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Author(s)	Nozaki, Masumi; Fujita, Ichiro; Saito, Noboru; Tsukahara, Tetsuo; Kobayashi, Hideshi; Ueda, Kazuo; Oshima, Kiyoshi
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Distribution of LHRH-like Immunoreactivity in the Brain of the Japanese Eel (*Anguilla japonica*) with Special Reference to the Nervus Terminalis

MASUMI NOZAKI, ICHIRO FUJITA^{1,3}, NOBORU SAITO^{2,4},
TETSUO TSUKAHARA², HIDESHI KOBAYASHI²,
KAZUO UEDA¹ and KIYOSHI OSHIMA

Primate Research Institute, Kyoto University, Inuyama, Aichi 484,

¹*Zoological Institute, Faculty of Science, University of Tokyo,*

Bunkyo-ku, Tokyo 113, and ²Department of Biology,

Faculty of Science, Toho University,

Miyama, Funabashi 274, Japan

ABSTRACT — The brain of the Japanese eel, *Anguilla japonica*, was studied immunocytochemically using antisera generated against the synthetic luteinizing hormone-releasing hormone (LHRH) of mammals. LHRH-positive perikarya were observed in both the distal and proximal ganglia of the nervus terminalis and in the ventrolateral portions of the ventral telencephalon and preoptic area. LHRH-positive perikarya of the distal and proximal ganglia of the nervus terminalis sent their fibers to the olfactory nerve, ventral telencephalon, preoptic area, and optic tectum. LHRH-positive fibers, originating from the ventral telencephalon and preoptic area, proceeded toward the olfactory bulb, dorsolateral part of the rostral telencephalon, preoptic area, neurohypophysis, and optic tectum. LHRH-positive fibers were also observed in the optic nerve. These findings are discussed in comparison with recent anatomical findings by others in other teleosts. Further, possible participation of nervus terminalis in reproductive behavior is discussed.

INTRODUCTION

A recent resurgence of interest in the nervus terminalis (terminal nerve) has resulted in new anatomical findings, suggesting participation of nervus terminalis in reproductive responses toward sex pheromones (see [1]). Particularly, Schwanzel-Fukuda and Silverman [2] presented the first evidence in the guinea pig that cells and fibers of the nervus terminalis contain LHRH-like immunoreactivity. Subsequently, Münz *et al.* [3, 4] reported LHRH-like immunoreactivity in the

nucleus olfactoretinalis (NOR) of cichlid, poeciliid, and centrarchid fishes, in which the olfactory bulb is situated close to the telencephalic hemisphere (sessile olfactory bulb). These teleosts with sessile olfactory bulbs possess both the distal and proximal ganglia of the nervus terminalis (DGNT and PGNT) [5], and the NOR seems to correspond to the PGNT [5]. On the other hand, teleosts with the pedunculated olfactory bulbs (e.g. cyprinid) possess only the DGNT [5], where LHRH-like immunoreactivity has recently been demonstrated in the goldfish [6]. Thus, evidence for the occurrence of LHRH-like immunoreactivity in the DGNT in teleosts with the sessile olfactory bulbs is lacking at this time. In order to explore a functional significance of the nervus terminalis, further detailed studies in teleosts with the sessile olfactory bulbs are urgently necessary.

The present study describes the distribution of

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³ Present address: Department of Biological Control System, National Institute for Physiological Science, Okazaki 444, Japan.

⁴ Present address: Department of Animal Physiology, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan.

LHRH-like immunoreactivity in the brain of the Japanese eel which possesses sessile olfactory bulbs, with special reference to the nervus terminalis.

MATERIALS AND METHODS

More than thirty cultured Japanese eels (*Anguilla japonica*) were obtained from a commercial source and were about 45 cm in total length, weighing 180 g. They were killed by decapitation without anesthesia. Following the rapid removal of the dorsal cranial bone, the dorsal surface of the brain was exposed and the head was immersed in Bouin's solution. A preliminary experiment showed that in the Japanese eel, substances immunoreactive toward anti-LHRH sera were more abundant in tissues fixed with Bouin's solution than those fixed with 10% formalin solution or Bouin's solution without acetic acid. During the first 30 min of fixation, the brain and the attached pituitary were dissected out carefully, and placed in Bouin's solution for about 24 hr. The tissue was dehydrated through a series of increasing concentrations of ethanol, embedded in paraplast, and serial sagittal or transverse sections of 6–10 μm in thickness were cut. In several specimens, all the serial sagittal sections ($n=10$) and serial transverse sections ($n=4$) of the brain were treated with LHRH-immunocytochemistry, and most of them were counterstained with Meyer's hematoxylin. Three other series of sagittal or transverse sections were stained with cresyl violet to examine normal cytoarchitecture. The present terminology of brain areas is mainly according to Nieuwenhuys [7], Peter and Gill [8] and Northcutt and Bradford [9].

