides were incubated with Alexa Flour 488 goat anti-rabbit IgG (1 : 400) for 1 h. Following washes in TBS, sections were mounted with ProLong Gold antifade reagent. Unless otherwise noted, all steps were performed at room temperature with the washes consisting of three incubations with buffer for 10 min each. To determine the specificity of immunostaining, anti-chicken GnIHR antibody (1 : 100) was preadsorbed with GnIHR synthetic peptide (25 $\mu$g GnIHR peptide/ml) for 18 h and centrifuged to collect the supernatant. As a negative control, the supernatant was substituted in place of the primary antibody during immunohistochemical staining. Green (GnIHR) fluorescent-cells were visualised by excitation of Alexa Flour 488 fluorophor with lasers using an Olympus Fluoview 300 Confocal Laser Scanning Microscope (Olympus, Center Valley, PA, USA).

## Colocalisation of GnIHR with LH$\beta$ mRNA or FSH$\beta$ mRNA in the chicken pituitary gland

### *Preparation of digoxigenin labelled cRNA probes*

Partial cDNA encoding chicken LH$\beta$ mRNA or FSH$\beta$ mRNA were generated using oligonucleotide primer sets (Table 1) using a chicken pituitary cDNA library as a template. The primers for amplifying chicken LH$\beta$ cDNA and FSH$\beta$ were derived from a previous study (29) and Gen Bank Accession No. AY029204, respectively. The PCR products were cloned into pGEM-T Easy vector and transformed into JM109 competent cells following manufacturer's protocol (Promega, Madison, WI, USA). The antisense and sense strands of plasmids were confirmed by nucleotide sequencing using Sp6 and T7 primers (Davis Sequencing, Davis, CA, USA). The plasmids were linearised using *Nco*I and *Nde*I restriction endonucleases and gel purified. The antisense and sense strands of FSH$\beta$ cDNA and LH$\beta$ cDNA were *in vitro* transcribed in a 20-$\mu$l

reaction using digoxigenin-labelled nucleotides (Roche Biochemicals Indianapolis, IN, USA) with Sp6 polymerase and T7 polymerase, respectively. Both sense and antisense cRNA probes were treated with DNaseI (Roche) at 37 °C for 15 min to remove the cDNA template. At the end of reaction, 180 $\mu$l of nuclease free water, 500 $\mu$l of ice-cold ethanol and 20 $\mu$l of 3 M sodium acetate (pH 5.2) were added and stored at −80 °C overnight. After incubation, tubes were centrifuged at 13 000 *g* for 30 min at room temperature. The resultant pellet was washed with 70% ethanol and vacuum dried for 10 min and reconstituted in 20 $\mu$l of nuclease-free water. The probes were subjected to agarose gel electrophoresis for confirming the quality and size. The probes were stored at −20 °C until used for *in situ* hybridisation histochemistry.

### *In situ* hybridisation histochemistry

Pituitary tissues collected and processed for immunohistochemistry were used for this study. Tissue sections were deparaffinised, hydrated, and treated with 0.2 N hydrochloric acid. Slides were rinsed in 0.3% triton-X-100 and treated with proteinase K (Roche Biochemicals; 5 $\mu$g/ml) in 0.1 M Tris HCl containing 50 mM ethelynediaminetetraceteic acid (EDTA) for 20 min. Tissue sections were then treated with 0.2% glycine for 5 min, fixed in 3.7% paraformaldehyde and acetylated with 0.25% acetic anhydride and 0.1 M triethanolamine. The slides were then incubated in prehybridisation buffer (50% formamide in 2 × SSC) for 90 min at 37 °C. Digoxigenin-labelled LH$\beta$ cRNA or FSH$\beta$ cRNA probe (50 ng of antisense or sense probe as negative control) were diluted in hybridisation buffer (10 mM Tris-HCL, 12.5% Denhardt's solution, 50% formamide, 0.5% SDS, 2 × SSC) and applied to the slides under a coverslip. The slides were incubated in a humidified chamber at 45 °C overnight. The slides were treated with RNAse (Invitrogen; 20 $\mu$g/ml) in 10 mM Tris-HCl, 5 mM EDTA, 0.3 M NaCl for 30 min at 37 °C. Slides were then washed twice in 2 × SSC for 15 min each time at room temperature, twice in 0.1 × SSC for 5 min each, and twice in 0.1 × SSC for 30 min each at 42 °C. Next, the slides were washed in TBS containing 0.1% triton X-100 (Sigma) for 10 min, and treated with 1 × blocking buffer (Roche Biochemicals) in TBS for 30 min. A monoclonal anti-digoxigenin antibody (Roche Biochemicals; 1 : 250) and affinity purified anti-chicken GnIHR antibody (1 : 100) were applied to the slides and incubated at 4 °C overnight. Following three washes in TBS for 5 min each, slides were treated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1 : 400) for 1 h at room temperature. Slides were washed three times in TBS and incubated with streptavidin-Alexa Flour 488 (1 : 100) and Alexa Flour 546 goat anti-mouse IgG (1 : 250) for 1 h at room temperature. Following three washes in TBS, slides were mounted with ProLong Gold Antifade Reagent (Invitrogen). GnIHR-ir (green) and LH$\beta$ mRNA- or FSH$\beta$ mRNA (red) cells were visualised by exciting the fluorophores sequentially with respective lasers using an Olympus Fluoview 300 Confocal Laser Scanning Microscope (Olympus).

### Quantification of diencephalic and pituitary GnIHR mRNA in sexually immature and mature chickens

Sexually mature (26 weeks old; n = 7) and immature chickens (16 weeks old; n = 7) were killed by decapitation to collect brains and pituitary glands. The brain was removed from the cranium and frozen in powdered dry ice. Coronal section of the brain were made to separate the entire diencephalon as described above. Total RNA was extracted from the diencephalon and pituitary gland using Trizol (Invitrogen) and/or RNeasy kit (Qiagen) as described above. Total RNA (1 $\mu$g) was reverse transcribed and subjected to qPCR analysis for the determination of GnIHR mRNA. Each sample was run in duplicate to obtain average $C_T$ values for GnIHR mRNA and $\beta$-actin mRNA. For negative controls, reverse transcription reactions using 1 $\mu$g total RNA without reverse transcriptase (−RT) were used as a template in place of single stranded cDNA. The relative amount of pituitary GnIHR mRNA in mature and immature birds was compared.

