Changes in the Gonadotropin-Releasing Hormone (GnRH) Neuronal System during the Annual Reproductive Cycle of the Horseshoe Bat, Rhinolophus ferrumequinum

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Changes in the Gonadotropin-Releasing Hormone (GnRH) Neuronal System during the Annual Reproductive Cycle of the Horseshoe Bat, *Rhinolophus ferrumequinum*

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ABSTRACT—Unique reproductive phenomena, such as delayed fertilization in females and asynchrony between spermatogenesis and mating behavior in males, are known in hibernating bats. The present study was undertaken to examine sex differences and seasonal changes in the gonadotropin-releasing hormone (GnRH)-immunoreactive (ir) neuronal system of Japanese bats, *Rhinolophus ferrumequinum*. GnRH-ir neurons were preferentially distributed in the medial preoptic area (POA) and medial basal hypothalamus (MBH). The number and immunoreactivity of GnRH-ir neurons decreased in summer (lactation period in females and spermatogenic period in males), whereas both increased dramatically in winter (hibernation period). The number and immunoreactivity of GnRH-ir neurons varied more in the MBH than in the POA throughout the annual reproductive cycle of both sexes. The changes in GnRH neurons in the MBH closely paralleled those of GnRH-ir fibers in the median eminence (ME), which is the release site for GnRH. In contrast, there was no sex difference in the number and immunoreactivity of GnRH-ir neuronal perikarya in either region except for the number of GnRH-ir neurons in the POA in spring and summer. These findings suggest that GnRH neurons in the MBH supply the major GnRH innervation to the ME and play a central role in seasonal regulation of gonadotropin secretion in this bat.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a hypothalamic neuropeptide that plays a central role in the control of reproductive function in vertebrates. This decapeptide is the primary signal that modulates the biosynthesis and secretion of pituitary gonadotropins required for gametogenesis and gonadal steroidogenesis. The structure of this peptide is conserved among mammals and exhibits only minor amino acid substitutions in some lower vertebrate groups (King and Millar, 1980, 1995; White *et al*., 1995). Although the distribution of GnRH-producing neurons and their function have been examined in several orders of mammals (Silverman *et al*., 1979; Hoffman, 1983; Shivers *et al*., 1983; Anthony *et al*., 1984), this neurosecretory system in bats, Chiroptera, the second largest order of the class Mammalia, has been described by only a few investigators (King *et al*., 1984; Mikami *et al*., 1988; Fernández *et al*., 1992; Oelschläger and Northcutt,1992).

Most bats living in the temperate zone show an unusual reproductive phenomenon (delayed implantation or delayed ovulation in females) that is temporarily interrupted by daily torpor in the late autumn and subsequent hibernation during winter. However, little information is available in either sex on the activity of the GnRH neuronal system associated with season and reproductive condition. We used the Japanese horseshoe bat, *Rhinolophus ferrumequinum nippon*, in the present study because they are common in ordinary dwellings in Toyama Prefecture and the sexually mature adults are easy to distinguish. This species exhibits the reproductive phenomenon of delayed ovulation (Oxberry, 1979; Uchida and Mōri, 1987), i.e., female bats begin hibernation after mating in autumn and store spermatozoa within their reproductive tracts until the next spring. After arousal in spring, ovulation and subsequent fertilization occur. In male bats, spermatogenesis occurs in summer, but mating behavior and maximal hypertrophy of accessory sex glands are delayed until autumn (Gustafson, 1979).

The present study investigated whether changes in the number and immunoreactivity of the GnRH neuronal system are associated with the annual reproductive cycle in bats of both sexes, using immunocytochemistry.

MATERIALS AND METHODS

Animals and tissue preparations

Eighteen adult male and 17 adult female horseshoe bats, *Rhinolophus ferrumequinum*, were collected at natural roosting sites (36°7'N, 147°E) in Takaoka City, Toyama Prefecture, from August 1995 through January 1997. The animals were captured in late spring (early June, 4 males and 4 pregnant females), summer (from late July to August, 4 males and 4 lactating females), autumn (October, 4 males and 4 females with copulation plugs) and winter (January, 6

