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≪**Review**≫

Gonadotropin-Inhibitory Hormone (GnIH): Discovery, Progress and Perspective

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Probing undiscovered neuropeptides that play important roles in the regulation of pituitary function is essential for the progress of avian endocrinology and neuroendocrinology. Neuropeptide control of gonadotropin secretion at the level of the anterior pituitary gland is primarily through the stimulatory action of the hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH). Until recently, any neuropeptide that directly inhibits gonadotropin secretion has not been identified. In 2000, we discovered a novel hypothalamic dodecapeptide that directly inhibits gonadotropin release in quail and termed it gonadotropin-inhibitory hormone (GnIH). A gonadotropin inhibitory system is an intriguing concept and provides us with an unprecedented opportunity to study the regulation of avian reproduction from an entirely novel standpoint. To elucidate the mode of action of GnIH, we have identified a novel G protein-coupled receptor for GnIH in quail. The GnIH receptor possessed seven transmembrane domains and specifically bound to GnIH. The GnIH receptor was found to be expressed in the pituitary and several brain regions including the hypothalamus. These results indicate that GnIH acts directly on the pituitary via GnIH receptor to inhibit gonadotropin release. GnIH may also act on the hypothalamus to inhibit GnRH release. To demonstrate the functional significance of GnIH and its potential role as a key neuropeptide involved in avian reproduction, we investigated GnIH actions on gonadal development and maintenance in quail. Chronic treatment with GnIH inhibited gonadal development and maintenance by the decreasing gonadotropin synthesis and release. Melatonin is a key factor for involved in GnIH neural function, because quail GnIH neurons contain melatonin receptor and melatonin treatment stimulates expression of GnIH mRNA and mature GnIH peptide. Thus GnIH is capable of transducing photoperiodic information via changes in the melatonin signal and to influence the reproductive axis of birds. It is concluded that GnIH, a newly discovered hypothalamic neuropeptide, acts as an important factor on avian reproduction.

Key words : hypothalamus, gonadotropin-inhibitory hormone (GnIH), gonadotropin-releasing hormone (GnRH), gonadotropins, melatonin

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Introduction

The neuropeptide control of gonadotropin secretion is primarily through the stimulatory action of the hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), which was originally isolated from mammals and subsequently from non-mammals including birds. Until recently, no neuropeptide acting at the level of the pituitary to negatively regulate gonadotropin secretion has been discovered in vertebrates, although gonadal sex steroids and inhibin can modulate gonadotropin secre-

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tion. We recently identified a novel hypothalamic dodecapeptide which directly inhibits gonadotropin release in the Japanese quail and termed it gonadotropin-inhibitory hormone (GnIH ; Tsutsui *et al.,* 2000). This was the first demonstration of a hypothalamic neuropeptide inhibiting gonadotropin release in any vertebrate. A gonadotropin inhibitory system is an intriguing concept and provides us with an unprecedented opportunity to study the regulation of avian reproduction from an entirely novel standpoint. Here I summarize the advances made in our understanding of GnIH, a newly discovered hypothalamic neuropeptide, in birds.

Background

Since the molluscan cardioexcitatory neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) was found in the ganglia of the venus clam *Macrocallista nimbosa* (Price and Greenberg, 1977), neuropeptides that possess the RFamide motif at their C-termini (i.e., RFamide peptides) have been characterized in various invertebrates. Subsequently, many immunohistochemical studies that used the antiserum against FMRFamide suggested that vertebrate nervous systems possess some unknown neuropeptides similar to FMRFamide (Raffa, 1988 ; Rastogi *et al.,* 2001). Immunohistochemical findings indicated that some of the FMRFamide-like immunoreactive neurons project to the hypothalamic region close to the pituitary gland, and thus were predicted to play an important role in the regulation of pituitary function. We therefore looked for a novel RFamide peptide in the avian brain.