### Effect of ovarian steroids on pituitary GnIHR mRNA expression

Sexually immature female chickens are utilised as their gonadal steroid levels in the circulation are expected to be low obviating the use of ovariectomised chicken for this experiment. Sexually immature chickens (16 weeks old; n = 7) were injected, intramuscularly, with peanut oil containing oestradiol-17$\beta$ [0.5 mg/kg body weight; four injections on alternate days (30)], progesterone [0.17 mg/kg bodyweight/day for seven consecutive days (31)], oestradiol and progesterone together at the above dosage, or no steroids (negative control). Seven days after the first dose, the chickens were killed by decapitation and the oviduct was isolated (infundibulum to shell gland) and weighed to confirm the efficacy of oestradiol and/or progesterone treatments. Anterior pituitary glands were snap frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from the pituitary glands using RNeasy kit and subjected to real-time qPCR for determination of GnIHR mRNA quantity as described above.

### Effect of GnIH on FSH and LH secretion from pituitary gland slices *in vitro*

Anterior pituitary glands from sexually immature (16 weeks old) and mature (26 weeks old) Leghorn chickens (n = 9) were collected after decapitation and placed in ice-cold M199 culture medium. Each pituitary gland was cut into three equal sized slices and all three slices were placed in a tissue culture plate well containing 0.5 ml of M-199 culture media supplemented with 10 mM HEPES buffer, 0.1% bovine serum albumin, penicillin and streptomycin in a 24-well plate. The culture plate was placed in an airtight container, equilibrated with 95% $O_2$ and 5% $CO_2$, and incubated at 37 °C with gentle shaking (60 revolutions/min) for a 90 min pre-incubation period. After the pre-incubation period, the culture medium from each well was replaced with a medium containing 0, $10^{-8}$ or $10^{-6}$ M GnIH synthetic peptide. The culture plate was placed in the airtight container, equilibrated with 95% $O_2$ and 5% $CO_2$ and incubated for 120 min with gentle shaking. At the end of 120 min incubation, the culture medium was recovered for determination of LH and FSH quantity by radioimmunoassay as described previously (32, 33). Intra-assay coefficients of variation for the FSH and LH radioimmunoassays were found to be 8.7% and 4.8%, respectively.

### Statistical analysis

GnIHR mRNA quantity in the anterior pituitary gland of sexually immature and mature chickens was compared using Student's t-test. Effect of oestradiol and/or progesterone treatments on GnIHR mRNA quantity, as well as the effect of GnIH treatment on FSH and LH secretion *in vitro* and oviduct weights were analysed by ANOVA using the General Linear Model Procedure of Statistical Analysis Software (SAS Institute, Cary, NC, USA). Relative GnIHR mRNA quantity to $\beta$-actin mRNA quantity was first converted from log-linear to linear term and then compared using general linear models procedure. $P < 0.05$ was considered statistically significant.

## Results

### Quantification of GnIHR mRNA

The GnIHR mRNA quantity was significantly higher in diencephalon compared with pituitary gland and total ovary ($P < 0.05$; Fig. 1).

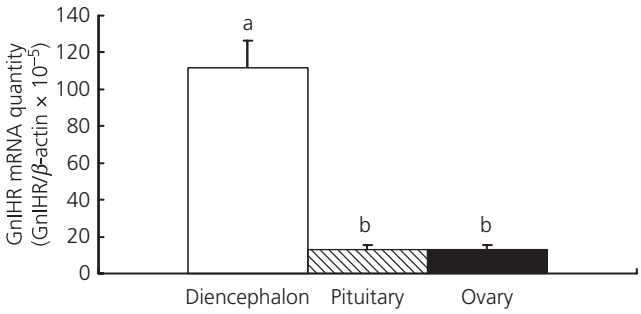


**Fig. 1.** Gonadotrophin-inhibitory hormone receptor (GnIHR) mRNA abundance relative to $\beta$-actin mRNA abundance in diencephalon, pituitary gland and ovaries of sexually immature chickens. Total RNA from each tissue was deoxyribonulcease-I treated and reverse transcribed. Approximately 150 ng of cDNA was used in the real-time quantitative polymerase chain reaction employing SYBR green as the dye to quantify GnIHR mRNA or $\beta$-actin mRNA in separate reactions. Different letters above each bar indicate significant difference at $P < 0.05$. Values represent the mean $\pm$ SE ($n = 6$).

However, GnIHR mRNA quantity in the anterior pituitary gland and ovary was not significantly different from each other ($P > 0.05$; Fig. 1). Melting curve analyses showed the presence of a single PCR product for GnIHR or $\beta$-actin product confirming the specificity of the reaction (data not shown). Total RNA from the diencephalon, pituitary gland, and ovary reverse transcribed in the absence of reverse transcriptase resulted in $C_T$ values not different from blanks (> 30 cycles), indicating that genomic DNA was not contributing to the GnIHR or $\beta$-actin mRNA quantity (data not shown). In addition, GnIHR cDNA sequence amplified in the real-time qPCR showed 100% homology to chicken GnIHR cDNA (Gen Bank Ref no.AB193127) (13) and did not share significant homology to any other chicken gene sequences.

## Localisation of GnIHR-ir cells in the pituitary gland

GnIHR-ir cells within the chicken pituitary gland were localised by immunohistochemical staining utilising a custom generated GnIHR antibody. GnIHR-ir cells were found to be distributed throughout the cephalic (Fig. 2A) and caudal lobes (Fig. 2B, C), particularly in the peripheral regions of the anterior pituitary gland. Higher magnification images of the pituitary gland tissue sections revealed punctate GnIHR-immunofluorescence, a pattern that is unique to transmembrane receptors, both in cell membrane (Fig. 2D) and/or in the cyto-