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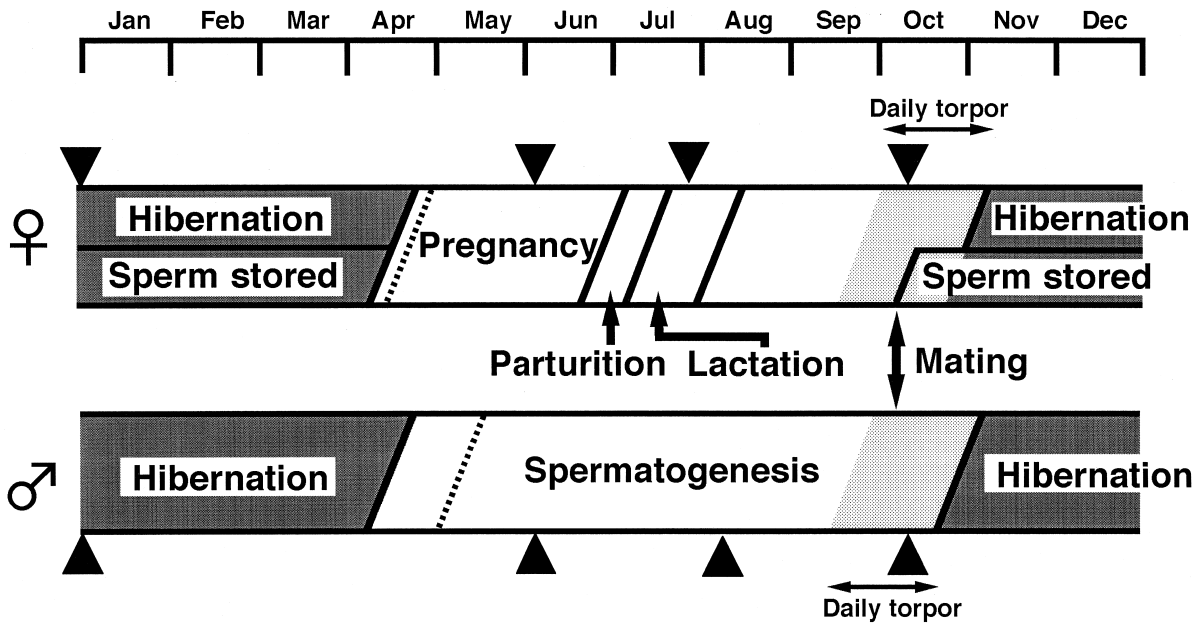

Fig. 1. Annual reproductive cycle of the horseshoe bat, *Rhinolophus ferrumequinum* in Toyama Prefecture. Arrowheads indicate the period of capture in this study. The beginning of pregnancy in the female and spermatogenesis in the male is not determined.

males and 5 females during hibernation). Figure 1 represents the annual reproductive cycle of the bats used in the present study. The cycles of their annual behavior and reproductive events were examined from June 1994 through April 1997.

After capture, the bats were carefully transported to the laboratory for minimized stress. The bats were killed by decapitation under ether anesthesia within 3 hr after capture and their blood was collected. Separated serum was stored at –30°C for separate experiments. Brains were removed, fixed in Bouin's fluid for 48 hr and embedded in paraffin after dehydration in a graded series of ethanol. The brain was serially sectioned at 8 μm thickness in the coronal plane. A series of sections from sixteen males and females, respectively, was used for immunocytochemistry of GnRH. Brain sections from two males and one female collected in winter were used for Nissl staining to produce a brain map and for an immunocytochemical absorption test.

Immunocytochemistry

GnRH neurons were immunocytochemically stained by the avidin biotin peroxidase complex (ABC) method using a commercial kit (Vector Laboratories, Inc., Burlingame, CA). The monoclonal mouse GnRH antibody (HAC-MM02-MSM84) was provided by Dr. K. Wakabayashi (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan). This antibody cross-reacts with chicken-I and -II and salmon GnRHs as well as mammalian GnRH (Park and Wakabayashi, 1986). The antibody was used at a final dilution of 1: 6,000 and sections were incubated at room temperature for 90 min. Immunological reagents were diluted with phosphate-buffered saline (PBS) containing 0.05% Tween-20. The reaction products were visualized with 0.01% 3,3'-diaminobenzidine (DAB) in PBS containing 0.005% H_2O_2 and 0.02% $NiCl_2$ (Hsu and Soban, 1982). Control immunocytochemical staining was performed by the addition of 10 μg synthetic GnRH (Peptide Institute, Inc., Osaka) to 1 ml of diluted antiserum.

