CHANGES IN THE PINEAL GLAND, LH-RH NEURON SYSTEM AND PITUITARY-GONADAL AXIS IN GOLDEN HAMSTERS UNDER ARTIFICIAL WINTER CONDITIONS

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ABSTRACT

Male golden hamsters were exposed gradually to low temperatures and short photoperiods in a climatic chamber, and kept finally in an environment of 5°C and 3 h light/21 h darkness (3L/21D) for more than 5 weeks. Control animals were maintained at 20°C and 14L/10D or 12L/12D. The following changes were induced in animals kept under the artificial winter conditions. 1) Pinealocytes showed proliferation of the smooth- and rough-surfaced endoplasmic reticulum (sER and rER) and marked development of the Golgi apparatus, indicating an enhanced cellular function. Secretory granules were increased in cell processes, and lipid droplets and myeloid bodies were frequently observed. 2) LH-RH-positive neurons in the brain were immunostained more numerous than in controls, in agreement with a significant increase in hypothalamic LH-RH demonstrated by radioimmunoassay. 3) Pituitary gonadotrophs showed signs of suppressed secretory activity, such as accumulation of granules, whereas mammotrophs showed signs of activation including a marked development of the rER and Golgi apparatus. 4) Testes and accessory sex organs exhibited significant atrophy, whose weight was decreased to 13% and to 39% of that in the respective controls. It was assumed that pineal hormones secreted excessively under the experimental winter condition inhibited the discharge of hypothalamic LH-RH, resulting in a suppression of gonadotropin release and subsequent gonadal atrophy.

The mammalian pineal gland is a neuroendocrine transducer, which secretes an antigonadotropic hormone, melatonin, in response to environmental light or temperature; a possible involvement of this organ in the annual rhythm of animals was postulated by several authors (5, 11, 18).

We have been very interested in the relationship between the pineal gland and the hypothalamus-pituitary-gonadal axis, and examined the changes in the pineal gland of gonadectomized or LH-RH-treated rabbits, as well as the pituitary gland of melatonin-treated rabbits (20).

In some hibernating rodents, the gonadal system involutes completely in the winter season, but the involution is prevented by pinealectomy (5). Because of a difficulty in obtaining naturally hibernating rodents in Japan, we exposed golden hamsters to artificial winter conditions in a climatic chamber, and examined their pineal glands, brains, pituitary glands and gonads (21-23).

MATERIALS AND METHODS

Animals and Artificial Winter Conditions

Seventy male golden hamsters (*Mesocricetus*

auratus) weighing about 100 g were used. They were housed individually in plastic cages and allowed free access to an MF pellet diet (Oriental Yeast, Tokyo) and water. After keeping them for some period in an animal room at 20°C and 14 h light/10 h darkness (14L/10D; lights on at 0700) or 12L/12D (lights on at 0800), the experimental group was transferred to a climatic chamber and exposed gradually to low temperatures and short photoperiods to avoid the effect of cold stress, according to the following protocol: week 1, 15°C and 9L/15D (lights on at 0930); week 2, 10°C and 6L/18D (lights on at 1100); weeks 3 to 8, 5°C and 3L/21D (lights on at 1230). The control group was maintained in the animal room stated above. At the end of the experiment, 10 to 12 animals from the experimental and control groups were killed at 1400 under pentobarbital anesthesia by decapitation or cardiac perfusion of a fixative. Pituitary glands, testes, accessory sex organs and adrenal glands were weighed, and changes in the relative weight were statistically evaluated using the Student's *t* test.

Electron Microscopy

Animals were perfused through the heart with 2% glutaraldehyde in 0.1 M phosphate buffer under pentobarbital anesthesia. Organs were removed, cut into small blocks, postfixed with 2% OsO₄, dehydrated, and embedded in the Epon 812. Ultrathin sections were cut on an LKB or a Reichert ultramicrotome, stained with uranyl acetate and lead hydroxide, and examined with a Hitachi electron microscope model HU-12.

Immunohistochemistry

For immunohistochemical examination of the LH-RH neuron system, animals were perfused through the heart with ice-cold saline, followed by Zamboni's fixative, under pentobarbital anesthesia. Brains were quickly removed, immersed in the same fixative for 12 h, and rinsed in phosphate buffer containing 30% sucrose. Serial frontal sections were cut in a cryostat at a thickness of 10 µm. For the demonstration of LH-RH-like immunoreactivity, the indirect immunofluorescence method (3) was used. All the sections were incubated in cold phosphate-buffered saline (PBS) for 10 min, and then incubated with an LH-RH antiserum (diluted to 1:2,000 with PBS) overnight at room temperature in a humid atmosphere. Sections were rinsed 3 times, each for 10 min, with PBS containing 0.05% Triton

X-100, and then incubated for 24 h with fluorescein isothiocyanate-conjugated anti-rabbit IgG goat serum diluted to 1:1,000 with PBS. Sections were rinsed 3 times, each for 10 min, with PBS and mounted in a PBS-glycerine mixture (1:1).

