



Gonadotropin-inhibitory hormone receptor signaling and its impact on reproduction in chickens

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ABSTRACT

In birds, as in other vertebrates, reproduction is controlled by the hypothalamo–pituitary–gonadal axis with each component secreting specific neuropeptides or hormones. Until recently, it was believed this axis is exclusively under the stimulatory control of hypothalamic gonadotropin-releasing hormone I (GnRH-I) which in turn, stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion from the pituitary gland. However, the discovery of a novel inhibitory hypothalamic peptide able to reduce LH secretion (gonadotropin-inhibitory hormone: GnIH) challenged this dogma. Furthermore, with the characterization of its specific receptor (GnIHR), progress has been made to clarify the physiological relevance of GnIH in birds. This short review discusses the recent advances in GnIHR signaling at the level of the pituitary gland and the gonads. GnIHR is a member of the G-protein coupled receptor (GPCR) family which couples to G_{α_i} and, upon activation inhibits adenylyl cyclase (AC) activity, thus reducing intracellular cAMP levels. This implies that GnIH interferes with signaling of any GPCR coupled to G_{α_s} , including GnRH, LH and FSH receptors. In the chicken pituitary gland, the GnRHR-II/GnIHR ratio changes during sexual maturation in favor of GnRHR-II that appears to result in hypothalamic control of gonadotropin secretion shifting from inhibitory to stimulatory, with corresponding changes in GnRH-induced cAMP levels. Within the gonads, GnIH and its receptor may act in an autocrine/paracrine manner and may interfere with LH and FSH signaling to influence ovarian follicular maturation and recruitment, as well as spermatogenesis.

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1. Introduction

Since their discovery in molluscs (Price and Greenberg, 1977), numerous neuropeptides belonging to the RF-amide family have been isolated from the brains of vertebrates (Tsutsui and Ukena, 2006). In birds, an LPXRF-amide (X = L or Q) peptide that specifically inhibits gonadotropin secretion from the pituitary gland was initially characterized from the Japanese quail (*Coturnix japonica*) brain, and named gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000). GnIH occurs exclusively in neurons in the posterior paraventricular nucleus (PVN) of the hypothalamus (Satake et al., 2001; Bentley et al., 2003; Ukena et al., 2003; Osugi et al., 2004), and nerve terminals containing GnIH peptide are present in the external layer of the median eminence (ME) indicating that this neuropeptide is released into the hypophysial portal vascular system (Tsutsui et al., 2000). Within the hypothalamus, GnIH fibers also directly contact GnRH-I neurons suggesting that GnIH may directly regulate the synthesis and release of GnRH-I at the level of the cell body (Bentley et al., 2003). Additionally, GnIH

fibers are distributed throughout the brain, possibly down to the brain stem (Ukena et al., 2003) suggesting that GnIH has multiple functions in the central nervous system. For example, it may regulate reproductive behavior since intra-cerebroventricular (icv) injection of GnIH in female White-crowned sparrows significantly lowers copulation solicitation (Bentley et al., 2006). Further, it may play a role in appetite control since icv injection stimulates food intake in layer-type chicks (Tachibana et al., 2005, 2008). In addition to the central nervous system, GnIH has also recently been found in the gonads of male and female passeriformes and Japanese quail, suggesting further roles in the regulation of reproductive function (Bentley et al., 2008).

The GnIH receptor (GnIHR) is a G-protein coupled receptor (GPCR) with characteristic seven transmembrane domains. In birds, GnIHR has been characterized in quail (Yin et al., 2005) and chickens (Ikemoto and Park, 2005) and occurs in several regions of the brain, the anterior pituitary gland, and the reproductive organs (Ikemoto and Park, 2005; Yin et al., 2005; Bedecarrats and Zeini, 2006; Bentley et al., 2008; Maddineni et al., 2008a,b). Members of the GPCR family typically couple to either G_{α_q} , G_{α_s} or G_{α_i} , with coupling to G_{α_q} resulting in stimulation of phospholipase C (PLC) and activation of Ca^{2+} channels, and coupling to G_{α_s} or