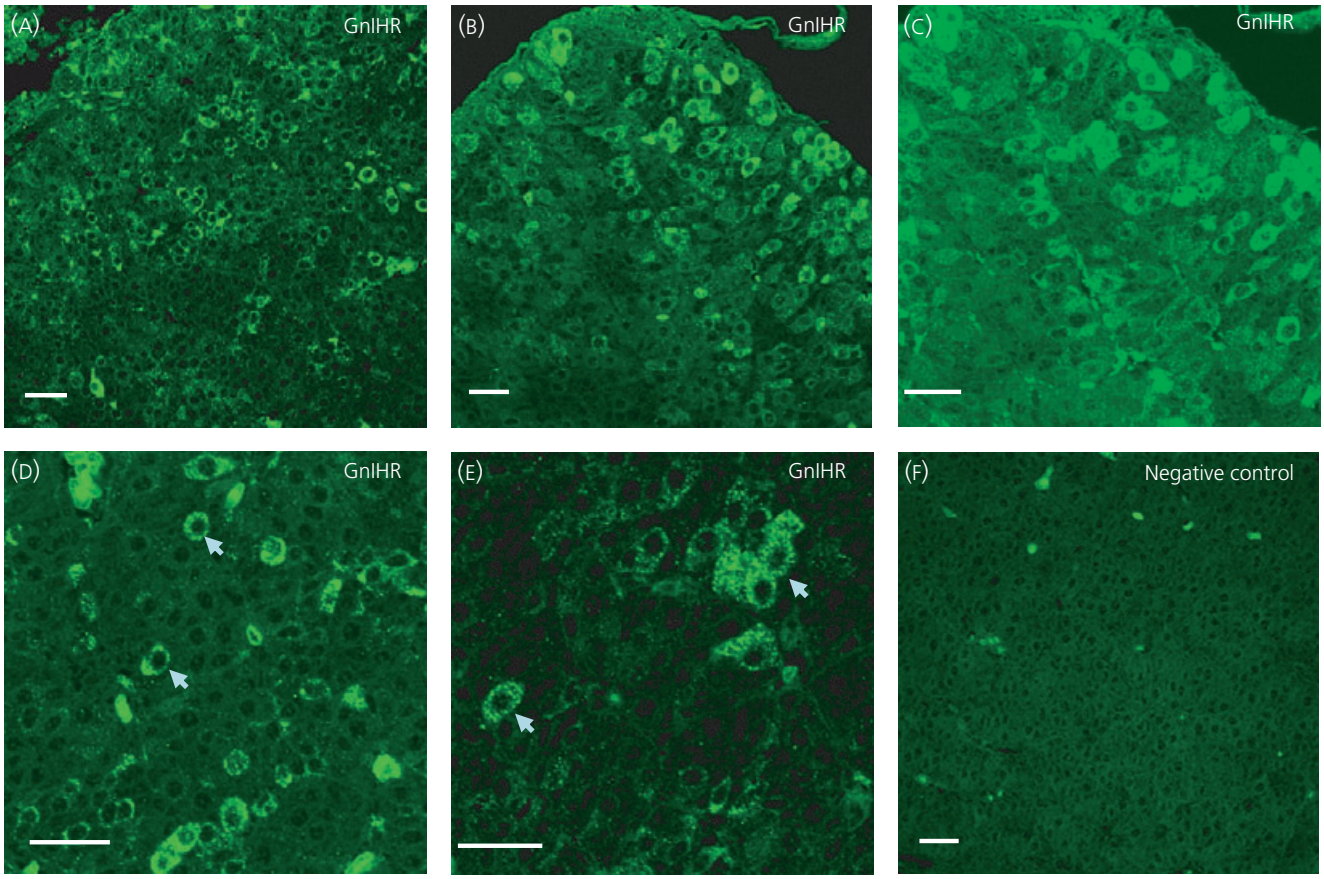


**Fig. 2.** (A–F) Representative confocal photomicrographs of the chicken pituitary gland showing gonadotrophin-inhibitory hormone receptor (GnIHR)-immunoreactive (ir) cells. Mid-sagittal pituitary tissue sections were probed with affinity purified anti-chicken GnIHR immunoglobulin G (A–E) or with primary antibody that was preadsorbed with synthetic GnIHR peptide (negative control; F). GnIHR-ir cells were found in the cephalic (A) and caudal (B, C) lobes of the pituitary gland. Punctate GnIHR-ir granules were found along the cell membrane as well as in the cytoplasm of several pituitary cells (D, E, arrows). Green (GnIHR)-fluorescent cells were visualised by exciting the fluorophore using a laser confocal microscope. Scale bar = 20 $\mu$m.

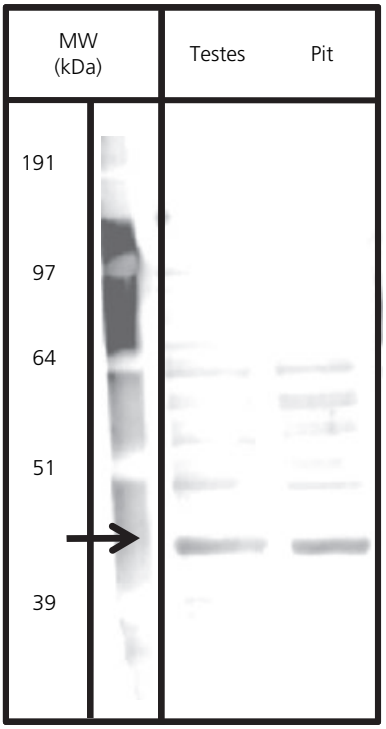


**Fig. 3.** Detection of gonadotrophin-inhibitory hormone receptor (GnIHR) protein in chicken pituitary gland and testis protein extracts. Approximately 10 $\mu$g of total protein from pituitary gland or testis was electrophoresed and blotted onto PVDF membrane. A 45-kDa protein (arrow), corresponding to the deduced molecular weight of GnIHR protein, was detected using rabbit anti-chicken GnIHR antiserum. The identities of higher molecular weight immunoreative products are not known. MW, protein molecular weight standards; Pit, pituitary gland.

plasm of the pituitary cells (Fig. 2E). Presence of GnIHR-ir granules in the cytoplasm suggests that not all of this protein is transported to the cell membrane after synthesis but is stored in the cytoplasm. Immunostaining in the pituitary gland was abolished when the primary antibody was replaced by a GnIHR antibody preadsorbed with GnIHR synthetic peptide (Fig. 2F). Furthermore, the anti-chicken GnIHR antibody was validated by a western blot analysis that detected a 45-kDa protein both in the pituitary gland and testis protein extracts in accordance with the molecular weight for the predicted GnIHR protein (Fig. 3). In addition to the 45-kDa band, a few other bands of higher molecular weight were also detected in the immunoblot with both testis and pituitary gland protein extracts, which may represent multimeric forms and/or post-translationally modified GnIHR protein.

## Colocalisation of GnIHR with LH$\beta$ mRNA or FSH$\beta$ mRNA in the chicken pituitary gland

Figure 4(A–L) shows representative photomicrographs of chicken pituitary gland tissue sections subjected dual *in situ* hybridisation and immunohistochemistry to reveal LH$\beta$ mRNA or FSH$\beta$ mRNA in GnIHR-ir cells. Punctate GnIHR-ir granules were found scattered throughout the cephalic and caudal lobes of the pituitary gland (green; Fig. 4A, D, G, J). LH$\beta$ mRNA (Fig. 4B) or FSH$\beta$ mRNA (Fig. 4H) staining was noticed throughout the cytoplasm of numerous cells in both cephalic and caudal lobes of the pituitary gland. Many of the LH$\beta$ mRNA stained cells also contained GnIHR-ir granules (Fig. 4C). Similarly, several FSH$\beta$ mRNA stained cells also contained GnIHR-ir granules (Fig. 4I). As a negative control, pituitary gland tissue sections were hybridised with cRNA probes corresponding to the sense strand of LH$\beta$ cDNA or FSH$\beta$ cDNA and found to exhibit greatly diminished staining or no staining at all (Fig. 4E, K).

