Photoperiodic Response of Serotonin- and Galanin-Immunoreactive Neurons of the Paraventricular Organ and Infundibular Nucleus in Japanese Quail, *Coturnix coturnix japonica*

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**ABSTRACT**—We investigated the photoperiodic response of serotonin- and galanin (GA)-immunoreactive (ir) cells in the paraventricular organ (PVO) and infundibular nucleus (IF) of the Japanese quail and the interaction of these cells with gonadotropin-releasing hormone (GnRH)-ir neurons in the hypothalamus. Serotonin-ir cells were located in series from the PVO to the IF, and were connected with each other. The number of serotonin-ir cells differed significantly between light and dark phases on the short days (SD), but did not differ between light and dark phases on long days (LD). GA-ir cells were also found in the PVO and IF. The number of GA-ir cells under SD conditions was significantly greater than under LD conditions but did not change diurnally. Both serotonin-ir and GA-ir fibers ran along the GnRH-ir cells in the nucleus commissurae pallii. Serotonin-ir and GA-ir fibers were connected with the GnRH-ir fibers in the external layer of the median eminence (ME). We confirmed that GA-ir fibers were closely associated with serotonin-ir neurons in the PVO and IF. GA-ir neurons have at least 2 routes of regulating GnRH neurons directly, and indirectly via the serotonin-ir cells in the PVO and IF.

**Key words:** photoperiodic-gonadal response, cerebrospinal fluid-contacting neurons, deep brain photoreceptor, immunohistochemistry, paraventricular organ

**INTRODUCTION**

In the vertebrates, reproductive activity and gonadal development are controlled by environmental cues such as light and temperature. The most common trigger of gonadal development or regression is daylength. Photic information (daylength) is transmitted to the endocrine system via the hypothalamo-hypophyseal-gonadal axis. In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus, onto which light impulses are projected directly from the retina, have been implicated as key circadian clock mechanism, and the eyes are photoreceptors for entrainment to the environmental light cycle (Moore and Lenn, 1972; Moore et al., 1995). The duration of melatonin secretion from the pineal, which receives inputs from the SCN, determines the level of gonadotropin-releasing hormone (GnRH) secretion and the time of seasonal breeding (Bartness et al., 1993; Goldman, 1999).

The photoreceptive organs of birds include not only eyes but also other tissues such as the pineal complex and deep brain. The pineal complex and deep brain photoreceptors are essential for seasonal reproduction (Benoit, 1964; Oishi and Kato, 1968; Oishi and Lauber, 1973; Oishi and Ohashi, 1993). Multi-photoreceptor and multi-oscillator systems are constructed from the eyes, pineal complex, and hypothalamus, and photoperiodic responses are controlled by these systems (Oishi et al., 2001). However, it is not known how photic information received by the photoreceptors is transmitted to the hypothalamo-hypophyseal axis of the endocrine system.

Yoshikawa and Oishi (1998) have reported that, in the
Japanese quail the rhodopsin-immunoreactivity is found in the paraventricular organ (PVO), which is located on the lateral wall of the third ventricle of the hypothalamus. Therefore, we consider that the PVO is a candidate for deep brain photoreceptor. Most of the cells that make up the PVO are cerebrospinal fluid (CSF)-contacting neurons. Many CSF-contacting neurons in the PVO of the Japanese quail display serotonin immunoreactivity (Cozzi et al., 1991; Oishi et al., 2001). However, their function is not yet known. We especially focused on serotonin-ir cells in the PVO and investigated the interaction between them and GnRH cells in order to prove their relation to the photoperiodic gonadotropin response. We used Japanese quail as experimental animals because they show a distinct and marked response to changing photoperiod (Oishi et al., 1966; Follett and Sharp, 1969).

