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Seasonal Morphological Changes in the Neuro-Glial Interaction between Gonadotropin-Releasing Hormone Nerve Terminals and Glial Endfeet in Japanese Quail

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In a previous study we showed that photoperiodically generated T_3 in the hypothalamus is critical for the photoperiodic response of gonads in Japanese quail. The expression of thyroid hormone receptors in the median eminence (ME) suggested that photoperiodically generated T_3 acts on the ME. Because thyroid hormone is known to play a critical role in the development and plasticity of the central nervous system, in the present study we have examined ultrastructure of the ME in Japanese quail kept in short-day and long-day environments. Immunoelectron microscopy revealed that GnRH nerve terminals are in close proximity to the basal lamina under long-day conditions, and conventional transmission electron microscopy demonstrated the encasement of the terminals by the endfeet of glia under short-day conditions. These morphological changes may regulate photoperiodic GnRH secretion. (*Endocrinology* 145: 4264–4267, 2004)

MOST BIRDS LIVING outside the tropics use changing day length to time their breeding seasons. In most avian species, long photoperiods stimulate gonadal maturation, and gonadal maturation is induced by secretion of GnRH. GnRH, which is essential for reproduction, is synthesized in neuronal cell bodies distributed diffusely in the hypothalamic preoptic area and is secreted from neuroendocrine terminals localized in the external zone of the median eminence (ME). GnRH secretion is induced under long-day (LD) conditions and inhibited under short-day (SD) conditions. However, the molecular mechanism for seasonally regulating GnRH secretion remains unclear. In birds, the mediobasal hypothalamus (MBH) is considered the center for photoperiodic time measurement for the following reasons: 1) lesioning blocks the photoperiodic response of gonads and LH secretion even though the GnRH system is intact (1–3); 2) c-Fos expression is induced by the LD condition (4, 5); 3) deep brain photoreceptors are thought to be localized (6); and 4) it contains expressed circadian clock genes (7). If the center for photoperiodic time measurement is localized in the MBH, it is expected that some molecular events should occur in the MBH when birds experience LD conditions. The Japanese quail (*Coturnix japonica*) is an excellent model for studying photoperiod, and many studies have been performed with this animal over several decades. Using this model animal, we have identified a gene that is responsible for the photoperiodic response of gonads by using differential subtractive hybridization analysis (8). We found that the expression of type 2 deiodinase is induced by LD stimulus in the MBH. Type 2 deiodinase is an enzyme that converts prohormone T_4 to bioactive T_3 within a narrow range (9). The T_3 content of the MBH was higher under LD than SD, whereas such differences were not observed in other brain regions and plasma. Furthermore, we found that the expression of thyroid hormone receptors (α, β, and retinoid X receptor α) in the basal tuberal hypothalamus, consisting of the ME and the infundibular nucleus (8). These results indicated that T_3 generated locally in response to the LD stimulus acts on the basal tuberal hypothalamus. Thyroid hormones are known to be critically involved in the development, plasticity, and function of the central nervous system (9). Because it was suggested that photoperiodic GnRH release could be controlled at the GnRH nerve terminals by glia (5, 10), it is expected that some morphological change between LD and SD may be produced by the T_3 in the ME. In the present study, therefore, we examined the ultrastructures of GnRH nerve terminals and glial processes in the ME of birds exposed to LD and SD conditions.

Materials and Methods

Animals and housing

Male 4-wk-old Japanese quail were obtained from a local dealer and kept under SD (8 h of light, 16 h of darkness) at 24 ± 1 C in light-tight boxes (55 × 210 × 62 cm). Light was supplied by fluorescent lamps with a light intensity of 200 lux measured at the level of the bird's head. The SD birds were kept in SD (8 h of light and 16 h of darkness) conditions, and LD birds were transferred to LD (16 h of light and 8 h of darkness) from SD for 2–3 wk. Food and water were provided *ad libitum*. Five birds from the SD and LD groups were used for immunoelectron microscopy; four birds from the SD and LD groups were used for conventional transmission electron microscopy (TEM). Birds were treated in accordance with the guidelines of Nagoya University.

Perfusion

Birds were anesthetized with diethyl ether and perfused intracardially with heparinized physiological saline for 5 min, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer at pH 7.4 for 5 min or Zamboni's fixative (4% PFA in 0.1 M phosphate buffer containing 15%

Abbreviations: LD, Long day; MBH, mediobasal hypothalamus; ME, median eminence; PFA, paraformaldehyde; SD, short day; TEM, transmission electron microscopy.