Discovery of GnIH

To isolate the RFamide peptide from the brain, Japanese quail (*Coturnix japonica*) was used and the peptidergic molecule was probed with a competitive enzyme-linked immunosorbent assay (ELISA), employing the antibody against the dipeptide, Arg-Phe-NH_2 (Tsutsui *et al.,* 2000). Acetic acid extracts of quail brain were passed through C-18 reversed-phase cartridges, and the retained material was subjected to reversed-phase and cation-exchange high performance liquid chromatography (HPLC). Amino acid sequence analysis of the isolated substance by automated Edman degradation with a gas-phase sequencer revealed the following sequence : Ser (62)-Ile (252)-Lys (233)-Pro (226)-Ser (38)-Ala (194)-Tyr (173)-Leu (148)-Pro (104)-Leu (108)-Arg (45)-Phe (52) with the detected amount (pmol) of each amino acid indicated in parentheses. A protonated molecule ion $(M+H)^+$ peak in the fast atom bombardment-MS (FAB-MS) of this peptide at *m/z* 1389.4 indicated that the peptide is amidated at the C-terminus. Synthetic and native peptides showed identical retention times on a C-18 reversed-phase column and a cation-exchange column. The mixture of the synthetic and native peptides eluted as a single peak from each column. Thus the isolated native peptide was confirmed as a 12 amino acid sequence (SIKPSAYLPLRFamide) with RFamide at the C-terminus (Tsutsui *et al.,* 2000). This neuropeptide had not been previously reported in vertebrates, although the C-terminal LPLRFamide was identical to chicken pentapeptide LPLRFamide peptide (Dockray *et al.,* 1983). The chicken peptide may be a degraded fragment of the dodecapeptide, as suggested by Dockray and Dimaline (1985).

Subsequently, the isolated novel peptide was shown to be located in the quail hypothalamo-hypophysial system and to decrease gonadotropin release from cultured anterior pituitary in a dose-dependent manner (Tsutsui *et al.,* 2000). We therefore designated this novel RFamide peptide as gonadotropin-inhibitory hormone (GnIH) (Tsutsui *et al.,* 2000 ; see Fig. 1).

Localization of GnIH in the Brain

As a first step to examine the localization of GnIH, we dissected out the quail brain into several regions, and quantified the concentration of GnIH by ELISA using a rabbit polyclonal antibody raised against GnIH (Tsutsui *et al.,* 2000). The concentration of GnIH in the diencephalon was much higher than that in the mesencephalon. In contrast, GnIH concentrations in the cerebrum and cerebellum were below the level of detectability. Subsequently, we investigated the precise localization of GnIH in the quail brain by immunohistochemistry (Tsutsui *et al.,* 2000 ; Ubuka *et al.,* 2003 ; Ukena *et al.,* 2003). Clusters of distinct GnIH-immunoreactive neurons were found in the paraventricular nucleus (PVN) in the hypothalamus (Fig. 2 A). In addition to the PVN, some scattered small cells were immunoreactive in the septal area. In contrast to the highly

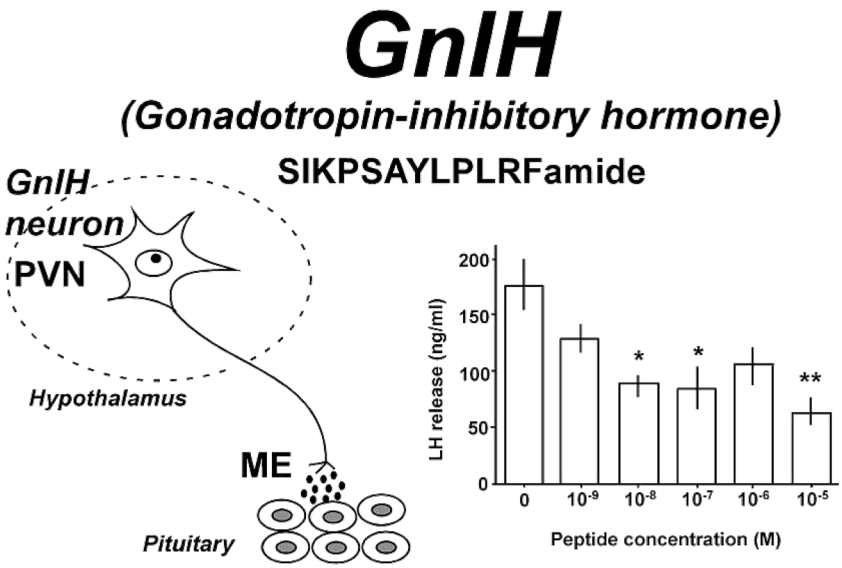

Fig. 1. **GnIH, a newly discovered hypothalamic neuropeptide, in the quail brain.** We isolated a novel hypothalamic dodecapeptide (SIKPSAYLPLR-Famide) inhibiting gonadotropin release in quail (Tsutsui *et al.,* 2000). Cell bodies and terminals containing the isolated novel neuropeptide were localized in the paraventricular nucleus (PVN) and median eminence (ME), respectively (Tsutsui *et al.,* 2000). The isolated novel neuropeptide was shown to decrease gonadotropin release from cultured anterior pituitary in a dose-dependent manner (Tsutsui *et al.,* 2000). We therefore designated this novel hypothalamic neuropeptide as GnIH (Tsutsui *et al.,* 2000).