## Effect of sexual maturation on diencephalic and pituitary GnIHR mRNA quantity

GnIHR mRNA abundance was significantly higher in the diencephalon of sexually mature chickens compared with sexually immature chickens ($P < 0.05$; Fig. 5A). By contrast to diencephalon, pituitary GnIHR mRNA quantity was significantly greater in the sexually immature chickens compared with sexually mature chickens ($P < 0.05$; Fig. 5B). Melting curve analyses showed the presence of a single PCR product for GnIHR or $\beta$-actin confirming the specificity of the reaction (data not shown). Total RNA from pituitaries reverse transcribed in the absence of reverse transcriptase resulted in $C_T$ values not different from blanks (> 30 cycles) indicating that genomic DNA did not contribute to the GnIHR or $\beta$-actin mRNA quantity (data not shown).

## Effect of oestradiol and/or progesterone treatment on pituitary gland GnIHR mRNA quantity

Using real-time qPCR, GnIHR mRNA abundance in the anterior pituitary gland was determined. Oestradiol or a combination of oestradiol and progesterone treatments significantly decreased the amount of GnIHR mRNA in the anterior pituitary gland when compared to that in vehicle-treated chickens ($P < 0.05$; Fig. 6). Progesterone treatment alone did not affect pituitary gland GnIHR mRNA quantity ($P > 0.05$; Fig. 6). The oviduct weight was significantly higher ($P < 0.01$) in chickens treated with oestradiol ($10.65 \pm 0.38$ g) or a combination of oestradiol and progesterone ($17.98 \pm 0.58$ g) compared to chickens that were treated with vehicle ($0.34 \pm 0.05$ g) or progesterone ($0.44 \pm 0.08$ g), confirming the efficacy of the steroid treatments. Melting curve analyses showed the presence of a single PCR product for GnIHR or $\beta$-actin confirming the specificity of the reaction (data not shown).

## Effect of GnIH treatment on pituitary LH and FSH secretion *in vitro*

Compared to controls, LH quantity was significantly less in the culture medium in which pituitary gland slices of sexually immature chickens were incubated for 120 min with $10^{-8}$ M, but not with $10^{-6}$ M GnIH peptide ($P < 0.05$; Fig. 7A). However, GnIH treatment of pituitary gland slices from sexually mature chicken did not result in significant changes in LH quantity in the culture medium ($P > 0.05$; Fig. 7A). Likewise, GnIH treatment had no significant

