Immunohistochemical Study on Gonadotropin-Releasing Hormone Neurons in the Shiba Goat Brain

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Abstract. Gonadotropin-releasing hormone (GnRH) neurons were examined immunohistochemically in the brain of mature female Shiba goats with a monoclonal antibody to GnRH (LRH-13, Park and Wakabayashi, *Endocrinol. Jpn.* 33: 257–272, 1986). In all animals, GnRH immunoreactive neurons exhibited a complex morphology consisting of round (49.6%), multipolar (39.8%) or fusiform (10.6%) cell bodies and fibers with beaded varicosities. GnRH cell bodies were distributed rostrally from the diagonal band of Broca and medial septum, passing through the medial and lateral preoptic areas, and caudally ending within the ventromedial anterior hypothalamic areas. A majority (62.6%) of GnRH cells were found in the preoptic areas adjacent to the organum vasculosum of the lamina terminalis, and relatively few cells (7.1%) were distributed in the arcuate nucleus or its vicinity. Preoptic GnRH neurons project their fibers to the tubero-infundibular sulcus of the median eminence by at least 2 routes: (1) the majority of fibers were observed in the periventricular area and the arcuate nucleus (periventricular pathway), and (2) less prominent ones were found in the anterior and ventrolateral hypothalamus (ventrolateral pathway). In addition to these hypothalamic regions, GnRH immunoreactive fibers were also found in the neurohypophysis.

Key words: GnRH, Immunohistochemistry, Hypothalamus, Shiba goat.

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The Shiba goat (*Capra hircus*) is a Japanese indigenous goat of which a closed colony has long been established for experimental uses [1]. This goat has been employed in various research fields including reproductive physiology [2]. Recently, we have developed a method for precisely approaching the central nervous system of the Shiba goat [3] based on the brain atlas with stereotaxic coordinates (Shimada *et al.*, unpublished), that enabled us to attempt a neuroendocrine

study on regulatory mechanisms of hypothalamic GnRH neurons [4, 5]. In the course of the study it became of exceptional importance to determine the distribution of GnRH neurons in the brain and to compare it with those reported in other mammalian species such as man [6], monkey [7, 8], sheep [9], cattle [10], rat [11], guinea pig [12] and mouse [13]. In the present study we examined the distribution pattern as well as the morphological characteristics of GnRH neurons immunohistochemically identified in the hypothalamus and adjacent areas of the female Shiba goat.

Materials and Methods

Five mature female Shiba goats (#934, #943, #952, #959, #961, 2–10 years old) were used. They were deeply anesthetized with 30 mg/kg sodium pentobarbital following systemic heparinization (5,000 units/head, i.v.), and then perfused bilaterally via the carotid arteries with 3 l of 0.9% NaCl (the first 1 l of saline containing 10 units/ml heparin), followed by 2.5 l of Zamboni's fixative (4% paraformaldehyde, 7% saturated picric acid in 0.1 M phosphate buffer, pH 7.4). Following perfusion, the brain and pituitary were carefully removed from the cranium and blocked. The blocks of brain were then stored in the fixative containing 15% sucrose at 4°C over-night.

The brain block including the hypothalamus was cut at 40 μ m coronally on the plane of the goat stereotaxic atlas (Shimada *et al.* unpublished data) on a freezing microtome, whereas the pituitary was cut sagittally. Every sixth section was processed for GnRH immunohistochemistry. Free-floating sections were washed in 0.1 M phosphate buffered saline containing 0.05% Triton X-100 (PBST) and then incubated in PBST containing 1% normal horse serum for 40 min to minimize nonspecific staining. Sections were exposed to the primary antiserum (LRH-13 [14]) at a dilution of 1: 3,000 in 0.1 M PBST for 40 h at 4°C, and were then treated at room temperature sequentially with a biotinylated horse antimouse IgG for 1 h, 0.3% hydrogen peroxidase for 30 min to reduce endogenous peroxidase activity and the avidin-biotin-horseradish peroxidase complex for 1 h, with a commercial kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA U.S.A.). The horseradish peroxidase activity was demonstrated using 0.05% 3,3'-diaminobenzidine tetrahydrochloride (POLYSCIENCES, Warrington, PA, U.S.A.) and 0.01% hydrogen peroxidase as the chromogen. Sections were then mounted, dehydrated, and coverslipped. Next every sixth section was stained with cresyl violet to identify nuclear boundaries.