localized clusters of cell bodies, GnIH-containing fibers were widely distributed in the diencephalic and mesencephalic regions particularly in the ventral paleostriatum, septal area, preoptic area, hypothalamus and optic tectum. The most prominent fibers were seen in the median eminence of the hypothalamus (Fig. 2B), and in the dorsal motor nucleus of the vagus in the medulla oblongata.

We further investigated GnIH localization in the brain of sparrows, seasonally breeding avian species (Bentley *et al.,* 2003 ; Osugi *et al.,* 2004). Dense populations of GnIH-immunoreactive neurons were also found in the PVN of these birds. The PVN was the only location where immunoreactive neurons were located (Bentley *et al.,* 2003 ; Osugi *et al.,* 2004). Thus the presence of GnIH in the PVN appears to be a conserved property among several avian species. In addition, a widespread distribution of GnIH-containing fibers was also found in the brain of seasonally breeding sparrows.

Interestingly, GnIH-containing fibers were further observed in extremely close proximity to GnRH neurons in the preoptic area (POA) in birds (Bentley *et al.,* 2003 ; Ukena *et al.,* 2003). It is therefore plausible that GnIH may act at the level of the hypothalamus to regulate gonadotropin release as well as at the pituitary.

Structure of GnIH Precursor Polypeptide

We further examined the precursor polypeptide for GnIH and localization of its transcript. A cDNA that encoded the GnIH precursor polypeptide was identified in the quail brain using a combination of 3′ and 5′ rapid amplification of cDNA ends (3′/5′ RACE) (Satake *et al.,* 2001). The deduced GnIH precursor consisted of 173 amino acid residues that encoded one GnIH and two putative GnIH-related peptide (GnIH-RP-1 and -RP-2) sequences that included -LPXRF (X＝L or Q) at their C-termini. All these peptide sequences were flanked by a glycine C-terminal amidation signal and a single basic amino acid on each end as an endoproteolytic site.

We also cloned a cDNA that encoded GnIH in the brain of Gambel's white-crowned sparrow (Osugi *et al.,* 2004). The deduced sparrow GnIH

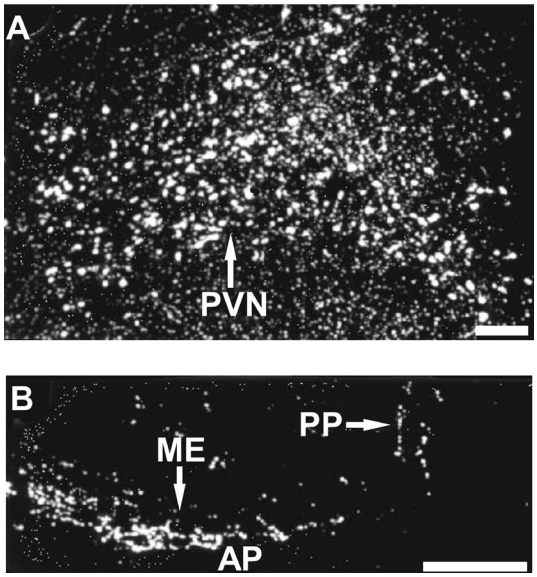

Fig. 2. **Localization of GnIH in the brain.** We investigated the localization of GnIH in the quail brain by immunohistochemistry (Tsutsui *et al.,* 2000 ; Ubuka *et al.,* 2003 ; Ukena *et al.,* 2003). Clusters of distinct GnIH-immunoreactive neurons were found in the paraventricular nucleus (PVN) in the hypothalamus (A). In addition to the PVN, some scattered small cells were immunoreactive in the septal area. In contrast to the highly localized clusters of cell bodies, GnIH-containing fibers were widely distributed in the diencephalic and mesencephalic regions particularly in the ventral paleostriatum, septal area, preoptic area, hypothalamus and optic tectum. The most prominent fibers were seen in the median eminence (ME) of the hypothalamus (B). Sagittal brain sections. AP, anterior pituitary ; PP, posterior pituitary. Scale bars, 100μm.

precursor also consisted of 173 amino acid residues, encoding one sparrow GnIH and two sparrow GnIH-related peptides (sparrow GnIH-RP-1 and GnIH-RP-2) that included -LPXRFamide (X=L or Q) at their C-termini. Although the homology of sparrow and quail GnIH precursors was approximately 66%, the C-terminal structures of GnIH, GnIH-RP-1 and GnIH-RP-2 were all identical in two species (Satake *et al.,* 2001 ; Osugi *et al.,* 2004). Subsequently, a cDNA encoding GnIH and GnIH-RPs was also reported in the chicken from a gene database.