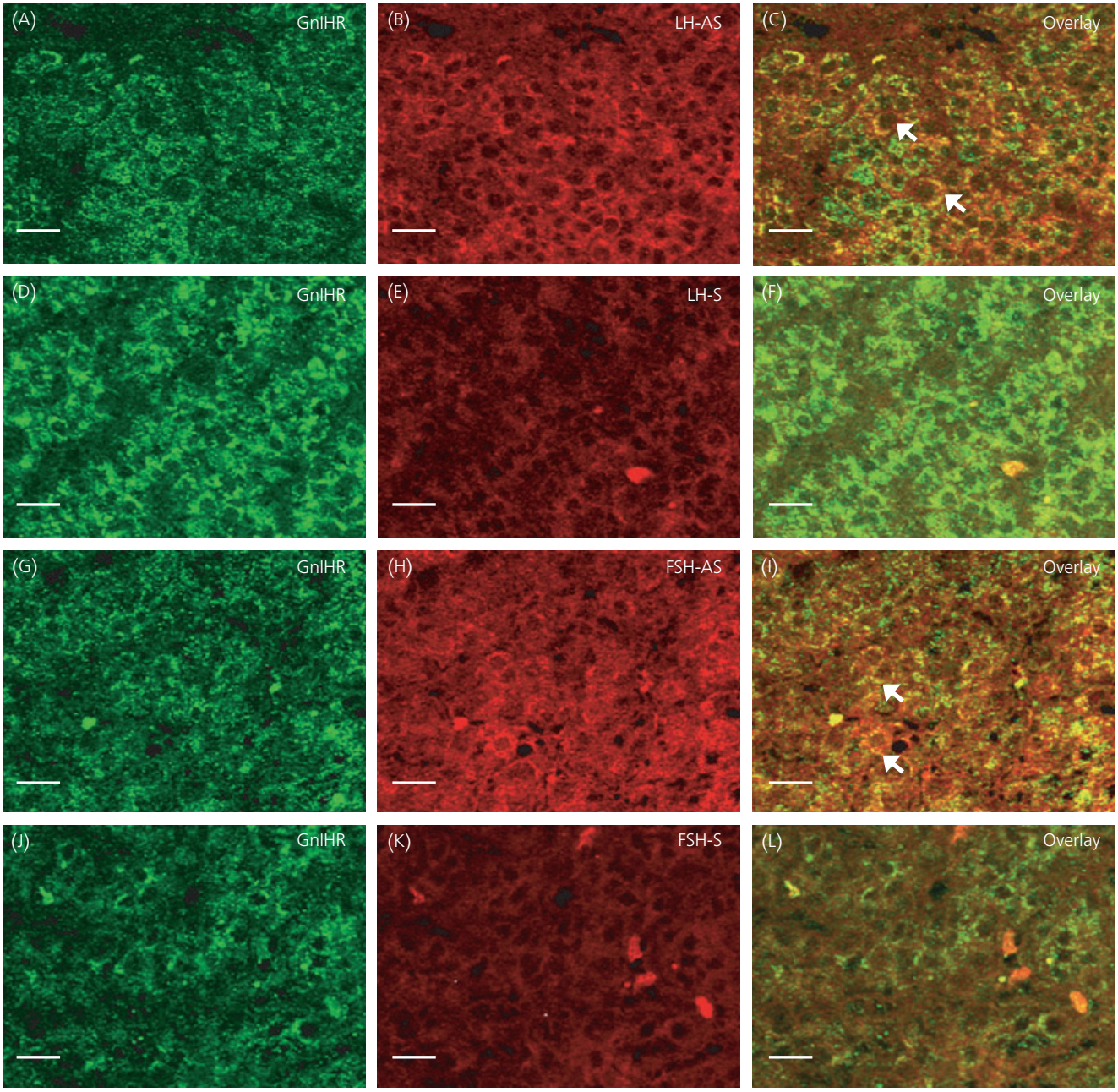


**Fig. 4.** (A–L) Representative confocal photomicrographs of the chicken pituitary gland tissue sections subjected to dual labelling of gonadotrophin-inhibitory hormone receptor (GnIHR) and luteinising hormone (LH)$\beta$ mRNA or follicle-stimulating hormone (FSH)$\beta$ mRNA. (A–C) Punctate GnIHR-immuoreactive granules (A) and cytoplasmic LH$\beta$ mRNA (B) are colocalised in many cells (C, arrows). (G–I) Punctate GnIHR-immuoreactive granules (G) and cytoplasmic FSH$\beta$ mRNA (H) are colocalised in many cells (I, arrows). (E, K) Note the absence of specific staining when pituitary sections were hybridised with cRNA probe made with sense strand of LH$\beta$ cDNA (E) or FSH$\beta$ cDNA (K). Pituitary tissue sections were probed with affinity purified anti-chicken GnIHR immunoglobulin G (GnIHR) and LH$\beta$ antisense cRNA probe (LH-AS), LH$\beta$ sense cRNA probe (LH-S), FSH$\beta$ antisense cRNA probe (FSH-AS), or FSH$\beta$ sense cRNA probe (FSH-S). Scale bar = 20$\mu$m.

effect on FSH quantity in the culture medium wherein pituitaries from sexually immature or mature chickens were incubated for 120 min ($P > 0.05$; Fig. 7B).

## Discussion

The present study is the first to characterise expression of GnIHR protein in the pituitary gland of any species. Using a custom developed antibody against a synthetic GnIHR peptide, chicken pituitary gland were found to contain GnIHR-immunoreactive granules and many of them were colocalised with LH$\beta$ mRNA or FSH$\beta$ mRNA-expressing cells. Such colocalisation suggests that GnIHR is expressed in gonadotrophs and is likely to mediate the inhibitory effect of GnIH on LH and FSH secretion.

We previously found that GnIHR mRNA was expressed in chicken pituitary gland, diencephalon, and ovary (24). In the present study,

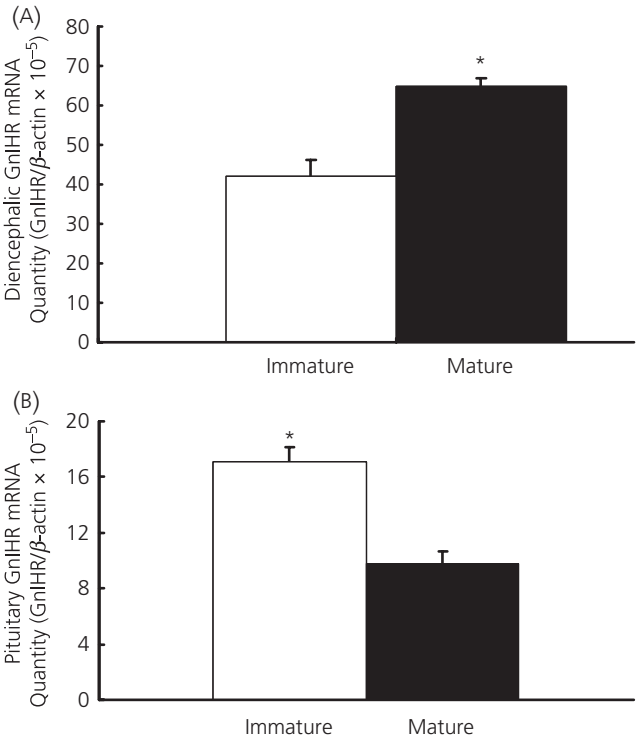


**Fig. 5.** (A–B) Gonadotrophin-inhibitory hormone receptor (GnIHR) mRNA abundance in diencephalon (A) and pituitary gland (B) of sexually immature (16 weeks old) and mature (26 weeks old) female chickens. Following reverse transcription of pituitary RNA, approximately 150 ng of cDNA was used in real-time quantitative polymerase chain reaction using SYBR green as the dye to quantify GnIHR mRNA or $\beta$-actin mRNA in separate reactions. An asterisk above the bar indicates significant difference at $P < 0.05$. Values represent the mean ± SE (n = 7, 4 in A and B, respectively).

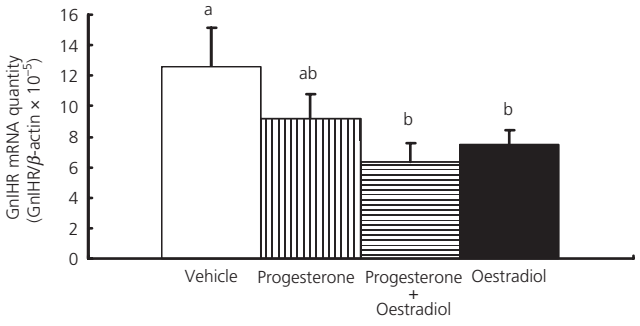


**Fig. 6.** Gonadotrophin-inhibitory hormone receptor (GnIHR) mRNA abundance in the pituitary gland of sexually immature chickens treated with vehicle, progesterone, oestradiol, or with a combination of progesterone and oestradiol. Total RNA extracted from the pituitary gland was treated with deoxyribonuclease-I and reverse transcribed. Approximately 150 ng of cDNA was used in real-time quantitative polymerase chain reaction using SYBR green as the dye to quantify GnIHR mRNA or $\beta$-actin mRNA in separate reactions from vehicle and steroid treated chickens. Different letters above each bar indicate significant difference at $P < 0.05$. Values represent the mean ± SE (n = 7).