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$G_{\alpha i}$ resulting in activation or inhibition of adenylyl cyclase (AC), respectively. In turn, PLC hydrolyses phosphatidyl inositol diphosphate (PIP₂) to inositol triphosphate (IP₃) and diacyl-glycerol (DAG) triggering cascades of activation of protein kinases, while AC regulates intracellular cAMP levels. In mammals, GnIHR orthologs have been shown to couple to $G_{\alpha i/\alpha}$ (Bonini et al., 2000; Hinuma et al., 2000) and reduce the forskolin-induced cAMP response when transfected in CHO cells (Hinuma et al., 2000). Similarly in chickens, we recently showed that activation of cGnIHR also inhibits the forskolin-induced cAMP response, demonstrating that cGnIHR couples to $G_{\alpha i}$ and inhibits AC activity (Shimizu et al., 2008b). In addition, since no effect was observed on IP₃ accumulation, cGnIHR does not seem to couple to $G_{\alpha q}$ (Shimizu et al., 2008b). This review evaluates the functions of GnIHR signaling in reproductive systems, focusing chiefly on the domestic chicken.

2. GnIHR signaling in the avian pituitary gland

The effects of GnIH on pituitary function were first investigated using quail anterior pituitary glands *in vitro*. While the neuropeptide had no significant effect on prolactin or FSH secretion, it significantly decreased LH secretion in a dose dependent manner (Tsutsui et al., 2000). Similarly, treatment of diced cockerel pituitaries with GnIH resulted in a reduced LH as well as FSH release, however, only at a dose below 10⁻⁶ M (Cicccone et al., 2004). *In vivo*, GnIH treatment of Gambel's White-crowned sparrow (Osugi et al., 2004) and quail (Ubuka et al., 2006) decreased circulating LH levels. At the molecular level, *in vitro* incubation of cockerel pituitary fragments with GnIH reduced levels of mRNAs encoding the common α -glycosylated subunit (α GSU) and the FSH- β subunit, but did not affect LH- β mRNA (Cicccone et al., 2004). On the other hand, intra-peritoneal infusion of GnIH in quail decreased both α GSU and LH- β mRNA levels (Ubuka et al., 2006). Thus, it is clear that GnIH regulates both the synthesis and release of gonadotropins. Since the key mediator of GnIH action is its specific receptor, changes in gonadotropin associated with changes in reproductive activity may result, at least in part, from a change in pituitary GnIHR. This has been shown to be the case in the chicken where concentrations of pituitary cGnIHR mRNA are high in juveniles, decrease after photostimulation, and progressively increase toward the end-of-lay in females, but stay depressed in aging males (Bedecarrats and Zeini, 2006; Maddineni et al., 2008a). Interestingly, levels of gonadotropin-releasing hormone receptor-2 (cGnRHR-II) mRNA, the main pituitary GnRHR in chickens, were inversely related to the concentration of cGnIHR mRNA (Shimizu and Bedecarrats, 2006). This observation indicates that in the domestic hen, at sexual maturation, the responses of the pituitary gland to GnIH and GnRH-I may shift towards increased responsiveness to GnRH, while at the end of the laying period, the reverse trend occurs. If this interpretation of the data is correct, both cGnRHR-II and cGnIHR might be expected to be present on cell types synthesizing either FSH or LH (Proudman et al., 1999) or both. We recently found this prediction to be correct by showing that both receptors colocalize in the anterior pituitary gland (Shimizu et al., 2008b), and that cGnIHR is expressed on the surface of both LH and FSH-producing cells (Maddineni et al., 2008a). This led us to hypothesize that GnRH and GnIH may interact with each others' intracellular signaling mechanisms. As shown in Fig. 1, although cGnRHR-2 does couple to $G_{\alpha q}$ leading to the activation of PLC and the subsequent increase in IP₃ and DAG (Shimizu and Bedecarrats, 2006), we recently showed that cGnRHR-II can also couple to $G_{\alpha s}$ to stimulate intracellular cAMP production (Shimizu et al., 2008a). Since cGnIHR couples to $G_{\alpha i}$, inhibiting AC and reducing intracellular cAMP, it is logical to assume that this pathway is the most likely candidate for an interaction between cGnRHR-II and cGnIHR sig-

