Melatonin Stimulates the Release of Gonadotropin-Inhibitory Hormone by the Avian Hypothalamus

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Gonadotropin-inhibitory hormone (GnIH), a neuropeptide that inhibits gonadotropin synthesis and release, was first identified in quail hypothalamus. GnIH acts on the pituitary and GnRH neurons in the hypothalamus via GnIH receptor to inhibit gonadal development and maintenance. In addition, GnIH neurons express melatonin receptor and melatonin induces GnIH expression in the quail brain. Thus, it seems that melatonin is a key factor controlling GnIH neural function. In the present study, we investigated the role of melatonin in the regulation of GnIH release and the correlation of GnIH release with LH release in quail. Melatonin administration dose-dependently increased GnIH release from hypothalamic explants *in vitro*. GnIH release was photoperiodically controlled. A clear diurnal change in GnIH release was observed in quail, and this change was negatively correlated with changes in plasma LH concentrations. GnIH release during the dark period was greater than that during the light period in explants from quail exposed to long-day photoperiods. Conversely, plasma LH concentrations decreased during the dark period. In contrast to LD, GnIH release increased under short-day photoperiods, when the duration of nocturnal secretion of melatonin increases. These results indicate that melatonin may play a role in stimulating not only GnIH expression but also GnIH release, thus inhibiting plasma LH concentrations in quail. This is the first report describing the effect of melatonin on neuropeptide release. **(*Endocrinology* 151: 271–280, 2010)**

In vertebrates, gonadotropin secretion is stimulated by the hypothalamic decapeptide GnRH (1–5). Until the discovery of gonadotropin-inhibitory hormone (GnIH), it was not known that gonadotropin secretion could be inhibited by a hypothalamic neuropeptide (6). This discovery that GnIH could inhibit pituitary LH release was the first known example of a hypothalamic neuropeptide inhibiting the release of pituitary gonadotropin in any vertebrate.

In birds, GnIH is located in neurons of the paraventricular nucleus (PVN) (6–8). These neurons project to the median eminence (ME), and are thus able to control anterior pituitary function (6–8). We also cloned a cDNA encoding the GnIH precursor polypeptide in quail brain (9). The GnIH precursor mRNA was expressed only in the PVN (7–9). Subsequently we identified a cDNA encoding GnIH in white-crowned sparrow (10) and European starling brain (11) and determined that gonadotropin release was inhibited *in vivo* by GnIH in both galliform and passeriform birds (10, 12). Addition of GnIH to cultured chicken pituitary also reduced gonadotropin synthesis and release (13). To uncover the mode of action of GnIH, we further identified a novel G protein-coupled receptor for GnIH in quail (14). The identified GnIH receptor was expressed in the anterior pituitary and specifically bound

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Abbreviations: GnIH, Gonadotropin-inhibitory hormone; LD, long day; ME, median eminence; PrRP, prolactin-releasing peptide; PVN, paraventricular nucleus; Px+Ex, pinealectomy with orbital enucleation; RFRP, RFamide-related peptide; SD, short day.

to GnIH in a concentration-dependent manner (14). In starlings, GnIH neurons also project to GnRH-I and -II neurons; it seems that GnIH probably acts on GnRH neurons via GnIH receptor to inhibit GnRH release centrally as well as to inhibit gonadotropin release at the anterior pituitary (11). The inhibitory effects of GnIH on gonadal development and maintenance have also been demonstrated in quail after chronic treatment with GnIH (12). Thus, it is considered that in birds GnIH acts on the anterior pituitary and GnRH neurons in the hypothalamus via GnIH receptor to inhibit gonadal development and maintenance by decreasing gonadotropin release and synthesis (15). Previous studies on quail suggested that GnIH acts on the anterior pituitary to inhibit gonadotropin release and synthesis during development and maturity (6, 12). Evidence from other bird species suggests that GnIH regulates GnRH and thus inhibits gonadotropin release, but there is as yet no published evidence that GnIH acts in this way before maturity in species other than quail (11, 16).

The inhibitory action of GnIH on gonadotropin secretion occurs in at least two orders of birds and thus may be an evolutionarily conserved property. Furthermore, very recently RFamide-related peptide (RFRP)-3 has been demonstrated as a potent mammalian homolog of GnIH (17, 18). RFRP-3 inhibits gonadotropin release *in vitro* and *in vivo* in hamsters (17, 19) and rats (20, 21). RFRP-3 also reduces GnRH-stimulated gonadotropin secretion in sheep (18) and pulsatile gonadotropin secretion in cattle (22). Thus, GnIH and RFRP-3, a mammalian GnIH homolog, are considered to act as important inhibitory hypothalamic neuropeptides to control reproduction in birds and mammals, respectively.