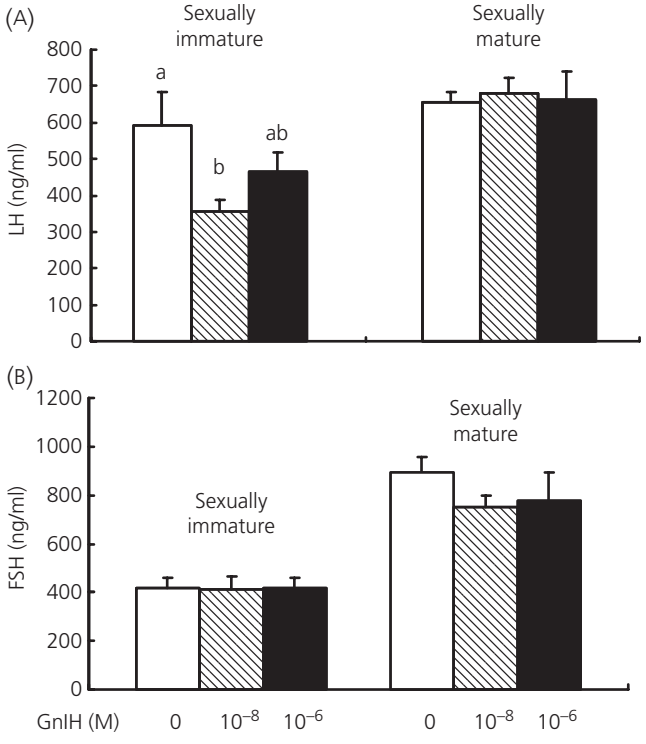


**Fig. 7.** Effect of gonadotrophin-inhibitory hormone (GnIH) on follicle-stimulating hormone (FSH) and luteinising hormone (LH) secretion from chicken pituitary gland slices *in vitro*. Pituitary gland collected from sexually immature (16 weeks old) or sexually mature (26 weeks old) chickens was cut into three equal sized slices and treated with GnIH (0, $10^{-8}$, $10^{-6}$ M) for 120 min. Culture media was collected and FSH and LH quantity were determined by radioimmunoassay. Each value represents the mean ± SE (n = 9). Different letters above each bar indicate significant difference at $P < 0.05$.

we hypothesised that the pituitary gland would contain greater quantities of GnIHR mRNA relative to diencephalon and the ovary based on the inhibitory effect of GnIH on gonadotrophin secretion (9). Refuting our hypothesis, we found that diencephalon GnIHR mRNA quantity was 8.8-fold higher than that in the anterior pituitary gland or ovaries. The physiological role of diencephalic GnIHR or the phenotypic identity of GnIHR-expressing neurones is presently unknown. However, GnIHR mRNA-containing neurones were reported to be colocalised with GnRH-I-ir neurones in the preoptic area and with GnRH-II-ir neurones in the midbrain of European starlings (34), suggesting that GnIHR is likely to be involved in controlling GnRH secretion. In addition, hypothalamic GnIHR is likely to be involved in GnIH signalling as it has been found that central administration of GnIH reduced solicited copulation behaviour in the white-crowned sparrows (18) while increasing feed intake in chicks (17). Previously, GnIH-ir neurones in the song sparrow and house sparrow hypothalamus were found to be colocalised with GnRH neurones and fibres in the preoptic area and median eminence, respectively (19). Similarly, GnIH-ir neuronal fibres were found in close proximity to GnRH-I neurones in the preoptic area and GnRH-II neurones in the midbrain of European starlings (15). Furthermore, GnIHR mRNA-expressing neurones were found to

 

be colocalised with both GnRH-I and GnRH-II cell bodies in the European starling hypothalamus (15). Therefore, it is possible that GnIH may be interacting with GnRH neurones through GnIHR in the hypothalamus for regulating gonadotrophin secretion from the anterior pituitary gland. The receptor for kisspeptin, GPR54, another RFamide receptor important for the control of reproduction in mammals, has been found to be expressed in the rat hypothalamus wherein it was colocalised with GnRH neurones in the preoptic area (35).

Using a highly specific antibody against a synthetic GnIHR peptide, we localised GnIHR-containing cells in the chicken anterior pituitary gland. Moreover, we found that GnIHR-ir cells were distributed throughout the anterior pituitary gland. The chicken pituitary gland can be divided into cephalic and caudal lobes, wherein lactotrophs are predominantly distributed in the cephalic lobe whereas somatotrophs are found in the caudal lobe (36–38). FSH-containing gonadotrophs were absent in the peripheral region of both cephalic and caudal lobes, while LH-containing gonadotrophs were present throughout cephalic and caudal lobes, with many of them distributed in the peripheral region (39). We found that a majority of GnIHR-ir cells in the chicken pituitary gland are distributed in the cephalic and caudal lobes, particularly in the peripheral regions. Based on the colocalisation of GnIHR and LH$\beta$ mRNA or FSH$\beta$ mRNA, GnIHR appears to be expressed in gonadotrophs. This possibility is also strengthened based on the fact that GnIH treatment decreased LH release from the pituitary gland *in vitro* (Fig. 7A). Such a decrease in LH secretion in response to GnIH treatment *in vitro* has been previously reported with chicken (21) and Japanese quail (9) pituitary glands. Our data on localising GnIHR-containing cells provides a basis for GnIH signalling within the pituitary gland. Similar to GnIH, several other RFamide peptides were found to regulate the secretion of pituitary hormones in frogs, mice, rats and Japanese quail. For example, RFamide related peptide-3 increases growth hormone but decreases LH secretion in rats (40), prolactin-releasing peptide increases prolactin release in rats (41), and frog growth hormone-releasing peptide (fGRP) stimulates growth hormone release in bull frog (4, 42). Another RFamide, fGRP related peptide-2, has also been found to stimulate growth hormone and prolactin release (42). Further studies are required to determine the phenotypic identity of pituicytes that express GnIHR in the chicken pituitary gland.

In the present study, we found 43% less GnIHR mRNA quantity in the pituitary glands of sexually mature chickens compared with that of sexually immature chickens. Our data suggest that GnIH signalling is most likely to be regulated at the receptor level, wherein GnIHR is downregulated in sexually mature chicken to allow LH secretion from the pituitary gland. In support of our findings, a recent study reported that GnIHR mRNA expression in pituitary gland was significantly reduced during a reproductive cycle in both male and female chickens with lowest levels observed around sexual maturity (43). These results suggest that GnIHR may play an important role during sexual maturation by allowing gonadotrophin secretion from the pituitary gland. This argument is further strengthened by a report that there was no change in hypothalamic GnIH mRNA levels in both laying and out-of-lay female chickens (44). In contrast to pituitary gland, diencephalic GnIHR mRNA abundance was greater in sexual mature chickens compared to immature chickens indicating that GnIHR expression is regulated differently in the brain. Nevertheless, sexual maturation in the chicken is associated with increased activity in the hypothalamo-pituitary-ovarian axis. During this period, there is an increase in the secretion of gonadal steroids into circulation as a result of follicular development (45, 46), possibly affecting expression of GnIHR mRNA. In order to test the influence of gonadal steroids on pituitary GnIHR mRNA expression, we injected oestradiol and progesterone to 16-week-old sexually immature female chickens. We found that oestradiol alone or in combination with progesterone reduced GnIHR mRNA abundance in the pituitary gland. These results suggest that higher levels of circulating oestradiol may be reducing the abundance of GnIHR mRNA and removing the inhibitory action of GnIH on gonadotrophin secretion in sexually mature chickens. We have previously reported that ovarian GnIHR mRNA quantity was significantly decreased with oestradiol and/or progesterone treatment (24), suggesting that gonadal steroids could down-regulate GnIHR gene expression in the pituitary-ovarian axis. In the present study, we found that progesterone treatment alone did not decrease pituitary GnIHR mRNA quantity. This lack of effect may be due to the lower amounts of progesterone receptors in the sexually immature chicken as reported in the pituitary gland of immature pullets (47) and expression of progesterone receptor may require priming with oestradiol. In support of this argument, we found that a combination of oestradiol and progesterone treatments resulted in a 50% decrease in pituitary GnIHR mRNA quantity in sexually immature chickens.

