Gonadotropin-inhibitory hormone (GnIH) receptor gene is expressed in the chicken ovary: potential role of GnIH in follicular maturation

Sreenivasa R Maddineni, Olga M Ocón-Grove, Susan M Krzysik-Walker, Gilbert L Hendricks III and Ramesh Ramachandran

Department of Poultry Science, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

Correspondence should be addressed to R Ramachandran; Email: rameshr@psu.edu

Abstract

Gonadotropin-inhibitory hormone (GnIH), an RFamide peptide, has been found to inhibit pituitary LH secretion in avian and mammalian species. The gene encoding a putative receptor for GnIH (GnIHR) was recently identified in the chicken and Japanese quail brain and pituitary gland. GnIHR appears to be a seven-transmembrane protein belonging to a family of G-protein-coupled receptors. In the present study, we have characterized the expression of GnIHR mRNA in the chicken ovary and demonstrate that GnIHR may exert an inhibitory effect on ovarian follicular development. By RT-PCR, we detected GnIHR mRNA in the chicken testis and in the ovary, specifically both thecal and granulosa cell layers. Real-time quantitative PCR analysis revealed greater GnIHR mRNA quantity in theca cells of prehierarchial follicles compared with that of preovulatory follicles. GnIHR mRNA quantity was significantly decreased in sexually mature chicken ovaries versus ovaries of sexually immature chickens. Estradiol (E_2) and/or progesterone (P_4) treatment of sexually immature chickens significantly decreased ovarian GnIHR mRNA abundance. Treatment of prehierarchial follicular granulosa cells *in vitro* with chicken GnIH peptide significantly decreased basal but not FSH-stimulated cellular viability. Collectively, our results indicate that the ovarian GnIHR is likely to be involved in ovarian follicular development. A decrease in ovarian GnIHR mRNA abundance due to sexual maturation or by E_2 and/or P_4 treatment would implicate an inhibitory role for GnIHR in ovarian follicular development. Furthermore, GnIH may affect follicular maturation by decreasing the viability of prehierarchial follicular granulosa cells through binding to GnIHR.

Reproduction (2008) **135** 267–274

Introduction

Gonadotropin-inhibitory hormone (GnIH), first isolated from quail hypothalamus, has been shown to inhibit luteinizing hormone (LH) secretion from the anterior pituitary gland both *in vitro* and *in vivo* (Tsutsui *et al.* 2000). GnIH is a dodecapeptide belonging to a family of RFamide peptides with a C-terminal Pro-Xaa-Arg-Phe-NH$_2$ motif. The gene encoding a precursor protein for GnIH in Japanese quail, chickens, and white-crowned sparrows has been cloned and found to share a high degree of homology (Satake *et al.* 2001, Osugi *et al.* 2004, Ikemoto & Park 2005). GnIH-immunoreactive (ir) neurons are exclusively found in the hypothalamic paraventricular nucleus while GnIH-ir fibers are found throughout the brain including the median eminence in Japanese quail (Ukena *et al.* 2003) and sparrows (Bentley *et al.* 2003). In addition to avian species, GnIH-like molecules have been identified in hamsters, rats, and mice (Kriegsfeld *et al.* 2006) wherein GnIH-ir cell bodies were found to be clustered in the mediobasal hypothalamus with pronounced projections and terminals throughout the

central nervous system. RFamide-related peptides (RFRPs) homologous to GnIH and its receptors have previously been identified in rats and mice (Hinuma *et al.* 2000, Ukena & Tsutsui 2001, Ukena *et al.* 2002). The extensive distribution of GnIH-ir fibers in the multiple brain areas is consistent with multiple functions of GnIH such as feeding behavior in chickens (Tachibana *et al.* 2005) and sexual behavior in white-crowned sparrows (Bentley *et al.* 2006). GnIH-ir neurons were found to have close appositions with GnRH-ir cells suggesting a possible interaction of GnIH and GnRH neurons (Kriegsfeld *et al.* 2006). 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 pinealectomized and enucleated birds (Ubuka *et al.* 2005).

Administration of GnIH either by intracerebroventricular or peripheral route to ovariectomized Syrian hamsters resulted in reduced plasma LH concentrations, providing compelling evidence for the role of GnIH as a gonadotropin inhibitory factor (Kriegsfeld *et al.* 2006). Treatment of male chicken pituitary slices *in vitro* with GnIH resulted in

decreased follicle-stimulating hormone (FSH) and LH secretion (Ciccone *et al.* 2004). Furthermore, GnIH was found to decrease plasma testosterone concentrations, induce testicular apoptosis and decrease testicular spermatogenic activity when continuously administrated to Japanese quail over a 2-week period (Ubuka *et al.* 2006). Based on the foregoing findings, GnIH appears to be an important neuropeptide involved in the control of gonadotropin secretion in both avian and mammalian species.