To understand the functional significance of GnIH and its physiological role as a key neuropeptide involved in the regulation of gonadotropin release and synthesis, it is essential to clarify the regulatory mechanism(s) that regulates its expression and release. There are many avian and mammalian species that are photoperiodic. In general, the annual changes in pineal melatonin secretion drive the reproductive responses of photoperiodic mammals (23). However, it has been almost universally accepted that melatonin is not responsible for changes in seasonal reproduction in birds (24, 25). Despite this general dogma, there is information available on regulation of seasonal processes by melatonin, including that of gonadal activity and gonadotropin secretion (26–31). It is possible that the avian hypothalamus can synthesize melatonin *de novo* (32). These researchers demonstrated the presence of melatonin in turkey hypothalamus along with tryptophan hydroxylase 1 and 5-hydroxytryptamine *N*-acetyl-transferase, key enzymes in melatonin biosynthesis. If this finding is applicable to birds in general, then it could explain the lack of effect of pinealectomy on the avian reproductive system. Based on these reports and taking into account the inhibitory effects of GnIH on gonadotropin release and synthesis (6, 10, 12, 13), we manipulated melatonin levels in quail by removing sources of melatonin and investigating the action of melatonin on GnIH expression in the quail brain (33). The pineal gland and eyes are the major sources of melatonin in quail (34). The expression of GnIH precursor mRNA and the mature GnIH peptide in the diencephalon, including the PVN and ME, were decreased by a manipulation that combined pinealectomy with orbital enucleation (Px+Ex) (33). The expression of GnIH precursor mRNA and production of mature peptide were dose-dependently increased by melatonin administration to Px+Ex birds (33). The duration of melatonin increases under the longer nights of short-day (SD) photoperiods (35), and SD increased GnIH expression (33). Critically, Mel_{1c}, a melatonin receptor subtype, was expressed in GnIH-immunoreactive neurons in the PVN (33). It seems that GnIH expression is induced by a direct action of melatonin on GnIH neurons via its receptor. Thus, GnIH is capable of detecting and transducing photoperiodic information via changes in the melatonin signal, thereby influencing the reproductive axis in birds. However, until now there is no information in any species whether melatonin is capable of causing GnIH secretion. Although we know that melatonin can stimulate GnIH mRNA expression *in vivo* (33), this does not necessarily mean that the GnIH peptide is actually secreted and secreted in quantities that can affect LH concentrations. Therefore, it is very important to clarify the effect of melatonin on GnIH secretion.

In this study, we investigated further the role of melatonin in the regulation of GnIH release in quail. We first analyzed the effects of melatonin on GnIH release and GnIH mRNA expression by using an *in vitro* model. Subsequently the release of GnIH was compared between the dark and light periods in hypothalamic explants from quail exposed to long-day (LD) photoperiods. Plasma LH concentrations were also examined during the dark and light periods. Diurnal changes in the release of GnIH and the concentrations of plasma LH and diencephalic and plasma melatonin were also analyzed in quail exposed to LD photoperiods. Finally, we examined the action of endogenous melatonin on the release and expression of GnIH by using hypothalamic explants from quail exposed to SD and LD photoperiods, thus varying the length of endogenous melatonin secretion by varying the length of the night (melatonin is secreted at night) (35, 36). Here we show that melatonin is a potent inducer of GnIH release as well as GnIH expression and that the duration of GnIH release parallels the duration of elevated melatonin *in vitro* and *in vivo*.

Materials and Methods

Animals

Adult male Japanese quail (*Coturnix japonica*) at 3 months of age were housed in a temperature-controlled room (25 ± 2 C) under daily photoperiods of 16 h light and 8 h dark (LD; lights on at 0700 h, off at 2300 h). All birds were isolated in individual cages, and the experimental protocol was approved in accordance with guidelines prepared by Waseda University (Tokyo, Japan). All efforts were made to minimize the number of animals used.

Experimental schedules

To determine the effect of melatonin on GnIH release, hypothalamic explants including GnIH neurons in the PVN and their terminals in the ME were collected from quail exposed to LD photoperiods during the light period (1500 h). Explants were collected into ice-cold Medium 199 (Life Technologies, Inc., Invitrogen, Carlsbad, CA) supplemented with 10 mM HEPES, 0.1% BSA, and 0.1 mM phenylmethylsulfonyl fluoride and preincubated for 90 min at 37 C in an atmosphere of 95% O_2-5% CO_2. Each hypothalamic explant was then transferred to a 24-well microplate, each well containing 1 ml medium supplemented with melatonin (Sigma-Aldrich, St. Louis, MO) at concentrations of 10^{-9}, 10^{-8}, or 10^{-7} M or vehicle only (n = 6 in each group). After 120 min incubation, each medium was collected and subjected to ELISA to quantify GnIH concentrations. We incubated hypothalamic explants for 120 min because our preliminary study showed that GnIH mRNA expression significantly increased by melatonin administration during 120 min incubation (see Fig. 2). Each hypothalamic explant was also subjected to competitive PCR analysis to quantify the expression of GnIH precursor mRNA.

To investigate the effect of light and dark on the release of GnIH and the expression of GnIH precursor mRNA, hypothalamic explants were collected in the same manner from quail exposed to LD photoperiods during the light period (1500 h) and the dark period (0300 h) (n = 6 in each group) and incubated in the medium for 120 min. We incubated hypothalamic explants under light or dark condition with respect to the ambient light and dark conditions of each quail just before the animals were killed. After incubation, each medium was collected and subjected to ELISA to quantify GnIH concentrations. Each hypothalamic explant was also subjected to competitive PCR analysis to quantify the expression of GnIH precursor mRNA. Trunk blood was collected into heparinized tubes and centrifuged at 1800 × *g* for 20 min at 4 C. Plasma was stored at −20 C. Plasma LH concentrations during the light and dark periods were quantified using RIA.

To investigate diurnal changes in GnIH release, hypothalamic explants were collected from quail exposed to LD photoperiods at intervals of 6 h during a 24-h period (n = 8 in each group) and incubated in the medium for 120 min. After incubation, each medium was collected and subjected to ELISA to quantify GnIH concentrations. Each hypothalamic explant was used to determine its own melatonin concentration. Trunk blood was collected into heparinized tubes and centrifuged at 1800 × *g* for 20 min at 4 C. Plasma was stored at −20 C. The concentrations of plasma LH and diencephalic and plasma melatonin were quantified using RIAs for LH and melatonin, respectively.

