Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling

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ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic peptide from the RFamide peptide family that has been identified in multiple avian species. Although GnIH has clearly been shown to reduce LH release from the anterior pituitary gland, its mechanism of action remains to be determined. The overall objectives of this study were (1) to characterize the GnIH receptor (GnIH-R) signaling pathway, (2) to evaluate potential interactions with gonadotropin releasing hormone type III receptor (GnRH-R-III) signaling, and (3) to determine the molecular mechanisms by which GnIH and GnRH regulate pituitary gonadotrope function during a reproductive cycle in the chicken. Using real-time PCR, we showed that in the chicken pituitary gland, GnIH-R mRNA levels fluctuate in an opposite manner to GnRH-R-III, with higher and lower levels observed during inactive and active reproductive stages, respectively. We demonstrated that the chicken GnIH-R signals by inhibiting adenylyl cyclase cAMP production, most likely by coupling to Gαi. We also showed that this inhibition is sufficient to significantly reduce GnRH-induced cAMP responsive element (CRE) activation in a dose-dependent manner, and that the ratio of GnRH/GnIH receptors is a significant factor. We propose that in avian species, sexual maturation is characterized by a change in GnIH/GnRH receptor ratio, resulting in a switch in pituitary sensitivity from inhibitory (involving GnIH) to stimulatory (involving GnRH). In turn, decreasing GnIH-R signaling, combined with increasing GnRH-R-III signaling, results in significant increases in CRE activation, possibly initiating gonadotropin synthesis.

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1. Introduction

In 2000, a novel hypothalamic peptide belonging to the RFamide family was isolated from the brain of the Japanese quail (Coturnix japonica) and was shown to specifically decrease gonadotropin release in vitro (Tsutsui et al., 2000). Subsequently, this gonadotropin-inhibitory hormone (GnIH) was reported to be present, conserved, and active in several other avian species (Urbanski, 1984; Satake et al., 2001; Osugi et al., 2004; Ciccone et al., 2005; Ikemoto and Park, 2005; Ubuka et al., 2006). More recently, mammalian equivalents have been characterized in the hamster (Kriegsfeld et al., 2006), rat (Johnson et al., 2007), sheep (Dardente et al., 2008), rhesus macaque (Ubuka et al., 2009a) and human (Ubuka et al., 2009b). Interestingly, in most seasonal breeding species, the expression of GnIH (or its mammalian orthologue) appears to be associated with the inhibition of reproduction and may be controlled by melatonin (Ubuka et al., 2005; Dardente et al., 2008; Revel et al., 2008), whereas in non-photoperiodic breeders, GnIH does not affect the onset of puberty (Dardente et al., 2008).