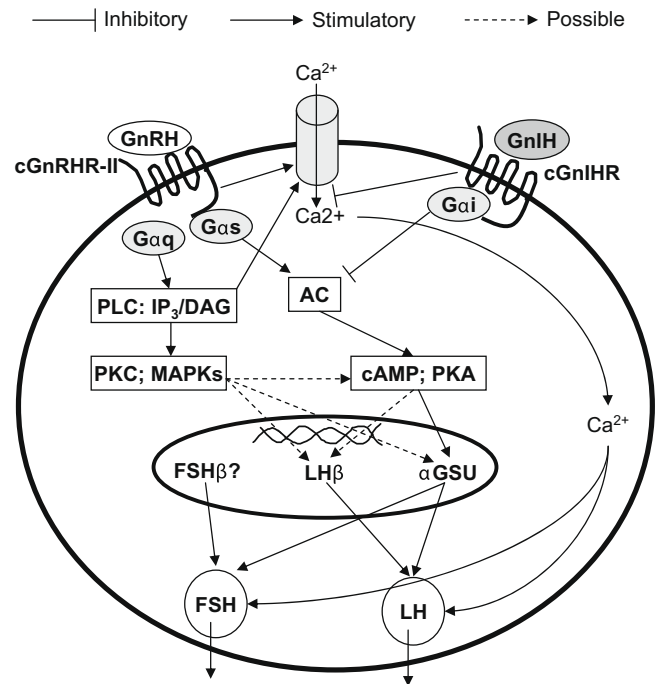


Fig. 1. Interactions between cGnRHR-II and cGnIHR in a pituitary gonadotrope. Upon binding to its specific receptor (cGnRHR-II), GnRH-I activates two pathways. Coupling to $G_{\alpha q}$ activates phospholipase C (PLC) thus increasing levels of diacylglycerol (DAG) and inositol triphosphate (IP₃). In turn, IP₃ stimulates Ca²⁺ entry and, in combination with DAG, activates a cascade of protein kinases from protein kinase C (PKC) to mitogen activated protein kinases (MAPKs). Increased intracellular Ca²⁺ stimulates the release of gonadotropins (LH or FSH), while kinases possibly stimulate gene transcription. Coupling to $G_{\alpha s}$ activates adenylyl cyclase (AC) thus increasing intracellular cAMP levels and stimulating protein kinase A (PKA). This results in gene promoter activation. In contrast, binding of GnIH to its receptor (cGnIHR) inhibits AC via $G_{\alpha i}$. This inhibition results in a decrease in GnRH-induced cAMP response, and the down-regulation of target genes (most likely the common α GSU). In addition, activation of GnIHR may also prevent the IP₃ stimulation of Ca²⁺ channels thus preventing the release of gonadotropins.

naling (Fig. 1). We therefore investigated whether cGnIH inhibits the GnRH-induced cAMP response in GH₃ cells (a rat somatotrophic cell line) transfected with both cGnIHR and cGnRHR-II. A significant dose dependent decrease was observed, with an ID₅₀ inhibitory dose of 1.62 × 10⁻⁸ M (Shimizu et al., 2008b). However, we were unable to achieve a complete inhibition suggesting that part of the GnRH-induced cAMP response bypasses AC and may involve cross talk at a lower signaling level. Nonetheless, our data show that GnIH significantly reduces GnRH-I stimulation of cAMP responsive elements on target genes (Fig. 1). Preliminary results obtained in our laboratory suggest that this inhibition mainly affects the common α GSU gene promoter, but not the FSH- β subunit promoter (unpublished data), partially confirming results obtained by Cicccone et al (2004). However, since we used a rat cell line to test chicken gene promoter activity, it is possible that any effect of GnRH and/or GnIH on FSH- β subunit gene promoter may require chicken specific factors, or depend indirectly on autocrine/paracrine factors released from gonadotropes. Although interactions at the cAMP level of the signaling pathway may explain changes in gene expression, it does not explain the inhibitory effect of GnIH on gonadotropin secretion. In most species, GnRH stimulation of pituitary gonadotropes involves both a direct and indirect (via IP₃) activation of Ca²⁺ channels (Stojilkovic and Catt, 1992), and exocytosis appears to be mainly under the influence of Ca²⁺ controlled by IP₃ (Izumi et al., 1989). Although we did not observe any effect of GnIH on PLC activity in our studies, stimulation of sheep pituitary gonadotropes with both GnRH and GnIH results

in decreased GnRH-induced intracellular Ca^{2+} (Clarke et al., 2008), suggesting a possible cross talk distal to the levels of AC and cAMP control in the signaling pathway (Fig. 1). However, the molecular mechanisms underlying this reduced Ca^{2+} entry are not known.