The specificity of the LRH-13 was previously characterized [14]. In the present experiment the immunohistochemical specificity of this antibody was verified with an antibody (antiserum to luteinizing hormone-releasing hormone-human thyroglobulin, UCB Bioproducts, S.A. Belgium, at

a dilution of 1: 5,000 in 0.1 M PBST) having a different epitope from LRH-13. Its immunoreactive products were the same as those shown with LRH-13 (data not shown). All sections were examined under a light microscope with a bright field, and sketched with a stereoscopic microscope and a camera lucida.

Postmortem examination of the ovaries of the perfused goats revealed that #952 had 2 small corpora lutea, #961 2 developed corpora lutea and 2 medium follicles, and #934, #943 and #959 small follicles without corpus luteum.

Results

Morphology of GnRH neurons

With the sensitive LRH-13 antibody to GnRH it was possible to visualize GnRH-immunoreactive (GnRH-IR) neurons in all the animals examined. GnRH-IR neurons were darkly stained against a low background. The cell bodies and fibers immunoreactive to LRH-13 are designated as GnRH-IR cell bodies and fibers, respectively. Immunoreactive products were observed in the cytoplasm but not in the nucleus. Most of the immunoreactive cell bodies had "dendrite-like" processes. These processes were irregular in outline and often branched (Fig. 1). Immunoreactive GnRH fibers appeared as thin fibers with beaded varicosities. In individual animals 256–420 GnRH-IR cells were observed, and the GnRH-IR cells were classified into 3 morphological types: fusiform, round, and multipolar, (Fig. 1A-F, Table. 1). The majority were round (49.6%) and multipolar (39.8%), but there were fewer fusiform cells (10.6%). Some GnRH-IR cell bodies and fibers

Table 1 Classification of immunoreactive GnRH cells

Goat No. (n) ^a	cell type (%)		
	fusiform	round	multipolar
934(256)	6.9	52.9	40.2
943(420)	10.7	50.2	39.1
952(268)	16.7	45.8	37.5
959(332)	8.4	47.3	44.3
961(408)	10.3	51.9	37.8
mean	10.6	49.6	39.8

a) Number of immunoreactive GnRH cells observed.

Fig. 1. Photomicrographs ($\times 560$) of GnRH-immunoreactive (IR) cells of the "round" (A, #961; B, #934), "multipolar" (C, #961; D, #934), and "fusiform" (E, #943; F, #934) types. Close somatic apposition between adjacent GnRH-IR cells (G, #961) and between GnRH-IR fiber and GnRH-IR cell body (D, #934; H, #943) in the MPOA are shown by arrows.

were seen in close association with others, i.e. the cell body-cell body (Fig. 1G) or fiber-cell body (Fig. 1H) contacts, but the frequency was rather low.

Distribution of GnRH cell bodies

The majority of GnRH-IR cell bodies were found in the medial preoptic area, at the level of the organum vasculosum of the lamina terminalis (OVLt, Fig. 2, 3A). GnRH-IR cells were distributed extending rostrally and dorsally with those in the medial septum and caudally with those in

the anterior hypothalamus and the ventromedial hypothalamus. Few cells were seen in and around the arcuate nucleus, and in and above the supraoptic nucleus. Considerable numbers of GnRH-IR cells were seen in the medial preoptic nucleus. Although the total number of observed GnRH-IR cell bodies varied from one animal to another, the pattern of distribution of GnRH-IR cells in the brain was similar in all of them (Table 2). The preoptic area consistently contained the highest proportion of GnRH cells (62.6%) and the arcuate nucleus the lowest (7.1%).