For immunohistochemical identification of the golden hamster gonadotrophs, animals were perfused with Zamboni's fixative containing 0.6% glutaraldehyde under pentobarbital anesthesia. The anterior pituitary gland was removed, immersed in the fixative without glutaraldehyde for 24 h, and cut at 20 µm with a vibratome (Oxford). Immunohistochemical staining was carried out using the peroxidase-antiperoxidase (PAP) method (24), incubating the sections successively in 1) normal goat serum at a dilution of 1:10, 2) rabbit antiserum against rat LH (1:1,000), 3) goat antiserum against rabbit IgG (1:40), 4) PAP complex solution (1:50), 5) 0.025% 3,3'-diaminobenzidine (DAB) in 0.05 M Tris buffer, and 6) DAB solution containing 0.005% H₂O₂. Sections were then postfixed with 1% OsO₄, dehydrated and embedded in the Epon 812. Ultrathin sections were examined without uranyl acetate or lead hydroxide staining.

Radioimmunoassay for LH-RH

Animals were decapitated under pentobarbital anesthesia and brains were removed quickly. The hypothalamus, extending from the rostral border of the optic chiasm to the caudal edge of the mamillary body, was excised, weighed, and homogenized in 3 ml of 0.1 N HCl, and centrifuged for 15 min at 9,000 g. The supernatant was mixed with 3 vol of methanol, evaporated to dryness under a stream of air in a water bath at 40°C, and stored frozen until assayed. After reconstitution with PBS, LH-RH was determined by radioimmunoassay (12). Results were evaluated using the Student's *t* test.

RESULTS

The Pineal Gland

The pinealocyte was irregular in shape and extended a long, thin process to the intercellular space (Fig. 1). The nucleus was ovoid or indented, and the nucleolus was usually prominent. The cytoplasm contained many mitochondria, most of which were cylindrical in shape, but some were spherical and had tubular cristae. The smooth-surfaced endoplasmic reticulum (sER), which was occasionally arranged concentrically, was more abundant than the rough-surfaced

endoplasmic reticulum (rER). The Golgi apparatus was moderately developed and located near the nucleus. Secretory granules were 80–110 nm in diameter and of high electron density.

On and after the 5th week of the artificial winter, most pinealocytes showed signs of enhanced

cellular function. The sER and rER proliferated strikingly (Fig. 2). The Golgi apparatus was markedly developed (Fig. 3), and secretory granules increased in number, especially in cell processes (Fig. 4).

Myeloid bodies were occasionally found in the

Fig. 1 An overall view of a golden hamster pinealocyte in the control group. Arrowheads indicate the outline of the cell. N, nucleus; P, process. $\times 4,900$

Fig. 2 Proliferation of the smooth-surfaced endoplasmic reticulum (sER) and rough-surfaced endoplasmic reticulum (rER) in the pinealocyte in the 6th week of the artificial winter. $\times 7,200$

Fig. 3 The well-developed Golgi apparatus (G) in the pinealocyte in the 5th week of the artificial winter. $\times 9,000$

Fig. 4 Increased secretory granules (S) in processes (P) of pinealocytes in the 7th week of the artificial winter. $\times 7,200$

control pinealocyte, but were more frequently observed in animals under the winter condition (Fig. 5). Lipid droplets also increased in number (Fig. 6).

The LH-RH Neuron System in the Brain

Immunohistochemistry LH-RH-positive perikarya were noted in the diagonal band, medial and lateral septal nuclei, medial preoptic area, and anterior hypothalamic nucleus. LH-RH-positive fibers were distributed, in addition to the above-mentioned areas, in the organum vasculosum laminae terminalis, medial hypothalamic area and most densely in the external layer of the median eminence. Extrahypothalamic LH-RH-positive fibers were observed in the medial and central amygdaloid nuclei, bed nucleus of stria terminalis, stria terminalis, and stria medullaris (Fig. 7).

When brain sections of the experimental and control groups were subjected simultaneously to the immunohistochemical procedure, LH-RH-positive perikarya and fibers were immunostained more numerous in the experimental group than in the control.