In situ hybridization further revealed the cellular localization of GnIH mRNA solely in the PVN of quail and sparrow hypothalami (Ukena *et al.,* 2003 ; Osugi *et al.,* 2004). As already described, immunohistochemical analysis using the quail and sparrow also showed that quail and sparrow GnIH-immunoreactive cell bodies and terminals were localized in the PVN and median eminence, respectively. Thus only the PVN expresses GnIH and, in birds, the immunoreactive peptide found in fibers in multiple brain areas including the median eminence appears to originate from the PVN only (Ukena *et al.,* 2003 ; Osugi *et al.,* 2004).

GnIH Action on Gonadotropin Release

In view of the immunohistochemical finding indicating that GnIH-immunoreactive neurons project to the median eminence close to the pituitary, we analyzed the effect of the isolated SIKPSAYLPLRFamide, GnIH, on the release of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin (PRL) using cultured quail anterior pituitaries (Tsutsui *et al.,* 2000). GnIH significantly inhibited LH release, after 100-min incubation. The inhibitory effect on LH release was dose-dependent and its threshold concentration ranged between 10^{-9} and 10^{-8} M. A possible similar tendency for GnIH to inhibit FSH release was also detected. However, there was no effect of GnIH on PRL release. Based on these results of this novel RFamide peptide isolated from the quail brain, we therefore named it GnIH (Tsutsui *et al.,* 2000).

We further showed that GnIH was effective in inhibiting circulating LH *in vivo.* When administered intraperitoneally to quail via osmotic pumps, GnIH significantly reduced plasma LH (Ubuka *et al.,* 2006). GnIH injected simultaneously with GnRH inhibited the surge of plasma LH above the baseline in *Zonotrichia* sparrows (Osugi *et al.,* 2004). Furthermore, GnIH injections also decreased breeding levels of LH in free-living sparrows (Osugi *et al.,* 2004).

In addition to the inhibitory effects of GnIH on gonadotropin release, there is evidence that GnIH inhibits gonadotropin synthesis *in vitro* (Ciccone *et al.,* 2004 ; Ubuka *et al.,* 2006). The suppressive effect of GnIH on gonadotropin mRNA was associated with an inhibition of both LH and FSH release in the chicken (Ciccone *et al.,* 2004) and quail (Ubuka *et al.,* 2006).