Cell counts and quantitative image analyses

The total numbers of GnRH-immunoreactive (ir) neurons with a nucleolus were counted in all serial sections from the rostral to caudal brain region. The numbers of GnRH-ir neurons in the medial preoptic area (POA) and medial basal hypothalamus (MBH) were also counted. Thirty GnRH-ir cells (in summer, all cells encountered in the MBH) per animal were selected in each region, and the immunoreactivity and cell body size of the GnRH-ir neurons were quantified with a light microscope equipped with a color video camera (KY-F55B, Victor, Tokyo, Japan) and NIH image software (NIH, Bethesda, MD). The immunoreactivity of individual GnRH-ir neurons was expressed as the mean density (gray level) per cell, which was obtained by subtracting the actual gray level from the gray level of the background. The immunoreactivity of fibers containing GnRH in the median eminence (ME) was examined as described above in 6 sections at 40 μm intervals and the results were expressed as the integrated density per unit area of the ME.

Statistical analyses were performed by the Kruskal-Wallis H test followed by the Mann-Whitney U test.

RESULTS

Distribution of GnRH-ir neurons

GnRH-ir neurons were observed from the diagonal band of Broca (DBB) to the mammillary body (MB), but were particularly abundant in the medial POA and MBH which contains the arcuate nucleus (ARC) (Fig. 2). Single GnRH-ir cells were also scattered in the septal area, DBB, and anterior hypothalamic area (AHA). A few GnRH-ir cells were occasionally observed in the supraoptic nucleus and the internal layer of the ME. Most of these neurons were bipolar with a smooth outline, but neurons with irregular shapes were also encountered, especially in the MBH. These GnRH-ir neurons were observed in all animals throughout the four seasons. GnRH-ir fibers were observed in the vicinity of the third ventricle in the POA and AHA, but they were densely distributed in the organum vasculosum lamina terminalis (OVLT) and ME, the major projection area of GnRH-ir cells.

The frequency with which GnRH-ir cells occurred along

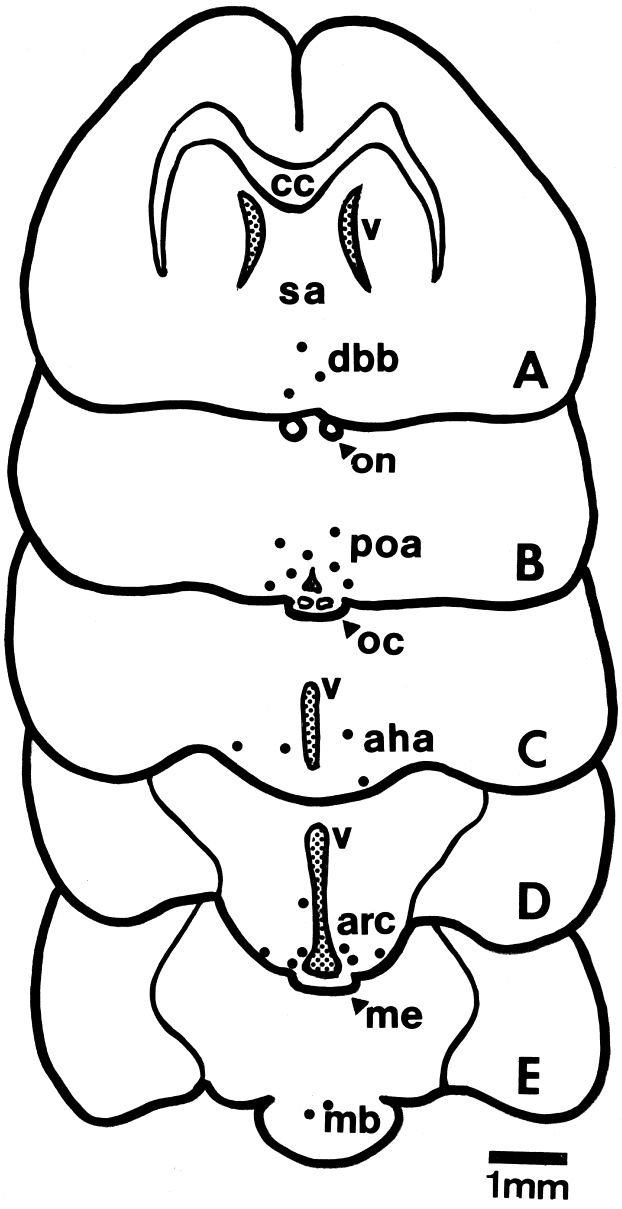

Fig. 2. The distribution of GnRH-ir cells in drawings of coronal sections from the rostral to caudal brain areas (A-E). GnRH-ir cell bodies are represented by solid circles. Abbreviations: aha = anterior hypothalamic area; arc = arcuate nucleus; cc = corpus callosum; dbb = diagonal band of Broca; mb = mamillary body ; me= median eminence; oc = optic chiasma; on = optic nerve; poa = preoptic area; sa = septal area; v = ventricle.