Two kinds of anti-LHRH sera were used. One was provided at the courtesy of Dr. K. Wakabayashi, Gunma University [10], and another was obtained commercially (Miles-Yeda, lot No. UZ-8). Both antisera were produced in rabbits by multiple intradermal injections of synthetic mammalian LHRH-bovine serum albumin conjugate. These antisera were used optimally at dilutions of 1:400 to 1:1,000.

The immunocytochemical staining procedure was the unlabeled antibody-enzyme method. In brief, paraplast was removed from sections

with xylene, and the sections were hydrated in a graded ethanol series, and washed in phosphate buffered saline (0.14 M NaCl, 0.01 M phosphate buffer, pH 7.3) containing 0.1% bovine serum albumin and 0.1% normal goat serum. This solution served as the diluent for antisera. Sections were treated with normal goat serum (1:20, Polysciences) for 1 hr to reduce non-specific staining. Primary antisera were applied to the sections for 2 to 16 hr, and goat anti-rabbit gamma globulin serum (1:40, kind gift of Dr. K. Wakabayashi, Gunma University), and rabbit peroxidase-anti-peroxidase complex (1:50, Polysciences) were each applied for 1 to 2 hr. Finally, 3,3'-diamino-benzidine tetrahydrochloride and 0.003% H_2O_2 were used for visualization of the immunoreaction. All the procedures were performed at room temperature.

Control staining for specificity of the reaction was performed (1) by replacing the primary antisera with normal rabbit serum or (2) by using the primary antisera previously absorbed with synthetic mammalian LHRH. For this purpose, histological sections prepared from both the rat median eminence and Japanese eel brain were used. The immunopositive reaction toward anti-LHRH sera in both the rat and Japanese eel was completely abolished by preabsorption with a low concentration of synthetic mammalian LHRH (50 $\mu\text{g}/\text{ml}$ antisera at a dilution of 1:500).

RESULTS

A specific reaction product was found in the neuronal perikarya and fibers of the brain by both anti-LHRH sera, and no difference could be detected in the distribution of LHRH-like immunoreactivity for either antisera used. The results obtained are schematically shown in Figures 1 and 2.

LHRH-positive perikarya

Neuronal perikarya containing LHRH-like immunoreactivity could be grouped into four according to location (Figs. 1 and 2). The first group of LHRH-positive perikarya was near the transition between the olfactory nerve and olfactory bulb (Figs. 1, 2a, 3a and 4). Ac-

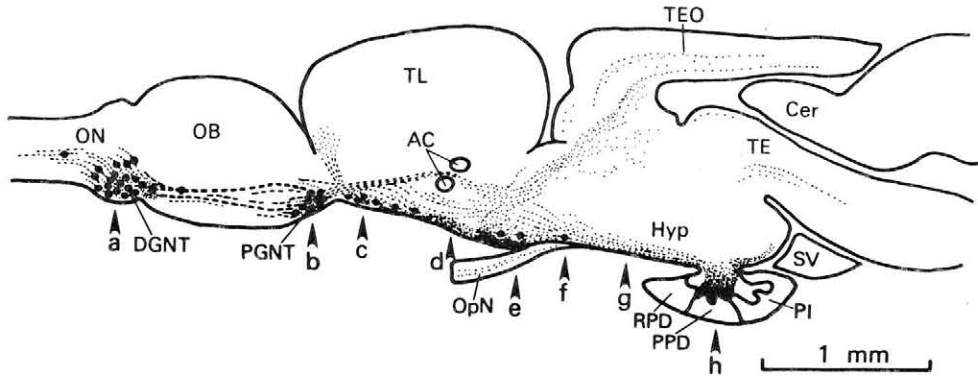


Fig. 1. Topographic distribution of LHRH-positive perikarya (black circles) and fibers (broken lines) projecting on a nearly midsagittal plane. Arrowheads with *a-h* represent the transverse planes of the sections illustrated in Fig. 2a-h.

ABBREVIATIONS IN FIGS. 1-10.