Finally, we manipulated endogenous melatonin to determine its action on GnIH release. Birds were exposed to SD (8 h light and 16 h dark; lights on at 0900 h, off at 1700 h) or LD (16 h light and 8 h dark; lights on at 0700 h, off at 2300 h) photoperiods. Melatonin is secreted during dark; thus, the duration of endogenous melatonin secretion was increased by SD treatment (35, 36). After 3 wk of exposure to SD or LD, birds (n = 6 in each group) were terminated at 1500 h, and the release of GnIH and the expression of GnIH precursor mRNA from hypothalamic explants were measured as before. Plasma LH concentrations were also analyzed.

ELISA of GnIH

To determine the release of GnIH in the medium, each medium was collected and subjected to complete evaporation followed by dilution with buffer [0.1% BSA in 10 mM PBS (pH 7.5)]. Then the adjusted sample was subjected to competitive ELISA by using rabbit antiquail GnIH antiserum (6) to quantify GnIH concentrations in the medium. Quail GnIH (SIKPSAYLPLRFamide) was synthesized and used as the standard (6, 8, 33). In brief, different concentrations of GnIH (1–1000 pmol/ml) and adjusted sample were added with the antiserum against GnIH (1:1000 dilution) to each antigen-coated well of a 96-well microplate (multiwell plate for ELISA, H-Type; Sumitomo Bakelite, Tokyo, Japan) and incubated for 1 h at 37 C. After the reaction with alkaline phosphatase-labeled goat antirabbit IgG, immunoreactive products were obtained in a substrate solution of *p*-nitrophenylphosphate, and the absorbance was measured at 415 nm on a microtiter plate reader (MTP-120; Corona Electric, Ibaraki, Japan).

To investigate the specificity of GnIH antiserum used in this study, we conducted a competitive ELISA using GnIH, RFRP-3, a homologous peptide of GnIH, and other RFamide peptides having a different C-terminal structure from GnIH. GnIH has a C-terminal LPLRFamide motif (6). RFRP-3, a mammalian homologous peptide of GnIH, has a C-terminal LPQRFamide motif (15). GnIH and RFRP-3 possess the same C-terminal LPXRF (X = L or Q) motif. Chicken pentapeptide, LPLRFa, is considered to be a fragment of GnIH (15, 37, 38). Neuropeptide FF (NPFF) and prolactin-releasing peptide (PrRP) are other RF-

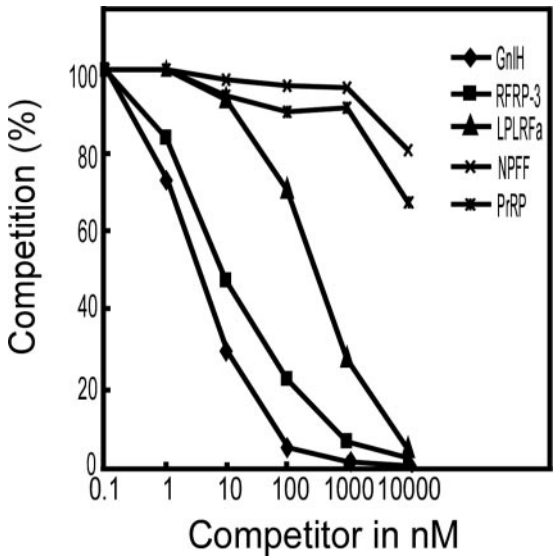

FIG. 1. Competition binding of quail GnIH to the antiserum raised against quail GnIH with various RFamide peptides as measured by competitive ELISA.

amide peptides, which have a different C-terminal structure from GnIH and RFRP-3. As shown is Fig. 1, GnIH antiserum highly cross-reacted only with GnIH and RFRP-3, a mammalian homologous peptide of GnIH, unlike other RFamide peptides (human NPFF and human PrRP). The IC_{50} values in the competitive ELISA were estimated as follows: 3.6 nM for quail GnIH (SIKPSAYLPLRF-amide), 7.8 nM for rat RFRP-3 (ANMEAGTMSHFPSLPQRF-amide), 254.1 nM for chicken LPLRFa, and more than 10,000 nM for other RFamide peptides, *e.g.* human NPFF (FLFQPQRF-amide) and human PrRP (TPDINPAWYASRGIRPVGRF-amide). Thus, the antiserum of GnIH showed a high specificity for binding with GnIH and its homologous peptide compared with its binding with other RFamide peptides.

Competitive PCR analysis of GnIH precursor mRNA

To quantify GnIH precursor mRNA in hypothalamic explants, we performed competitive PCR analysis as described previously (8, 33). Briefly, hypothalamic explants were removed and snap frozen immediately in liquid nitrogen. Total RNA (including rRNA and mRNA) was isolated by the Sepasol extraction method (Sepasol-RNA I Super; Nacalai Tesque, Kyoto, Japan) from each hypothalamic explant and reverse transcribed by using oligo(deoxythymidine)$_{12–18}$ primer (Amersham Pharmacia Biotech, Piscataway, NJ) and reverse transcriptase (Moloney murine leukemia virus reverse transcriptase; Promega, Madison, WI).