In photoperiodic birds, an increase in day-length may induce sexual maturation by stimulating the release of GnRH from the hypothalamus, which in turn stimulates the release of gonadotropins from the pituitary gland (for a review: Dawson et al., 2001). This is followed by reproductive tract maturation in both males and females, and the initiation of sperm and egg production. In chickens, hens and roosters reach sexual maturity with a fully functional reproductive axis in an average of 4 weeks following an increase in photoperiod (photostimulation). After an active laying period (ranging from several months to over a year, depending on the breed), females progressively cease laying eggs and stop responding to stimulatory photoperiods. This phase, leading to photorefractoriness, is characterized by decreasing levels of LH and the involution of the reproductive tract, despite stimulatory input from hypothalamic GnRH (Ciccone et al., 2005). We previously cloned a novel GnRH receptor in chickens (initially labeled cGnRHR-2) and have shown that its mRNA levels fluctuate during a reproductive cycle, with low levels associated with immature and end-of-lay stages and high levels associated with sexual activity (Shimizu and Bedecarrats, 2006), suggesting pituitary responsiveness to GnRH changes. Recently, it was further confirmed that this receptor is the predominant form expressed in the chicken pituitary gland, and that it is a type III receptor (Joseph et al., 2009).
We will therefore refer to this receptor as cGnRH-R-III. To date, GnIH receptors (GnIH-Rs) have been cloned in several species, including chickens and Japanese quail (Ikemoto and Park, 2005). Similar to GnRH-Rs, they belong to the G-protein coupled receptor family. Therefore, although the signal transduction pathway of GnIH-Rs has not been characterized, they likely signal either through G\(_{a11}\), G\(_{a16}\), or G\(_{a20}\). However, the anti-gonadotrophic effects of GnIH suggest that this peptide may interfere with GnRH-R signaling to alter the synthesis and release of gonadotropins. To date, the only insights into GnIH signaling indicate a decrease in G\(_{a1}\) mRNA following stimulation of COS-7 cells transfected with the cGnIH-R (Ikemoto and Park, 2005), and a reduced GnRH-induced mobilization of intracellular calcium in sheep primary gonadotropes (Clarke et al., 2008). However, a decrease in G\(_{a1}\) mRNA is not indicative of receptor coupling, and the molecular mechanisms involved in the inhibition of calcium mobilization have not been investigated. Therefore, in the current study, we first determined the expression pattern of the cGnIH-R in the pituitary gland of male and female chickens at various reproductive stages. We then characterized the signaling pathway utilized by cGnIH-R and investigated whether GnIH interferes with GnRH signaling.

2. Materials and methods

2.1. Animals and tissue collection

Tissue samples used for this study correspond to the ones used by Shimizu and Bédécarrats (Shimizu and Bedecarrats, 2006). Briefly, White Leghorn chickens were raised under an 8-h photoperiod up until the time when they were photostimulated by an abrupt transfer to a 14-h photoperiod at 19 weeks of age. Five brain tissues (pituitary gland, diencephalon, brain stem, cerebrum, and cerebellum) were collected from birds of both sexes. For females, samples were collected at the immature (14 weeks old, n = 5), peak-of-lay (30 weeks old, n = 5), mid-lay (1 year old, n = 5), and end-of-lay (over 1 year old, n = 5) stages. For males, samples were collected at the immature (14 weeks old, n = 5), 4 weeks post-photostimulation (23 weeks old, n = 5), and old mature (14 months old, n = 5) stages. After collection, individual tissues were snap-frozen in liquid nitrogen, and stored at -80°C until use. All animal procedures were conducted under the guidelines of the Canadian Council for Animal Care, and were approved by the University of Guelph Animal Care Committee.

2.2. Isolation of RNA and cDNA synthesis

Total RNA was extracted from tissue samples using Tri-Reagent (Sigma–Aldrich, Inc. Missouri, USA) according to the manufacturer’s instructions. After extraction, total RNA was treated with DNAse I (Ambion, Austin, TX) to eliminate DNA contamination and samples were stored at -80°C until use. RNA pools were generated for each tissue at each stage by mixing an equal amount of total RNA from individual samples. In addition, a general pool (GP) was generated by mixing an equal amount of RNA from every individual brain tissue sample. For cDNA synthesis, 5 μg of total RNA (from individual and pooled samples) were reverse transcribed using an Oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

2.3. Cloning of the chicken GnIH-R cDNA and tissue expression profiling

Primers were designed to amplify the cGnIH-R cDNA from the start to the stop codon (GnIH-R-start/GnIH-R-stop, Table 1) based on the published sequence (Ikemoto and Park, 2005). PCR amplification (95°C, 5 min; 40 cycles of 95°C for 30 s, 63°C for 1 min, 72°C for 1 min; and a final extension step at 72°C for 10 min) was then performed on cDNA from the GP. A 1.2 kb amplicon was excised, cloned into a pCR2.1 vector (Invitrogen, Carlsbad, CA), and sequenced. After verifying the integrity of the sequence, cGnIH-R cDNA was subcloned into the pcDNA3.1 expression vector (Invitrogen).