Our previous study showed that galanin (GA)-immunoreactive (ir) fibers were closely associated with serotonin-ir cell bodies and fibers in the PVO and in the infundibular nucleus (IF) (Haida et al., 2002). GA is a C-terminally amidated 29-residue peptide first discovered in extracts of the gastrointestinal tract of pigs (Tatemoto et al., 1983). In mammals, GA is widely distributed in the brain and has been implicated in the control of reproductive function (for reviews see Merchenthaler et al., 1993; Finn et al., 1998). GA-ir neurons are co-expressed in the GnRH-ir and growth hormone-releasing hormone-ir neurons of rats (Merchenthaler et al., 1990, 1993; Marks et al., 1992; Hohmann et al., 1998). GA stimulated the release of GnRH from arcuate nucleus - median eminence (ME) fragments in vitro (López and Negro-Vilar, 1990; Sahu et al., 1994). GA infused directly into the brain of rats stimulates luteinizing hormone (LH) secretion (Sahu et al., 1994). In birds, there have been few studies for GA. In the peripheral function, GA acts to cause contractions of uterine muscles of the quail (Li et al., 1996; Tsutsui et al., 1997, 1998). GA-ir perikarya are located in the preoptic area, lateral septal nucleus, periventricular nucleus (PVN), and IF in the chicken (Józsa and Mess, 1993) and Japanese quail (Azumaya and Tsutsui, 1996). Gonadotropin-inhibitory hormone (GnIH) was isolated and identified as a novel dodecapeptide containing a C-terminal RFamide sequence from the quail brains. GnIH inhibited gonadotropin release from cultured quail anterior pituitaries (Tsutsui et al., 2000). We, therefore, investigated that the relation of GA-ir cells with not only GnRH- but GnIH-ir cells.

We investigated the effects of photoperiod on the number of serotonin- and GA-ir cells in the PVO and IF of the Japanese quail to demonstrate their association in the PVO and IF with the photoperiodic gonadotropin response.

**MATERIALS AND METHODS**

**Photoperiodic effect**

Fifty-two male Japanese quail (Coturnix coturnix japonica), 4 weeks old at the beginning of the experiment, were housed under short-day (SD) conditions (8:16 light-dark schedule with lights on at 0800) and long-day (LD) conditions (16:8 light-dark schedule with lights on at 0400) for 25 days at 25°C. The animals were euthanized in about the middle of the light period (between 1030 and 1330) or the dark period (between 2230 and 0130), respectively. They were anesthetized with fluothane (halothane, Takeda, Osaka, Japan) and perfused via the left ventricle of the heart with heparinized saline followed by Zamboni’s fixative (4% paraformaldehyde in 0.1 M phosphate-buffer (PB) containing 0.2% picric acid at pH 7.4). After fixation, the brains were removed and post-fixed at least overnight in the same fixative at 4°C. Both right and left testes were weighed after fixation.

Frozen serial sections (30 µm) were prepared using a cryostat (Bright, Cambridge, England). Sections were mounted on gelatin-coated slides, and air-dried. Sections were treated with primary antibodies against serotonin (diluted 1:1000) (Diasorin, Stillwater, MN, USA) and GA (diluted 1:500) (Azumaya and Tsutsui, 1996). Binding of the antisera was visualized by means of the strept-avidin-biotin method. Specimens were incubated overnight at room temperature with the respective primary antisera, rinsed in 0.1 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBS-T) for 30 min, incubated with biotin-labeled anti-rabbit IgG (diluted 1:1000 in PBS-T) (Biosource, Camarillo, CA, USA), rinsed in PBS and then incubated with horseradish peroxidase-labeled streptavidin (diluted 1:250 in PBS-T) (ICN, Aurora, Ohio, USA). The immunoreaction was visualized by use of diaminobenzidine (DAB) (Sigma, St. Louis, Mo, USA). Sections were incubated with 0.025% DAB in Tris buffer containing 0.05% H2O2 for about 8 min. After the sections had been washed with Tris buffer for 20 min, the staining was stopped by washing in distilled water. Sections were dehydrated in an alcohol xylene column, and then cleared and mounted using HSR solution (KokusaiShiyaku, Kobe, Japan). Total numbers of serotonin- and GA-ir cell bodies of the PVO and IF in each preparation were counted under a differential interference microscope (BX51, Olympus, Tokyo, Japan).

**Immunofluorescence**

Six male Japanese quail (Coturnix coturnix japonica), 4 weeks old, were housed under a 12:12-h light-dark schedule with lights on at 0600 for 2 to 4 weeks at 25°C. The animals were euthanized in about the middle of the light phase (between 1030 and 1330). The same protocols as described above were used for anesthesia and for tissue, fixation, and frozen sectioning.

**Single immunostaining**

In the first step, the sections were incubated with first antibodies against serotonin (diluted 1:1000) and GA (diluted 1:500) overnight at room temperature. After being washed with PBS-T, the sections were incubated with Alexa 488 or 594-conjugated anti-rabbit IgG (diluted 1:330 in PBS-T) (Molecular Probes, Eugene, OR, USA) for 5 h, rinsed with PBS 3 times for 10 min, and then mounted using Gel/Mount (Biomeda Corp, Foster City, CA, USA).