AC, anterior commissure; *Cer*, cerebellum; *D*, area dorsalis telencephali; *DGNT*, distal ganglion of the nervus terminalis; *Hyp*, hypothalamus; *LFB*, lateral forebrain bundle; *LOT*, lateral olfactory tract; *MFB*, medial forebrain bundle; *MOT*, medial olfactory tract; *NAPv*, nucleus anterioris periventricularis; *NDL*, nucleus dorsolateralis thalami; *NDM*, nucleus dorsomedialis thalami; *NE*, nucleus entopeduncularis; *NG*, nucleus glomerulosus; *NH*, nucleus habenularis; *NLT*, nucleus lateralis tuberis; *NOH*, nucleus opticus hypothalamicus of Ekström (1982) [33]; *NPG*, nucleus preglomerulosus; *NPO*, nucleus preopticus; *NPP*, nucleus preopticus periventricularis; *NRL*, nucleus recessus lateralis; *OB*, olfactory bulb; *OC*, optic chiasma; *ON*, olfactory nerve; *OpN*, optic nerve; *OT*, optic tract; *PGNT*, proximal ganglion of the nervus terminalis; *PI*, pars intermedia; *PIT*, pituitary; *PPD*, proximal pars distalis; *RPD*, rostral pars distalis; *SCO*, subcommissural organ; *SV*, saccus vasculosus; *TE*, mesencephalic tegmentum; *TEO*, optic tectum; *TL*, telencephalon; *Vc*, central nucleus of area ventralis telencephali; *Vd*, dorsal nucleus of area ventralis telencephali; *Vl*, lateral nucleus of area ventralis telencephali; *Vp*, post-commissural nucleus of area ventralis telencephali; *Vs*, supra-commissural nucleus of area ventralis telencephali; *Vv*, ventral nucleus of area ventralis telencephali.

cordingly, most of the LHRH-positive perikarya of this group were distributed in both the most proximal portion of the olfactory nerve and most rostral portion of the olfactory bulb (Figs. 1, 2a and 3a, b). Some could be found in the olfactory nerve and olfactory bulb which were situated some distance from the transition (Figs. 1 and 4c). The LHRH-positive perikarya of this group were distributed near the ventromedial surface (Figs. 2a and 3a), and were moderate in number (10 to 30 cells for one side of the brain). They were bipolar or multipolar and were distributed loosely (Figs. 3 and 4). These LHRH-positive cells were usually spindle shaped and about 10 μm along the minor axis, and were larger than the mitral cells. Some of them sent thick (about 3 μm in diameter) axon-like fibers in the caudal direction (Fig. 3b). From the size and the

characteristic location, these cells appeared to correspond to the DGNT as described by Rossi *et al.* [5], and to the nervus terminalis ganglion cells described by several earlier authors [11-13].

The second group of LHRH-positive perikarya was found in a cluster in the caudalmost part of the olfactory bulb (Figs. 1, 2b, 5 and 6). These cells were distributed near the ventromedial surface of that region (Figs. 5a and 6) and were usually ovoid and appeared unipolar (Figs. 5a and 6), though some were bipolar (Fig. 5b). The diameter of these LHRH-positive cells ranged from 7 to 20 μm (Fig. 6). The immunoreactivity of these cells varied according to cell and specimen, and was generally weaker than that of other three groups. In some specimens, a considerable number of moderately stained cells were found (about 80 cells for each side; Fig. 5a), whereas

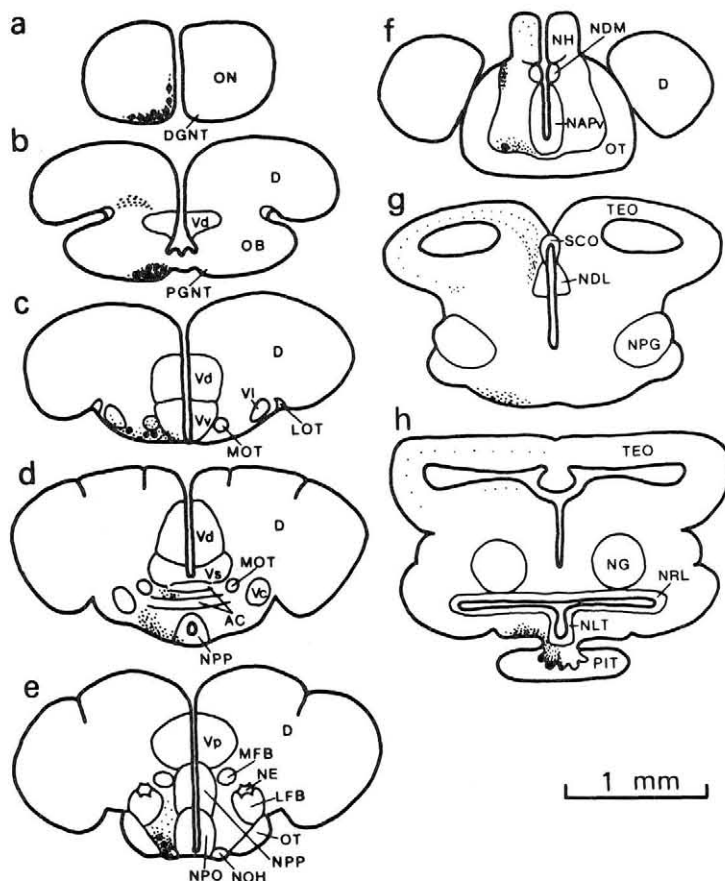


FIG. 2. Schematic representation of LHRH-positive perikarya (black circles) and fibers (dots) at eight transverse levels of *a-h* in Fig. 1.