Since we have shown *in vivo*, that concentrations of pituitary cGnRHR-II and cGnIHR mRNAs are inversely related, and that this relationship changes with reproductive activity, we investigated whether changing the ratios of the receptors are of functional significance. To address this question, we altered the ratios of cGnIHR/cGnRHR-II transfected into our GH₃ cell line, and determined whether this affected the inhibitory effect of GnIH on GnRH-induced cAMP accumulation. Using a cGnIHR/cGnRHR-II ratio of 2:1, cGnIHR induced a 40% reduction in the cAMP response. This corresponds to the ratio of receptors in the pituitary of juvenile hens when cGnIHR mRNA level are the highest and cGnRHR-II mRNA levels are low. Conversely, at a cGnIHR/cGnRHR-II ratio of 1:2, corresponding to the ratio in the pituitary of laying hens with low levels of cGnIHR mRNA and high levels of cGnRHR-II, only a 20% reduction in the cAMP response was observed (Shimizu et al., 2008b). It therefore appears that developmental changes in the cGnIHR/cGnRHR-II ratio in chicken gonadotropes may play a key role in the regulation of their function.

3. Relationship between photoperiod, hypothalamic peptides and pituitary responsiveness

In the chicken and other birds, it is well established that photostimulation induces an increase in hypothalamic GnRH-I peptide or gene expression (Sharp et al., 1990; Dunn and Sharp, 1999; Dawson et al., 2002; Bedecarrats et al., 2006). Conversely in quail, GnIH gene expression is increased in the hypothalamus after exposure to short-day photoperiods (Ubuka et al., 2005). In birds, during dark phases, melatonin is synthesised and released by both the pineal gland and the retina of the eye. Although the pineal gland is the major source of melatonin present in the systemic circulation, both the retina and the pineal gland contribute to melatonin present within the hypothalamus. Since removing the source of melatonin (pinealectomy and enucleation) results in a decrease in GnIH peptide concentration within the diencephalon, and melatonin administration can restore GnIH production (Ubuka et al., 2005), melatonin appears to be a key regulator of GnIH synthesis and release. These observations suggest a model to explain how GnIH and GnRH might interact in photoperiodic birds, including the domestic chicken, to control reproduction (Fig. 2). In birds exposed to short days, increased levels of hypothalamic melatonin stimulate GnIH production while minimal exposure to light reduces the release of GnRH-I. Interactions between these low and high concentrations of hypothalamic GnRH-I and GnIH, and the occurrence of a high GnIHR/GnRHR-II ratio in pituitary gonadotropes, all result in low gonadotropin secretion (Fig. 2). After photostimulation, increased light exposure directly stimulates the hypothalamic release of GnRH-I while indirectly reducing GnIH release by inhibiting hypothalamic melatonin levels. Meanwhile, the responsiveness of the pituitary gland to GnIH/GnRH-I switches in favor of GnRH-I as levels of GnRHR-II and GnIHR increase and decrease, respectively (Fig. 2). Once sexual maturity is reached, high amounts of hypothalamic GnRH-I combined with low amounts of GnIH and a low GnIHR/GnRHR-II ratio in the pituitary gland promote effective synthesis and release of gonadotropins (Fig. 2). Although this model adequately describes events we and others observed in domestic avian species such as chickens and Japanese quail, it may require some modifications for many temperate zone wild photoperiodic species in which the greatest LH response to GnRH-I may not occur after photostimulation but

may rather depend on the initiation and dissipation of photorefractoriness (Balthazart et al., 1980; Bluhm, 1985; Dawson, 2005).

In our model of the roles of GnRH-I and GnIH and their receptors in the control of avian reproductive function (Fig. 2), one key question is, what mechanism controls the GnIHR/GnRHR-II ratio in the pituitary gland? One possibility is that GnRH-I and GnIH, in part, control the gene expression of their own receptors. However, we also recently showed that in the chicken pituitary gland, cGnIHR mRNA levels are significantly down-regulated by estradiol (E_2) (Maddineni et al., 2008a). Thus, at least in females, the increase in E_2 production during the onset of sexual maturation might be the factor determining the GnIHR/GnRHR-II ratio in the pituitary gland (Fig. 2).

