Gonadotrophin Inhibitory Hormone Depresses Gonadotrophin α and Follicle-Stimulating Hormone β Subunit Expression in the Pituitary of the Domestic Chicken

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Abstract

Studies performed in vitro suggest that a novel 12 amino acid RF amide peptide, isolated from the quail hypothalamus, is a gonadotrophin inhibitory hormone (GnIH). The aim of the present study was to investigate this hypothesis in the domestic chicken. Injections of GnIH into nest-deprived incubating hens failed to depress the concentration of plasma luteinizing hormone (LH). Addition of GnIH to short-term (120 min) cultures of diced pituitary glands from adult cockerels depressed follicle-stimulating hormone (FSH) and LH release and depressed common α and FSHβ gonadotrophin subunit mRNAs, with no effect on LHβ subunit mRNA. Hypothalamic GnIH mRNA was higher in incubating (out-of-lay) than in laying hens, but there was no significant difference in the amount of hypothalamic GnIH mRNA in out-of-lay and laying broiler breeder hens at the end of a laying year. It is concluded that avian GnIH may play a role in controlling gonadotrophin synthesis and associated constitutive release in the domestic chicken.

A novel RF amide, SIKPSAYLPLRF-NH2, isolated from the quail hypothalamus, inhibits luteinizing hormone (LH) but not prolactin secretion from the male quail pituitary in vitro. Accordingly, the peptide was named gonadotrophin inhibitory hormone (GnIH) (1). This peptide has a transiently depressive effect on plasma LH in the white crowned sparrow in vivo (2). Immunocytochemical studies in quail (1, 3) and song sparrow (4) show that GnIH is located in cells in the paraventricular nucleus of the hypothalamus, with terminals in the median eminence, with a potential to control anterior pituitary function. The cDNA sequence encoding GnIH has been cloned in the Japanese quail (5), domestic chicken (NCBI accession number AB120325) and white crowned sparrow (2). The predicted amino acid sequence for chicken GnIH (SIRPSAYLPLRF-NH2) differs from quail GnIH at position 3 where arginine conservatively substitutes lysine. It is unknown whether the expression of the gene encoding GnIH in the avian hypothalamus changes with reproductive state consistent with an inhibitory action on gonadotrophin secretion. Furthermore, it is not known whether, in addition to inhibiting LH release, GnIH also inhibits gonadotrophin synthesis. This appears to be possible because, by analogy, gonadotrophin-releasing hormone-I (GnRH-I) stimulates both the release and synthesis of chicken (6, 7) and mammalian gonadotrophins (8, 9). The present study investigated the role of GnIH in chicken reproductive function by determining whether GnIH decreases plasma LH concentrations in vivo, whether GnIH depresses gonadotrophin subunit mRNAs in vitro and whether there is an increase in hypothalamic GnIH mRNA associated with the atrophy of the ovary in incubating hens (10, 11) and in broiler breeders at the end of a laying period (12).

Materials and methods

Animals

All experimental procedures were carried out under UK Home Office regulations. Incubating and laying domestic hens required for studies performed in vivo were obtained from the Roslin Institute’s breeding flocks. The hens were a hybrid between White Leghorn and Silkie breeds. Pituitary glands for studies performed in vitro were from adult ISA Brown cockerels (ISA Poultry Services Ltd, Peterborough, UK) purchased at 1 day old. All birds obtained from Roslin Institute were held under a LD 16 : 8 h light/dark cycle with free access to food and water.

Broiler breeder hens (Cobb Vantress Inc., East Hanningfield, UK) were obtained from a flock of pedigree broiler breeders at the end of a commercial laying period at 58 weeks of age. The birds were fed a commercial restricted feeding programme to maximize egg laying as recommended by Cobb Vantress and were held under a LD 16 : 8 h light/dark cycle.
The objective was to investigate in vivo the effect of GnIH on LH release in nest-deprived incubating hens. Nest removal results in an increase in plasma LH (10) and could be a consequence of reduced GnIH release, which was predicted to be reversed by exogenous administration of GnIH. Quail GnIH was synthesized in the laboratory of K. Tsutsui (1), and dissolved in physiological saline and injected (100 μl) into a brachial vein of a nest deprived incubating hen. Incubating hens were given three injections of 50 μg quail GnIH per kg body weight or saline at 5.5, 6.5 and 7.5 h after nest deprivation. The dose of GnIH was chosen based on analogous experiments in the incubating hens using GnRH analogues, which suggested that injections of reproductive neuroendocrine peptides of 50 μg/kg are likely to affect gonadotrophin secretion (11). This is similar to the dose of GnIH shown to be effective in depressing LH in vivo in the white crowned sparrow (2). Three injections of GnIH were given at hourly intervals to cover the possibility that an inhibitory effect of the peptide on LH release may be secondary to a longer term inhibitory effect on synthesis. Blood samples (approximately 1 ml) were taken from a brachial vein before nest deprivation at 0 h, and at 5.5, 6.5, 7.5 and 8.5 h thereafter for LH assay. GnIH or saline injections were given immediately after withdrawing blood samples at 5.5, 6.5 and 7.5 h after nest deprivation.