To examine the distribution of cGnIH-R at different reproductive stages, cDNA samples from tissue pools at each stage were amplified by PCR using the GnIH-R-start/GnIH-R-stop primers. PCR conditions were identical to the ones used for cloning the receptor. In addition, the plasmid encoding cGnIH-R was used as positive control.

2.4. Quantification of chicken GnIH-R mRNA levels in the pituitary gland and diencephalon

Relative quantification of cGnIH-R mRNA levels in individual pituitaries and diencephalon was performed by real-time PCR using a Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA) in an ABI prism 7000 thermocycler (Applied Biosystems, Foster city, CA) using the 2^(-ΔΔCt) method as described by Livak and Schmittgen (Livak and Schmittgen, 2001). This method compares the Ct (cycle threshold) values obtained from samples with the value obtained from a calibrator after normalization with an endogenous housekeeping gene. In our case, the β-actin housekeeping gene was utilized, as we did not observe any changes in mRNA levels between the different reproductive stages. When comparing GnIH-R mRNA levels in pituitary glands, values obtained for the immature stages were used as calibrators, while for the comparison of mRNA levels between pituitary glands and diencephalon (performed in separate PCR runs), a common internal control sample (GP) was used as calibrator. Each individual sample was analyzed in triplicate. GnIH-R gene-specific primers were designed to span intron 2 in order to detect any possible genomic DNA amplification (GnIH-RF1/GnIH-R1, Table 1). β-actin cDNA, which was used as internal control to normalize for minor variations in RNA input or RT efficiency, was amplified using the β-actin-F and β-actin-R primers (Table 1). Three microgram of total RNA were reverse transcribed in a total volume of 20 μl as described above. Individual cDNA samples were then diluted 1/4 with ddH2O and 1 μl was used for real-time PCR (initial denaturation at 95°C for 10 min followed by 45 cycles consisting of 95°C for 15 s, 61°C for 30 s, 72°C for 30 s). Real-time PCR reactions were carried out in 96-well plates, and for each sex, pituitary and diencephalon samples were analyzed separately (in different plates). An aliquot of the GP was included in every run to allow for comparison between plates. To ensure the validity of the calculation before analysis, the amplification efficiencies of the target (GnIH-R) and the endogenous reference (β-actin) genes were first compared and confirmed to be similar using a serial dilution of the general pool as template.

2.5. Cell culture and transfection

In order to determine the individual and combined effect of GnIH and GnRH on their respective receptors, we opted to use a...
cell line that does not possess endogenous GnIH or GnRH receptors. Although we initially considered using COS-7 cells, preliminary experiments showed that this cell type does not effectively support our luciferase assay system, and a pituitary cell type was therefore selected. Since no avian pituitary cell line is available, cells from a rat pituitary somatolactotrope line (GH3 cells) were used for all functional analyses (inositol phosphate and luciferase assays). Furthermore, this cell line has previously been used to study mammalian GnRH-R signaling (Bedecarrats et al., 2003) and therefore appeared to be an ideal model for the current study. Cells were cultured in Dulbecco’s modified Eagle medium with low glucose (DMEM, HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

2.6. Inositol phosphate accumulation assay

The protocol for measuring total inositol phosphate (IP) production was adapted from Panchenko et al. (1998) as previously described (Shimizu and Bedecarrats, 2006). Briefly, GH3 cells cultured in 6-well plates were transiently transfected with 2 μg per well of either cGnRH-R-III, cGnIH-R, or empty pcDNA3.1 (negative control) expression vectors. Twenty-four hours post-transfection, culture medium was replaced with 1 ml of inositol-free DMEM (MP Biomedicals, Aurora, OH) supplemented with 1% penicillin/streptomycin without FBS. After a 2 h-incubation, 1 ml of the same medium containing 2 μCi of myo-(2-3H) inositol (MP Biomedicals) was added to each well. Cells were then incubated at 37 °C for 15 min, and 10 mM lithium chloride was added to each well. After an overnight incubation at 37 °C, cells were stimulated with 45 min with either 10^{-6} M of custom-synthesized cGnRH-I (pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH2; Sigma Genosys, Woodlands, TX) or cGnIH (NH2-Ser-Ile-Arg-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH2; Sigma Genosys). Cells were then lysed and total IPs were purified as previously described (Shimizu and Bedecarrats, 2006) and quantified by counting radioactivity in a scintillation counter.