the rostral-caudal axis of the brain was examined in each group (Fig. 3). In both sexes collected in late spring (pregnant period in females and early stage of spermatocytogenesis in males), two evident peaks in cell number were found at levels corresponding to the POA and MBH. These peaks were more prominent in males than in females. However, in summer (lactation period in females and the active period of spermatocytogenesis in males), the distribution frequency of GnRH-ir cell bodies showed a peak at the level where the POA is located. This change was due to a decrease in the number of GnRH-ir cells located in the MBH, especially in the ARC (Fig. 3). In autumn (mating period), the distribution frequency again showed a bimodal pattern. This distribution pattern was more pronounced in winter (hibernation period) than in the other seasons.

Number of GnRH-ir neurons in the brain

Except for animals captured in summer, no sex difference in the total number of GnRH-ir neurons in the whole brain was detected in any season (Fig. 4). The number of GnRH-ir neurons in animals collected in summer was almost the same as that in spring, although the number increased rapidly from autumn to winter ($p < 0.05$ vs. spring or summer) (Fig. 4). In the selected brain regions, the POA and MBH, a sex difference in the number of GnRH-ir neurons was detected in the POA in spring and summer ($p < 0.05$); however, there were no sex differences in the POA during the other seasons or in the MBH at any season (Fig. 5). In contrast, seasonal changes in the number of GnRH-ir neurons were found in both areas. Significant differences in the cell number in the MBH were detected among the four seasons ($p < 0.05$), and the difference was especially marked in summer, when the number decreased dramatically in both sexes (Fig. 5). The number of GnRH-ir neurons in the POA was constant from autumn to winter in both sexes. A significant difference in both sexes was detected only between animals collected in spring and winter ($p < 0.05$) . Thus, seasonal changes in the number of GnRH-ir neurons were greater in the MBH than in the POA of both sexes.

GnRH-immunoreactivity and the size of neuronal perikarya

GnRH-immunoreactivity of the neuronal perikarya was considerably decreased in summer compared with that in spring ($p < 0.05$), specially in the MBH (Figs. 6 and 7). In contrast, the immunoreactivity of neuronal perikarya located in both the POA and MBH was markedly elevated during the hibernation period (winter), especially in neurons located in the MBH ($p < 0.05$ vs. other seasons). In hibernating animals, all neurons in the POA, MBH and other regions accumulated GnRH-ir material in their cell bodies (Fig. 6). Seasonal differences in the GnRH-immunoreactivity of the cell bodies were also more marked in the MBH than in the POA, but these changes were not so marked as those in the cell number.

In contrast, there were no sex differences in the immunoreactivity of neuronal perikarya throughout the four seasons. In addition, there were neither seasonal changes nor sex differences in the size of GnRH-ir neurons located in the POA and MBH.

The density of GnRH-ir fibers in the ME

The density of GnRH-ir fibers in the ME changed considerably with the season in both sexes, although there were no sex differences throughout the annual seasonal cycle (Figs. 6 and 8). GnRH-immunoreactivity per unit area of the ME was very low during the reproductively active season (summer),