Experiment 2

The objective was to confirm in adult male chickens, the observation in male quail (1) that GnIH inhibits LH secretion from pituitary fragments incubated in vitro for 120 min, and to determine correlated effects on gonadotrophin subunit mRNAs. Pituitary glands were dissected from eight cockerels and pooled into chilled phosphate-buffered saline. The pituitaries were collectively diced into fragments and distributed randomly into the wells of 12-well cell culture plates (Corning Costa, Amsterdam, The Netherlands). Each well contained one pituitary fragment, 1 ml of DMEM culture medium with phenol red (Invitrogen Life Technologies, Paisley, UK) supplemented with 3.75% foetal calf serum, 6% horse serum and antibiotics: 100 μg/ml streptomycin and 100 μg/ml penicillin. The culture plates were placed in an airight container equilibrated with 95% O₂ and 5% CO₂, which was transferred to an orbital incubator maintained at 37 °C and shaken gently (60 r.p.m.) for a 90-min preincubation. The culture medium was then removed and 1 ml of fresh medium was added to each well containing GnIH peptide (1 × 10⁻⁴, 1 × 10⁻⁵, 1 × 10⁻⁶ M) or no peptide (control) in a Latin square design. The culture plates were re-equilibrated with 95% O₂ and 5% CO₂ and replaced in the orbital incubator for a 120-min incubation. Pituitary fragments were harvested and snap frozen in liquid nitrogen and stored at −80 °C before RNA extraction. The experiment was replicated on a separate occasion.

Experiment 3

An important factor to consider, when evaluating the predicted inhibitory effect of GnIH on gonadotrophin subunit mRNAs in a short-term pituitary gland culture, is the half-lives of the mRNAs. The objective of this experiment was to measure the half-life of each gonadotrophin subunit mRNA using the transcriptional inhibitor actinomycin D (13). The experimental conditions were as in Experiment 2. Cockerel pituitary fragments were incubated without (control) or with 8 μM actinomycin D (Sigma-Aldrich, Poole, UK) and collected for gonadotrophin subunit mRNA assay at 1, 3, 6 and 9 h.

Observations were made at each time point in triplicate.

Experiment 4

The objective was to test the prediction that hypothalamic GnIH mRNA content is higher in incubating than in laying hens, and that this is inversely related to pituitary gonadotrophin subunit mRNAs and plasma LH. Hypothalamic GnRH-I mRNA was also measured to test the alternative prediction that ovarian regression in incubating hens is a consequence of reduced GnRH-I mRNA. The reproductive status of both laying and incubating hens was confirmed by post-mortem dissection: the ovaries and pituitaries were collectively pooled into chilled phosphate-buffered saline. The pituitaries were collectively dissected, collectively pooled into chilled phosphate-buffered saline. The pituitaries were collectively pooled into chilled phosphate-buffered saline. The pituitaries were collectively pooled into chilled phosphate-buffered saline.