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saturated picric acid) at pH 7.6 for 5 min. All perfusions were performed at the midpoint of the light phase. After perfusion, brains were removed and postfixed overnight in PFA at 4 C.

Immunocytochemistry

The brain was blocked and embedded in a yolk-gelatin matrix to facilitate Vibratome (Technical Products International, St. Louis, MO) sectioning. Egg gelatin-embedded blocks were cut coronally on a Vibratome at 50 μm. Sections containing the ME were collected and washed several times in PBS. Free-floating sections were incubated with 10% normal goat serum for 30 min at room temperature. The sections were incubated with primary antibodies against GnRH (LRH-13, supplied by Dr. Park, University of Tokyo, Tokyo, Japan; 1:2000) for 72 h at 4 C. Thereafter, sections were treated with secondary antibodies of biotinylated antimouse IgG (1:50; DakoCytomation, Carpinteria, CA) for 1.5 h at room temperature and then with streptavidin labeled with peroxidase (1:80, DakoCytomation) for 1.5 h at room temperature. The coloring reaction was performed with a 0.1% solution of 3,3-diaminobenzidine containing 0.015% H_2O_2. Between each step, sections were carefully rinsed in 0.1 M PBS, except for the incubations in normal serum and primary antibodies.

Electron microscopy

Vibratome sections run for immunoelectron microscopy and conventional TEM were placed in 1% osmium tetroxide in 0.1 M PBS for 1.5 h at 4 C, washed three times for 10 min each time in PBS, dehydrated through a series of graded alcohols and propylene oxide, and infiltrated overnight in Araldite. Osmicated sections were flat-embedded in Araldite on glass slides with silicone rubber (1 mm thick). The regions of the ME were cut out of the Araldite-embedded sections with a pointed piece of razor blade and pyramid-embedded in Araldite for ultrathin (90 nm) sectioning. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEM-1210; JEOL, Peabody, MA).

Analysis

Image analysis was carried out using NIH Image software run on a Macintosh computer (Apple Computer, Cupertino, CA). All measurements were confined to a distance of 5 μm or less from the basal lamina. The distance of GnRH nerve terminals in the external zone of the ME to the basal lamina was measured by drawing a line on the NIH image from the GnRH nerve terminal to the closest basal lamina. A total of 30 immunoreactive terminals, which were selected at random, were analyzed for each animal (*i.e.* a total of 150 immunoreactive terminals were analyzed in SD and LD birds, respectively). After examination of individual variations in each group by one-way ANOVA, mean values were compared between LD and SD animals (n = 5). In the conventional TEM analysis, five representative photomicrographs for each animal were analyzed (*i.e.* a total of 20 representative photomicrographs for each light condition were analyzed). Glial processes were distinguished according to the method described by Ganten and Pfaff (11). The lengths of the basal lamina and glial processes contacting the basal lamina were measured with a curvimeter (Uchida, Tokyo, Japan). The area of glial processes was measured by tracing perimeters of glial processes on NIH image. After examination of individual variations in each group by one-way ANOVA, mean values were compared between LD and SD animals (n = 4).

Results

Distance between GnRH nerve terminal and the basal lamina

Immunoelectron microscopy revealed the difference in the distance between GnRH nerve terminals and the basal lamina in the external zone of the ME in SD and LD birds (Figs. 1 and 2). No statistically significant individual variation was observed in any group [by one-way ANOVA: LD, $F(4,145) = 1.791$; $P > 0.05$; SD, $F(4,145) = 2.427$; $P > 0.05$]. The mean distance in LD birds (2.30 ± 0.16 μm; n = 5) was shorter than that in SD birds (2.91 ± 0.16 μm; n = 5; Figs. 1 and 2A; by Mann-Whitney *U* test, $P < 0.05$). The proportion of GnRH nerve terminals localized within 1 μm of the basal lamina was larger in LD than in SD birds (Fig. 2B).

