General and Comparative Endocrinology 167 (2010) 331–337

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

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

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ARTICLE INFO

*Article history:*
Received 24 November 2009
Revised 15 March 2010
Accepted 25 March 2010
Available online 27 March 2010

*Keywords:*
Reproduction
Gonadotropin-inhibitory hormone
Gonadotropin releasing hormone
Avian species

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 $G_{\alpha i}$. 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.



## 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).

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0016-6480/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2010.03.029

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 $G_{\alpha q/11}$, $G_{\alpha s,}$ or $G_{\alpha i}$. However, the anti-gonadotrophic effects of GnIH suggest that this peptide may interfere with GnRH-Rs signaling to alter the synthesis and release of gonadotropins. To date, the only insights into GnIH signaling indicate a decrease in $G_{\alpha i}$ 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 $G_{\alpha i}$ 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, 3 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 (GnIHR-start/GnIHR-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).

**Table 1**
List of primers used.

| Name | Sequence (5′–3′) |
|---|---|
| β-actin-F | GTATGGAGTCCTGTGGTAT |
| β-actin-R | CACATCTGCTGGAAGGTGG |
| GnIHR-start | ATGCAAGCGCTGCAGCACCCGGAGCCA |
| GnIHR-stop | TCAGCCATTCCACGCGGGGATGCCAG |
| GnIHRF1 | CAACATGTTCATCCTCAACCTT |
| GnIHRR1 | GAAAACAGAGGCGGAGACAGACAT |

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 GnIHR-start/GnIHR-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 $2^{-\Delta\Delta Ct}$ 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 (GnIHRF1/GnIHRR1, 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 $ddH_2O$ 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