Experiment 5

The objective was to test the prediction that hypothalamic GnIH mRNA is higher in out-of-lay than in laying hens, and that this is inversely related to pituitary gonadotrophin subunit mRNAs and plasma LH. Hypothalamic GnRH-I mRNA was also measured to test the alternative prediction that ovarian regression in out-of-lay hens is a consequence of reduced GnRH-I mRNA. This experiment took advantage of the natural presence of laying and out-of-lay hens in a flock of broiler breeder hens at the end of a commercial laying period at 58 weeks old. Neuroendocrine tissues were collected at a commercial broiler breeding unit. Out-of-lay hens were identified by the absence of hierarchical yellow yolky ovarian follicles and regressed oviducts, and laying hens by the presence of fully developed oviducts and six or seven yellow yolky follicles in the ovary. Whole hypothalami, containing the GnIH and GnRH-I neurons, and pituitary glands were dissected into 1 ml of ‘R’NA Later’ (Ambion, Huntingdon UK). The neuroendocrine tissues were stored at −20 °C before RNA extraction. Blood samples for LH RIA were taken from a brachial vein. Collected blood samples were centrifuged and blood plasma was stored at −20 °C before RIA.

RNA extraction

Total RNA was extracted from neuroendocrine tissues in Matrix D tubes (Q-biogene-Alexis Ltd, Bingham, Nottingham, UK) containing 600 μl Trizol (Invitrogen Life Technologies) for pituitary fragments or 1 ml Trizol for hypothalami. The tissues were disrupted using a FastPrep FP120 homogeniser (Q-biogene-Alexis Ltd). Final precipitation of pituitary RNA was facilitated by addition of 2 μl glycogen solution (20 mg/ml, Roche Diagnostics Ltd, East Sussex, UK). The total RNA pellet was briefly dried under vacuum and reconstituted in 100–150 μl of dH₂O. The yield of RNA was quantified by measuring the optical density of a sample diluted to 1 : 50 at 260 nm and 280 nm, and its quality was confirmed by running a sample out on a formaldehyde gel.

Reverse transcription of total RNA

A 4 μl sample of total RNA was reverse transcribed using a First Strand synthesis kit (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). Reverse transcribed samples were diluted to 40 μl of dH₂O.

Quantitative competitive QC RT-PCR assays for pituitary gonadotrophin subunits, common alpha, follicle-stimulating hormone (FSH)b, Lhb, and for GnIH and GnRH-I

Chicken GnRH-I mRNA was measured by quantitative competitive reverse transcription-polymerase chain reaction (QC RT-PCR) (14), and the same methodology was used to develop and validate assays for gonadotrophin common α, FSHb, and LHb subunit mRNAs, and for GnIH mRNA. Oligonucleotide primers for the amplification of FSHb, LHb, common α gonadotrophin subunits and GnIH were designed using the ‘primer’ computer package version 0.5 (Whitehead Institute for Biomedical Research Cambridge, MA, USA) with sequences of published cDNA transcripts (Table 1). Sequence information for all these genes was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez). All primers were designed to span at least one exon-intron boundary, to allow detection of any contaminating gDNA.

All plasmids used as standards in the QC RT-PCR assays were constructed by cloning the product of RT-PCR amplification into the pBSK-II+ (Stratagene Europe, Amsterdam, The Netherlands) cloning vector using chicken pituitary (for common α, FSHb, LHb subunits) or hypothalamic (for GnIH) cDNAs as templates. Cloned fragments were transformed into XL1 Blue competent Escherichia coli cells (Stratagene Europe). Plasmid DNA was purified using a Qiagen QIAprep Spin MidiPrep kit (Qiagen Ltd, Crawley, West Sussex, UK) and sequenced to check gene identity.

Construction of competitor plasmids

Competitor plasmids were made by shortening (common α and FSHb subunit, or by inserting a fragment of foreign DNA (LHb subunit, GnIH) into the cloned standard cDNAs. Primer specific sequences were retained at the 5’ and 3’ ends to allow PCR amplification.

The common α subunit competitor was constructed using a Bpl (New England Biolabs, Hitchin, UK) and AflII (New England Biolabs) double

enzyme digest of the cloned alpha standard. Both endonucleases cut once within the standard sequence to produce a competitor fragment of 335 bp after blunt ending and religation. The FSHß competitor was constructed by digestion with PvuII (Roche Diagnostics) and BglI (Roche Diagnostics Ltd) followed by blunt ending and religation. Both LHß subunit and GnIH competitors were constructed by insertion of a 200 bp fragment of a pBSK II+ vector backbone produced by a HaeIII digestion (Roche Diagnostics Ltd). The LHß standard was cut by PflM I (New England Biolabs) and GnIH standard was cut using BgIII (Roche Diagnostics Ltd). Both restriction enzymes cut once within each respective gene sequence in preparation for the subcloning of the foreign DNA insert. The standard and competitor bp sizes, respectively, for each gene were: α subunit: 521 and 335; FSHß subunit: 341 and 115; LHß subunit: 239 and 439; GnIH: 552 and 752, and GnRH-I: 358 and 463.