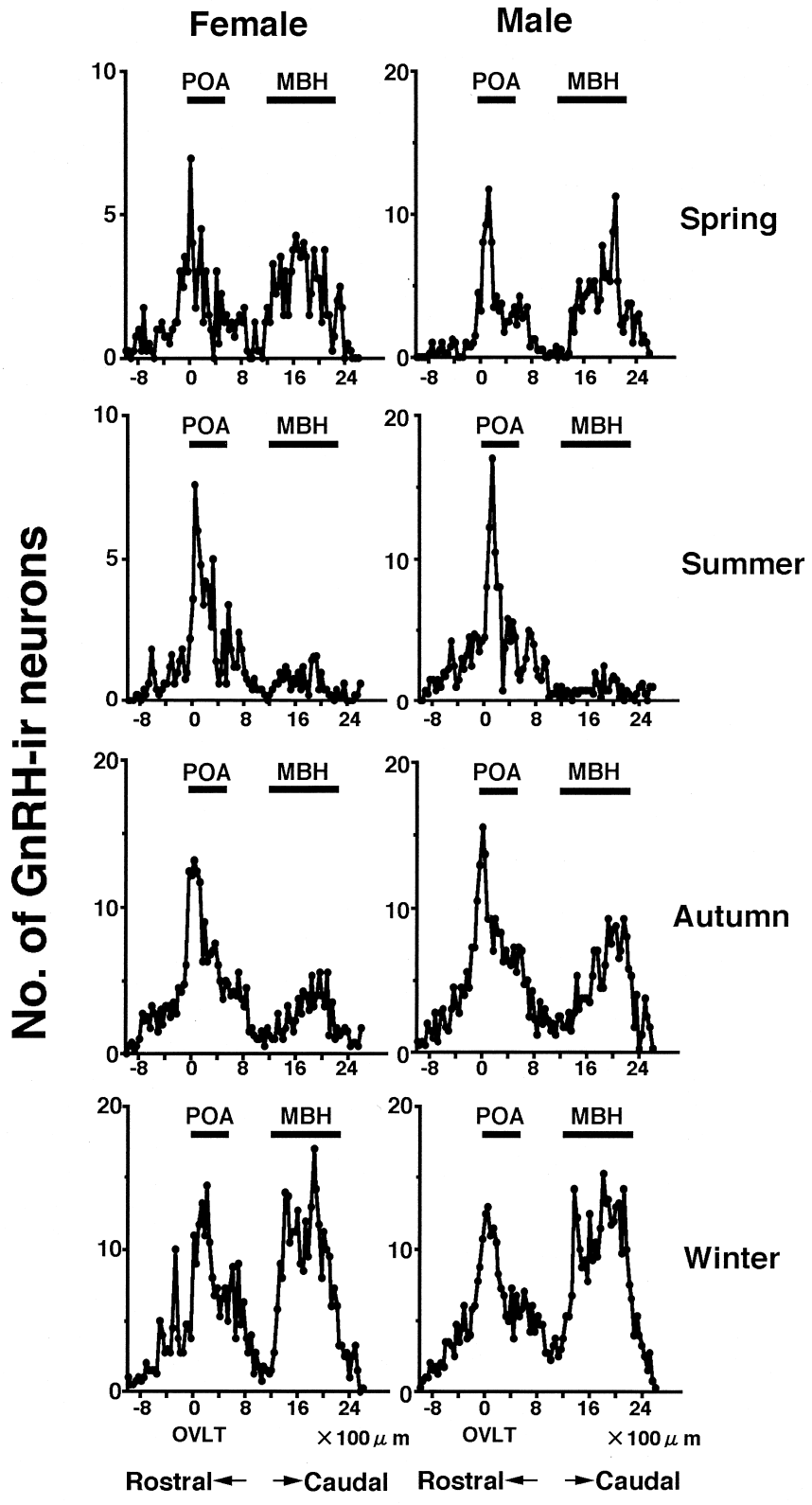

Fig. 3. Mean number of GnRH-ir cell bodies counted per 40-μm interval plotted along the rostral-caudal brain axis in female and male bats. The abscissa shows the distance from the organum vasculosum lamina terminalis (OVLT). Each solid circle indicates the mean value of 4 animals in each season. Bars on the top indicate the position corresponding with the POA and the MBH.

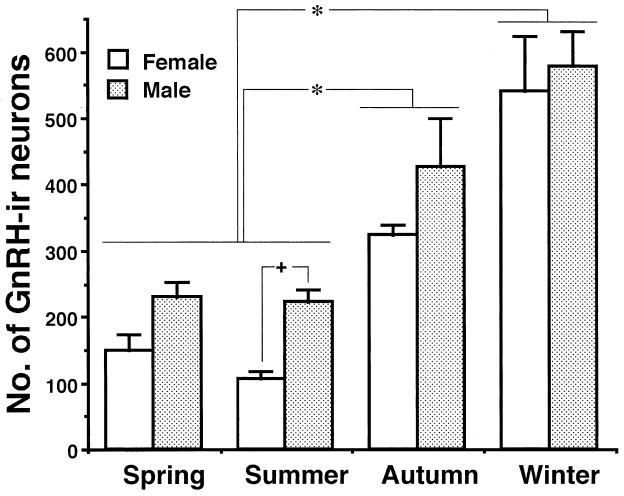

Fig. 4. Total number of GnRH-ir cell bodies in the whole brain area. Each column indicates the mean ± SE of 4 animals in each season. Difference between seasons in the same sex: *, $p < 0.05$. Difference between male and female: +, $p < 0.05$.

but the immunoreactivity was not significantly different from that in the mating period (autumn). GnRH-immunoreactivity in the ME during the hibernation period was about 4 times higher than that in summer. These changes resembled those of the neuronal perikarya in the MBH.