Recently, the chicken and Japanese quail GnIH receptor (GnIHR) cDNAs were cloned and found to be 97% similar to each other (Ikemoto & Park 2005, Yin *et al.* 2005). The deduced protein sequence of Japanese quail GnIHR appears to be a seven-transmembrane receptor belonging to a family of G-protein-coupled receptors (Yin *et al.* 2005). The membrane fraction of COS-7 cells wherein putative quail GnIHR was overexpressed showed a dose-dependent high-affinity binding to GnIH (Yin *et al.* 2005). Furthermore, GnIH treatment of COS-7 cells overexpressing chicken GnIHR resulted in a dose-dependent decrease in the accumulation of $G_{\alpha i2}$ mRNA (Ikemoto & Park 2005). At the tissue level, GnIHR cDNA was found to be expressed in the diencephalon and anterior pituitary gland in chickens and Japanese quail (Ikemoto & Park 2005, Yin *et al.* 2005). However, regulation of *GnIHR* gene expression or its possible expression in the gonads has not been investigated in any species. In this report, we present novel evidence that the *GnIHR* gene is expressed in the chicken ovary and testes in addition to the diencephalon and anterior pituitary gland. We found that ovarian *GnIHR* gene expression is influenced by gonadal steroids, follicular, and sexual maturation. Furthermore, we present evidence that GnIH may affect follicular maturation by decreasing the viability of granulosa cells in the prehierarchial ovarian follicles.

Results

Detection of GnIHR mRNA by RT-PCR

A single band of 364 bp partial GnIHR cDNA was detected in the total RNA of diencephalon, anterior pituitary gland, ovary, and testes (Fig. 1). No GnIHR cDNA amplicon was observed in skeletal muscle and liver RNA (Fig. 1). We also amplified a partial GnIHR cDNA in both theca and granulosa layers of all preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles (Fig. 2). The partial GnIHR cDNA amplified from the ovary RNA had identical nucleotide sequences to diencephalon and pituitary-derived GnIHR cDNA sequence (GenBank accession no. AB193127) and did not share any homology with a receptor for neuropeptide FF (NPFFR) cDNA (GenBank accession no. AB193125) expressed in the chicken ovary that was found to have low-affinity binding to GnIH (Ikemoto & Park 2005). The use of RNA in place of reverse-transcribed RNA failed to produce any PCR product
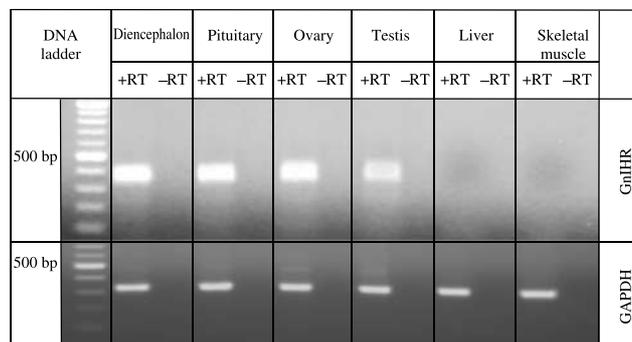

Figure 1 RT-PCR analysis of *GnIHR* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression in various tissues in the chicken. Total RNA extracted from each tissue was deoxyribonuclease-I treated. Approximately, 200 ng cDNA (+RT) were used as a template to amplify a 364 bp product of chicken GnIHR cDNA. Contamination controls consisted of RNA from each tissue without reverse transcriptase (−RT). bp, base pair.

(Figs 1 and 2), confirming the absence of genomic DNA contamination. Furthermore, RNA and cDNA integrity was confirmed by successfully amplifying a fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA (Fig. 1).

Effect of follicular development on GnIHR mRNA quantity

The diameters of preovulatory and prehierarchial follicles ($n=6$ chickens) included in this experiment are as follows: 36.2 ± 0.65 mm for F1 follicles, 27.0 ± 0.84 mm for F3 follicles, 10.5 ± 0.43 mm for F6 follicles, and 3–5 mm for prehierarchial follicles. GnIHR mRNA abundance in F1, F3, and F6 were expressed as a ratio of GnIHR mRNA abundance in 3–5 mm follicles from each animal. GnIHR mRNA abundance was significantly decreased in the theca layer of F1 and F3 preovulatory follicles compared with 3–5 mm prehierarchial follicles
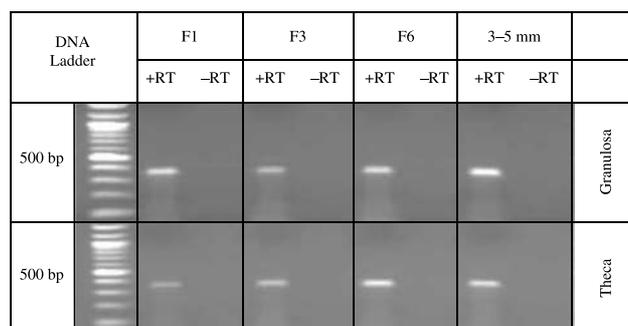

Figure 2 RT-PCR analysis of *GnIHR* gene expression in granulosa and thecal cell layers of preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles. Approximately, 200 ng cDNA (+RT) were used as a template to amplify a 364 bp product of GnIHR. Contamination controls consisted of RNA from each tissue without reverse transcriptase (−RT). bp, base pair.

(*P*<0.05; Fig. 3A). In the granulosa cell layer, GnIHR mRNA abundance was not significantly different among preovulatory or prehierarchial follicles (*P*>0.05; Fig. 3B). In every preovulatory or prehierarchial follicle tested, GnIHR mRNA quantity was significantly higher in the granulosa cell layer compared with thecal cell layer (*P*<0.05; Fig. 3C).