2.7. Luciferase assay for the indirect measurement of cAMP levels

Signaling through Gαi or Gai results in the activation or inhibition of adenyl cyclase (AC), which is associated with an increase or decrease in intracellular cAMP levels, respectively. In turn, cAMP activates cAMP-dependent protein kinase (PKA), and triggers the binding of cAMP response element binding protein (CREB) to cAMP response element (CRE) on target genes. The plasmid pCRE-luc (Stratagene, La Jolla, CA) is a synthetic promoter construct containing four copies of CRE fused to firefly luciferase cDNA. In this system, luciferase expression is under the control of CRE, and any increase or decrease in luciferase activity is indicative of changes in cellular cAMP levels. In addition, as an internal control, the pRL-TK construct (Promega, Madison, WI) encoding Renilla luciferase driven by the herpes simplex virus thymidine kinase promoter was used. To determine whether cGnIH-R has the ability to signal through Gαi, cells were transfected with 2 μg of cGnIH-R expression construct plus 1 μg of pCRE-luc and 10 ng of pRL-TK. Cells transfected with 2 μg of cGnRH-R-III encoding cDNA were used as a positive control, while cells transfected with 2 μg of empty pcDNA3.1 were used as a negative control. Six hours post-transfection, media was replaced with low-glucose DMEM supplemented with 5% FBS and 1% penicillin/streptomycin. After 48 h, cells were stimulated for 4 h with either 10^{-6} M cGnIH or 10^{-5} M cGnRH-I. To determine whether cGnIH-R can signal via Gαi, cells transfected with 1 μg of cGnIH-R, 1 μg of pCRE-luc, and 10 ng of pRL-TK were stimulated with 10^{-6} M of cGnIH alone or in combination with 10^{-6} M forskolin. Forskolin is an activator of AC and thereby increases intracellular levels of cAMP. Therefore, inhibition of the effect of forskolin by cGnIH would indicate coupling to Gαi. To study a possible effect of cGnIH on cGnRH activation of the cAMP pathway, cells were cotransfected with 1 μg of cGnIH-R and pcDNA3.1 empty vector, or 1 μg of cGnIH-R and cGnRH-R-III, and stimulated simultaneously with 10^{-6} M of cGnIH and cGnRH-I. To study the effect of receptor ratios, cells were cotransfected with a constant amount of cGnRH-R-III (1 μg/well) and various amounts of cGnIH-R (0.5, 1, and 2 μg). The total overall amount of DNA was kept constant in the transfection mixture by adding the appropriate amount of empty pcDNA3.1 vector. For all assays, luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI) with a Wallac Victor 1420 multilabel counter (Perkin Elmer, Waltham, MA). All assays were performed in triplicate, and each experiment was repeated three times.

2.8. Computational and statistical analysis

Messenger RNA levels measured by real-time PCR in individual samples were compared using a one-way ANOVA followed by a Tukey multiple comparison test. Mean changes in CRE-luciferase activity was calculated from at least three independent experiments and were analyzed by one-way ANOVA followed by a Tukey multiple comparison test. For CRE-luciferase dose response analyses, data from each set of experiments was subjected to nonlinear regression analysis, and ED50 values were calculated using the GraphPad Prism 4.00 software (GraphPad Software, San Diego, CA). All statistical analyses were performed using the GraphPad InStat 3.00 software (GraphPad Software). P < 0.05 was considered significant.