QC RT-PCR assays

The assays were carried out in tubes containing plasmid DNA standard and competitor for standard curves, or experimental sample cDNA and the competitor.

Standard curves were made up of eight, two-fold dilutions of the respective standard plasmid. The concentration of standard plasmids ranged between 2.60 × 10^{-14} and 1.79 × 10^{-19} mol, depending on the assay, in a volume of 5 μL. The competitor plasmids were diluted to a concentration that fell in the middle of the standard range. A fresh standard curve and competitor dilutions were made on the day of each assay. Each tube for all assays contained standard or cDNA neuroendocrine sample (5 μL) and competitor (5 μL) made up to a PCR mix of 20 μL. The PCR assays were carried out in PCR buffer (× 10) with 1.5 mM MgCl₂ (Roche Diagnostics Ltd), 2 mM dNTP (Advanced Biotechnologies Ltd (Abgene), Epsom, Surrey, UK), 0.025 U/μL Taq DNA polymerase [Advanced Biotechnologies Ltd (Abgene)] and 0.5 μM of assay specific forward and reverse primers (Sigma-Genosys, Cambridge, UK). The PCR amplification was carried out in a Thermo-Fast® low profile 96-well plate [Advanced Biotechnologies Ltd (Abgene)] on a Hybaid MBS 0.2G programmable heating block (Hybaid Ltd, Ashford, Middlesex, UK). The PCR conditions were 30 cycles (94 °C, 20 s; 62 °C, 20 s; 72 °C, 20 s) for FSHß, LHß, common α subunits and GnRH-I, and 30 cycles (94 °C, 20 s; 60 °C, 20 s; 72 °C, 20 s) for GnIH. After the PCR amplification was completed, a final incubation step of 80 °C for 20 min was added to ensure heterodimers of amplified competitor and standard or cDNA were eliminated.

The product (10 μL) was loaded with 3 μL of gel loading dye [30% glycerol, 70% 1× Tris-Acetate/EDTA (TAE), 0.4% Orange G] on a 3% agarose gel (BDH Laboratory Supplies, Poole, UK) containing ethidium bromide (0.2 μg/ml) and electrophoresed (6 V/cm) in 1× TAE running buffer until band separation was visible. The relative amounts of cDNA in the bands were measured as a function of ethidium bromide fluorescence produced by UV illumination via a gel transilluminator at 312 nm (UVP, Cambridge, UK.), and captured using a video camera linked to a personal computer running the Multi-Analyst program (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplified cDNA in each lane was quantified using gel plotting macros in the Scion Image computer package (Scion Corporation, Frederick, MD, USA). To obtain a linear standard curve, the ratio of the two bands, standard and competitor, was calculated, log-transformed and plotted against the number of moles of standard plasmid present in each sample.

Chicken gonadotropin radioimmunoassays

FSH and LH were measured by homologous radioimmunoassays (15, 16). The inassay coefficients of variation were 8 and 5.4%, respectively. Culture medium samples were diluted serially in assay diluent. The sensitivity of the FSH assay was too low to measure FSH reliably in the plasma of adult hens.

Statistical analysis

All analyses were carried out using Genstat 6 edition (VSN International Ltd, Oxford, UK). Differences between experimental groups were considered significant at P < 0.05. Data from experiments 1, 2, 4 and 5, log-transformed where necessary to normalize variance, were analysed using analysis of variance (ANOVA). In Experiment 1, treatment and time were used as variables and bird as a block in the analyses. In Experiment 2, the treatment and location of pituitary fragments within a tissue culture plate were used as variables for ANOVA, followed by Student’s t-test, if appropriate, to test for significance between means. For Experiment 3, degradation of gonadotrophin subunit mRNA was calculated by subtraction of the control values from the actinomycin D-values to give the change in the concentration of mRNA due to degradation. The value for change in the concentration of mRNA was log₂-transformed and plotted against time. Half-lives were calculated by taking the reciprocal of the slope of the regression between the change in the concentration of mRNA and time, because a change of 1 in log₂-transformed data equals a decrease in concentration of a half.