in other specimens only few such cells could be distinguished. Those cells of the second group apparently correspond to the PGNT [5], and to the NOR [3-4].

The LHRH-positive perikarya of the third group were scattered about in the precommissural, ventrolateral part of the ventral telencephalon, where LHRH-positive perikarya were present in the white matter ventral to the medial olfactory tract (MOT; Figs. 1, 2c and 7). Most of these cells appeared bipolar, and sent fibers in the rostral and caudal directions (Fig. 7b), but some were unipolar. They were a few in number (about 10 cells for one side of brain), spindle shaped (5 to 7 μm along the minor axis), and smaller than those of the first group.

LHRH-positive perikarya of the fourth group were located in the ventrolateral part of the

preoptic area, where they were distributed in the neuropil lateral to the nucleus preopticus and nucleus anterior periventricularis (Figs. 1, 2e, f and 8). LHRH-positive cells of this group were occasionally observed (about three for each brain side). They appeared bipolar, sending fibers in the rostral and caudal directions (Fig. 8a, c) and were similar to those of group 3 in the shape and size.

LHRH-positive fibers

Olfactory nerve Many LHRH-positive fibers were present in the most proximal part of the olfactory nerve, where the olfactory nerve formed a pair of thick bundles (Figs. 1, 2a and 3). Rostrally, number of LHRH-positive fibers decreased remarkably, and in the region where the olfactory nerve was separated into several