Effect of sexual maturation on ovarian GnIHR mRNA abundance

The ovaries of sexually immature chickens were found to have significantly greater GnIHR mRNA abundance than those of sexually mature birds (*P*<0.05; Fig. 4). Melting curve analyses showed the presence of a single PCR product for GnIHR and β-actin confirming the specificity of the reaction (data not shown). Ovarian total RNA
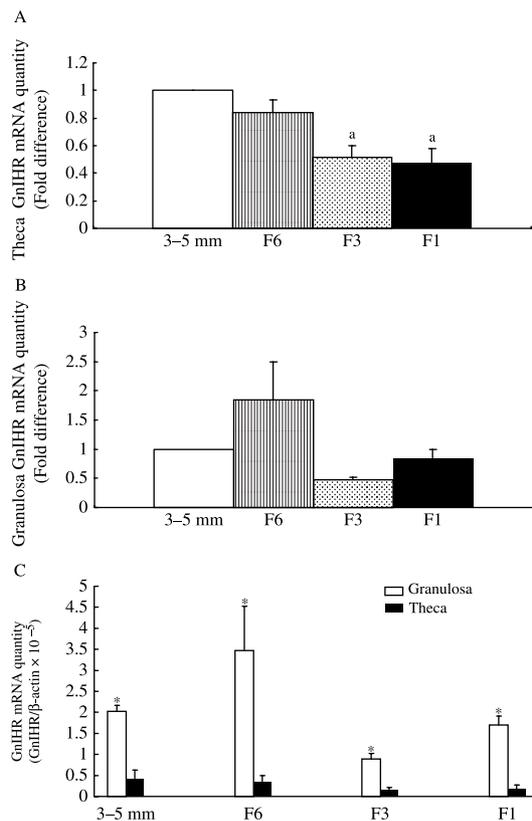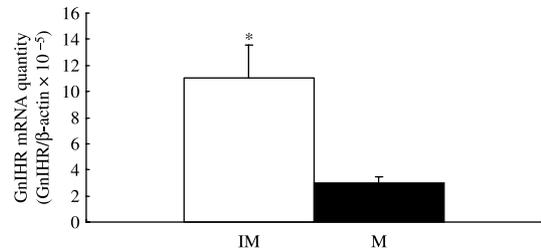

Figure 4 GnIHR mRNA quantity relative to β-actin mRNA quantity in the ovary of sexually immature (IM) and mature chickens (M; *n*=6). Total RNA extracted from the ovary was reverse transcribed and subjected to real-time quantitative PCR to determine GnIHR mRNA quantity as described in Fig. 3. Data represent mean±s.e.m. An asterisk above each bar indicates significant difference at *P*<0.05.

reverse transcribed in the absence of reverse transcriptase resulted in C_T values similar to blanks (>33 cycles or none detected) indicating that genomic DNA was not contributing to the GnIHR or β-actin mRNA quantity (data not shown).

Effect of ovarian steroids on ovarian GnIHR mRNA quantity

Estradiol (E_2) and/or progesterone (P_4) treatment for 7 days resulted in a significant increase in oviduct weights (data not shown). GnIHR mRNA quantity was significantly decreased (*P*<0.05; Fig. 5) in the ovaries of chickens treated with E_2 and/or P_4 compared with vehicle-treated controls. Melting curve analyses showed the presence of a single PCR product for GnIHR mRNA or β-actin mRNA confirming the specificity of the reaction (data not shown).

Effect of chicken GnIH and FSH on granulosa cell viability

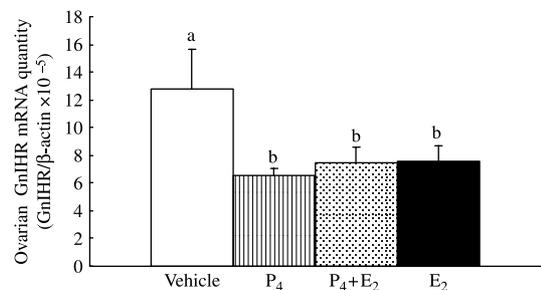In order to determine the role of GnIH on cell viability in prehierarchial follicles (6–8 mm), granulosa cells were

Figure 3 (A–C) GnIHR mRNA abundance in the granulosa and theca cells of preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles as measured by real-time quantitative PCR. Total RNA from granulosa or thecal cell layers was treated with DNase-I and reverse transcribed. Approximately, 100 ng cDNA were used in real-time quantitative PCR using SYBR green as the dye to quantify GnIHR mRNA or β-actin mRNA in separate reactions. Each reaction was run in duplicate and threshold (C_T) values for GnIHR mRNA were subtracted from values of β-actin mRNA and converted from log-linear to linear term. A comparison of GnIHR mRNA abundance in the thecal cell layer (A), in granulosa cell layer (B), and between granulosa and thecal cell layers (C) of prehierarchial and preovulatory follicles. Data in (A–C) represent mean±s.e.m. (*n*=5–6). [a]*P*<0.05; *P*<0.05.