For competitive PCR analysis of GnIH precursor, competitor DNA was produced by PCR, using cDNA generated from the cerebrum and the following primers: sense primer, 5′GAGCTTCCTAACTGAGCTTCAGCTATGTTGGTGATGAAGC-3′, and antisense primer, 5′-CACGGTGCATCTTTTCTGGGGGTGAAGCTGTAGCCTCTCT-3′. By using these primers, a native β-actin gene of a 450-bp fragment (nucleotides 91-540; GenBank accession no. AF199488) with additional 20-bp fragments of GnIH precursor where primers for competitive PCR anneal (designated by *underlining*) (9). The PCR was conducted at 94 C for 3 min and then 30 cycles at 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min, with an additional incubation at 72 C for 3 min. Quantification of the PCR production was performed spectrometrically, and aliquots were used as standards for competitive PCR analysis. For competitive PCR, an aliquot of the cDNA solution corresponding to 1 μg of initial total RNA of each sample and competitor standard (1-100 amol per tube) were used as templates.

Oligonucleotides used as competitive PCR primers based on nucleotide sequences of quail GnIH precursor (9) were as follows: sense primer, 5′-GAGCTTCCTAACTGAGCTTC-3′ [identical with nucleotides 10–29 in Satake *et al.* (9)], and antisense primer, 5′-CACGGTGCATCTTTTCTGGG-3′ [complementary to nucleotides 615–634 in the report by Satake *et al.* (9)]. A native 625-bp fragment was amplified by the sense and antisense primers of the GnIH precursor gene and the primers coamplified competitor standard (490 bp) diluted in the reaction mixture. The PCR was conducted at 94 C for 3 min and then 35 cycles at 94 C for 1 min, 54 C for 1 min, and 72 C for 1 min, with an additional incubation at 72 C for 10 min.

PCR products were quantified by fluorescence of ethidium bromide on a 3UV transilluminator (UVP, Upland, CA) followed by two-dimensional analysis of the gel image with an IMAGE software package (National Institutes of Health, Bethesda, MD). Derived intensity data were subjected to quantitative analysis to calculate the GnIH precursor mRNA concentration. For competitive PCR analysis of β-actin, competitor DNA was also produced by PCR, using cDNA generated from the cerebrum and following primers: sense primer, 5′-ATCATGTTCGAGACCTTCAAACTGTGCCCATCTATGAAGG-3′, and antisense primer, 5′-GACAGAGTATTTGCGCTCGG-3′. By using these primers, a 100-bp fragment of β-actin gene (nucleotides 321–420; GenBank accession no. AF199488) was detected from the native 654-bp fragment of β-actin gene (nucleotides 301-954; GenBank accession no. AF199488). The underlined sequence is the part in which annealing occurs in sense primer for competitor PCR. The antisense primer was also used for β-actin competitive PCR.

For competitive PCR, an aliquot of the cDNA solution corresponding to 1 μg of initial total RNA of each sample and competitor standard (10–1000 amol per tube) were used as templates. Oligonucleotides used as competitive PCR primers based on nucleotide sequences of quail β-actin were as follows: sense primer, 5′-ATCATGTTCGAGACCTTCAA-3′ (identical with nucleotides 301–320; GenBank accession no. AF199488), and antisense primer, 5′-GACAGAGTATTTGCGCTCGG-3′ (complementary to nucleotides 935-954; GenBank accession no. AF199488). A native 654-bp fragment was amplified by the sense and antisense primers of the β-actin gene, and the primers coamplified competitor standard (554 bp) diluted in the reaction mixture. PCR products were then quantified and the β-actin mRNA concentration was calculated. GnIH precursor mRNA level was normalized with the expression of the housekeeping gene β-actin and expressed as a ratio of GnIH precursor mRNA concentration to β-actin mRNA concentration in the corresponding total RNA derived from each hypothalamic explant.

RIA of LH

A highly purified chicken LH (USDA-cLH-I-3) (39) was radioiodinated by our previous method (40, 41). The RIA was performed as described previously (12, 42, 43), using rabbit antichicken LH serum (USDA-AcLH-5) (39) supplied by the U.S. Department of Agriculture-Agricultural Research Service (ARS), Biotechnology and Germplasm Laboratory (Beltsville, MD). The antiserum cross-reacted with quail LH. Chicken LH preparation (USDA-cLH-K-3) was used as the standard (39), and LH concentrations were expressed in nanograms of chicken LH (USDA-cLH-K-3) per milliliter of plasma. The purity of USDA-cLH-K-3 was approximately a third of the most purified chicken LH fraction (USDA-cLH-I-3) (39).

RIA of melatonin

Melatonin was extracted from each hypothalamic explant by using chloroform, as described previously (34). Plasma melatonin was measured directly without performing extraction. The RIA was performed as described previously (33) by using rabbit antimelatonin serum supplied by the Institute for Molecular and Cellular Regulation (Gunma University, Maebashi, Japan).

Statistical analysis

Results of the PCR, ELISA, RIA, and testicular weight were expressed as the mean ± SEM and analyzed for significance by one-way ANOVA. When the F value of ANOVA for multiple comparisons was significant, then Duncan's multiple range test was subsequently conducted. Correlation coefficients of the interactions of time courses of incubation and GnIH mRNA expression, melatonin concentrations, and GnIH release as well as

melatonin concentrations and GnIH mRNA expression were calculated. We also conducted Student's *t* test to analyze for significance if the experiment consisted of only two groups.