In the present study, we found that treatment of 16-week-old chicken pituitary gland slices *in vitro* with GnIH decreased LH but not FSH quantity in the culture media. This inhibitory effect was noticed with a $10^{-8}$ M but not $10^{-6}$ M GnIH concentration in the culture media. The lack of an effect in response to higher GnIH concentration on LH secretion may be due to receptor desensitisation. Our findings on LH release are in agreement with previous reports on the inhibitory effect of GnIH on LH secretion in Japanese quail and chicken pituitary gland (9, 21). An earlier study has reported that GnIH treatment *in vitro* also decreased FSH secretion from the chicken pituitary gland (21). However, we did not observe a decrease in the FSH release in response to GnIH treatment *in vitro*, possibly due to lack of releasable pool of FSH in the pituitary gland and/or variations in the strain of chickens used in the present study. Similarly, GnIH treatment had no effect on either FSH or LH release from pituitaries of sexually mature chickens possibly due to lesser GnIHR expression in the pituitary gland as demonstrated by our data showing decreased GnIHR mRNA in the pituitary gland of sexually mature chickens. Previously, we reported that GnIH treatment decreased the viability of granulosa cells isolated from prehierarchial follicles, thereby possibly preventing them from entering into the preovulatory hierarchy (24). Collectively, GnIHR appears to play an anti-gonadal role at both the pituitary and ovarian levels by affecting LH secretion and follicular maturation, respectively.

In summary, the results obtained in the present study indicate that there was a greater GnIHR mRNA abundance in the chicken diencephalon than in the pituitary gland. GnIHR-ir cells were colocalised with FSH$\beta$ mRNA or LH$\beta$ mRNA throughout the pituitary gland and are likely to mediate the inhibitory effect of GnIH on pituitary LH secretion. Furthermore, pituitary GnIHR abundance was significantly decreased by sexual maturation and by oestradiol, or a combination of oestradiol and progesterone treatments. Further studies are required to characterise GnIHR-expressing neurones as well as to determine the role of GnIHR in gonadotrophin secretion.

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## References

1 Amano M, Moriyama S, Iigo M, Kitamura S, Amiya N, Yamamori K, Ukena K, Tsutsui K. Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. *J Endocrinol* 2006; **188**: 417–423.

2 Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, Kitada C, Masuo Y, Asano T, Matsumoto H, Sekiguchi M, Kurokawa T, Nishimura O, Onda H, Fujino M. A prolactin-releasing peptide in the brain. *Nature* 1998; **393**: 272–276.

3 Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, Fujii R, Watanabe T, Kikuchi K, Terao Y, Yano T, Yamamoto T, Kawamata Y, Habata Y, Asada M, Kitada C, Kurokawa T, Onda H, Nishimura O, Tanaka M, Ibata Y, Fujino M. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nat Cell Biol* 2000; **2**: 703–708.

4 Koda A, Ukena K, Teranishi H, Ohta S, Yamamoto K, Kikuyama S, Tsutsui K. A novel amphibian hypothalamic neuropeptide: isolation, localization, and biological activity. *Endocrinology* 2002; **143**: 411–419.

5 Moriyama S, Ito T, Takahashi A, Amano M, Sower SA, Hirano T, Yamamori K, Kawauchi H. A homolog of mammalian PRL-releasing peptide (fish arginyl-phenylalanyl-amide peptide) is a major hypothalamic peptide of PRL release in teleost fish. *Endocrinology* 2002; **143**: 2071–2079.

6 Navarro VM, Fernandez-Fernandez R, Nogueiras R, Vigo E, Tovar S, Chartrel N, Le Marec O, Leprince J, Aguilar E, Pinilla L, Dieguez C, Vaudry H, Tena-Sempere M. Novel role of 26RFa, a hypothalamic RFamide orexigenic peptide, as putative regulator of the gonadotropic axis. *J Physiol* 2006; **573**(Pt 1): 237–249.

7 Sakamoto T, Oda A, Yamamoto K, Kaneko M, Kikuyama S, Nishikawa A, Takahashi A, Kawauchi H, Tsutsui K, Fujimoto M. Molecular cloning and functional characterization of a prolactin-releasing peptide homolog from *Xenopus laevis*. *Peptides* 2006; **27**: 3347–3351.

8 Sawada K, Ukena K, Satake H, Iwakoshi E, Minakata H, Tsutsui K. Novel fish hypothalamic neuropeptide. *Eur J Biochemistry/FEBS* 2002; **269**: 6000–6008.

9 Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, Ishii S, Sharp PJ. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun* 2000; **275**: 661–667.

10 Tsutsui K, Ukena K. Hypothalamic LPXRF-amide peptides in vertebrates: identification, localization and hypophysiotropic activity. *Peptides* 2006; **27**: 1121–1129.

11 Ukena K, Ubuka T, Tsutsui K. Distribution of a novel avian gonadotropin-inhibitory hormone in the quail brain. *Cell Tissue Res* 2003; **312**: 73–79.

12 Ubuka T, Ukena K, Sharp PJ, Bentley GE, Tsutsui K. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology* 2006; **147**: 1187–1194.

13 Ikemoto T, Park MK. Chicken RFamide-related peptide (GnIH) and two distinct receptor subtypes: identification, molecular characterization, and evolutionary considerations. *J Reprod Dev* 2005; **51**: 359–377.

14 Satake H, Hisada M, Kawada T, Minakata H, Ukena K, Tsutsui K. Characterization of a cDNA encoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release. *Biochem J* 2001; **354**: 379–385.

15 Ubuka T, Kim S, Huang YC, Reid J, Jiang J, Osugi T, Chowdhury VS, Tsutsui K, Bentley GE. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. *Endocrinology* 2008; **149**: 268–278.

16 Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K, Silver R. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proc Natl Acad Sci USA* 2006; **103**: 2410–2415.

17 Tachibana T, Sato M, Takahashi H, Ukena K, Tsutsui K, Furuse M. Gonadotropin-inhibiting hormone stimulates feeding behavior in chicks. *Brain Res* 2005; **1050**: 94–100.

18 Bentley GE, Jensen JP, Kaur GJ, Wacker DW, Tsutsui K, Wingfield JC. Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH). *Horm Behav* 2006; **49**: 550–555.