Figure 5 Effect of estradiol (E_2) and/or progesterone (P_4) on GnIHR mRNA quantity in the ovary. Sexually immature female chickens were treated with E_2, P_4, E_2+P_4, or vehicle (*n*=7 per treatment) for 7 days. The chickens were killed by decapitation at the end of treatments. Total RNA extracted from the ovary was reverse transcribed and subjected to real-time quantitative PCR to determine GnIHR mRNA quantity as described in Fig. 3. Data represent mean±s.e.m. [a,b]*P*<0.05.

cultured for 12 h with various concentrations of chicken GnIH with or without recombinant human FSH, with viability determined by the CellTiter-Blue assay. The viability of granulosa cells was significantly decreased by treatment with GnIH at 10^{-6} and 10^{-8} M concentrations ($P<0.05$; Fig. 6A). No significant changes in cellular viability were observed in response to a combination of FSH and GnIH treatments (Fig. 6B). Also, there was no change in the cellular viability following 24-h incubation with GnIH (data not shown).

Discussion

The present study is the first to characterize GnIHR expression in the ovary of any vertebrate species. We found that GnIHR mRNA is expressed in the ovary and testis but not in skeletal muscle or liver. In the ovary, both theca and granulosa cell layers of prehierarchial and preovulatory follicles were found to express GnIHR mRNA. Assuming that GnIHR mRNA is translated into a functional receptor protein in the ovary and testis, it is

Figure 6 (A and B) Effect of GnIH and/or FSH on granulosa cell viability *in vitro*. Granulosa cells were dispersed from the 6–8 mm follicles of two hens, pooled, and treated in triplicate with chicken GnIH (0, 10^{-10}, 10^{-8}, and 10^{-6} M) and recombinant human FSH ((A) 0 or (B) 100 ng/ml) for 12 h. Cell viability was measured using the CellTiter-Blue assay, and viability expressed as percentage of vehicle control±s.e.m. ($n=5$, replicates of two animals each). [a]$P<0.05$.

possible that GnIH could directly affect ovarian and testicular functions in addition to influencing its receptor previously described in the brain and anterior pituitary gland (Ikemoto & Park 2005, Yin *et al.* 2005).

GnIHR gene expression has been reported in the diencephalon, cerebrum, mesencephalon, spinal cord, and pituitary gland of Japanese quail (Yin *et al.* 2005). A similar study in chickens has identified expression of the *GnIHR* gene in various regions of the brain and anterior pituitary gland but not in gonads (Ikemoto & Park 2005). The present study employed a different PCR protocol from that used by Ikemoto & Park (2005) for the successful amplification of partial GnIHR cDNA from the chicken ovary and testis. In support of our findings, a homolog of chicken GnIHR that binds to an RFRP was found to be expressed in the rat testis, ovary, and placenta in addition to brain and anterior pituitary gland (Hinuma *et al.* 2000). Other hormones affecting reproduction, such as GnRH and kisspeptin, have been found to exert a direct effect in the ovaries of several mammalian species by activating their cognate receptors. For instance, GnRH and its receptor are expressed in granulosa cells of developing follicles in the rat ovary (Whitelaw *et al.* 1995) and may play an important role in oocyte maturation (Dekel & Shalgi 1987), follicular atresia or selection (Whitelaw *et al.* 1995), and fertilization processes (Morales 1998). G-protein coupled receptor (GPR)54, a receptor for kisspeptin, has been found to be expressed in the human testis (Kotani *et al.* 2001) and rat ovary (Castellano *et al.* 2006).

In the present study, thecal cells of prehierarchial follicles (3–5 mm) were found to have greater GnIHR mRNA abundance compared with that of preovulatory follicles. The reproductively active chicken ovary consists of a hierarchy of four to six preovulatory follicles (designated as F1–F6) that are typically >10 mm in diameter. In addition, the ovary also contains several prehierarchial follicles that, after undergoing maturation, enter the hierarchy of preovulatory follicles. The preovulatory follicles, particularly the F1 follicle, are the major source of P_4 secretion, whereas the prehierarchial follicles predominantly secrete estrogen and androgen (Robinson & Etches 1986). In the present study, we observed a 49–52% decrease in the GnIHR mRNA quantity in the thecal cell layer of F3 and F1 follicles. This data may indicate that *GnIHR* gene expression is possibly downregulated as the prehierarchial follicles are selected to undergo further maturation and recruitment into the hierarchy of preovulatory follicles. Greater expression of *GnIHR* gene in the prehierarchial follicles than in the preovulatory follicles may also be viewed as one of the factors that inhibit the prehierarchial follicles from undergoing maturation and entering into the hierarchy. In support of this theory, the present study also found a 73% reduction in GnIHR mRNA abundance in sexually mature chicken ovaries compared with that of
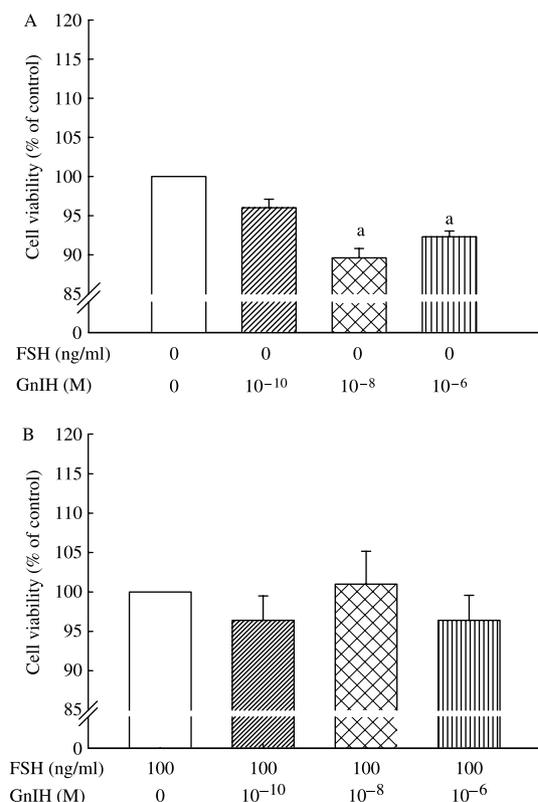
sexually immature chickens. A decrease in GnIHR mRNA abundance in the ovary could possibly result from the influence of gonadal steroids, particularly E_2 and P_4.