4. Significance of GnIHR signaling in the gonads

Several hypothalamic neuropeptides involved in the regulation of reproductive function, such as GnRH and kisspeptin are present in mammalian gonads and other sexual organs and, since their specific receptors are also often present, it is likely they have autocrine/paracrine functions in these organs (Dekel and Shalgi, 1987; Whitelaw et al., 1995; Morales, 1998; Kotani et al., 2001; Castellano et al., 2006). Similarly, since a mammalian homolog of cGnIHR that binds an RFamide related peptide (RFRP) is expressed in the rat testis, ovary, and placenta (Hinuma et al., 2000), it is possible that in birds, GnIH and its receptor may also be involved in the control of the gonadal function. This possibility was recently investigated in the testis and ovary of White Leghorn chickens (Maddineni et al., 2008b), Japanese quail, White-crowned sparrow, and European starlings (Bentley et al., 2008). In Japanese quail, GnIHR mRNA occurs in cells of the internal pseudostratified columnar epithelium of the epididymis, in seminiferous tubular interstitium, and within the seminiferous tubules (Bentley et al., 2008). In the European starling and White-crowned sparrow, putative binding sites for GnIH have been detected in ovarian follicular granulosa cell layer suggesting the presence of GnIHR protein (Bentley et al., 2008). Similarly in chicken ovarian follicles, cGnIHR mRNA is present in both theca and granulosa cell layers (Maddineni et al., 2008b). Since GnIH has not yet been reported in the systemic blood circulation of any species, GnIH or a homolog belonging to the RFRP family is likely to be present in the gonads to activate gonadal GnIHR. This is indeed the case in mammals where RFRP has been detected within the rat testis but not the ovary (Hinuma et al., 2000). Although cGnIHR mRNA could not be amplified by PCR from the White Leghorn chicken ovary or testis (Maddineni et al., 2008b), GnIH immunoreactivity (ir) has been observed in the gonads of both male and female European starlings and Japanese quail (Bentley et al., 2008). In male Japanese quail, GnIH-ir cells occur in the testicular interstitium, spermatocytes and spermatids within seminiferous tubules. In female European starlings and White-crowned sparrows, GnIH-ir cells have been localized in the theca and granulosa layers of ovarian follicles (Bentley et al., 2008).

In chickens, the fully developed ovary contains a hierarchy of 4–6 yellow-yolky preovulatory follicles (designated as F1–F6) that are typically greater than 10 mm in diameter. In addition, the ovary also contains many prehierarchial follicles some of which are destined to enter preovulatory follicular hierarchy. The preovulatory follicles, particularly the F1, are the major source of ovarian progesterone (P_4) secretion, whereas prehierarchial follicles predominantly secrete estrogen and androgen (Robinson and Etches, 1986). Using real-time quantitative PCR, the concentration of thecal cellular GnIHR mRNA was found to be 55–57% lower in F3 and F1 preovulatory follicles compared to prehierarchial (3–5 mm) follicles (Maddineni et al., 2008b).