Results

Effect of an intravenous injection of GnIH on plasma LH in nest deprived incubating hens

Three intravenous injections of 50 μg GnIH per kg given at hourly intervals after nest deprivation of incubating hens for...
5.5 h failed to attenuate or block the increase in plasma LH observed (Fig. 1).

**Effect of GnIH on gonadotrophin subunit mRNAs and gonadotrophin secretion in vitro**

Incubation of cockerel pituitary fragments for 120 min with GnIH at doses of $1 \times 10^{-7}$ and $1 \times 10^{-6}$ M but not at $1 \times 10^{-5}$ M depressed the concentration of common $\alpha$ subunit mRNA (Fig. 2A). GnIH had a similar depressive effect on FSH$\beta$ mRNA, although this effect was seen with doses of $1 \times 10^{-7}$ and $1 \times 10^{-5}$ M but not $1 \times 10^{-6}$ M (Fig. 2B). By contrast, GnIH did not depress LH$\beta$ subunit mRNA (Fig. 2C). GnIH inhibited both FSH and LH release into the incubation medium at the lowest but not the highest doses (Fig. 2D,E). The experiment was repeated, and the combined results are shown.

**Estimation of the half-lives of gonadotrophin subunit mRNAs**

The half-lives of the gonadotrophin $\alpha$, FSH$\beta$ and LH$\beta$ subunit mRNAs were calculated to be 8.6, 4.1 and 13 h, respectively, although the error estimates are relatively large (Fig. 3A–C).

**Comparison of hypothalamic GnIH, GnRH-I and pituitary gonadotrophin subunit mRNAs and plasma LH in incubating and laying hens**

Concentrations of common $\alpha$ and LH$\beta$ subunit mRNAs were lower in incubating than in laying hens (Fig. 4A) and were directly correlated with depressed plasma LH (Fig. 4B). No significant difference in the amount of FSH$\beta$ mRNA was observed between incubating and laying hens (Fig. 4A). Hypothalamic GnRH-I mRNA was lower, whereas GnIH

![Graphs showing the effects of GnIH on gonadotrophin subunit mRNAs and gonadotrophin secretion in vitro.](image-url)
Fig. 3. Estimations of the half-lives for chicken gonadotrophin subunit mRNAs. Cockerel anterior pituitary fragments were incubated in the presence or absence of 8 μM actinomycin D for 1–9 h and subunit mRNAs were measured during this period. The half-lives were calculated by taking the reciprocal of the slope of the fitted line. Regression analyses are shown for (A) the common α subunit, (B) follicle-stimulating hormone (FSH) β subunit and (C) luteinizing hormone (LH) β subunit mRNAs. Values are mean ± SEM (n = 3).

![Graph](image)

Fig. 4. Comparison of hypothalamic gonadotrophin inhibitory hormone (GnIH), gonadotrophin-releasing hormone-I (GnRH-I) and gonadotrophin subunit mRNAs and plasma luteinizing hormone (LH) in incubating and laying hens.

![Bar chart](image)

Fig. 5. Comparison of hypothalamic gonadotrophin inhibitory hormone (GnIH), gonadotrophin-releasing hormone-I (GnRH-I) and gonadotrophin subunit mRNAs and plasma luteinizing hormone (LH) in laying and out-of-lay broiler breeder hens

![Bar chart](image)