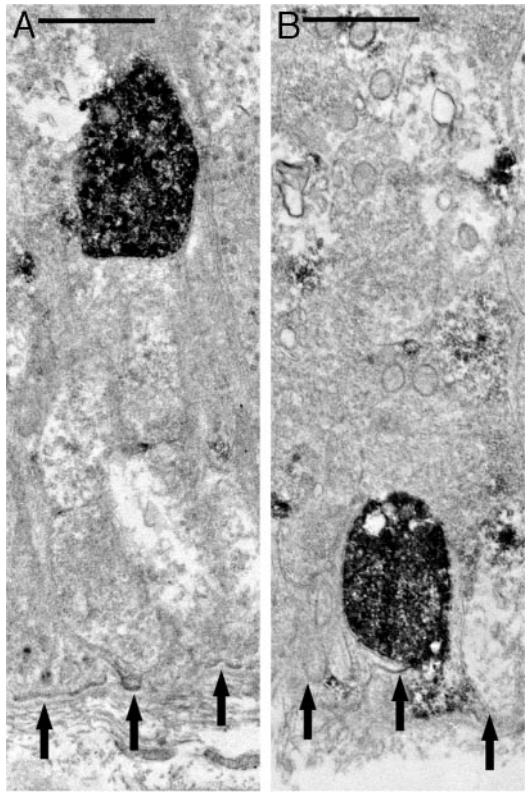

FIG. 1. Representative electron micrographs of GnRH terminals in the ME under SD (A) and LD (B) conditions. *Arrows* indicate the basal lamina. *Scale bar*, 1 μm.

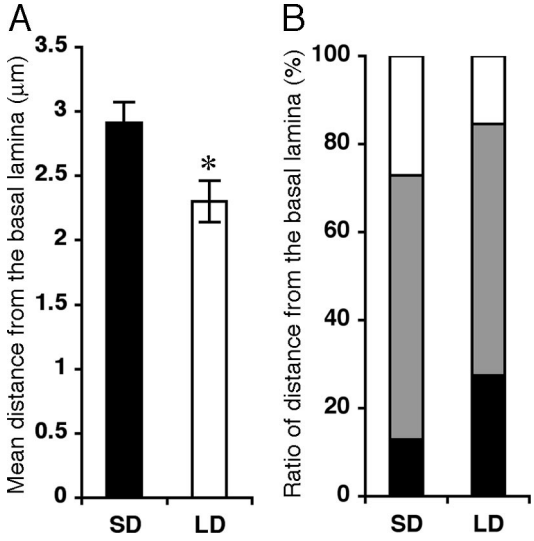

FIG. 2. Changes in the distance of GnRH nerve terminals to the basal lamina between SD and LD conditions. A, Mean ± SEM distance of GnRH terminals (n = 5) from the basal lamina. *, $P < 0.05$, by Mann-Whitney *U* test. B, Distribution of GnRH terminal (percentage localized: ■, 0–1 μm; ▨, 1–4 μm; □, 4–5 μm from the basal lamina).

Morphological changes in glial processes

Conventional TEM demonstrated a dynamic change in glial processes in the external zone of the ME between SD and LD birds. In SD conditions, nerve endings were literally embedded within endfeet of glial processes (Fig. 3, A and C). In contrast, such a phenomenon could hardly be observed under LD conditions (Fig. 3, B and D). The endfeet of glial processes make physical contact with 84.0 ± 2.98% (n = 4) of the basal lamina under SD conditions (Fig. 4A). In contrast, this percentage was reduced to 63.9 ± 3.82% (n = 4) in birds subjected to LD conditions (Fig. 4A; by Mann-Whitney *U* test, $P < 0.05$; n = 4). No statistically significant individual variation was observed in each group [by one-way ANOVA: LD, $F(3,16) = 2.97$; $P > 0.05$; SD, $F(3,16) = 1.585$; $P > 0.05$]. Furthermore, glial processes accounted for 31.7 ± 0.97% (n = 4) of the external zone of the ME in SD conditions, whereas the area of glial processes was reduced to 24.3 ± 1.22% (n = 4) in LD conditions (Fig. 4B; by Mann-Whitney *U* test, $P < 0.05$; n = 4). No statistically significant individual variation was observed in any group [by one-way ANOVA: LD, $F(3,16) = 0.526$; $P > 0.05$; SD, $F(3,16) = 0.33$; $P > 0.05$].

Discussion

In the present study we found dynamic seasonal morphological changes in the ME. GnRH nerve terminals were in close proximity to the basal lamina in birds subjected to LD conditions. The encasement of the GnRH terminal by the endfeet of glia that was observed in SD birds could barely be seen in LD birds. It is considered that neuroendocrine terminals containing GnRH terminals need to contact the pericapillary space (*i.e.* the basal lamina) so that they can secrete the neurohormone into portal blood (12). Therefore, these morphological changes seem designed to allow the neurons to secrete GnRH into the perivascular area surrounding the fenestrated portal capillaries. Several groups have proposed that glia may be involved in regulating the release of hypothalamic factors by controlling the space in the superficial area abutting the portal vessels (12–19). Our data from the present study provide additional evidence to support their hypothesis.