Fig. 2. Distribution of GnRH-IR cells (solid circles) and fibers (short solid lines) in drawings of coronal brain sections, rostral to caudal (A-F), through the POA and the hypothalamus. The figures show the rostro-caudal distance of the corresponding plate from the vertical plane passing through the interaural line. *Abbreviations:* ac, anterior commissure; ACB, septal accumbens nucleus; AHA, anterior hypothalamic area; ARC, arcuate nucleus; CA, caudate nucleus; fx, fornix; ir, infundibular recess; is, infundibular stalk; LS, lateral septal area; MPOA, medial preoptic area; MPON, medial preoptic nucleus; MRe, mammillary recess; MS, medial septum; mt, mammillothalamic tract; oc, optic chiasm; ot, optic tract; OVLt, organum vasculosum of the lamina terminalis; PD, pars distalis; PI, pars intermedia; PN, pars nervosa; POA, preoptic area; PT, pars tuberalis; PVN, paraventricular nucleus; PVT, paraventricular thalamic nucleus; SON, supraoptic nucleus; VMH, ventromedial hypothalamic nucleus; 3V, third ventricle.

Fig. 3. A) A photomicrograph ($\times 42$) of GnRH-IR cells in the MPOA at the level of the OVLT. GnRH-IR cells were densely located in this region. #943. B) A photomicrograph ($\times 21$) of GnRH-IR fibers in the ME. Many GnRH-IR fibers terminated in this region. #961. Abbreviations, see Fig. 2.

Table 2 Distribution of immunoreactive GnRH cells (%)

Goat No. (n) ^a	hDBB-MS	POA	AHA-LH	ARC-ME	others
934(256)	7.0	56.2	23.0	9.0	4.8
943(420)	10.2	59.8	21.2	6.9	1.9
952(268)	4.1	66.1	20.1	7.8	1.9
959(332)	5.7	60.6	25.3	6.0	2.4
961(408)	3.2	70.1	16.9	5.9	3.9
mean	6.0	62.6	21.3	7.1	3.0

a) Number of immunoreactive GnRH cells observed.

hDBB-MS, horizontal limb of diagonal band of Broca-medial septum; POA, preoptic area; AHA-LH, anterior hypothalamic area-lateral hypothalamus; ARC-ME, arcuate-median eminence

Distribution of GnRH fiber tracts and terminal fields

GnRH-IR fibers which probably originated in the preoptic area and anterior hypothalamus projected to the median eminence and infundibular stalk (Fig. 3B) by at least 2 separate pathways:

one is the periventricular pathway, and the other the ventrolateral one. The former, which contained the majority of fibers leaving in the preoptic area, coursed periventricularly in the medio-ventral part of the preoptic area and the anterior hypothalamus. These fibers travelled caudally along the wall of the third ventricle and then

Fig. 4. Camera lucida drawing of sagittal section of the pituitary showing the distribution of GnRH-IR fibers described as short solid lines and photomicrographs of higher magnification of boxed areas in the drawing, demonstrating GnRH-IR fibers in the ME-infundibular stalk region (A; $\times 79$) and the neurohypophysis (B, C; $\times 98$). #959. Abbreviations, see Fig. 2.

coursed ventrally to the median eminence. The other fibers in the periventricular pathway were also seen in the arcuate nucleus, and more caudally in the mammillary body. Some fibers were seen in close association with the ependymal layer of the third ventricle in the periventricular area, especially in the OVLT.

The second major pathway of GnRH-IR fibers (the ventrolateral pathway), probably originated in the preoptic area and the anterior hypothalamus including the supraoptic nucleus, passed through the region just above the supraoptic nucleus and the lateral hypothalamic area, and terminated in the median eminence and other caudal targets.

In the neurohypophysis a considerable number of GnRH-IR fibers entered from the infundibular stalk into deeper portions of the posterior hypophysis (Fig. 4). These fibers coursed along the dorsal border of the pars intermedia and some of them reached ventrocaudal portions of the neurohypophysis.

Discussion

In the present study, GnRH neurons were examined immunohistochemically in the hypothalamus of the mature female Shiba goat. Immunoreactive GnRH was found in the cytoplasm of the cell body and in the fibers with beaded varicosities as reported in other mammalian species [15], suggesting that GnRH is synthesized and stored in the cytoplasm and transported through the fibers with beaded varicosities to the nerve terminal for neurosecretion.