We found that E_2 and/or P_4 treatment to sexually immature chickens caused a 50% decrease in the abundance of GnIHR mRNA in the ovaries. Sexually immature chickens were selected for this study as they have very low endogenous E_2 and P_4 secretion. Our findings suggest that E_2 and/or P_4 treatment is likely to downregulate *GnIHR* gene expression in the chicken ovary and possibly decrease the inhibitory effect of GnIH at ovarian level. In contrast, estrogen was found to be necessary for the expression GnRH receptor mRNA in granulosa cells of both atretic and healthy follicles in the rat ovary (Kogo *et al.* 1999). In previous reports, GnIH neurons in the Syrian hamster hypothalamus were found to co-express estrogen receptor-α, and E_2 treatment has been found to increase the activity of GnIH-ir neurons, demonstrated by an increased number of cFOS-ir neurons co-localized with GnIH-ir neurons (Kriegsfeld *et al.* 2006).

In order to determine whether GnIH would directly affect follicular maturation in the chicken ovary, we treated granulosa cells dispersed from prehierarchial follicles with chicken GnIH and/or FSH. We found that GnIH significantly decreased the viability of granulosa cells when cultured in the absence of FSH. Our results indicate that GnIH may decrease cellular viability through decreased mitotic activity (Springer *et al.* 1998) thereby affecting proliferation (Nakayama *et al.* 1997) and maturation of granulosa cells in the absence of FSH. We found that the granulosa cellular viability was unaffected by a combination of GnIH and FSH treatments. GnIH and FSH are likely to activate antagonistic signaling pathways mediated by $G_{\alpha i2}$ and $G_{\alpha s}$ respectively (Reiter *et al.* 2001, Ikemoto & Park 2005). Such antagonism may, at least in part, explain the lack of inhibitory effect of GnIH on granulosa cellular viability in the presence of FSH. It is plausible that GnIH may favor ovarian regression and/or prevent maturation of prehierarchial follicles at a time when circulating FSH concentration declines. Although we found a significant decrease in cellular viability in response to GnIH treatment following 12 h incubation, the inhibitory effect was not observed following 24 h incubation, possibly due to degradation of GnIH peptide in the culture medium. A lack of effect in response to GnIH at 24 h may also be due to overcompensatory increase in the metabolic activity of granulosa cells that was repressed by GnIH at 12 h and/or due to possible induction of differentiation in undifferentiated granulosa cells over time rendering them less sensitive to GnIH.

In the present study, we found that the chicken testes also expressed GnIHR mRNA whose functional significance remains to be explored. A recent study (Ubuka *et al.* 2006) suggests that continuous administration of GnIH to Japanese quail induced testicular apoptosis and decreased spermatogenic activity in the testis of adult males while causing suppression of normal testicular growth in juvenile males. Although the above responses were attributed to a decrease in LH secretion by a direct effect of GnIH on the pituitary gland, exogenous GnIH administration may have activated testicular GnIHR and possibly contributed to the testicular suppression observed. As chicken and Japanese quail GnIHR cDNAs are highly conserved, we expect that GnIHR is also expressed in the Japanese quail testis as in the chicken.

Although GnIHR is localized in the chicken ovary, the GnIH precursor protein gene is not expressed in the chicken ovary (data not shown). While the presence of GnIH in the blood circulation has not yet been reported in the chicken, GnIH in the systemic blood circulations have been detected by RIA in sparrows (Dr George Bentley, University of California, Berkeley; personal communication). Based on this observation, we hypothesize that GnIHR found in the ovary or testes is possibly activated by blood-borne GnIH in the chicken. Alternatively, chicken gonads may be expressing other RFamide ligand(s) that are structurally similar to GnIH that could activate GnIHR. In support of this theory, RFRP and its receptor have been found to be expressed in the rat testis (Hinuma *et al.* 2000). Kisspeptin, another RFamide, has been found to be expressed in thecal cells of rat ovarian follicles and has been implicated as a putative controller of ovulation (Castellano *et al.* 2006).

Taken together, our results indicate that a receptor for GnIH is expressed in the chicken testis and ovary. Ovarian *GnIHR* gene expression is regulated by sexual maturation as well as gonadal steroids. Furthermore, GnIH may exert a direct effect on the viability of granulosa cells *in vitro*. Additional studies are required to determine the source of GnIH for activation of gonadal GnIHR and other physiological functions of GnIH and GnIHR in the gonads.