DISCUSSION

In the present study, GnRH-ir neurons of the horseshoe bat were encountered from the DBB to the MB, although they were localized preferentially in the POA and MBH. Immunocytochemical localization of GnRH neurons in the bat brain has been reported by several investigators (Mikami *et al*., 1988; Fernández *et al*., 1992; Oelschläger and Northcutt, 1992). They pointed out that the majority of GnRH-ir cells are localized in the MBH, particularly in the region of the ARC, and that their distribution closely resembles that in higher primates (Parker *et al*., 1980; Silverman *et al*., 1982). In contrast, GnRH-ir neurons in rodent species are concentrated more rostrally, ie., within the DBB, POA and AHA, particularly in the medial POA which is the major source of GnRH fiber terminals in the ME (Silverman *et al*., 1979; Jennes and Stumpf, 1980; Witkin *et al*., 1982; Hoffman, 1983). In rats, the majority of perikarya are located throughout the region from the medial septumn to the DBB and within the medial POA adjacent to the OVLT, but no GnRH neurons are found in the basal hypothalamus (ventromedial hypothalamus and ARC) (Witkin *et al*., 1982). Thus, compared to the variation seen in other mammals including the other species of bats previously studied, the distribution of GnRH-ir cells in the bat used for this study seems to show a characteristic feature.

In the present study, the number and immunoreactivity of GnRH-ir neurons were decreased in animals collected during the reproductively active season (summer), whereas both were dramatically increased in hibernating bats. These changes may be closely related to GnRH neuronal activity. The present study showed that the variation in immunoreactivity and number of GnRH-ir cells was greater in the MBH (ARC) than in the POA throughout the annual seasonal cycle. These findings suggest that GnRH neuronal activity in the MBH is more closely associated with the reproductive function of this bat than that in the POA. The role of the MBH as a hypophysiotrophic region involved in the regulation of gonadotropin secretion has been demonstrated for several species of mammals (Jackson *et al*., 1978; Krey and Silverman, 1978; Thind and Goldsmith,1988; Merchenthaler *et al*., 1989). Previous investigators have found that in several species of bats the GnRH-ir neurons in the ARC mostly project axons to the posterior ME and infundibular stalk adjacent to the dorsal area of the pars distalis, where gonadotrophs are particulaly abundant, and they suggested that these gonadotrophs may receive GnRH both via portal vessels and through simple diffusion (Anthony *et al*.,1984; King *et al*.,1984; Anthony, 1987). Although we have not yet made similar observations and did not directly determine whether the major source of the hypophysiotrophic GnRH fiber terminals is cell bodies in the POA or MBH, parallel fluctuations between the number of GnRH-ir cells or their immunoreactivity in the MBH and the immunoreactivity of the ME during the annual cycle strongly

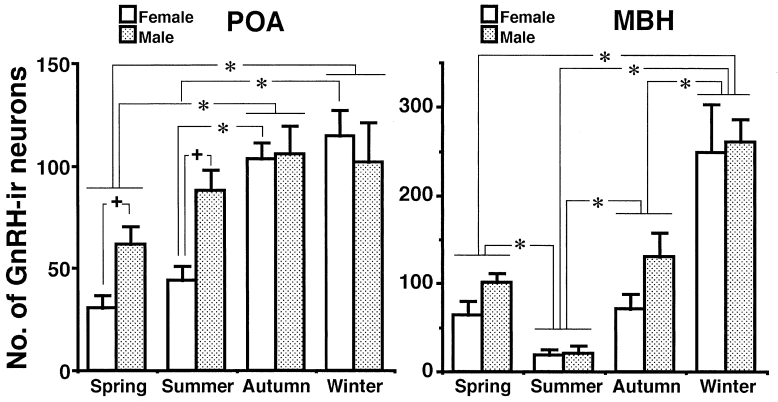

Fig. 5. Total number of GnRH-ir cell bodies in the POA and MBH. Other explanations are the same as for Fig. 4.