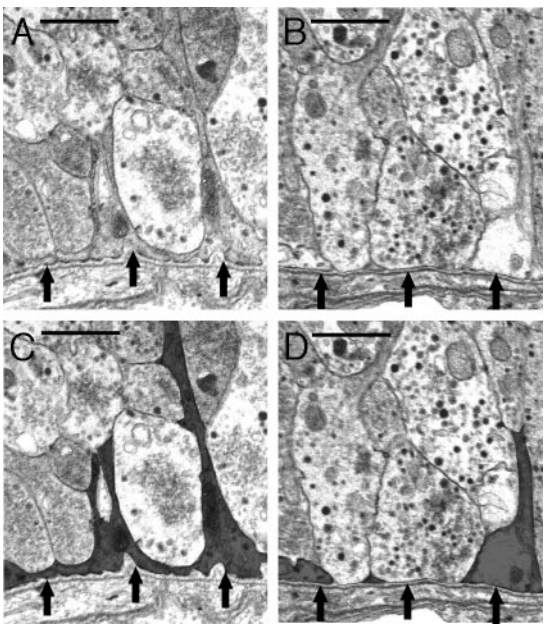

FIG. 3. The encasement of nerve terminals by the endfeet of glia under SD conditions. A and B, Representative electron micrographs analyzed by conventional TEM under SD (A) and LD (B) conditions. C and D, Glial endfeet are indicated by *gray painting*. *Arrows* indicate the basal lamina. *Scale bar*, 1 μm.

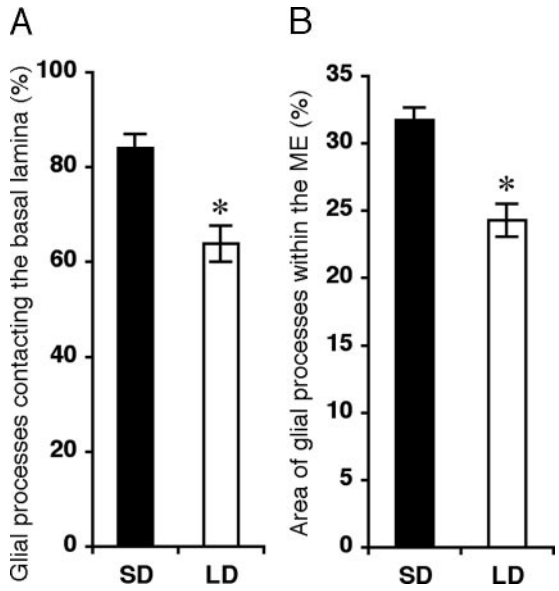

FIG. 4. Dynamic changes in glial processes in the external zone of the ME. A, Quantification of the proportion of the endfeet of glia contacting the basal lamina. B, Calculated percent area of the ME occupied by glial processes. Values indicate the mean ± SEM (n = 4). *, $P < 0.05$, by Mann-Whitney *U* test.

Meddle and Follett (5) have shown that c-Fos is expressed in the ME under the stimulus of LD conditions. It is particularly noteworthy that this photoperiodic activation of Fos-like immunoreactivity is observed within the glia. In mammals, seasonal information is converted to the humoral factor melatonin, which is secreted from the pineal gland during the night. Melatonin is critical for the regulation of seasonal reproduction, and studies of the seasonal plasticity of the GnRH system have focused on the ultrastructure of GnRH cell bodies, but not the terminals (20, 21). In birds, however, melatonin has no role in the photoperiodic regulation of reproduction (22), and photic stimuli modulating seasonal reproduction are directly detected by nonretinal, nonpineal, deep brain photoreceptors (23). Saldanha *et al.* (24) reported the close apposition of the opsin terminals, GnRH terminals, and glial endfeet in the ME and proposed that secretion from the opsin terminal modulates the release of GnRH. Together with the present study, these reports support the hypothesis that the photoperiodic response in birds is regulated primarily at the level of secretion of GnRH rather than by the synthesis of GnRH in the cell bodies (5, 10).

In the present study we reported seasonal morphological changes in the relationship between GnRH nerve terminals and the endfeet of glial processes. Thus, neuro-glial plasticity may regulate GnRH release, gonadotropin secretion, and gonadal growth. The molecular link between this neuro-glial

plasticity and photoperiodically generated T_3 remains to be clarified in future studies.

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