Results

Effects of melatonin administration on the release and expression of GnIH

To measure the release of GnIH, we conducted competitive ELISA by using an antiserum raised against quail GnIH. The binding of GnIH to the antiquail GnIH antiserum used in this study was completely inhibited by GnIH and RFRP-3, a mammalian GnIH homologous peptide, but not by other RFamide peptides (NPFF and PrRP) having a different C-terminal structure from GnIH and RFRP-3, indicating a high specificity of the antiserum (Fig. 1). As shown in Fig. 2, 120 min of melatonin administration significantly stimulated the release of GnIH from hypothalamic explants. This effect of melatonin on GnIH release was dose-dependent ($P < 0.05$, vehicle *vs.* 10^{-7} M melatonin; 10^{-9}*vs.* 10^{-7} M melatonin), and the minimum effective concentration ranged between 10^{-9} and 10^{-7} (Fig. 3A). Our preliminary study showed a significant increase of GnIH mRNA expression as result of melatonin administration during 120 min incubation (Fig. 2). Thus, we administered melatonin at different doses in the medium for 120 min. Melatonin administered in this way increased the expression of GnIH precursor mRNA in the hypothalamic explant in a dose-dependent manner ($P < 0.05$, vehicle *vs.* 10^{-7} M melatonin; 10^{-9}*vs.* 10^{-7} M melatonin; 10^{-8} *vs.* 10^{-7} M melatonin; Fig. 3B). The interactions of time courses of incubation and GnIH mRNA expression (r = 0.97; Fig. 2), melatonin concentrations and GnIH release (r = 0.85; Fig. 3A) as well as melatonin concentrations and GnIH mRNA expression (r = 0.93; Fig. 3B) were positively correlated.

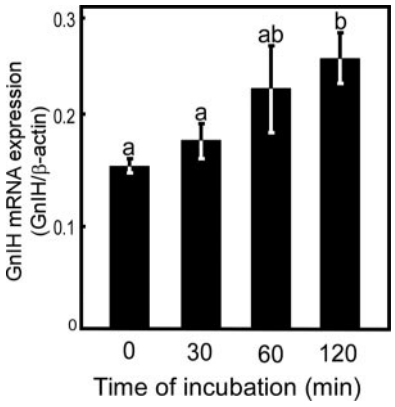

FIG. 2. GnIH precursor mRNA expression caused by melatonin administration after different periods of incubation. Birds were housed under LD photoperiods (16 h light and 8 h dark; lights on at 0700 h, off at 2300 h). Hypothalamic explants were collected at 1500 h and incubated for 0, 30, 60, and 120 min in medium containing 10^{-7} M melatonin for the determination of the appropriate time to incubate hypothalamic explants with melatonin in the main study. Each *column* and the *vertical line* represent the mean ± SEM (n = 6 samples; one sample from one bird). Groups with *different letters* are significantly different ($P < 0.05$) by one-way ANOVA (F value = 3.2, $P < 0.05$), followed by Duncan's multiple range test.

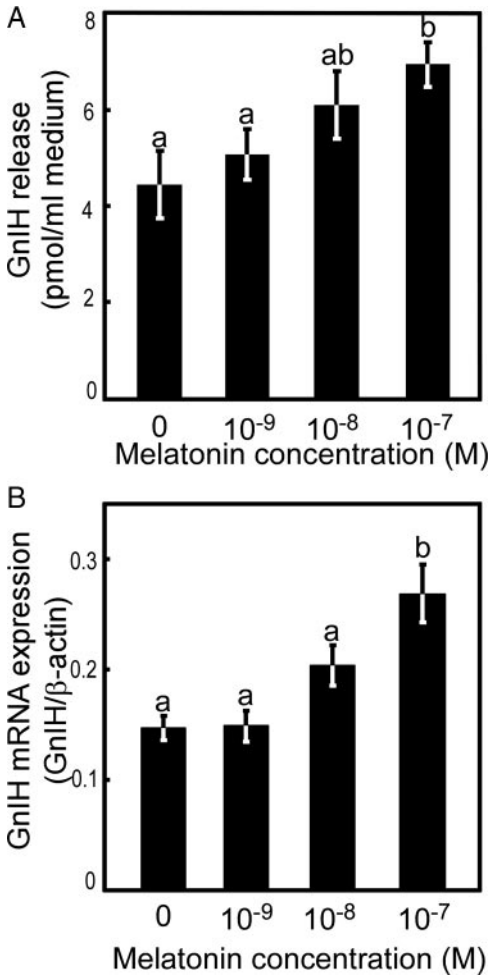

FIG. 3. Effects of melatonin administration on the release and expression of GnIH. A, Effects of melatonin administration on the release of GnIH from hypothalamic explants. B, Effects of melatonin administration on the expression of GnIH precursor mRNA in hypothalamic explants. Birds were housed under LD photoperiods (16 h light and 8 h dark; lights on at 0700 h, off at 2300 h). Hypothalamic explants were collected at 1500 h and incubated for 120 min in medium containing various concentrations of melatonin. Each *column* and the *vertical line* represent the mean ± SEM (n = 6 samples; one sample from one bird). Groups with *different letters* are significantly different ($P < 0.05$) by one-way ANOVA (F value = 4.0, $P < 0.05$ for GnIH release by different doses of melatonin; F value = 7.0, $P < 0.01$ for GnIH expression by different doses of melatonin), followed by Duncan's multiple range test.