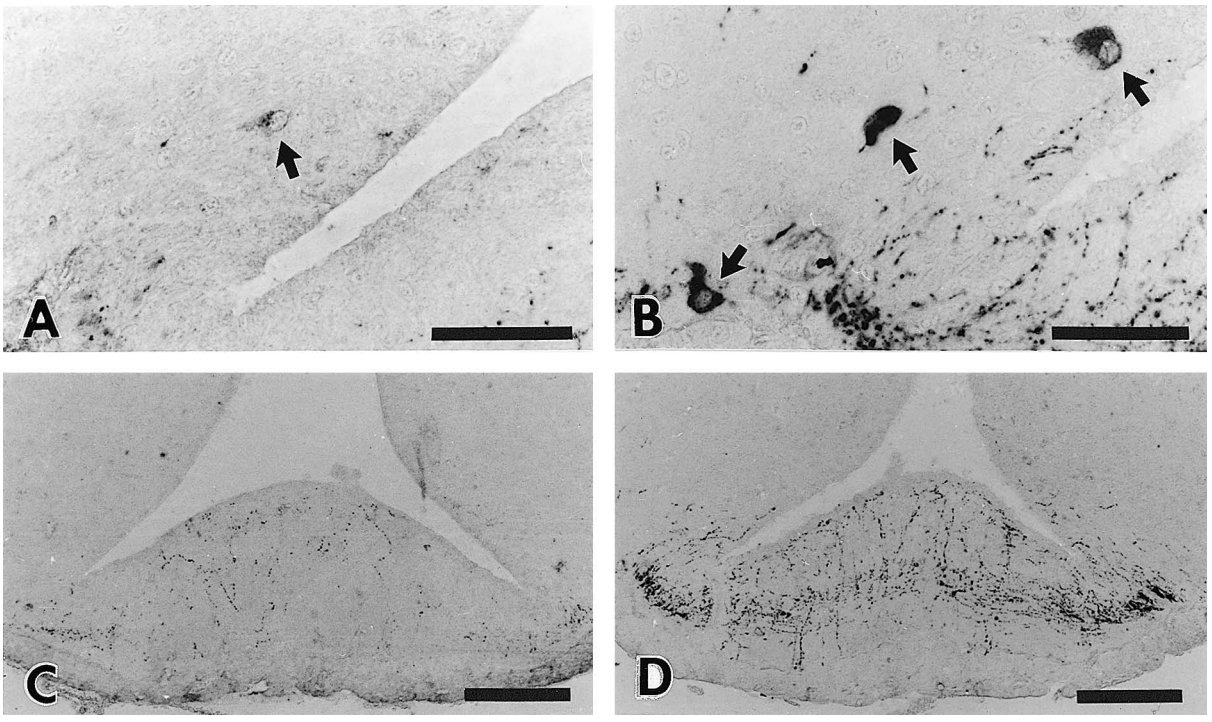

Fig. 6. GnRH-ir neuronal perikarya (arrows) and fibers of the ME in *Rhinolophus ferrumequinum*. GnRH-ir cells in the MBH of lactating (summer) (**A**) and hibernating (winter) (**B**) female bats. GnRH-ir fibers in the ME during the spermatogenic (summer) (**C**) and hibernation (winter) (**D**) periods of male bats. Note the increased immunoreactivity of the cell bodies and fibers in hibernating animals. Bars in **A** and **B**: 20 μm. Bars in **C** and **D**: 100 μm.

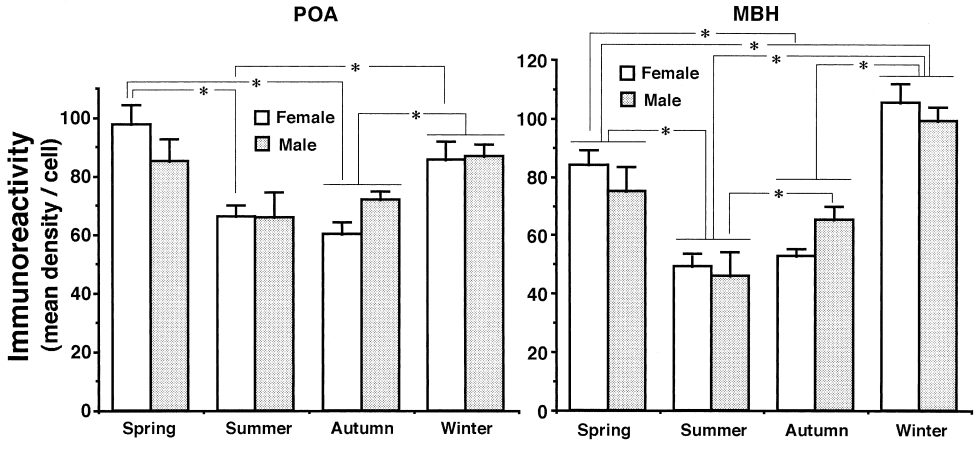

Fig. 7. GnRH-immunoreactivity of the neuronal perikarya in the POA and MBH. Other explanations are the same as for Fig. 4.

suggest that most GnRH neurons in the MBH contribute to the control of gonadotropin secretion in this species. This suggestion may be reinforced by the finding that changes in GnRH-ir neurons in the MBH correlated well with those (in immunoreactivity and cellular size) in immunocytochemically stained gonadotrophs of the pars distalis in this bat (unpublished data).