Discussion

This study confirms, in the cockerel, the observation in male quail (1), that GnIH inhibits LH secretion in vitro. However repeated injections of GnIH failed to suppress the increase in plasma LH that occurs in incubating hens after nest deprivation (Fig. 1). These observations are in contrast to a study in the white crowned sparrow where an intravenous injection of GnIH transiently suppressed plasma LH after 2 min but not 10 min, and an injection of 1000 ng GnIH antagonized the stimulatory effect of 10 ng GnRH on LH release (2). The sampling procedure used in the present study would not have detected a transient depression in plasma LH after 2 min. The failure to demonstrate a long-term inhibitory effect of GnIH in vivo in nest deprived incubating hens (Fig. 1) may be explained by an inappropriate dose or timing of GnIH administration. Alternatively, it is possible that the quail GnIH used in the study, which differs from the predicted sequence of chicken GnIH by a conservative substitution of lysine by arginine at position three, may be inactive in the chicken in vivo. This is unlikely because quail and white crowned sparrow GnIH mRNA was higher in incubating than in laying hens (Fig. 4b).
However, in the cockerel pituitary, the concentrations of LH in the chicken (26). If GnIH inhibited LH release providing further evidence for a constitutive release of LH in birds, this is supported by the observation reported in mammals (22, 23). Gonadotrophin subunit released without concurrent GnRH stimulation has been demonstrated whether this suppressive effect of GnIH is a short-acting effect, as suggested by the in vitro observations of Osugi et al. (2). Whereas a second, long-acting mechanism is suggested by the observations in vivo reported in the present study: GnIH may inhibit LH release as a consequence of decreased LH synthesis associated with a decrease in constitutive LH release. The possibility that LH is depression in the concentrations of common α and FSHβ subunit mRNAs. It remains to be demonstrated whether this suppressive effect of GnIH is a consequence of an inhibition of common α and FSHβ subunit gene transcription, or of decreased stability of the gonadotrophin subunit mRNAs. The possibility that GnIH also depresses LHβ mRNA cannot be excluded because the half-life of chicken LHβ mRNA was calculated to be 13 h (Fig. 3c) which is much longer than the 120 min experiment performed in vitro. Mammalian LHβ mRNA has a long half-life, 44 h in the rat (17), whereas the shorter, 4–8 h half-lives of common α and FSHβ subunit mRNAs calculated for the chicken (Fig. 3A,b) are similar to those reported for the corresponding mammalian mRNAs (17–21).

It is possible that the suppressive action of GnIH on LH release is mediated by more than one mechanism: the first appears to be a short-acting effect, as suggested by the observations of Osugi et al. (2), whereas a second, long-acting mechanism is suggested by the observations in vivo reported in the present study: GnIH may inhibit LH release as a consequence of decreased LH synthesis associated with a decrease in constitutive LH release. The possibility that LH is released without concurrent GnRH stimulation has been reported in mammals (22, 23). Gonadotrophin subunit mRNA levels are maintained for at least 3 h after GnRH deprivation (24). In birds, this is supported by the observation that, in the male turkey, increased baseline plasma LH during sexual maturation is not associated with a change in GnRH pulse frequency or amplitude (25). It is relevant to note that baseline concentrations of LH in laying hens are not pulsatile, providing further evidence for a constitutive release of LH in the chicken (26). If GnIH inhibited LH release in vitro as a consequence of inhibition of synthesis, LH synthesis would have been depressed within 120 min of the pituitary fragment incubation experiment. There is no information on the rate of LH synthesis in birds but, in mammals, synthesis to processing and packaging of a mature LH dimer takes 1.5 h (27). This was within the time-frame of the incubation experiment and supports the view that GnIH may suppress LH release secondarily to a reduction in synthesis.

The possibility that the GnIH-induced depression in the common α subunit mRNA is responsible for the reduction in LH synthesis and release would be unlikely in mammals where the common α subunit protein is always in excess of the LHβ subunit protein (28, 29). The synthesis of gonadotrophin β subunit is therefore seen as the limiting factor for mammalian gonadotrophin synthesis (30), because pituitary concentrations of common α subunit mRNAs are three- to four-fold greater than levels of LHβ in the rat (31, 32). However, in the cockerel pituitary, the concentrations of common α subunit mRNAs and the two gonadotrophin β subunit mRNAs are similar in concentration, ranging between $1 \times 10^{-18}$ and $1 \times 10^{-19} \ M$ in vitro (Fig. 2A–c) and this does not take into account the concentration of TSHβ subunit mRNA, for which no information is available. The view that the common α rather than the LHβ mRNA levels is predictive of LH secretion in birds is supported by observations in Japanese quail where changes in plasma LH induced by feed restriction and refeeding were correlated with common α, but not LHβ subunit mRNAs (33, 34). This is in contrast to mammals where neither the common α or LHβ subunit mRNAs are predictive of plasma LH concentrations (35).

LH release into the incubation medium and gonadotrophin α subunit mRNA were inhibited in the presence of $1 \times 10^{-7} \ M$ and $1 \times 10^{-6} \ M$ but not of $1 \times 10^{-5} \ M$ GnIH (Fig. 2A, E). This correlation further strengthens the view that GnIH inhibits LH release secondarily to a depression in common α subunit mRNA. The lack of an effect of a high dose of GnIH on LH release can be ascribed to a desensitization of the gonadotrophs. By analogy, the phenomenon of desensitization has been demonstrated for the response of the chicken (11, 36) and mammalian (24, 37–39) gonadotroph to continuous GnRH-I exposure.