3. Results

3.1. Pituitary levels of chicken GnIH-R mRNA fluctuate during a reproductive cycle

Tissue distribution of cGnIH-R mRNA was examined by reverse transcription PCR (RT-PCR) in the pituitary gland and brain from female and male chickens at different reproductive stages. Although not quantitative, RT-PCR analysis revealed that cGnIH-R is expressed widely in most tissues examined (Fig. 1), consistent with what has been previously reported (Ikemoto and Park, 2005). In addition to the amplicon corresponding to the full-length receptor (band at 1200 bp in Fig. 1), additional bands of lower molecular weight were also observed when PCR was performed using this set of primers (Fig. 1). However, no consistent pattern was obtained for these additional bands, which may likely correspond to non-specific amplification or formation of secondary structure during PCR. While the additional bands were not sequenced, it is also possible that they may correspond to splice variants, which have been previously observed for cGRHR-R-III (Shimizu and Bedecarrats, 2006).

In order to determine whether mRNA levels fluctuate during a reproductive cycle in the pituitary gland and diencephalon, which are the two main components of the reproductive axis, cGnIH-R mRNA was quantified in individual samples by real-time PCR. In the pituitary gland, the highest levels of mRNA were observed at the immature stage in both males and females (Fig. 2A). After photostimulation, a stage associated with the maturation of the reproductive tract and the initiation of egg and sperm production, levels significantly dropped. Interestingly, while levels remained low in older roosters, they progressively increased toward the end of the egg-laying period in females (Fig. 2A). In contrast, although levels of cGnIH-R mRNA tended to decrease after photostimulation in females, no significant change was observed in the diencephalon.
between stages (Fig. 2B). However, when the control GP was used as calibrator to allow comparison between PCR runs, levels of mRNA appeared to be, on an average, 5 (immature animals) to 10 (sexually mature animals) times higher in the diencephalon than the pituitary gland (Table 2).

### 3.2. Chicken GnIH-R signals through Gi but not Gq or Gs

No response in IP accumulation was observed when GH3 cells transfected with cGnIH-R were stimulated with a high dose ($10^{-6}$ M) of cGnIH (data not shown). Similarly, no increase in CRE-luc activity was observed (Fig. 3A), suggesting cGnIH-R does not couple to $G_q$ or $G_s$. Since no significant reduction in luciferase activity could be observed when cells were treated with GnIH alone, the possibility that AC requires an initial stimulation was investigated by simultaneously stimulating cells with both forskolin and cGnIH. Stimulation with forskolin alone resulted in a large increase in luciferase activity (23.47 ± 4.54-fold), while stimulation with both GnIH and forskolin resulted in a drastic, significant reduction in forskolin-induced CRE-luc activation (down to 3.82 ± 0.81-fold) (Fig. 3A).

### 3.3. Chicken GnIH reduces GnRH-induced CRE activation in a dose-dependent manner

Based on the results obtained with forskolin, the possibility that cGnIH-R may impair GnRH-induced CRE signaling was examined by cotransfecting GH3 cells with both cGnIH-R and cGnRH-R-III

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### Table 2

Relative amount of cGnIH-R mRNA in pituitary and diencephalon samples expressed as% of the general pool (GP).

<table>
<thead>
<tr>
<th>Reproductive Stage</th>
<th>Levels of cGnIH-R mRNA in the pituitary (% of GP, mean ± SEM)</th>
<th>Levels of cGnIH-R mRNA in the diencephalon (% of GP, mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immature</td>
<td>5.10 ± 1.64</td>
<td>20.22 ± 2.55</td>
</tr>
<tr>
<td>Post-photostimulation</td>
<td>0.84 ± 0.14</td>
<td>18.13 ± 2.73</td>
</tr>
<tr>
<td>Old-mature</td>
<td>0.92 ± 0.14</td>
<td>19.55 ± 4.25</td>
</tr>
<tr>
<td>Immature</td>
<td>6.62 ± 2.92</td>
<td>38.9 ± 5.89</td>
</tr>
<tr>
<td>Peak-of-lay</td>
<td>1.23 ± 0.14</td>
<td>21.39 ± 3.25</td>
</tr>
<tr>
<td>Mid-lay</td>
<td>1.93 ± 0.88</td>
<td>18.19 ± 5.04</td>
</tr>
<tr>
<td>End-of-lay</td>
<td>3.41 ± 0.76</td>
<td>41.48 ± 10.45</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immature</td>
<td></td>
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<tr>
<td>Post-photostimulation</td>
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<td>End-of-lay</td>
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</tbody>
</table>