Effects of light and dark on the release and expression of GnIH

As shown in Fig. 4A, the release of GnIH from hypothalamic explants into the incubation medium during the middle of the dark period (0300 h) was significantly higher than that during middle of the light period (1500 h) ($P <$

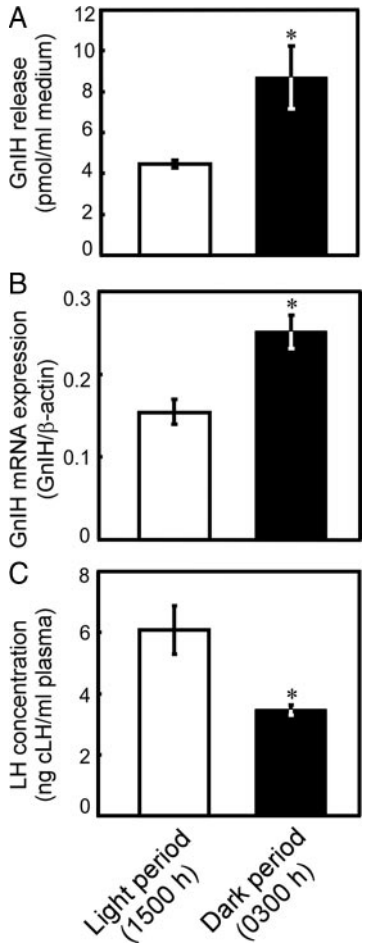

FIG. 4. Effects of the light and dark periods on the release of GnIH from hypothalamic explants (A), the expression of GnIH precursor mRNA in hypothalamic explants (B), and plasma LH concentrations (C). Birds were housed under LD photoperiods (16 h light and 8 h dark; lights on at 0700 h, off at 2300 h). Hypothalamic explants were collected at 1500 h (light period) and 0300 h (dark period) and incubated in the medium for 120 min. Each *column* and the *vertical line* represent the mean ± SEM (n = 6 samples; one sample from one bird). *, $P < 0.05$ *vs.* light period by Student's *t* test.

0.05, dark period *vs.* light period). The expression of GnIH precursor mRNA in the hypothalamic explant was also significantly higher during the middle of the dark period (0300 h) than during the middle of the light period (1500 h) ($P < 0.05$, dark period *vs.* light period; Fig. 4B). Conversely, plasma LH concentrations during the middle of the dark period were significantly lower than during the middle of the light period (1500 h) ($P < 0.05$, dark period *vs.* light period; Fig. 4C).

Diurnal changes in the release of GnIH and plasma LH concentrations

To understand the functional significance of melatonin action in the regulation of GnIH release, diurnal changes in the release of GnIH alongside changes in the plasma LH concentration and the diencephalic and plasma melatonin concentrations were analyzed. Hypothalamic explants were collected in the middle of the light phase (1500 h), early night (2100 h), middle of the dark period (0300 h), morning (0900 h), and again at the middle of the light period (1500 h) of the next day and incubated in the medium for 120 min to measure the release of GnIH. The concentrations of plasma LH and diencephalic and plasma melatonin were also measured at the above-described times. As shown in Fig. 5A, the GnIH concentration in the medium during the middle of the dark period (0300 h) was significantly higher than those during the middle of the light period (1500 h), early night (2100 h),

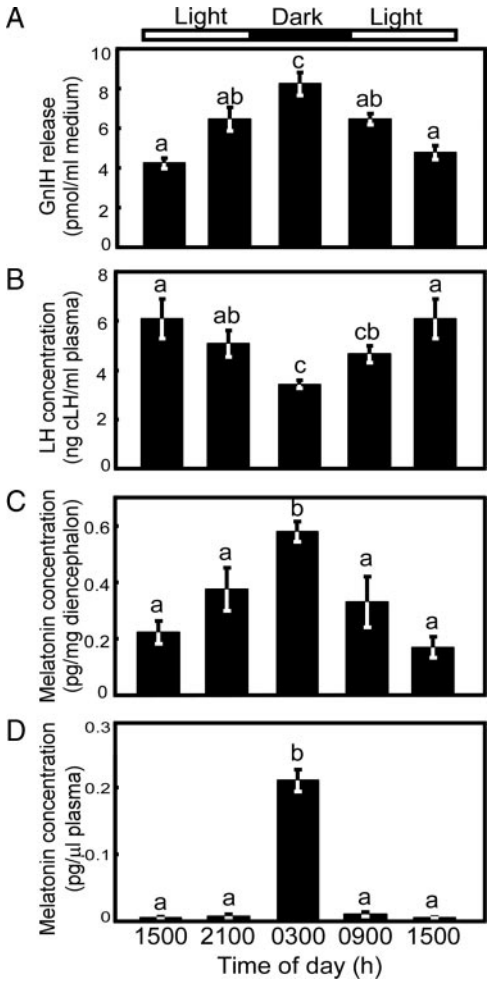

FIG. 5. Diurnal changes in the release of GnIH from hypothalamic explants (A), plasma LH concentrations (B), and diencephalic and plasma melatonin concentrations (C and D). Birds were housed under LD photoperiods (16 h light and 8 h dark; lights on at 0700 h, off at 2300 h). Hypothalamic explants were collected in the day at 1500 h, early night (2100 h), middle of the night (0300 h), morning (0900 h) and again at 1500 h the next day and incubated in the medium for 120 min. Each *column* and the *vertical line* represent the mean ± SEM (n = 8 samples; one sample from one bird). Groups with *different letters* are significantly different ($P < 0.05$ or $P < 0.01$ in case of the plasma melatonin concentration at 0300 h) by one-way ANOVA (F value = 8.5, $P < 0.01$ for GnIH release; F value = 4.9, $P < 0.01$ for LH concentration; F value = 7.1, $P < 0.01$ for diencephalic melatonin concentration; F value = 140.0, $P < 0.01$ for plasma melatonin concentration), followed by Duncan's multiple range test.

morning (0900 h), and middle of the light period (1500 h) of the next day ($P < 0.05$, 0300 *vs.* 0900, 1500, 2100, and 1500 h of the next day). Conversely, plasma LH concentrations during the middle of the dark period (0300 h) were significantly lower than those during the light periods at different times ($P < 0.05$, 0300 *vs.* 1500, 2100, and 1500 h of the next day; Fig. 5B). The diurnal changes of diencephalic and plasma melatonin concentrations (Fig. 5, C and D) showed a positive correlation with the release of GnIH (Fig. 5A) and a negative correlation with plasma LH concentrations (Fig. 5B).