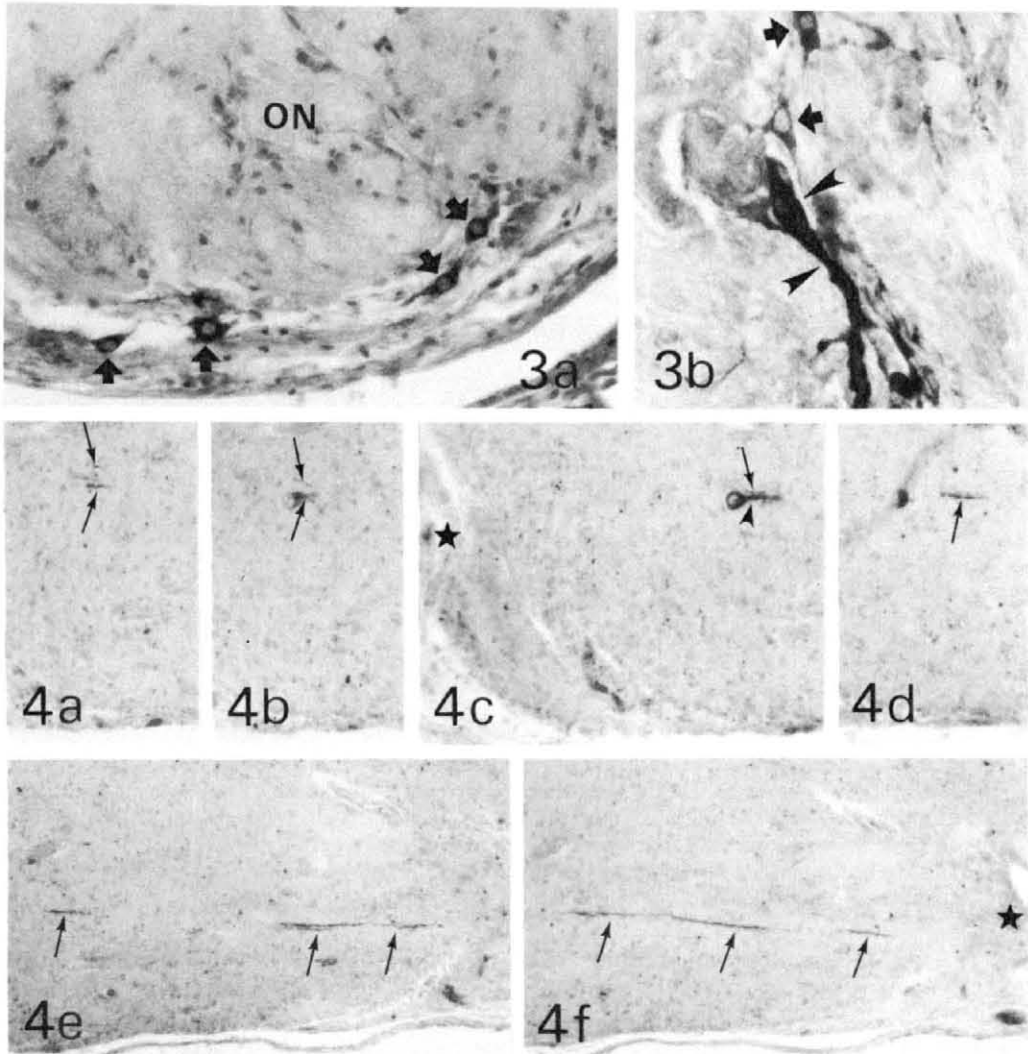


FIG. 3. Transverse (a) and sagittal (b) sections of the most proximal part of the olfactory nerve (ON) showing the LHRH-positive cells (arrows) of the DGNT. a, Note the LHRH-positive perikarya in the most peripheral region of the ON. b, Note the LHRH-positive perikarya (arrowheads) with a thick axon-like fiber. a, $\times 320$; b, $\times 350$.

FIG. 4. Six successive parasagittal sections through the ventromedial part of the OB. The scale for a-d and e-f are not the same. Several LHRH-positive fibers (arrows in a-c), along with a new fiber originating in the perikarya in the rostral olfactory bulb (arrowhead in c), aggregate and proceed caudally. Asterisks in c and f indicate transitions between ON and OB, and between OB and TL, respectively. a-d, $\times 160$; e-f, $\times 100$.

small bundles, LHRH-positive fibers were rarely observed.

Olfactory bulb In the olfactory bulb, LHRH-positive fibers were in the ventromedial portion running, in most cases, rostro-caudally over the

entire length of the olfactory bulb (Figs. 1 and 4). LHRH-positive fibers, which aggregated densely into bundles, were occasionally observed (Fig. 4). Some LHRH-positive fibers could be followed rostrally to the LHRH-positive perikarya of the

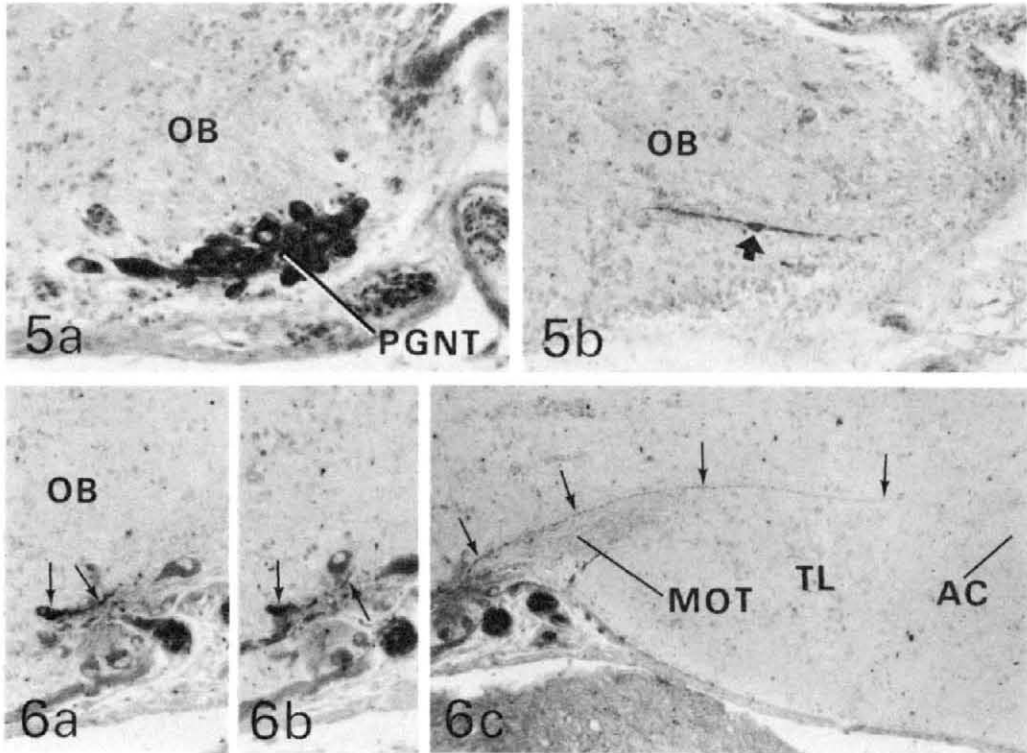


FIG. 5. Transverse (a) and sagittal (b) sections passing through the caudalmost part of the OB showing LHRH-positive cells of the PGNT. a, Note the mass of LHRH-positive cells. b, Note the apparently bipolar LHRH-positive cell sending fibers rostrally and caudally (arrow). a, $\times 210$; b, $\times 160$.