**Double immunostaining**

In the first step, the sections were incubated with first primary antibodies overnight at room temperature. After being washed with PBS-T, the sections were incubated with Alexa 488 or 594-conjugated anti-mouse IgG (diluted 1:330 in PBS-T) for 5 h and then rinsed 3 times with PBS-T. In the second step, the same sections were incubated with the second primary antibodies overnight at room temperature. After being rinsed in PBS-T, they were incubated with Alexa 594 or 488-conjugated anti-rabbit IgG (diluted 1:330 in PBS-T) for 5 h, rinsed with PBS 3 times for 10 min, and then mounted using Gel/Mount.

As the first primary antisera, we used rat antisera to chicken GnRH-I (diluted 1:1000), GnIH (diluted 1:500) (Ukena et al.,...
2003), serotonin (diluted 1:50) (Chemicon, Temecula, CA, USA). The rabbit antiserum to GA (diluted 1: 500) and serotonin (diluted 1:1000) were used for second primary antisera. Since the antisera against GnIH (Ukena et al., 2003), GA (Azu-maya and Tsutsui, 1996) and serotonin (DiaSorin, Cozzi et al., 1991) had been used in the quail brains, the sections for control staining were incubated in normal serum or buffer without the primary antisera. Control sections for GnRH and serotonin (Chemicon) were incubated in the antisera absorbed with GnRH and buffer without the primary antisera, respectively. In all control preparations, no immunoreactivity was observed (date not shown). The sections immunostained with both antisera against serotonin (DiaSorin, Chemicon) showed the same results.

We used antiserum to chicken GnRH-I, because chicken GnRH-I is homologous to mammalian GnRH and is regarded as an important hormone in the release of gonadotropin in birds.

The preparations stained by immunofluorescence were observed under a confocal laser scanning microscope (TCS-NT, Leica, Bensheim, Germany). The procedures used for optical sectioning, superimposing, and reconstructing are described in a previous study (Tamotsu et al., 1997).

RESULTS

Photoperiodic effects

Testis weight

Both right and left testes of males exposed to LD for 25 days were well developed, with a mean weight of 3.75±0.17 g (mean±SEM). In contrast, the testes of males exposed to SD for 25 days were small, with a mean weight of 0.08±0.02 g (mean±SEM). The weight of LD males was significantly greater than that of SD males (P<0.001, Fig. 1A).

Number of serotonin-ir cells

Serotonin-ir neurons were located in the PVO and IF (Fig. 2C, 3A, B) of the hypothalamus. Most of the serotonin-ir cells extended knob-like structures into the third ventricle and had the characteristics of CSF-contacting neurons (Fig. 3C). The axon of the serotonin-ir cells had many varicosities on the collateral branches that were connected to each other (Fig. 3D). Serotonin-ir neurons were distributed from the PVO to the IF in series (Fig. 3E). The serotonin-ir CSF-contacting neurons of the PVO were similar in shape to those of the IF. We counted these cells in the PVO and IF together, because it was difficult to distinguish them.

Under SD conditions the number of serotonin-ir cells in the PVO and IF in the light and dark phase was 1286±91.
and in the dark phase 739±18, respectively. Under LD conditions, there were 954±96 cells in the light phase and 901±59 cells in the dark phase. The number of serotonin-ir cells of the PVO and IF under SD conditions was significantly greater in the light phase than in the dark phase (P<0.05, Fig. 1B). Under LD conditions there was no significant difference in the number of these cells between the light and dark phases (Fig. 1B).

**Numbers of GA-ir cells**

GA-ir cells were observed in the PVO and in the ventral region of the IF (Fig. 2C, 4A, PVO; 4B, IF). Furthermore, GA-ir cells were apparent in the posterior region of the IF (pIF) (Fig. 2D, 4C, D). Most of the GA-ir cells in the pIF were bipolar cells and extended knob-like structures into the third ventricle (Fig. 4C, D). These characteristics showed that the GA-ir cells were CSF-contacting neurons.