Radioimmunoassay In agreement with immunohistochemical results, radioimmunoassay confirmed a significant rise in LH-RH in the hypothalamus of the experimental group (Table 1).

The Anterior Pituitary Gland

Gonadotrophs Golden hamster gonadotrophs, identified immunohistochemically by us, were characterized by the vacuole-dilated rER, well-developed Golgi apparatus, and secretory

granules of medium electron density, about 180–200 nm in diameter (Fig. 8).

After the 5th week of the artificial winter, secretory granules predominantly accumulated in the cytoplasm of most gonadotrophs, whereas the lumen of the rER was no longer dilated and the Golgi apparatus was very poor, indicating suppressed secretory activity (Fig. 9).

Mammotrophs Golden hamster mammotrophs, classified by Dekker (4) and Thompson *et al.* (25), were characterized by large, sometimes irregular-shaped secretory granules of low electron density, about 400–600 nm in diameter. The rER and the Golgi apparatus were moderately developed (Fig. 10).

After the 5th week of the artificial winter, the Golgi apparatus was developed markedly and secretory granules decreased in number in most mammotrophs, indicating enhanced synthesis and release of prolactin (Fig. 11).

The Gonadal System

On and after the 5th week of the artificial winter, a drastic gonadal atrophy was noticed even on macroscopic examination (Fig. 12). Statistical analysis revealed that the relative weights of one testis and its accessory sex organs in the 7th week of the experiment decreased to 13% and 39% of their respective controls (Table 2).

Seminiferous tubules of the control animal were lined by thick epithelium comprised of various stages of spermatogenic cells (Fig. 13). Leydig cells were characterized by plenty of mitochondria and well-developed sER. The nucleus was fairly large and the nucleolus was usually prominent (Fig. 15).

After the 5th week of the artificial winter, the diameter of seminiferous tubules was strikingly reduced in accordance with the testicular weight. The germinal epithelium was very thin and no spermatozoon was noticed (Fig. 14). Leydig cells markedly shrunk with a dark, irregular nucleus. Mitochondria appeared deformed and dark, and the sER was no longer seen, indicating suppressed cellular function (Fig. 16).

Table 1 *The Hypothalamic LH-RH Content*

Group	n	LH-RH (pg/hypothalamus)
Control	10	456.6±36.8
Artificial winter	12	642.5±47.1*

Means ± SE are given. * $P < 0.01$

Table 2 *Organ Weights in Golden Hamsters in the 7th Week of the Artificial Winter*

Group	n	Body weight (g)	Anterior pituitary gland†	Testis (unilateral)†	Accessory sex organs†	Adrenal gland (unilateral)†
Control	12	102.8±3.5	2.4±0.2	1,307.5±74.5	961.3±96.8	10.6±0.3
Artificial winter	12	97.8±1.8	2.0±0.1	167.4±8.4*	374.3±34.2**	11.7±0.4

Means ± SE are given. † mg/100 g of body weight. * $P < 0.001$. ** $P < 0.01$

Fig. 5 Myeloid bodies (M) were often seen in the pinealocytes of the experimental group. The same animal as in Fig. 3. $\times 7,200$

Fig. 6 Increased lipid droplets (L) in the pinealocyte of the experimental group. The same animal as in Fig. 4. $\times 7,200$

Fig. 7 Distribution of LH-RH-positive perikarya (large dots on the left) and fibers (small dots on the right) in the serial frontal sections of the golden hamster brain, arranged rostrally from a to l. AH, anterior hypothalamic nucleus; CA, central amygdaloid nucleus; DB, diagonal band (Broca); LS, lateral septal nucleus; MA, medial amygdaloid nucleus; ME, median eminence; MS, medial septal nucleus; PO, medial preoptic area; SM, stria medullaris; ST, stria terminalis; STb, bed nucleus of stria terminalis.

Other Findings

Spontaneous movements of golden hamsters measured by Animex (LKB) were higher in the dark than in the light. However, when animals were introduced in the environment of 5°C and 3L/21D, movements in both dark and light periods were remarkably reduced. They seemed to sleep continuously, but were awakened by touch-

ing them with fingers. The mean body (colonal) temperature of golden hamsters in the 8th week of the artificial winter was 36.85°C, whereas that in the control was 37.5°C.

No significant difference was noted between the two control groups, *i.e.* 14L/10D and 12L/12D.