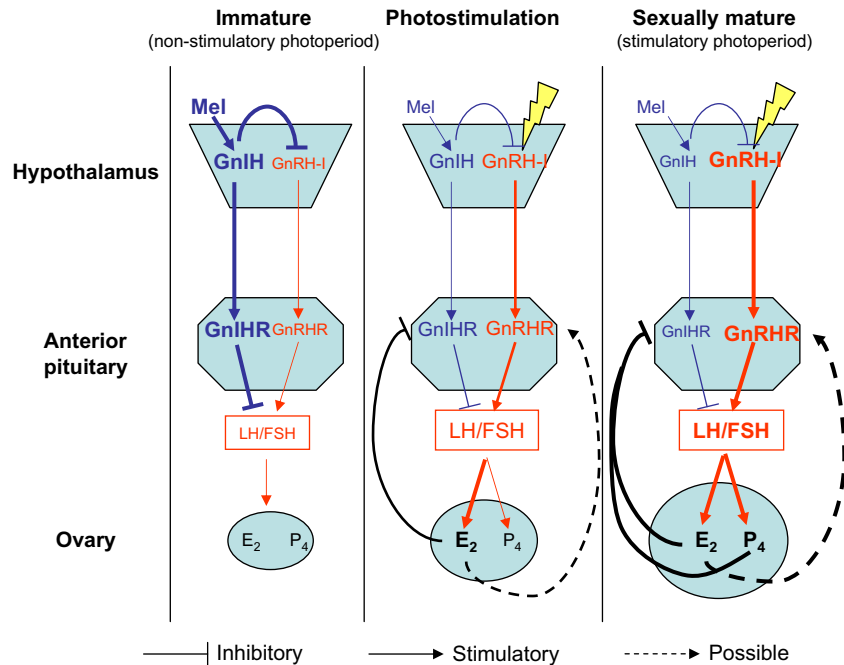


Fig. 2. Control of the reproductive axis by GnIH and GnRH during sexual maturation in the hen. In juvenile hens, under a non-stimulatory photoperiod, the prolonged nocturnal increase in melatonin stimulates gonadotropin inhibiting hormone (GnIH) neurons that send inhibitory inputs to gonadotropin-releasing hormone-I (GnRH-I) neurons. Additionally, the concentration of GnIH receptor (GnIHR) in the pituitary gland are at their highest while those of GnRH receptor II (GnRHR-II) are at their lowest. In this way, GnIH and its receptor maintain the hen in a juvenile state. After photostimulation (represented by the yellow flash) the duration of the nocturnal increase in melatonin decreases resulting in a decrease in GnIH neuronal activity and consequently an increase in GnRH-I neural activity. Simultaneously, direct light stimulation of the hypothalamus increases the production of GnRH-I. This triggers an initial, limited, release of gonadotropins by the pituitary gland, which initiates folliculogenesis and estradiol (E₂) production. In turn, E₂ down-regulates the synthesis of GnIHR at the pituitary level. As maturation progresses, GnRH-I release dominates GnIH, the pituitary GnIHR/GnRHR-II ratio switches toward GnRHR-II and the axis becomes fully functional. In mature hens, high levels of E₂ and progesterone (P₄) maintain the inhibition of the GnIHR. E₂ may also contribute to maintain high levels of GnRHR-II. Amounts of various factors are represented by the font size. Similarly, intensity of stimulatory and inhibitory actions is represented by line thickness. Effects of the inhibitory pathway (resulting from GnIH) are represented in blue while effects of the stimulatory pathway (resulting from GnRH) are represented in red.

These observations suggest that cGnIHR gene expression is down-regulated when the prehierarchial follicles enter the yellow-yolky preovulatory follicular hierarchy. The greater cGnIHR gene expression in the prehierarchial than in preovulatory follicles may also be one of the factors that inhibit the further maturation of preovulatory follicles. In support of this hypothesis, the same study also found that the concentration of cGnIHR mRNA in the ovaries of sexually immature chickens is 73% less than in the ovaries of laying hens. The expression of cGnIHR appears to be regulated by estradiol (E₂) and P₄ since treatment of sexually immature chickens with E₂ and/or P₄ caused a 50% decrease in the concentration of ovarian cGnIHR mRNA (Maddineni et al., 2008b). In order to further investigate how cGnIHR might regulate follicular maturation in the chicken ovary, cultured granulosa cells from prehierarchial follicles were treated with cGnIHR in the absence of FSH. This treatment resulted in decreased viability of granulosa cells (Maddineni et al., 2008b), suggesting that GnIH may decrease mitotic activity, thereby affecting proliferation and maturation of granulosa cells in the absence of FSH. Viability, however, was unaffected by a combination of GnIH and FSH treatments. Since cGnIHR and FSH receptors signal through G_{αi} and G_{αs}, respectively (Reiter et al., 2001; Ikemoto and Park, 2005; Shimizu et al., 2008b), it is likely that in chicken granulosa cells, GnIH and FSH activate antagonistic signaling pathways and thus, in the presence of FSH, the inhibitory effect of GnIH is abrogated. This is an attractive hypothesis as, at a time when circulating FSH concentrations are declining as in aging laying broiler breeder hens (Ciccione et al., 2005), increased ovarian GnIH may play a role in inducing

ovarian regression and/or prevent maturation of prehierarchial follicles. Since LH receptor also signals through G_{αs} it is also possible that increased ovarian GnIH in preovulatory follicles may inhibit final follicular maturation and ovulation.

5. Conclusion

Since its discovery, GnIH has been shown to act at multiple levels of the reproductive axis in birds. Within the hypothalamus, GnIH may down-regulate the synthesis and release of GnRH-I, and this effect may be controlled by melatonin and thus by photoperiod. At the level of the anterior pituitary gland, GnIH appears to interfere with GnRH signaling by inhibiting AC activity thus partially blocking the cAMP signaling pathway. This effect is suggested to result in inhibition of gonadotropin synthesis and possibly, release. Since cGnRHR-II and cGnIHR utilise antagonistic signaling pathways, the GnRHR-II/GnIHR ratio within gonadotrope is proposed to be a key to the activation of the hypothalamic-pituitary axis at the onset of sexual maturation, possibly under the influence of increasing E₂. In the gonads, GnIH and its receptor may play an autocrine/paracrine role in regulating the viability and maturation of ovarian granulosa cells, gonadal steroidogenesis, differentiation of spermatocytes, and maturation of spermatozoa. Although great progress has been made in determining the biological significance of GnIH much more needs to be done to elucidate the signaling events occurring in response to the activation of GnIHR at all levels of the reproductive axis.

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