Materials and Methods

Reagents

Trizol and RNeasy kits for RNA extraction were obtained from Invitrogen and Qiagen respectively. Moloney murine leukemia virus (M-MuLV) reverse transcriptase and Taq polymerase used for RT-PCR were purchased from New England Biolabs (Beverly, MA, USA). Additional PCR and RT materials (Platinum SYBR Green qPCR Super Mix-UDG, RNaseOut) were obtained from Invitrogen. E_2 17-β and P_4 were purchased from Sigma–Aldrich. Chicken GnIH, a 12 amino acid peptide with a C-terminal amide (SIRPSAYLPLRF-NH_2), was custom synthesized by Sigma Genosys (Austin, TX, USA). Recombinant human FSH was obtained from the National Hormone and Peptide Program (Torrance, CA, USA). The Cell Titer-Blue Cell Viability Assay kit was purchased from Promega.

Animals

Female white Leghorn chickens (Hyline W36 strain) and male broiler chickens (Cobb strain) were housed in cages and were provided water and feed *ad libitum*. Both male broiler and female Leghorn chickens were placed under a 16 h light:8 h darkness photoperiod starting at 12 weeks of age. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee approved protocol.

RT-PCR

Approximately, 4–6 h prior to ovulation, hens ($n=6$; Hyline W36 strain; 60 weeks old) were killed by decapitation using a guillotine. Prior to euthanasia, the presence of a hard-shelled egg in the shell gland was confirmed by cloacal examination. Immediately following killing, preovulatory (F1, F3, and F6) and prehierarchial follicles (3–5 mm) were separated from the ovary and placed in ice-cold 0.75% saline solution. The granulosa and theca cell layers from each follicle were separated using a dissection microscope following the method previously described (Gilbert *et al.* 1977), snap-frozen in liquid nitrogen, and stored at −80 °C until processed. Granulosa and theca cell layers obtained from two to three prehierarchial follicles were pooled from each animal. Diencephalon, anterior pituitary gland, liver, skeletal muscle, and testis from male broiler chickens were also harvested for RNA extraction. All the tissues including granulosa and thecal layers were homogenized separately, and total RNA was extracted using Trizol and RNeasy kits. After DNase-I treatment, first-strand cDNA was synthesized by reverse transcribing 1 µg total RNA using oligo d(T)$_{30}$ A/G/C A/G/C/T primer and 2 U M-MuLV reverse transcriptase. Approximately, 200 ng single-stranded cDNA were used as template to amplify a 364 bp GnIHR cDNA using forward and reverse primers (forward: 5′-AGTGGCCTGGTACAGGGCATGTCT-3′ and reverse: 5′-CAATGCGGGCATACATGACGACAA-3′). The forward and reverse primers were selected from exons 3 and 4 respectively, from the chicken RFRP receptor (*GnIHR*) gene (GenBank accession no. AB193127). Exons 3 and 4 are separated by an intron of 312 bp (Ikemoto & Park 2005) that will aid in detecting genomic DNA contamination by amplifying a longer PCR product of 676 bp. The PCRs were performed using SYBR GreenER qPCR SuperMix (Invitrogen) in 50 µl reaction mixture containing 500 nM forward and reverse primers that were subjected to the following thermocycle parameters: 50 °C for 2 min, 94 °C for 10 min, 34 cycles of 94 °C for 10 s, 65 °C for 10 s, and 72 °C for 10 s with a final extension at 72 °C for 10 min in a DNA Engine Opticon II (MJ Research, Reno, NV, USA). For positive controls, a portion of the chicken glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified using 300 nm the following primers (GenBank accession no. K01458): forward: 5′-AGTCA TCCCTGAGCTGAATG-3′ and reverse: 5′-ACCATCAAGTCCA-CAACACG-3′, with the following thermocycle conditions: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 10 s, and 68 °C for 1 min with a final extension at 68 °C for 5 min. The PCR products (30–50 µl volume) were subjected to agarose gel electrophoresis and ethidium bromide staining for visualization. For negative controls, reverse-transcribed RNA with no reverse transcriptase was used as template in place of single-stranded cDNA. The PCR products were sequenced (Davis Sequencing, Davis, CA, USA) to confirm the authenticity of the amplified products.