Mode of Action of GnIH

Identification of the receptor for GnIH is crucial

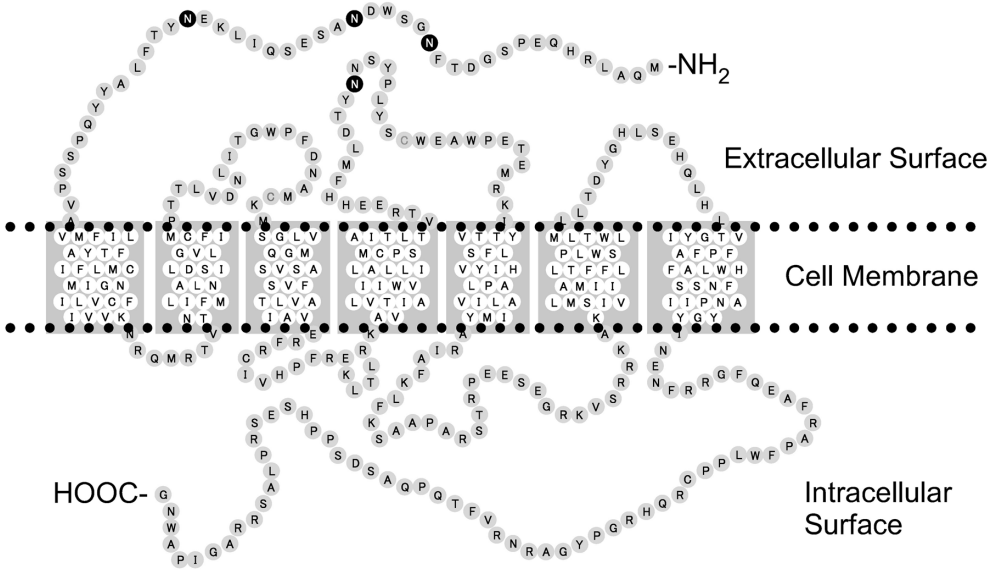

Fig. 3. **A Model of the identified GnIH receptor.** We cloned a cDNA encoding the receptor for GnIH as a new member of the G protein-coupled receptor (GPCR) superfamily in quail (Yin *et al.,* 2005). The nucleotide and amino acid sequences of GnIH receptor revealed a full length of 1,479 base pairs (bp) and an open reading frame (ORF) of 1,197 bp encoding 399 amino acid residues with a calculated molecular mass of 45.7 kDa. Analysis of this protein for regional hydrophobicity revealed seven putative transmembrane domains, connected by three cytosolic and three extracellular loops, extracellular amino-terminal and cytosolic carboxyl-terminal domains that are characteristic of GPCRs (Yin *et al.,* 2005). Binding experiments using the crude membrane fraction of COS-7 cells transfected with the GnIH receptor cDNA indicated that this membrane protein specifically binds to GnIH and possesses high-affinity binding sites for GnIH (Yin *et al.,* 2005).

to elucidate the mode of action of GnIH. We therefore identified the receptor for GnIH in the quail diencephalon and characterized its expression and binding activity (Yin *et al.,* 2005). We first cloned a cDNA encoding a putative GnIH receptor by a combination of 3′ and 5′ rapid amplification of cDNA ends (RACE) using PCR primers designed from the sequence for the receptor for rat RFamide-related peptide (RFRP), an orthologous peptide of GnIH. Hydrophobic analysis revealed that the putative GnIH receptor possessed seven transmembrane domains, indicating a new member of the G protein-coupled receptor (GPCR) superfamily (Yin *et al.,* 2005 ; see Fig. 3). The crude membrane fraction of COS-7 cells transfected with the putative GnIH receptor cDNA specifically bound to GnIH and GnIH-RPs in a concentration-dependent manner (Yin *et al.,* 2005). Scatchard plot analysis of the binding showed that the identified GnIH receptor possessed a single class of high-affinity binding sites (K_d = 0.752 nM). Southern blotting analysis of reverse-transcriptase-mediated PCR products revealed the expression of GnIH receptor mRNA in the pituitary and several brain regions including diencephalon in the quail (Yin *et al.,* 2005). These results indicate that GnIH acts directly on the pituitary via GnIH receptor to inhibit gonadotropin release (see Fig. 4). GnIH may also act on the hypothalamus to inhibit GnRH release, because GnIH fibers have been observed to contact GnRH cell bodies (Bentley *et al.,* 2003 ; 2006).

Functional Significance of GnIH

To demonstrate the functional significance of GnIH and its potential role as a key neuropeptide involved in avian reproduction, we investigated GnIH actions on gonadal development and maintenance in male quail (Ubuka *et al.,* 2006). In mature birds, chronic treatment with GnIH via osmotic pumps decreased gonadotropin synthesis and release

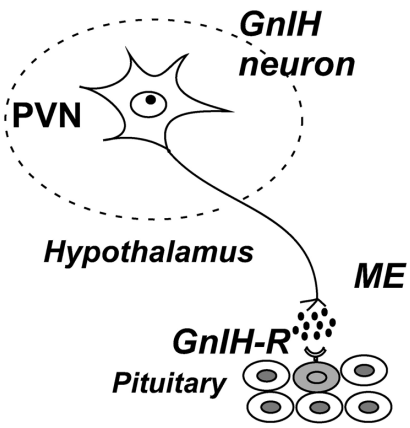

Fig. 4. **The mode of action of GnIH on gonadotropin release.** We characterized the expression of GnIH receptor in quail (Yin *et al.,* 2005). The GnIH receptor was found to be expressed in the pituitary and several brain regions including the hypothalamus (Yin *et al.,* 2005). Thus GnIH acts directly on the pituitary via GnIH receptor to inhibit gonadotropin release. GnIH may also act on the hypothalamus to inhibit GnRH release, because GnIH fibers have been observed to contact GnRH cell bodies (Bentley *et al.,* 2003 ; 2006).

in a dose-dependent manner (Ubuka *et al.,* 2006). Plasma testosterone concentrations were also decreased dose-dependently. Further, administration of GnIH to mature birds induced testicular apoptosis and decreased spermatogenic activity in the testis. In immature birds, chronic treatment with GnIH suppressed normal testicular growth and rise in plasma testosterone concentrations (Ubuka *et al.,* 2006). Our findings clearly show that GnIH inhibits testicular development and maintenance by decreasing gonadotropin synthesis and release. Thus GnIH appears to act as an important factor on avian reproduction. GnIH is the first identified hypothalamic neuropeptide inhibiting reproductive function in any vertebrate class.