19 Bentley GE, Perfito N, Ukena K, Tsutsui K, Wingfield JC. Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. *J Neuroendocrinol* 2003; **15**: 794–802.

20 Ubuka T, Bentley GE, Ukena K, Wingfield JC, Tsutsui K. Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. *Proc Natl Acad Sci USA* 2005; **102**: 3052–3057.

21 Ciccone NA, Dunn IC, Boswell T, Tsutsui K, Ubuka T, Ukena K, Sharp PJ. Gonadotrophin inhibitory hormone depresses gonadotrophin alpha and follicle-stimulating hormone beta subunit expression in the pituitary of the domestic chicken. *J Neuroendocrinol* 2004; **16**: 999–1006.

22 Yin H, Ukena K, Ubuka T, Tsutsui K. A novel G protein-coupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*): identification, expression and binding activity. *J Endocrinol* 2005; **184**: 257–266.

23 Bentley GE, Ubuka T, McGuire NL, Chowdhury VS, Morita Y, Yano T, Hasunuma I, Binns M, Wingfield JC, Tsutsui K. Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. *Gen Comp Endocrinol* 2008; **156**: 34–43.

24 Maddineni SR, Ocon-Grove OM, Krzysik-Walker SM, Hendricks GL III, Ramachandran R. Gonadotropin-inhibitory hormone (GnIH) receptor gene is expressed in the chicken ovary: potential role of GnIH in follicular maturation. *Reproduction* 2008; **135**: 267–274.

25 Kuenzel WJMM. *A Steriotaxic Atlas of the Brain of the Chick (Gallus domesticus)*. Baltimore, MD: John Hopkins University Press, 1988.

26 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif)* 2001; **25**: 402—408.
27 Krzysik-Walker SM, Ocon-Grove OM, Maddineni SR, Hendricks GL III, Ramachandran R. Is visfatin an adipokine or myokine? Evidence for greater visfatin expression in skeletal muscle than visceral fat in chickens. *Endocrinology* 2008; **149**: 1543—1550.
28 Maddineni SR, Krzysik-Walker SM, Ocon-Grove OM, Motch SM, Hendricks GL III, Ramachandran R. Calcitonin is expressed in the chicken pituitary gland: influence of gonadal steroids and sexual maturation. *Cell Tissue Res* 2007; **327**: 521—528.
29 Noce T, Ando H, Ueda T, Kubokawa K, Higashinakagawa T, Ishii S. Molecular cloning and nucleotide sequence analysis of the putative cDNA for the precursor molecule of the chicken LH-beta subunit. *J Mol Endocrinol* 1989; **3**: 129—137.
30 Dunn IC, Lewis PD, Wilson PW, Sharp PJ. Acceleration of maturation of FSH and LH responses to photostimulation in prepubertal domestic hens by oestrogen. *Reproduction* 2003; **126**: 217—225.
31 Liu HK, Bacon WL. Changes in egg production rate induced by progesterone injection in broiler breeder hens. *Poult Sci* 2005; **84**: 321—327.
32 Krishnan KA, Proudman JA, Bolt DJ, Bahr JM. Development of an homologous radioimmunoassay for chicken follicle-stimulating hormone and measurement of plasma FSH during the ovulatory cycle. *Comp Biochem Physiol* 1993; **105**: 729—734.
33 Proudman JA, Scanes CG, Opel H, Ottinger MA. Two avian luteinizing hormone radioimmunoassay procedures compared by measurement of changes during the ovulatory cycle of turkey and broiler hens. *Poult Sci* 1984; **63**: 1269—1275.
34 Ubuka T, Kim S, Huang YC, Reid J, Jiang J, Osugi T, Chowdhury VS, Tsutsui K, Bentley GE. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. *Endocrinology* 2008; **149**: 268—278.
35 Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology* 2004; **80**: 264—272.
36 Hansen GN, Hansen BL. Light and electron microscopic identification of pituitary cells containing growth hormone and prolactin in the pigeon [*Columba livia*], using the immunoglobulin-enzyme bridge technique. *Gen Comp Endocrinol* 1977; **32**: 99—107.
37 Mikami S, Yamada S. Immunohistochemistry of the hypothalamic neuropeptides and anterior pituitary cells in the Japanese quail. *J Exp Zool* 1984; **232**: 405—417.
38 Ravona H, Snapir N, Perek M. Histological changes of the pituitary gland in cockerels bearing basal hypothalamic lesions and demonstration of its anti-HCG immunofluorescence reacting cells. *Gen Comp Endocrinol* 1973; **20**: 490—497.
39 Proudman JA, Vandesande F, Berghman LR. Immunohistochemical evidence that follicle-stimulating hormone and luteinizing hormone reside in separate cells in the chicken pituitary. *Biol Reprod* 1999; **60**: 1324—1328.
40 Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. *Horm Behav* 2007; **51**: 171—180.
41 Matsumoto H, Noguchi J, Horikoshi Y, Kawamata Y, Kitada C, Hinuma S, Onda H, Nishimura O, Fujino M. Stimulation of prolactin release by prolactin-releasing peptide in rats. *Biochem Biophys Res Commun* 1999; **259**: 321—324.
42 Ukena K, Koda A, Yamamoto K, Kobayashi T, Iwakoshi-Ukena E, Minakata H, Kikuyama S, Tsutsui K. Novel neuropeptides related to frog growth hormone-releasing peptide: isolation, sequence, and functional analysis. *Endocrinology* 2003; **144**: 3879—3884.
43 Bedecarrats GYSM, Zeini M. *Pituitary Levels of a Putattive Gonadotropin Inhibiting Hormone Receptor mRNA Fluctuate During a Reproductive Cycle in the Chicken*. Poultry Science Association, 95th Annual Meeting. Edmonton, Canada 2006: 120.
44 Ciccone NA, Sharp PJ, Wilson PW, Dunn IC. Changes in reproductive neuroendocrine mRNAs with decreasing ovarian function in ageing hens. *Gen Comp Endocrinol* 2005; **144**: 20—27.
45 Williams JB, Sharp PJ. Age-dependent changes in the hypothalamo-pituitary-ovarian axis of the laying hen. *J Reprod Fertil* 1978; **53**: 141—146.
46 Sechman A, Paczoska-Eliasiewicz H, Rzasa J, Hrabia A. Simultaneous determination of plasma ovarian and thyroid hormones during sexual maturation of the hen (*Gallus domesticus*). *Folia Biol (Krakow)* 2000; **48**: 7—12.
47 Kawashima M, Kamiyoshi M, Tanaka K. Effects of progesterone, estradiol and testosterone on cytoplasmic progesterone receptor concentrations in the hen hypothalamus and pituitary. *Biol Reprod* 1979; **21**: 639—646.