The numbers of GA-ir cells of the PVO and IF in the light and dark phases in the SD conditions were 1046±38 and 1066±136, respectively. Under LD conditions the number of GA-ir cells in the light phase was 564±141 and in the dark phase 397±38. There was no significant difference between light and dark phase cell numbers in either photoperiod (Fig. 1C). However, the mean total numbers of GA-ir cells of the PVO and IF in both light and dark phases under

**Fig. 3.** Superimposed (A, B) and reconstructed (C, D) images of the serotonin-ir neurons in the PVO and IF. Serotonin-ir cells were located in the PVO (A) and IF (B). Scale bars=100 μm. Most of the serotonin-ir neurons have basal processes and one apical process that extends to the third ventricle (C). Serotonin-ir cells have many collateral branches and are connected with each other (D). Arrows indicate close points of contact. Scale bars=20 μm. E: Light micrograph of serotonin-ir cells. Serotonin-ir cells are present in series along the third ventricle from the PVO to the IF. Scale bar=100 μm.

**Fig. 4.** Superimposed images of GA-ir cells in the PVO, IF, and pIF. A: GA-ir cells in the PVO. Arrows indicate the GA-ir cell bodies. B: GA-ir cells in the IF. C: GA-ir cells in the pIF. D: High magnification of (C). GA-ir CSF-contacting cells were observed in the pIF. Scale bars=100 μm, except in (D), which indicates 20 μm. V, third ventricle.
Fig. 5. Confocal optical sections, of double-immunostained preparations. A: Serotonin (green) and GnRH (magenta) immunoreactivities in the nCPa. B: GnRH (green) and GA (magenta) immunoreactivities in the nCPa. C: GnIH (green) and GA (magenta) immunoreactivities in the IF. D: GnIH (green) and GA (magenta) immunoreactivities in the PVN. E: Serotonin (green) and GA (magenta) immunoreactivities in the PVO. Arrows indicate close points of contact. Scale bars=20 μm
SD conditions (1056±66) were significantly (P<0.001) greater than those under LD conditions (480±75) over the 25 days (Fig. 1C).

Neural interaction
Serotonin -GnRH
GnRH-ir cells were present around the nucleus commissurae pallii (nCPa) (Fig. 2B, 5A, B). Serotonin-ir fibers had many varicosities and ran along the GnRH-ir cell bodies (Fig. 5A). GnRH-ir fibers and serotonin-ir fibers innervated the external layer of the ME (Fig. 6A).

GA -GnRH
The localization of GA-ir cells in the basal hypothalamus was described above. These cell bodies were round or spindle shaped. Many GA-ir nerve fibers were located in the nCPa, the nucleus anterior hypothalami (AM), IF and ME (Fig. 5B, C, D, 6B-1, C-1). GnRH-ir cells were found in the nCPa and were spindle shaped (Fig. 5A, B). GA-ir fibers were seen in close apposition to GnRH-ir cell bodies and fibers in the nCPa (Fig. 5B). GA-ir and GnRH-ir fibers were observed in the same sections, and these fibers were associated with the external layer of the ME (Fig. 6B) and AM.

GA -GnRH
GnRH-ir fibers ran along the GA-ir cell bodies or fibers in the IF (Fig. 5C). GnRH-ir cell bodies were observed in the PVN and were associated with GA-ir nerve fibers (Fig. 5D). Many GnRH-ir fibers were observed in the AM and the inner layer of the ME (Fig. 6C-2). In the AM and the external layer of the ME, GA-ir fibers overlapped with GnRH-ir fibers (Fig. 6C).

Serotonin -GA
GA-ir beaded fibers ran along the serotonin-ir neurons in the PVO and IF (Fig. 5E).

This observation confirmed the results of our previous study (Haida et al., 2002).

DISCUSSION
We used immunohistochemistry to investigate the distribution of serotonin, GA, GnRH, and GnIH neurons in the hypothalamus of the Japanese quail. Our finding with regard to the general distribution of these substances in the posterior hypothalamus are in agreement with those of previous reports (serotonin: Cozzi et al., 1991; Oishi et al., 2001, GA: Azumaya and Tsutsui, 1996, GnRH: Mikami et al., 1988; van Gils et al., 1993; Teruyama and Beck, 2000, GnIH: Tsutsui et al., 2000; Ukena et al., 2003; Ubuka et al., 2003), except that the distribution of GA-ir cell bodies was wider in the IF. We demonstrated that some of the GA-ir neurons in the IF had the characteristics of CSF-contacting neurons. Therefore, they may be sensory cells, because the CSF-contacting neurons of the IF possess sensory cilia (Vigh and Vigh-Teichman, 1973, 1998).