FIG. 6. Three successive parasagittal sections passing through the transition between the OB and TL. The scale is different for a-b and c. Note that an LHRH-positive fiber (arrows) originating in the perikaryon of the PGNT runs caudally in the MOT. a-b, $\times 180$; c, $\times 120$.

DGNT (Fig. 4), whereas others could be followed caudally to those of the PGNT (Fig. 5b). Thus, in the olfactory bulb, both ascending and descending LHRH-positive fibers could be observed. LHRH-positive fibers terminated in the olfactory bulb were not observed. In the caudalmost part of the olfactory bulb, LHRH-positive fibers were situated medial to or in the PGNT. These fibers, as well as fibers originating from the PGNT, proceeded caudally toward the telencephalon through the junction between the olfactory bulb and telencephalon (Figs. 1 and 6).

Telencephalon In the telencephalon, many LHRH-positive fibers were found in the ventral portion (Figs. 1 and 2c, d). These fibers were grouped into two according to either origin and/or location.

The fibers of the first group originated from both

the DGNT and PGNT and proceeded caudally in association with the MOT (Figs. 1, 2c and 6). In the region rostral to the anterior commissure, the fibers diverged medially from the MOT, and passed through the region between the dorsal and ventral limbs of the anterior commissure (Figs. 1, 2d and 8a). Beyond the anterior commissure, they separated in two directions: dorso-caudally toward the optic tectum, and ventrally toward the preoptic area to join the second group of LHRH-positive fibers mentioned below (Fig. 1). Some LHRH-positive fibers which coursed in association with the MOT separated ventrally from the others at the level of the rostral telencephalon, and joined the second group of LHRH-positive fibers (Fig. 1).

LHRH-positive fibers of the second group originated primarily from the ventral telenceph-

alone, but some originated from both the DGNT and PGNT, as mentioned above. Most of these ran rostrocaudally in the neuropil surrounded by the lateral border of the ventral nucleus of the area ventralis (Vv), the ventral border of the MOT, and the medial border of the lateral nucleus of the area ventralis (VI). Some, however, were located within the Vv and VI (Fig. 2c). In the pathway of these LHRH-positive fibers, there were LHRH-positive perikarya of the ventral telencephalic group (Fig. 1). Most of this group of LHRH-positive fibers proceeded dorsolaterally toward the most rostral portion of the area

dorsalis, and terminated therein (Figs. 1 and 2b). It was also found that some LHRH-positive fibers originating from the ventral telencephalon, entered the MOT and proceeded rostrally to the olfactory bulb.

The second group of LHRH-positive fibers could also be followed caudally to the commissure of Goldstein, and further toward the preoptic area (Figs. 1, 2c-e, 7 and 8). In the preoptic area, many LHRH-positive fibers were observed near the bottom of the preoptic recess and in the lateral preoptic area dorsolateral or lateral to the preoptic recess (Figs. 1, 2d-f and 9). In