Effects of photoperiodic manipulation on the release and expression of GnIH

GnIH was released into the medium at significantly higher concentration from hypothalamic explants of birds previously exposed to SD photoperiods than from explants of birds previously exposed to LD photoperiods ($P < 0.05$, SD *vs.* LD; Fig. 6A). The expression of GnIH precursor mRNA in the hypothalamic explants was also significantly higher in birds previously exposed to SD photoperiods than in those from LD photoperiods ($P < 0.05$, SD *vs.* LD; Fig. 6B). Importantly, plasma LH concentration and testicular weight also decreased significantly in birds exposed to SD ($P < 0.05$, SD *vs.* LD; Fig. 6, C and D).

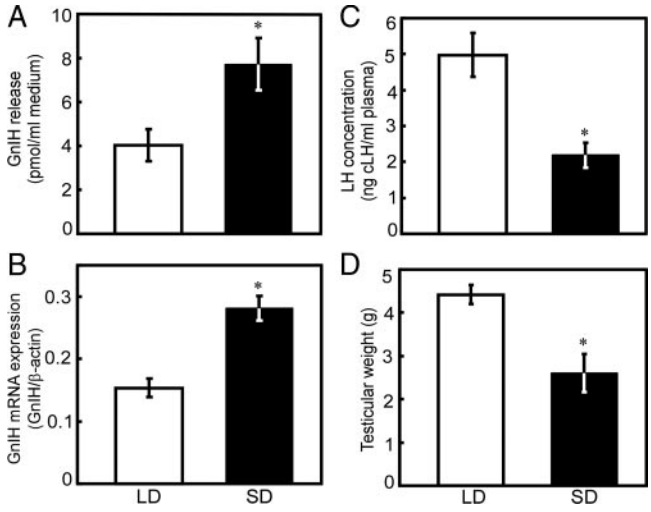

FIG. 6. Effects of photoperiodic manipulation on the release of GnIH from hypothalamic explants (A), the expression of GnIH precursor mRNA in the hypothalamic explants (B), plasma LH concentrations (C). and combined testicular weight (D). Birds were exposed to either LD (16 h light and 8 h dark; lights on at 0700 h, off at 2300 h) or SD (8 h light and 16 h dark; lights on at 0900 h, off at 1700 h) photoperiods for 3 wk. Hypothalamic explants of LD and SD birds were collected at 1500 h and incubated in the medium for 120 min. Each *column* and the *vertical line* represent the mean ± SEM (n = 6 samples; one sample from one bird). *, $P < 0.05$ *vs.* LD by Student's *t* test.

Discussion

The purpose of the present study was to clarify whether melatonin could regulate GnIH release in quail. Melatonin is involved in the regulation of GnIH expression. Considering GnIH's inhibitory effects on gonadotropin secretion (6, 10, 12, 13) and the role of photoperiod in avian reproduction, we hypothesized that melatonin may also be involved in the regulation of GnIH release, thus influencing gonadal activity. The present study investigated the effects of melatonin on the release of GnIH by using an *in vitro* model. Melatonin dose-dependently increased the release of GnIH from hypothalamic explants *in vitro*. Notably, the concentration of melatonin used to induce changes in GnIH release was within the physiological range. Measured concentrations of melatonin are 0.2–0.6 pg melatonin per milligram diencephalon (8×10^{-9} to 2.6×10^{-8} M per milligram diencephalon) (Fig. 5C) and the used doses of melatonin were 10^{-9} to 10^{-7} M. If we calculate this figure, then the available concentration of melatonin in a hypothalamic explant (average 100 mg) will be around 8×10^{-8} to 2.6×10^{-7} M. Thus, our used doses of melatonin (10^{-9} to 10^{-7} M) did not exceed the physiological range. In a further experiment, GnIH release during the dark period was greater than that during the light period in quail exposed to LD *in vivo*. Conversely, plasma LH concentrations decreased during the dark period when GnIH release increased *in vivo*. Furthermore, marked diurnal changes in GnIH release were observed in quail exposed to LD, and these changes were negatively correlated with changes in plasma LH concentration. In addition, the release of GnIH from hypothalamic explants and the expression of GnIH precursor mRNA in hypothalamic explants increased under SD photoperiods *in vivo*. Thus, melatonin stimulates not only GnIH expression but also GnIH release in quail. This is the first report describing an effect of melatonin on neuropeptide release and a negative correlation of GnIH release with LH release.