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Fig. 1. Distribution of cGnIH-R mRNA in brain tissues from female and male chickens at different reproductive stages. Representative agarose gel electrophoresis images of RT-PCR performed on pooled RNAs from various brain tissues. P: pituitary gland; D: diencephalon; B: brain stem; CR: cerebrum; CL: cerebellum; -: no template negative control; +: plasmid encoding the chicken GnIH-R cDNA. The arrow corresponds to the cGnIH-R cDNA amplicon from the start to stop codon (1200 bp).

Fig. 2. Real-time PCR analysis of cGnIH-R mRNA levels in the pituitary gland and diencephalon from birds at different reproductive stages. Real-time PCR analyses were performed on individual pituitary gland (A) or diencephalon (B) cDNA samples from females and males at different reproductive stages. Im.: immature; P.l.: peak-of-lay; M.l.: mid-lay; E.l.: end-of-lay; P.p.: post-photostimulation; O.m.: old mature. Each data point represents the mean ± SEM of five individual samples, each assayed in triplicate. Lower case letters (a-c) indicate significant differences between stages ($P < 0.05$).
expression constructs followed by stimulation with both cGnIH (10^{-6} M) and cGnRH-I (10^{-6} M). Co-stimulation with both ligands reduced CRE-luc activity by 17.9% when compared to stimulation with cGnRH-I alone (Fig. 3B). We therefore investigated whether this reduction occurs in a GnIH dose-dependent manner. Cells cotransfected with cGnRH-R-III and cGnIH-R encoding cDNAs were stimulated with 5 \times 10^{-8} M cGnIH and serial doses of cGnRH-I ranging from 10^{-10} M to 10^{-6} M. The dose of cGnRH-I was chosen close to the ED_{50} based on previous dose–response experiments to eliminate saturation of the response (Shimizu et al., 2008). As GnIH doses increased, the response of cGnRH-R-III to cGnRH-I decreased in a dose-dependent manner, with a maximum reduction of 27.77 \pm 3.58% observed at a dose of 10^{-7} M GnIH and an inhibitory dose 50 (ID_{50}) of 1.62 \times 10^{-8} M (Fig. 3C).

3.4. GnIH effect is dependent on cGnRH-R/cGnRH-R-III receptor ratio

Compared to our previous data (Shimizu and Bedecarrats, 2006), pituitary levels of cGnRH-R-III and cGnIH-R mRNAs fluctuate in an opposite pattern in the pituitary gland during a reproductive cycle. In the current study, we therefore examined how various ratios of cGnRH-R-III and cGnIH-R affect CRE activation in vitro. As the ratio of cGnIH-R to cGnRH-R-III increased, CRE-luc activity decreased (Fig. 4). This effect was already significant at a 1:2 (cGnIH-R:cGnRH-R-III) ratio, and a 40% reduction in luciferase activity was observed at a 2:1 (cGnIH-R:cGnRH-R-III) ratio. However, a total inhibition could not be achieved at any ratio, suggesting that the cGnRH-R-III-induced CRE response may partially be AC/cAMP independent. In addition, since cGnIH had no effect on the response of cells transfected with the cGnRH-R-III construct alone, it is clear that each peptide acts via its own receptor.