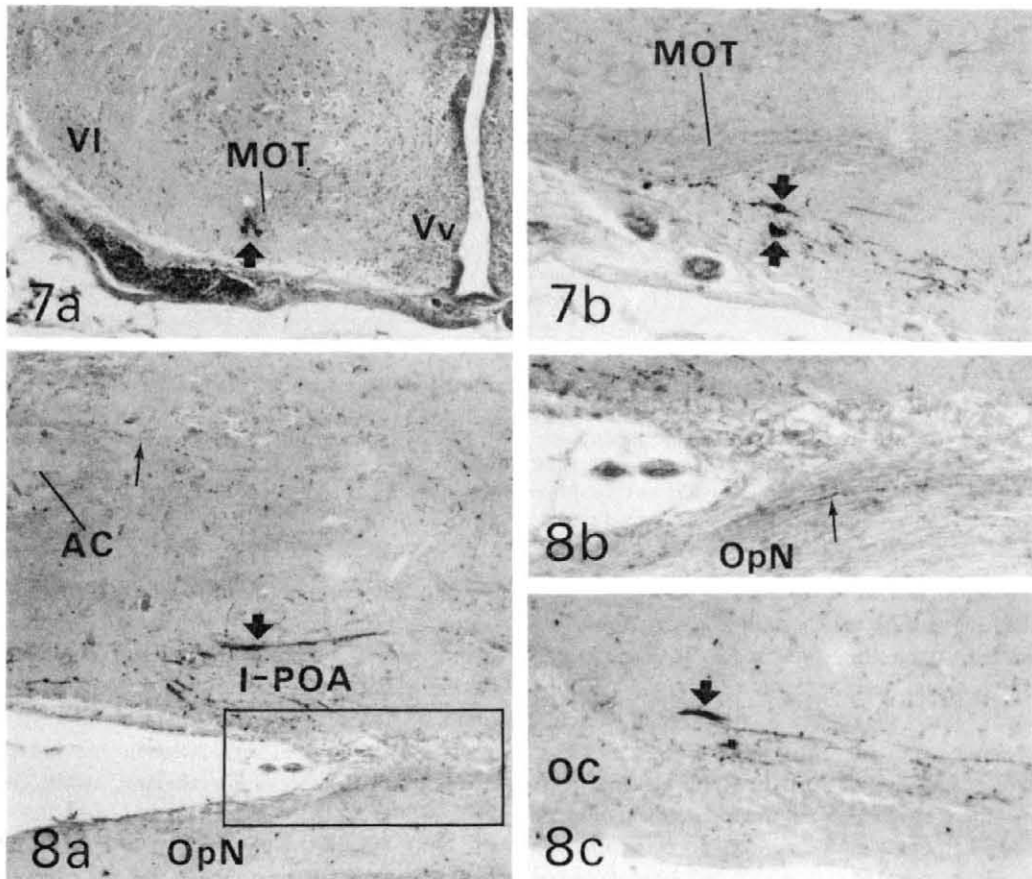


FIG. 7. Transverse (a) and sagittal (b) sections passing through the rostral telencephalon. In both a and b, note the LHRH-positive perikarya (arrows) distributed just ventral to the MOT. a, $\times 100$; b, $\times 150$.

FIG. 8. Two parasagittal sections (a and c) passing through the lateral preoptic area (l-POA). The rectangular area is enlarged and shown in b. Note the LHRH-positive perikarya (thick arrows in a and c) distributed in the l-POA. In a, there is an LHRH-positive fiber (thin arrow) near the AC, and in b an LHRH-positive fiber (arrow) in the OpN. a, $\times 150$; b, $\times 300$; c, $\times 170$.

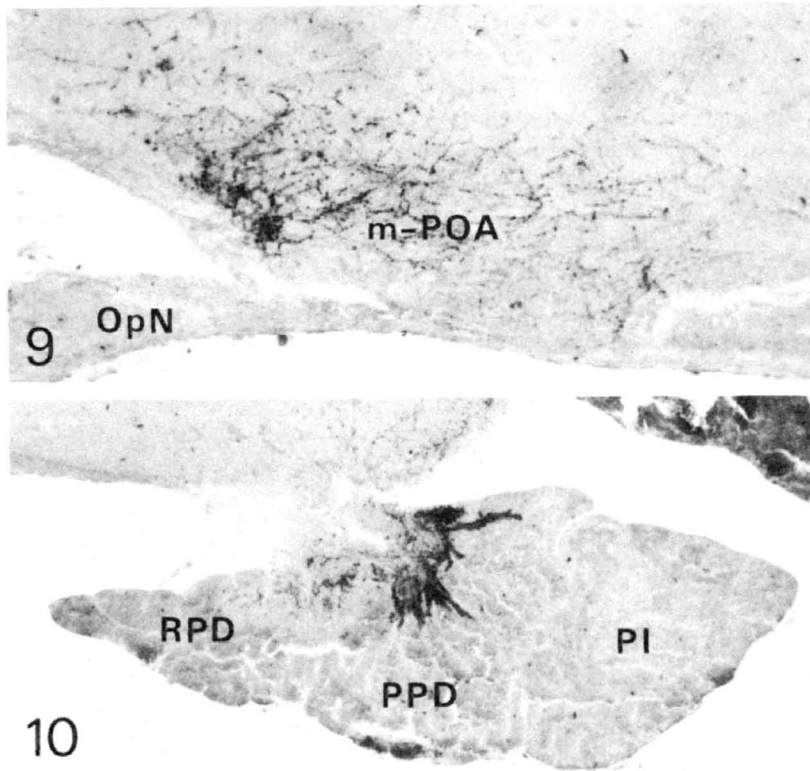


FIG. 9. Sagittal section passing through the medial preoptic area (m-POA) showing the distribution of LHRH-positive fibers in this region. $\times 200$.