Quantification of GnIHR mRNA in theca and granulosa layers

The granulosa and theca cell layers from both preovulatory and prehierarchial follicles were separated as described above. Granulosa and thecal cell layers were homogenized, and total RNA was extracted using Trizol and RNeasy kits. After DNase-I treatment, total RNA (1 µg) was reverse transcribed using d(T)$_{30}$A/G/C A/G/C/T, 2 U M-MuLV reverse transcriptase in a 20 µl reaction. Both GnIHR and chicken β-actin mRNAs were quantified using ∼150 ng single-stranded cDNA as template in the real-time quantitative PCR (qPCR). A 140 bp chicken GnIHR was amplified using the following set of primers: forward: 5′- CACTGATGCTGCTGA-CAGACTA-3′ and reverse: 5′- CTCATTGAAGTAGCCGTAGAT-GAT-3′. Similarly, a 123 bp product of chicken β-actin corresponding to nucleotides 1026–1148 (GenBank accession no. L08165) was amplified using the following primer set: forward: 5′-CTGGCACCTAGCACAATGAA-3′ and reverse: 5′-CTGCTTGCTGATCCACATCT-3′. The qPCR mixture consisted of Platinum SYBR Green qPCR Super Mix-UDG and 500 nm forward and reverse primers in 50 µl reaction mixture. The reactions were performed in the DNA Engine Opticon II (MJ Research) with the following thermocycle: 50 °C for 2 min, 95 °C for 2 min, followed by 35 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 done by heating the PCR products to 65–95 °C and held 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 C_T values for GnIHR mRNA and β-actin mRNA. For negative controls, reverse-transcribed RNA with no reverse transcriptase was used as template in place of single-stranded cDNA in the qPCR. The log-linear threshold values (C_T) during the exponential phase of the PCR for GnIHR mRNA were subtracted from that of β-actin mRNA. GnIHR mRNA quantity was expressed as a proportion of β-actin mRNA quantity following the $2^{-\Delta\Delta C_T}$ method for converting log-linear C_T values to linear term as previously described (Krzysik-Walker *et al.* 2007).

Effect of sexual maturation on ovarian GnIHR mRNA abundance

Sexually mature (26 weeks old; $n=7$) and immature (16 weeks old; $n=7$) female chickens were killed by decapitation, and the total ovary was collected and frozen immediately in liquid nitrogen. Large preovulatory follicles from the ovary of sexually mature chickens were removed before storing in liquid nitrogen. Total RNA was extracted from the ovary using Trizol and RNeasy kits. Total RNA (1 µg) was reverse transcribed and subjected to qPCR for quantification of GnIHR mRNA as described above. Each sample from sexually mature and

immature chickens was run in duplicate to obtain average C_T values for GnIHR mRNA and β-actin mRNA. The relative amount of ovarian GnIHR mRNA in sexually mature and immature birds was then compared.

Effect of ovarian steroids on ovarian GnIHR mRNA quantity

Sexually immature female chickens (16 weeks old; $n=7$) were injected, intramuscularly, with peanut oil containing 17β-E$_2$ (0.5 mg/kg body weight; four injections on alternate days; Dunn *et al*. 2003), P$_4$ (0.17 mg/kg body weight/day for 7 consecutive days; Liu & Bacon 2005), E$_2$ and P$_4$ together (E$_2$+P$_4$) at the above dosage, or no steroids (negative control). After 7 days of the first dose, the chickens were killed by decapitation and the oviduct isolated (infundibulum to shell gland) and weighed to confirm the efficacy of E$_2$ and/or P$_4$ treatments. The total ovary from each animal was collected and snap-frozen in liquid nitrogen. Total RNA from the ovaries of each chicken was extracted and subjected to qPCR analysis for the quantification of GnIHR mRNA as described above. The amount of GnIHR mRNA was expressed as a proportion to β-actin mRNA and compared among the treatment groups.

Effect of GnIH on granulosa cell viability

Granulosa cell isolation from prehierarchial follicles

Approximately, 2 h prior to ovulation, chickens (Hyline W36 strain; 70 weeks old) were killed by decapitation. Prior to killing, the presence of a hard-shelled egg in the shell gland was confirmed by cloacal examination. The granulosa layers of 10–12 prehierarchial follicles (6–8 mm in size) were removed from two hens each, pooled, and dispersed by treatment with 0.3% collagenase at 37 °C for 10 min with gentle agitation in a spinner flask (Tilly & Johnson 1987). A Trypan blue exclusion test was conducted for every batch of dispersed granulosa cells to confirm viability of cells.

Cell viability assay

Cell viability was measured by the CellTiter-Blue Cell Viability Assay following manufacturer's protocol, which measures metabolic activity of cells based on their ability to reduce resazurin to the fluorescent product resorufin. Approximately, 15 000 cells were seeded into each well of a 96-well black wall plate (Perkin–Elmer, Waltham, MA, USA) coated with 0.1% gelatin. After culturing for 6 h in 100 µl culture media (M199 with Hanks salts, 0.2% BSA, 2.5% fetal bovine serum, 0.2% α-D(+) glucose, 0.01% trypsin inhibitor (lima bean, type II-L), and 1% antibiotic–antimycotic solution) at 40 °C with 5% CO_2, media were removed and treatments applied. Chicken GnIH (0, 10^{-10}, 10^{-8}, and 10^{-6} M) with or without 100 ng/ml recombinant human FSH (Li & Johnson 1993) dissolved in 100 µl culture medium was applied and the plates were incubated for 12 h. All treatments were done in triplicate. At the end of 12 h incubation, 20 µl CellTiter-Blue was added to each well and incubation continued for an additional 1 h. Following incubation, resorufin fluorescence (544_{Ex}/590_{Em}) was measured using Victor[3] 1420 Multilable Counter (Perkin–Elmer), and cell viability was calculated as a proportion of viability recorded from vehicle-treated cells. The experiment was repeated five times ($n=5$).