4. Discussion

Consistent with what was previously reported in quail (Yin et al., 2005) and chickens (Ikecomo and Park, 2005), we observed that GnIH-R mRNA was widely present in most brain tissues examined. This is not surprising, given that GnIH-containing fibers have previously been shown to project into multiple areas (for a review: Tsutsui et al., 2007). For example, GnIH neurons have been shown to be in close proximity to and even contact GnRH-I and -II neurons in the preoptic area in European starlings (Ukuka et al., 2008), and fibers of both GnIH and GnRH-I neurons co-localize in the median eminence of song sparrows (Bentley et al., 2003). This strongly suggests that, in addition to its action on pituitary gonadotropes, GnIH may also control the synthesis and release of GnRH at the level of the hypothalamus. As reported by Maddineni et al. (Maddineni et al., 2008), we found that the amount of cGnRH-R mRNA present in the diencephalon is significantly higher than in the pituitary gland. However, although levels tended to decrease after sexual...
maturation in females, we did not detect any significant difference in mRNA levels between reproductive stages.

In the pituitary gland, cGnIH-R mRNA levels were highest in immature birds and decreased during sexual maturation in both females and males. This pattern is opposite to what we previously reported for cGnRH-R-III in the same samples (Shimizu and Bedecarrats, 2006), which suggests that, around the time of photostimulation, the pituitary sensitivity to hypothalamic peptides switches from tonic inhibition (involving GnIH) to stimulation (involving GnRH). The mechanisms responsible for such a switch in gene expression remain unknown. However, it has been established that direct photostimulation of the hypothalamic neurons results in an increase in GnRH-I release (for a review: Dawson et al., 2001; Bedecarrats et al., 2006). In such a scenario, the initial increase in GnRH would partially stimulate the pituitary–gonadal axis and the subsequent release of steroids. Since estradiol can reduce cGnIH-R mRNA levels in the pituitary gland of chickens (Maddineni et al., 2008), this initial stimulation would lift a tonic inhibition by reducing cGnIH-R expression, thereby “paving the way” for complete sexual maturation. Toward the end of an egg-laying cycle, the opposite may be true. As hens become photorefractory, levels of LH and thus ovarian steroids decrease, facilitating an increase in cGnIH-R gene expression and the restoration of tonic inhibition. Our results support this hypothesis, at least in females, and we recently proposed a novel model to describe the control of the reproductive axis by GnIH and GnRH during sexual maturation in the hen (Bedecarrats et al., 2009). Interestingly, in males, levels of GnIH-R mRNA remained low even in older individuals, suggesting a possible differential regulation. Nonetheless, whether GnIH is a causal factor or a mediator of the termination of reproduction remains to be clearly established. For example, at the beginning of the breeding season, capture-handling stress in house sparrows results in an increased GnIH neurons number while no increase is observed in animals captured at the end of the breeding season (Calisi et al., 2008). However, the number of GnIH cells was significantly higher in control animals at the end of the breeding season when compared to control animals at the beginning of the breeding season (Calisi et al., 2008).

Although cGnIH-R is a member of the GPCR family, its intracellular signal transduction pathways have not yet been characterized. A study by Ikemoto and Park (2005) suggested that in chickens, cGnIH-R might couple to Gαi. However, only ligand-induced Gαi mRNA expression was measured, at most indicating that GnIH regulates Gαi, but does not address coupling. Therefore, to determine which pathway is activated in chickens, we investigated whether GnIH increases intracellular IPs (indicative of coupling to Gαq), or modulates cAMP production (indicative of coupling to Gαs or Gαq). cGnIH treatment of GH3 cells transfected with cGnIH-R failed to elicit any increase in IPs or activation of CRE-luc, suggesting that the receptor does not couple to or signal through Gαq. In contrast, we reported on the Gαs pathway. Interestingly, cGnIH stimulation of GH3 cells transfected with cGnIH-R failed to show any response. This could be attributed to the fact that Gαq pathway blocks the activity of AC, thereby inhibiting the de novo production of cAMP rather than reducing pre-existing cellular levels. In addition, since only a small proportion (~50%) of cells was successfully transfected with receptor constructs, an effect of cGnIH on reducing basal cellular levels of cAMP may not have been detected. Therefore, to test the possible blocking effect of GnIH on AC activity, cells transfected with cGnIH-R were stimulated simultaneously with forskolin (a known activator of AC) and cGnIH. Compared to cells treated only with forskolin, a significant reduction in CRE-luc activity was observed in cells co-stimulated with forskolin and GnIH, suggesting that GnIH exerts its effect through Gαq and that the inhibition occurs by blocking AC.