In the brains of rats, GA co-expressed in a subset of GnRH (Merchenthaler et al., 1990; Hohmann et al., 1998), suggesting that GA has an important role in reproduction in mammals. We did not observe the co-expression of GA and GnRH in the Japanese quail. However, GA-ir fibers were connected with GnRH-ir neurons in the nCPa and ME. GA-ir neurons were connected to GnIH-ir neurons in the PVN, IF, and ME. D'Hondt et al. (1998) reported interaction between GA- and GnRH-ir cells in the chicken hypothalamus. These results suggest that these GA-ir neurons participate in the modulation of reproduction in birds. The number of GA-ir neurons was affected by the photoperiod over 25-day study period. These results suggest that GA-ir cells in the PVO and IF receive light information from the photoreceptors and regulate the activity of GnRH- and GnIH-ir cells in the hypothalamus.

Serotonin-ir cells were found in the PVO and IF. Serotonin-ir cells are common in the PVO of submammalian species (Sano et al., 1983), and serotonin-ir cells in the IF have been reported in the frog (Shimizu et al., 1983) and turtle (Sano et al., 1983). In our quail, the serotonin-ir CSF-con-
tacting neurons in the PVO had the same features as those in the IF. Besides, the serotonin-ir CSF-contacting neurons were connected to each other in series from the PVO to the IF and organized a neural group.

We showed that serotonin-ir fibers ran along the surface of GnRH-ir cells in the nCPa, and that both serotonin- and GnRH-ir fibers heavily innervated the ME. These results show histologically that the serotonin-ir cells of the PVO and IF participate in reproduction.

We also showed that GA-ir fibers connected with both the serotonin-ir neurons in the PVO and IF and the GnRH-ir cells of the nCPa. These findings show that GA-ir neurons projected into the GnRH cells both directly and indirectly via the serotonin-ir neurons in the PVO and IF. Moreover, the GA-ir neurons mutually connected with the GnIH-ir neurons. GnIH inhibits gonadotropin release from cultured quail anterior pituitary (Tsutsui et al., 2000). Bentley et al. (2003) reported that GnIH-ir fibers ran along the GnRH-ir cell bodies, and GnIH- and GnRH-ir fibers innervated in the ME of the sparrow hypothalamus. These results suggest that GnIH neurons regulate the GnRH neurons in birds. Our result suggests that GA-ir cells indirectly regulate the GnRH-ir cells through the GnIH-ir neurons. The present and previous results suggest that serotonin-ir neurons and GA-ir neurons in the PVO and IF regulate the GnRH-ir cells and thereby regulate gonadal response (Fig. 7).

The numbers of serotonin-ir cells in the PVO and IF showed significant diurnal changes only under SD conditions, but not under LD conditions. This result suggests that serotonin-ir cells in the PVO and IF integrate 2 pieces of photic information: the photoperiod and the diurnal changes in light intensity. The GA-ir neurons receive only photic information on the photoperiod, SD or LD, because they increased in number under SD conditions but showed no diurnal changes. These results suggest that serotonin-ir CSF contacting neurons in the PVO and IF receive 1 of 2 pieces of photic information regarding photoperiod from GA-ir neurons. This possibility is strongly supported by our histological finding that the GA-ir fibers contacted the serotonin-ir neurons in the PVO and IF (Fig. 7).

We found that the serotonin- and GA-ir cells in the PVO and IF changed in number with changing environmental light conditions and were associated with the GnRH- and GnIH-ir cells. These results suggest that in the Japanese quail the GA- and serotonin-ir cells in the PVO and IF receive photic information and play an important role in transmitting it to the reproductive axis. In particularly, serotonin-ir CSF-contacting neurons of the PVO might be photosensitive, because in the Japanese quail opsin immunoreactivity is localized in the PVO (Yoshikawa and Oishi, 1998). To our knowledge, it has not been proved that the PVO is directly photosensitive and responds to light. This issue is open to future electrophysiological investigation.

Recently, type 2 iodothyronine deiodinase, which is involved in the conversion of thyroxine prohormone to the active 3,5,3'-triiodothyronine, was induced by light in the mediobasal hypothalamus of the Japanese quail (Yoshi-Mura et al., 2003). These and our results suggest that the PVO and the mediobasal hypothalamus are very important place in the regulation of the photoperiodic-gonadal response.

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