Statistical analysis

GnIHR mRNA quantity in ovaries of immature and mature chickens was compared using Student's *t*-test using Statistical Analysis System (SAS; SAS Institute, Cary, NC, USA). The effect of E$_2$ and/or P$_4$ on GnIHR mRNA expression was analyzed using general linear model (GLM) procedure of the SAS package. Relative GnIHR mRNA quantity to β-actin mRNA quantity was first converted from log-linear to linear term and then compared using the GLM procedure of the SAS package. GnIHR mRNA abundance in granulosa and thecal cell layers of F1, F3, and F6 follicles was expressed as a ratio of GnIHR mRNA quantity in 3–5 mm prehierarchial follicle in each animal. A probability level of $P<0.05$ was considered statistically significant. For DNA sequence analysis and PCR primer designing, Vector NTI suite 9.1 (Invitrogen) was used. Data on cell viability were expressed as a proportion to that of the vehicle-treated cells. All data are represented as mean ± S.E.M.

References

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

Bentley GE, Jensen JP, Kaur GJ, Wacker DW, Tsutsui K & Wingfield JC 2006 Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH). *Hormones and Behavior* **49** 550–555.

Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sanchez-Criado JE, Pellicer A et al. 2006 Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? *Endocrinology* **147** 4852–4862.

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

Dekel N & Shalgi R 1987 Fertilization *in vitro* of rat oocytes undergoing maturation in response to a GnRH analogue. *Journal of Reproduction and Fertility* **80** 531–535.

Dunn IC, Lewis PD, Wilson PW & Sharp PJ 2003 Acceleration of maturation of FSH and LH responses to photostimulation in prepubertal domestic hens by oestrogen. *Reproduction* **126** 217–225.

Gilbert AB, Evans AJ, Perry MM & Davidson MH 1977 A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility* **50** 179–181.

Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, Fujii R, Watanabe T, Kikuchi K, Terao Y et al. 2000 New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nature Cell Biology* **2** 703–708.

Ikemoto T & Park MK 2005 Chicken RFamide-related peptide (GnIH) and two distinct receptor subtypes: identification, molecular characterization, and evolutionary considerations. *Journal of Reproduction and Development* **51** 359–377.

Kogo H, Fujimoto T & Mori T 1999 Evidence for gonadotropin-releasing hormone receptor mRNA expression by estrogen in rat granulosa cells. *Cell and Tissue Research* **297** 459–465.

Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F et al. 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *Journal of Biological Chemistry* **276** 34631–34636.

Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K & Silver R 2006 Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *PNAS* **103** 2410–2415.

Krzysik-Walker SM, Ocon-Grove OM, Maddineni SB, Hendricks GL III & Ramachandran R 2007 Identification of calcitonin expression in the chicken ovary: influence of follicular maturation and ovarian steroids. *Biology of Reproduction* **77** 626–635.

Li Z & Johnson AL 1993 Regulation of P450 cholesterol side-chain cleavage messenger ribonucleic acid expression and progesterone production in hen granulosa cells. *Biology of Reproduction* **49** 463–469.

Liu HK & Bacon WL 2005 Changes in egg production rate induced by progesterone injection in broiler breeder hens. *Poultry Science* **84** 321–327.

Morales P 1998 Gonadotropin-releasing hormone increases ability of the spermatozoa to bind to the human zona pellucida. *Biology of Reproduction* **59** 426–430.

Nakayama GR, Caton MC, Nova MP & Parandoosh Z 1997 Assessment of the Alamar Blue assay for cellular growth and viability *in vitro*. *Journal of Immunological Methods* **204** 205–208.

Osugi T, Ukena K, Bentley GE, O'Brien S, Moore IT, Wingfield JC & Tsutsui K 2004 Gonadotropin-inhibitory hormone in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*): cDNA identification, transcript localization and functional effects in laboratory and field experiments. *Journal of Endocrinology* **182** 33–42.

Reiter E, Marion S, Robert F, Troispoux C, Boulay F, Guillou F & Crepieux P 2001 Kinase-inactive G-protein-coupled receptor kinases are able to attenuate follicle-stimulating hormone-induced signaling. *Biochemical and Biophysical Research Communications* **282** 71–78.

Robinson FE & Etches RJ 1986 Ovarian steroidogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). *Biology of Reproduction* **35** 1096–1105.

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

Springer JE, Azbill RD & Carlson SL 1998 A rapid and sensitive assay for measuring mitochondrial metabolic activity in isolated neural tissue. *Brain Research. Brain Research Protocols* **2** 259–263.

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

Tilly JL & Johnson AL 1987 Presence and hormonal control of plasminogen activator in granulosa cells of the domestic hen. *Biology of Reproduction* **37** 1156–1164.

Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, Ishii S & Sharp PJ 2000 A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and Biophysical Research Communications* **275** 661–667.

Ubuka T, Bentley GE, Ukena K, Wingfield JC & Tsutsui K 2005 Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. *PNAS* **102** 3052–3057.

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

Ukena K & Tsutsui K 2001 Distribution of novel RFamide-related peptide-like immunoreactivity in the mouse central nervous system. *Neuroscience Letters* **300** 153–156.

Ukena K, Iwakoshi E, Minakata H & Tsutsui K 2002 A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry. *FEBS Letters* **512** 255–258.

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

Whitelaw PF, Eidne KA, Sellar R, Smyth CD & Hillier SG 1995 Gonadotropin-releasing hormone receptor messenger ribonucleic acid expression in rat ovary. *Endocrinology* **136** 172–179.

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