We previously reported that cGnRH-R-III signals through Gαq and can also activate CRE (Shimizu and Bedecarrats, 2006; Shimizu et al., 2008). Thus, we hypothesized that the stimulation of the CRE target gene by cGnRH-I may be modulated by cGnIH-R signaling. In the present study, when cells transfected with both cGnIH-R and cGnRH-R-III were stimulated simultaneously with GnIH and cGnRH-I, a dose-dependent reduction in cGnRH-mediated CRE-luc activity was observed. Furthermore, our data also indicate that each hypothalamic peptide acts via its own receptor. Although specific, the inhibitory effect of cGnIH on cGnRH-induced CRE-luc activity was less dramatic than what was observed with forskolin. This may be explained by the fact that stimulation of more than one pathway can result in the activation of CRE (reviewed in Naor, 2009)). For example, crosstalk between phospholipase C (PLC) and AC pathways has been reported, and two novel types of protein kinase C (PKCδ and PKCcα) that activate AC, thereby increasing cAMP levels, have been identified (Lariviere et al., 2007). In COS-7 cells, the human GnRH-R is able to stimulate cAMP production via Ca2+/calmodulin crosstalk only when AC-I is introduced (Grosse et al., 2000). Similarly, it has also been reported that activation of Gαq, which results in increase in intracellular Ca2+, may lead to the stimulation of CRE-binding protein (CREB) (Matthews et al., 1994). Therefore, although forskolin specifically activates AC, cGnRH-I stimulation (possibly involving both Gαq and Gαs) may result in additional CRE-binding protein activation, which is independent of AC. Nonetheless, our results clearly suggest that the proposed anti-gonadotropic effect of cGnIH in the pituitary (Cicone et al., 2004; Osugi et al., 2004; Ubuka et al., 2006) could occur by inhibiting GnRH-induced CRE signaling. Recently, it has been shown that in sheep pituitary gonadotropes, GnIH reduces GnRH-stimulated mobilization of intracellular Ca2+ (Clarke et al., 2008). Although we did not observe any effect of cGnIH on IP accumulation, it is possible that activation of the GnRH-R may affect Ca2+ channels, and while decreased CRE-Luc activity is indicative of a transcriptional effect, inhibition of GnRH-induced Ca2+ mobilization could account for a decrease in gonadotropin release.

As pituitary levels of cGnIH-R and cGnRH-R-III mRNAs fluctuate in an inverse pattern during a reproductive cycle in chickens, we further investigated whether these changes could impact intracellular signaling. Upon transfecting GH3 cells with increasing ratios of cGnIH-R to cGnRH-R-III, cGnIH inhibited cGnRH-I signaling more effectively. Using our in vitro model, it would be expected that in immature birds, when cGnIH-R mRNA levels are highest and cGnRH-R-III mRNA levels are low (simulated by the 2:1 ratio), cGnRH-induced CRE signaling would be inhibited by cGnIH. On the other hand, in mature birds, when cGnIH-R mRNA levels are low and cGnRH-R-III mRNA levels are the highest, GnRH stimulation would be maximum with less/no cGnIH inhibition.

In conclusion, this study is the first to characterize the GnIH signaling pathway in the chicken and to demonstrate a potential interaction between GnIH and GnRH in the regulation of pituitary function. In addition, we have shown that a fluctuation in the cGnIH-R and cGnRH-R-III ratio is likely the key that triggers a switch in sensitivity from inhibitory to stimulatory.

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