Fig. 8 Part of a gonadotroph of a control animal. The vacuole-dilated rER and well-developed Golgi apparatus (G) are noted. S: secretory granules. $\times 9,000$

Fig. 9 Part of a gonadotroph of an animal in the 8th week of the experiment. Massive accumulation of secretory granules (S) is seen. $\times 9,000$

Fig. 10 Mammotroph in a control animal. S, secretory granules. $\times 9,000$

Fig. 11 Mammotroph in an animal in the 8th week of the experiment. The same animal as in Fig. 9. The Golgi apparatus (G) is well developed. $\times 9,000$

DISCUSSION

The pineal gland of the golden hamster kept under artificial winter conditions showed morphological signs of enhanced cellular function. Melatonin, a pineal hormone, is secreted exclusi-

vely during the dark period of the day. Serotonin-*N*-acetyltransferase which synthesizes melatonin is suppressed by light stimulation (7), that reaches the pineal gland from the retina via the postganglionic sympathetic nerve fibers and/or via the recently postulated central pinealopetal

Fig. 12 Macroscopic changes in testes observed from the first week to the 6th week of the artificial winter, as compared with the control (Cont). In each week group, bilateral testes from 2 animals are shown.

Fig. 13 Seminiferous tubules in a control animal. $\times 140$

Fig. 14 Atrophied seminiferous tubules in an animal in the 6th week of the experiment. $\times 140$

Fig. 15 Leydig cell in a control animal. Many mitochondria (Mt) and prominent sER are noted. $\times 10,800$

Fig. 16 Leydig cell in an animal in the 7th week of the experiment. Only dark mitochondria (Mt) are contained. $\times 10,800$

fibers (8, 13-15). In our experiment, a prolonged dark period (21 h per day) seemed to stimulate pineal hormone secretion intensively. Low temperature is also reported to stimulate pineal activity independently from lighting conditions (17). In our experiment, a lowered temperature

(5°C) was considered to enhance pineal function in addition to a prolonged dark period. Similar morphological changes in the pineal gland, suggesting an augmented function, were reported in male hamsters 8 weeks after blinding (10), and in female hamsters after blinding or afternoon

melatonin injection (26); gonadal atrophy was prominent in both cases.

Numbers of secretory granules in the pinealocyte fluctuate within a day, being largest at 1200 and lowest at 2400 (19). In our experiment, it is likely that numerous granules were synthesized during the prolonged dark period, but their release from the process terminals was suppressed, because animals were killed at 1400, the mid-point of the light period. The significance of an increase in myeloid bodies is not yet clear, but the increase was noticed also in the pinealocyte in cold-exposed hamsters (1), and in the hedgehog pinealocyte in November and December, when the animal is sexually quiescent (16). Changes in the amount of lipid droplets in pinealocytes under various conditions have been extensively reviewed (27), but the physiological significance of these changes is still unknown. An increase in the number of lipid droplets in pinealocytes was reported in rats kept under continuous darkness (9), and female hamsters after blinding or melatonin treatment (26). It seems likely that an increase in the number of myeloid bodies or lipid droplets in pinealocytes of hamsters under artificial winter conditions reflects enhanced cellular metabolism accompanied by an excessive hormone secretion.

Effects of melatonin in mammals is now widely accepted to be antigonadotropic in nature, especially in long-day breeding animals, but the precise site of its action is not clear. However, because the direct infusion of melatonin into the anterior pituitary gland was without effect (6), and because specific binding of melatonin was found in the basal medial hypothalamus (2), melatonin seems to act on the hypothalamic level. Distribution of LH-RH-positive neural structures in golden hamsters was essentially similar to that in other rodents. Besides the neural inputs to these neurons, humoral factors including melatonin, gonadotropin or sex steroids, may also play important roles in modulating activity, directly or indirectly.

In the present study, immunohistochemistry and radioimmunoassay revealed a significantly enhanced level of LH-RH in animals under artificial winter conditions. It is assumed that melatonin intensively secreted under these conditions inhibits the discharge of LH-RH into the hypophyseal portal vessels, while a decrease in sex steroids stimulates LH-RH synthesis. Suppressed function of the gonadotrophs in the experimental group can be explained by the inhibition of LH-RH discharge, but the hyperfunction of the mammothrophs can not be explained

at present, because we have no data on the hypothalamic PIH or PRH. Although there is a controversy about pineal effects on prolactin secretion (18), the above finding is in agreement with our previous results that mammothrophs exhibit signs of increased function after chronic administration of pineal hormone in rabbits (20). The severe involution of testes may be caused by a deficiency of the pituitary gonadotropin, and the atrophy of the accessory sex organs may result from a decreased secretion of sex steroids from Leydig cells.

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