GnIH also inhibited FSH release in vitro (Fig. 2B), which confirmed a similar, but non-significant inhibitory effect on FSH release in vitro in the quail (1). However, it was not possible to demonstrate whether GnIH also inhibits FSH in vivo in adult hens because the chicken FSH assay was not sufficiently sensitive to measure it. The inhibitory effect of GnIH on FSH release in vitro correlated with a depression in common α and FSHβ subunits. The highest doses of GnIH did not depress FSH release, again suggesting, as for LH release, a desensitization of gonadotroph function to GnIH. The inhibitory effect of GnIH on FSH release is suggested to be a consequence of reduced common α or FSHβ mRNAs, resulting in reduced synthesis. In the chicken, FSH release is partially constitutive (40), as it is in mammals (41–43), and a reduction in FSH synthesis is predicted to result in a rapid decrease in release, as observed in the present study (Fig. 2B).

The physiological relevance of the depressive effect of GnIH in gonadotrophin secretion in vitro was evaluated by determining whether ovarian regression in the hen in two physiological conditions is correlated with changes in GnIH and gonadotrophin subunit mRNAs. In the first of two physiological conditions chosen for study (i.e. the incubating hen), ovarian regression was associated with increased GnIH mRNA, decreased common α and LHβ mRNA, and decreased plasma LH (Fig. 4A, a, b). This cascade of reduced neuroendocrine gene expression is consistent with the predicted increase in GnIH release into the hypophysial portal vasculature, resulting in a depression in LH synthesis and constitutive release. This is also consistent with the depressive effect of GnIH on common α mRNA and LH release in vitro (Fig. 2A, E). However, ovarian regression in the incubating hen, in confirmation of an earlier study (14), is also associated with a decrease in hypothalamic GnRH-I mRNA (Fig. 4B), which could also be responsible for decreased GnRH-I release and, consequently, decreased gonadotrophin subunit mRNA synthesis and LH secretion. In support of this view, it has been demonstrated in the chicken that GnRH-I stimulates...
common α subunit mRNA (6; N. A. Ciccone, unpublished observation). There was no significant difference in concentration of FSHβ mRNA between laying and incubating hens, although it was not possible to determine whether this was related to plasma FSH concentrations because FSH cannot be reliably measured in laying hens.

By contrast to incubating hens, ovarian regression in broiler hens at the end of a laying year was not associated with a change in plasma LH, nor in hypothalamic GnIH and GnRH-I mRNAs (Fig. 5a). No information was available for plasma FSH. Ovarian regression in these hens therefore appears to be downstream of a change in GnIH or GnRH-I gene transcription. However, it cannot be ruled out that ovarian regression is not due to altered patterns of GnRH-I or GnIH release controlled independently of steady-state concentrations of GnRH or GnIH mRNAs. However, it appears to be unlikely that ovarian regression at the end of laying year is a result of increased GnIH release and associated depressed plasma FSH release because, in contrast to the studies performed in vitro (Fig. 2), FSHβ subunit mRNA in vivo was increased with no change in common α subunit mRNA (Fig. 5a). The increase in FSHβ mRNA in the out-of-lay hens can be most readily ascribed to the removal of the inhibitory effect of circulating ovarian steroids. In support of this view, FSHβ subunit gene transcription is highly sensitive to the inhibitory effect of oestrogen and progesterone in mammals (44, 45) and, in the juvenile female chicken, pituitary FSH content is more responsive to the depressive action of oestrogen than is LH content (46). The depression in LHβ mRNA in out-of-lay birds (Fig. 5a) could be due either to removal of the trophic influence of GnRH-I or to an increase in inhibitory effect of GnIH, although there is no evidence that chicken LHβ mRNA is controlled by either neuropeptide.

In conclusion the results of this study provide sufficient evidence to suggest that GnIH may play a role in the neuroendocrine control of reproductive function. It remains to be established whether GnIH plays a pivotal role in avian reproduction. GnIH may have a modulatory function and be particularly important in the control of the onset of puberty, as well as the regulation of the timing of seasonal breeding.

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