The majority of GnRH-IR cell bodies were found in the medial preoptic area, at the level of the OVLT, but a few cells were observed in the mediobasal hypothalamus (MBH). The distribution in goat was similar to those reported in sheep [9], cattle [10], rat [11], guinea pig [12] and mouse [13] brain, but different from those in man [6] and monkey [7, 8] in which many GnRH cells are present in the MBH. GnRH-IR fibers were found mainly in the OVLT, periventricular regions, median eminence, and lateral basal hypothalamus at the level of the supraoptic nucleus as reported in sheep [9] and other mammals [15, 16]. These pathways, namely the septo-preoptico-infundibular pathways [15], were categorized into 2 routes [9, 16]: the periventricular and the

ventrolateral pathways. In the Shiba goat most of the fibers coursed periventricularly in the preoptic area and anterior hypothalamus projecting to the median eminence, whereas in the sheep the major pathway was a ventrolateral one [9] although the distribution of GnRH cell bodies was similar to that in the Shiba goat brain.

GnRH-IR cell bodies were morphologically classified into 3 types: round (49.6%), multipolar (39.8%) and fusiform (10.6%), as shown in Fig. 1 and Table 1. In the rat brain, GnRH-IR cell bodies are mostly fusiform but they are further classified into 2 sub-types: "smooth" and "thorny" of highly irregular outline [17–24]. It was suggested that the "thorny" GnRH-IR neurons with irregular surfaces might receive a larger number of synaptic inputs than the "smooth" neurons [18, 19], but no difference has been found either in the incidence of synapses or in the density of the synaptic input as measured per unit of neuronal membrane [23]. It has been suggested that the "thorny" type could be more actively engaged in peptide processing and packaging since the content of various sub-cellular organelles was shown to be different in the 2 morphological types [23]. In the Shiba goat, many GnRH cells have branching dendrites and very irregular content. Similar results were reported in the sheep [9]. If the spreading pattern and the number of the processes reflect the neuronal activity or secretory activity, multipolar neurons could be more active than any other cell types. However, there was no particular distribution pattern of GnRH cells of each category implying any functional-morphological relationship.

The effects of gonadal steroids on the morphology of GnRH-IR cells were reported in rats. The proportion of thorny cells was increased in both males and females at puberty [22] and decreased after gonadoectomy [20]. The effects of estradiol on the GnRH immunoreactive neurons were examined in ovariectomized ewes treated with estradiol-17 β (E₂), and the percentage of GnRH cell bodies of multipolar type was approximately twice that in untreated control ewes [25]. In this study, the proportion of GnRH cell bodies was similar in all the animals, and this may be because no animal was thought to be under a strong influence of E₂ according to the results of post-mortem examination of ovaries.

Regarding the cell-cell or cell-fiber contacts of

GnRH-IR neurons, similar results were reported in the man [6], sheep [9], cattle [10] and rat [11] at light microscopic levels. Synaptic contacts were demonstrated between GnRH neurons in the rat at electron microscopic levels, implying the morphological basis for autoregulation of the GnRH system [26, 27]. It was also observed that the GnRH-IR terminals made synaptic contact on estrogen receptor immunoreactive perikarya in the guinea pig [28]. Thus, these results suggest that GnRH not only plays a role as a gonadotropin releasing hormone, but also as a neuromodulator.

In the Shiba goat, GnRH-IR fibers were found in the neurohypophysis along the pars intermedia, some of them reaching caudal and ventral portions of the neurohypophysis (see Fig.4). Similar results were reported in other mammals, such as man, monkey, bat and ferret [29], although the function of the pituitary GnRH-IR fibers remains largely unknown. In the rat, however, the neurohypophysis lacks the GnRH-IR fibers, and this may have been one of the reasons why the functional study on neurohypophysis GnRH was hindered. Hypothesized functions are as follows, (1) GnRH acts as a neuromodulator affecting secretion of hormones of the pars nervosa and/or pars intermedia, (2) GnRH neurons contact

neurovascular system in the neural-lobe to release GnRH into the peripheral blood as a source of immunoreactive and biologically active GnRH [30–32], (3) GnRH reaches the gonadotropes of the pars distalis through the vessels from the pars nervosa to the pars distalis. GnRH has also been demonstrated in the neurohypophysis of fish among submammalian vertebrates [33–35]. In some teleost fishes, a direct innervation of GnRH terminals to pituitary gonadotropes has been demonstrated [36, 37] whereas GnRH is delivered to gonadotropes of the pars distalis by portal vessels in higher vertebrates. GnRH fibers of the neurohypophysis may, therefore, be more primitive neuroendocrine derivatives.

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