Regulation of GnIH Expression

Until now, a regulatory mechanism(s) governing GnIH expression has remained unclear. Although many bird species are photoperiodic, a dogma has existed that birds do not use seasonal changes in melatonin secretion to time their reproductive effort, and a role for melatonin in birds has remained enigmatic (Wilson, 1991 ; Juss *et al.,* 1993). Despite the accepted dogma, there is strong evidence that melatonin is involved in regulation of several seasonal processes, including gonadal activity and gonadotropin secretion (Ohta *et al.,* 1989 ; Bentley *et al.,* 1999 ; Bentley and Ball, 2000 ; Bentley, 2001 ; Guyomarc'h *et al.,* 2001 ; Rozenboim *et al.,* 2002). In light of these reports and considering GnIH's inhibitory effects on gonadotropin secretion (Tsutsui *et al.,* 2000 ; Osugi *et al.,* 2004), we manipulated melatonin levels in quail by removing sources of melatonin and investigated the action of melatonin on GnIH expression in the quail brain (Ubuka *et al.,* 2005). Pinealectomy combined with orbital enucleation (Px+Ex) decreased the expression of GnIH precursor mRNA and the mature peptide GnIH in the diencephalon including the PVN and median eminence. Melatonin administration to Px +Ex birds caused a dose-dependent increase in expression of GnIH precursor mRNA and production of mature peptide. The expression of GnIH was photoperiodically controlled and increased under short day (SD) photoperiods (Ubuka *et al.,* 2005), when the duration of melatonin secretion increases (Cockrem and Follett, 1985 ; Kumar and Follett, 1993). Interestingly, Mel_{1c}, a melatonin receptor subtype was expressed in GnIH-ir neurons in the PVN (Ubuka *et al.,* 2005). Melatonin receptor autoradiography further revealed specific binding of melatonin in the PVN. Melatonin appears to act directly on GnIH neurons via its receptor to induce GnIH expression (see Fig. 5). Thus GnIH is capable of transducing photoperiodic information via changes in the melatonin signal and to influence the reproductive axis of birds.

Conclusions and Future Directions

It is concluded that GnIH, a newly discovered hypothalamic neuropeptide, acts as an important factor on avian reproduction. The discovery of GnIH has opened avenues for a new research field in avian endocrinology. Future directions should focus on the mode of action of GnIH on the inhibition of gonadotropin synthesis and release. We also need to clarify the action of GnIH-RPs on the synthesis and release of pituitary hormones. Furthermore, the widespread distribution of GnIH indicates that this neuropeptide may participate not only in neuroendocrine functions but also in behavioral (Tachibana *et al.,* 2005 ; Bentley *et al.,* 2006) and autonomic mechanisms in birds. Future studies

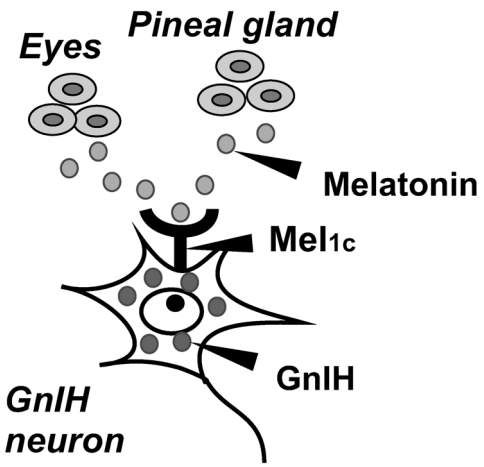

Fig. 5. **The mode of action of melatonin on GnIH expression.** Melatonin originating from the pineal gland and eyes induced GnIH expression in the quail brain (Ubuka *et al.,* 2005). Melatonin receptor (Mel_{1c}) was expressed in GnIH neurons (Ubuka *et al.,* 2005). Thus melatonin acts directly on GnIH neurons via its receptor to induce GnIH expression.

will bring to light previously unknown physiological functions of GnIH and GnIH-RPs.

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