It has been demonstrated in seasonally breeding mammals that the immunoreactivity and number of GnRH-ir cells, and the amount of GnRH in the brain, vary depending on the animals' reproductive condition (Polkowska *et al.*, 1980; Hart *et al.*, 1984; Jackson *et al.*, 1984; Glass, 1986; Ronchi *et al.*, 1992a). These findings demonstrate that the GnRH neurons of animals in a reproductively active state show not only a decrease in the amount of GnRH in their cell bodies, but also a reduction in the number of cell bodies and the neuronal processes which contain immunoreactive GnRH. Conversely, somal accumulation of GnRH followed by reduced release of this peptide may account for the increased number of cell bodies and fibers visualized. Glass (1986) reported that exposure of the white-footed mouse to a short photoperiod, which resulted in gonadal regression, increased the number and immunoreactivity of GnRH neurons in the POA/ AHA. In the

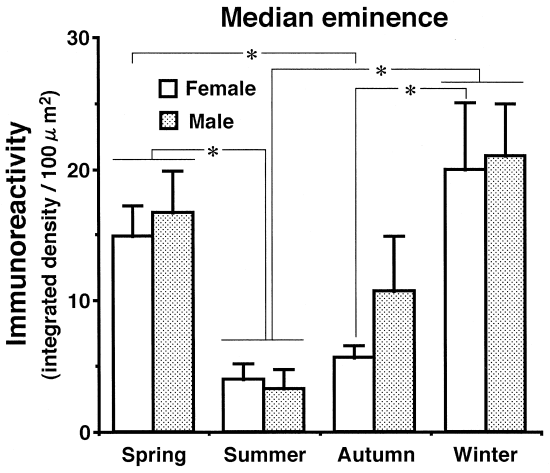

Fig. 8. GnRH-immunoreactivity of fibers per unit area of the ME. Other explanations are the same as for Fig. 4.

syrian hamster, Ronchi *et al*. (1992a, b) also made similar observations on GnRH neurons of the DBB/medial septum and further demonstrated that no decrease in the number of GnRH gene-expressing neurons or in the signal intensity per cell was ever observed. Their findings suggest that the increased immunoreactivity of the GnRH-ir cell bodies in the hamster forebrain results from backfilling of GnRH into the soma due to its continued synthesis and reduced secretion. The present study showed that the number and immunoreactivity of GnRH-ir neurons increased from autumn to winter, indicating decreased secretory activity from GnRH neuronal terminals. However, it remains to be elucidated whether these events are linked to alterations in the synthesis of this peptide.

The present study revealed a sex difference in the number of GnRH-ir neurons in the POA in spring and summer. Since we found that there were no significant differences between males and females in other seasons, the above difference is presumably due to a variation between the sexes in the functional state of GnRH neurons in the POA. From May to August, pregnancy, parturition and lactation occur in adult female bats, and therefore enhanced GnRH neuronal activity in the POA may be associated with female-specific reproductive events. The reduced number of GnRH-ir neurons in this brain area was, however, not reflected in alterations in the immunoreactivity of the GnRH cell bodies, indicating that quantitative differences in image analyses of the stainability were due to the limitation in visualizing GnRH neurons immunocytochemically. Therefore, a quantitative approach for specifically evaluating the biosynthesis of GnRH in these brain areas is required because the existence of other molecular forms of GnRH has been reported in early-evolved eutherian mammals (musk shrews and moles) and guinea pigs (Dellovade *et al*., 1993; King *et al*.,1994; Jimenez-Liñan *et al*., 1997). The antibody against GnRH used in the present study also reacts with several forms of GnRH in lower vertebrates and therefore may cross-react with the unknown additional form in this bat.

In conclusion, the immunoreactivity and number of GnRH-ir cells showed greater variation in the MBH (ARC) than in the POA throughout the annual seasonal cycle, suggesting that GnRH-ir neurons located in the MBH supply the major GnRH innervation to the ME and play a more important role in seasonal regulation of gonadotropin secretion than those in the POA. Further studies are needed on the release and synthesis of GnRH in the bat brain during the annual reproductive cycle.

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