Melatonin secretion in quail is dependent on the length of the night, just as in other vertebrates (35), and melatonin secretion starts soon after lights are turned off (36). Therefore, the increase of GnIH release under SD is presumably due to the increase in the duration of endogenous melatonin secretion. Accordingly, GnIH is capable of transducing photoperiodic information via changes in the melatonin signal. This is a mechanism by which changes in day length can influence the reproductive axis in birds. In addition to birds, it has been reported that the expression of RFRP, a mammalian GnIH homologous peptide is also regulated by melatonin in hamsters (44) and sheep (45, 46). However, the regulatory mechanism governing RFRP

release is still unclear in mammals. Future study is needed to clarify whether melatonin also regulates the release of RFRP in mammals just as the present study has shown in birds. Furthermore, additional research is required to elucidate how the interactions of daily rhythms of melatonin, GnIH, and LH affect seasonal reproduction in birds and mammals. Although we have provided evidence for a novel physiological mechanism of GnIH release by melatonin in quail, future studies are also needed to clarify the molecular mechanisms underlying melatonin's regulation of GnIH release. For example, data are required on melatonin receptor specificity, its mode of action (direct *vs.* indirect effects), and second-messenger activity and whether melatonin acts at the promoter level or affects RNA stability during the process of GnIH expression and release.

Seasonally breeding birds and mammals provide excellent models to understand the roles of GnIH and RFRP in regulating changes in reproductive status. The expression of RFRP is also regulated by changing photoperiod in seasonally breeding mammals. In Syrian and Siberian hamsters, the expression of RFRP mRNA and the number of RFRP-immunoreactive cell bodies were decreased in sexually quiescent animals on SD compared with sexually active animals maintained under LD (44). Furthermore, photoperiodic modulation of RFRP was prevented by pinealectomy, and melatonin injections reduced RFRP expression in hamsters. Thus, in these hamster species, the RFRP neurons appear to be modulated via melatonin signaling. In contrast to hamsters, sheep are SD breeders. In Blackface sheep, the number of RFRP neurons decreased during SD (45). In Soay sheep, the expression of RFRP is also increased overall with exposure to LD, and increased markedly in the ependymal cells surrounding the base of the third ventricle (46). In LD breeding birds, the expression of GnIH was increased under SD, and melatonin administration caused a dose-dependent increase in the expression of GnIH mRNA and the production of mature peptide (33). Further studies are needed to clarify the functional significance of the species difference in the direction of regulation of RFRP in mammals. Notwithstanding these differences, there is consistency in quail and mammals in terms of the regulation of GnIH and RFRP by changing photoperiod and melatonin.

It is very important to gather knowledge on the distribution of melatonin receptors in relation to GnIH neurons to understand the mode of melatonin action on GnIH release and synthesis. It has been reported that there are three avian melatonin receptor subtypes, Mel_{1a}, Mel_{1b}, and Mel_{1c}, which comprise a specific subfamily within the G protein-coupled receptor superfamily. In our previous study, we cloned quail Mel_{1c} cDNA (33) because it was reported that Mel_{1c} mRNA was differentially expressed in the chicken hypothalamus (47). We then conducted *in situ* hybridization of Mel_{1c} combined with GnIH immunocytochemistry (33). Colocalization of GnIH immunoreactive cells and Mel_{1c} mRNA provide strong evidence that GnIH neurons express Mel_{1c} in the quail (33). Melatonin receptor autoradiography further revealed specific binding of melatonin in the PVN (33). Based on these findings, we consider that melatonin acts directly on GnIH neurons through Mel_{1c}-mediated mechanisms to stimulate GnIH release and synthesis. However, there is expression of melatonin receptors in cells of the PVN in quail, which did not contain GnIH. Thus, it could be deduced that there may exist an indirect effect of melatonin on GnIH neurons via synaptic connections (33). This possibility requires further study as does the possibility that melatonin receptor subtypes other than Mel_{1c} are also involved in regulating GnIH release and synthesis. It will also be important to examine the expression of melatonin receptor protein in addition to mRNA. Studies on the clock gene expression in GnIH neurons and their involvement in the photoperiodic regulation of GnIH release will also be of great interest.

We previously studied developmental changes in GnIH expression in the hypothalamo-hypophysial system in the quail (8). Because GnIH begins its function around the time of hatching, it is thought that during this time it starts to act as a hypothalamic factor to regulate gonadotropin release in quail. Furthermore, melatonin synthesis and secretion begin during late-embryonic life in chickens (48–51). Based on these findings, we suspect that melatonin action on GnIH release and synthesis begins around the time of hatching and remains throughout development and adulthood. In the present study, GnIH release from hypothalamic explants of mature quail increased in response to changes in melatonin. We propose that most of the melatonin concentration that we detected in the hypothalamic explants originated from the pineal gland and eyes because in our previous study, we measured melatonin concentration in the diencephalon of Px+Ex quail and found it to be much lower than in sham operated quail, suggesting that the majority of melatonin comes from sources outside the brain (33). We consider that this melatonin exerts its effect on GnIH neurons through melatonin receptor to stimulate GnIH release in mature quail.

It is considered that GnRH-I controls reproductive function in birds (52). However, there is no association between gonadal regression due to decreased photoperiod with a decrease in hypothalamic GnRH-I in quail (53, 54). In this study, we are the first to show that the release of GnIH from hypothalamic explants was increased in quail previously exposed to SD, and was associated with a de-

crease in plasma LH levels and gonadal regression. GnIH inhibits gonadotropin synthesis and release in quail (12). Therefore, it could be assumed that the stimulatory action of melatonin on the release and synthesis of GnIH may be one of the main causes for decreasing plasma LH and thereby gonadal regression in birds under SD photoperiods. If this hypothesis is correct, spontaneous gonadal development could be induced by the inhibition of GnIH release on SD photoperiods. Further experimentation, such as immunoneutralization of GnIH by intracerebroventricular administration of GnIH antiserum or GnIH gene silencing by administration of an inhibitory small interfering RNA would be a logical next step from the present study. Overall, our data imply an important role for melatonin in photoperiodic regulation of the reproductive axis via changes in GnIH synthesis and release.

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