FIG. 10. Sagittal section passing through the hypothalamo-hypophysial region showing the distribution of LHRH-positive fibers in the neurohypophysis. Nearly all the LHRH-positive fibers terminate in the caudal part of the anterior neurohypophysis just dorsal to the PPD. $\times 95$.

the neuropil lateral to the nucleus periventricularis (NPP), branching and bouton-like structures were observed, suggesting that some of the LHRH-positive fibers terminate in this area. LHRH-positive fibers were occasionally found in the optic chiasma or the optic nerve (Fig. 8b).

Other areas Most of the LHRH-positive fibers distributed in the lateral preoptic area could be followed caudally to the neurohypophysis through the ventrolateral portion of the hypothalamus (Figs. 1 and 2f-h). In this pathway, LHRH-positive fibers were accompanied by LHRH-positive perikarya of the preoptic group (Fig. 1). In the neurohypophysis, they were converged in the caudal region of the anterior neurohypophysis just dorsal to the proximal pars distalis (Fig. 10).

Several other LHRH-positive fibers in the lateral preoptic area proceeded dorsocaudally to join those passing through the anterior commissure, and further proceeded toward the optic tectum and the habenula (Fig. 1). Some of these LHRH-positive fibers were terminated in the habenula or nucleus dorsolateralis thalami, while most entered the optic tectum (Figs. 1 and 2g). In the optic tectum, they were distributed mainly in the stratum album centrale and stratum fibrosum et griseum superficiale.

A few LHRH-positive fibers were observed in the mesencephalic tegmentum, although their origins and destinations could not be determined (Fig. 1).

DISCUSSION

Determination of the primary structure of LHRH of the salmon [14] has been made recently. Salmon LHRH differs from mammalian LHRH in the amino acids in positions 7 and 8: Leu⁷ and Arg⁸ of mammalian LHRH take the place of Trp⁷ and Leu⁸ in the salmon. Thus, the length of the chain, the N-terminal six amino acids, and the C-terminal two amino acids have been stable during the process of evolution. In the present study, we used antisera generated against mammalian LHRH and the specificity of the immunoreaction was examined by preabsorption of the antisera with synthetic mammalian LHRH: the positive reaction in both the rat and eel brain was equally eliminated by preabsorption with a low concentration of synthetic mammalian LHRH. Although preabsorption of the antisera with salmon LHRH was not performed in the present study, our antisera seem to react with mammalian and eel LHRH.

The region in which LHRH-positive perikarya were demonstrated so far in the teleostean brain are: 1) ganglion cells of the nervus terminalis of the goldfish [6], in which only the DGNT is present [5], 2) NOR (PGNT) of the cichlid, poeciliid and centrarchid fishes [3, 4, 15], 3) dorsal telencephalon of the rainbow trout [16], 4) ventral telencephalon of the platyfish [17], 5) preoptic area of the platyfish [3, 15], carp [18], stickleback [19], and goldfish [20], 6) nucleus lateralis tuberis of the platyfish [3, 15] and goldfish [6, 20], 7) dorsomedial and ventromedial thalamus of the stickleback [19], and 8) dorsal midbrain of the platyfish [3]. In the Japanese eel, however, LHRH-positive perikarya were found in the DGNT, PGNT, ventral telencephalon and preoptic area, but in other regions only LHRH-positive fibers could be observed. Thus, our results are in partial agreement with previously published reports on other teleosts.

The precise origins and destinations of LHRH-positive fibers coursing through the olfactory and optic nerves could not be determined in this study. Through the application of LHRH-immunocytochemistry and horseradish peroxidase (HRP) tracing techniques, however, Münz *et al.*

[3, 4] found that, in the platyfish and cichlid, LHRH-positive fibers of the NOR pass into (1) the olfactory bulb and olfactory nerve, (2) the retina and (3) the brain, although their termination is uncertain. It is also reported that the nervus terminalis of the goldfish sends peripheral processes into the olfactory epithelium and central processes into the supracommissural nucleus of the telencephalon and retina through the MOT [1, 21]. Taking these findings into considerations, it seems probable that LHRH-positive cells of the DGNT and PGNT of the Japanese eel project their fibers into the olfactory epithelium and retina, as well as the ventral telencephalon, peri-commissural region, preoptic area and optic tectum.

Demski and Northcutt [1] proposed that, in the goldfish, the nervus terminalis may mediate various reproductive responses toward sex pheromones. Evidences which support this hypothesis are: (1) electrical stimulation of the MOT in reproductively active male goldfish elicits sperm release, whereas bilateral transection of the MOT in goldfish drastically reduces male responses to pheromones of reproductively active females (see [1]). The nervus terminalis ganglion cells of the goldfish are located in the rostral part of the olfactory bulb and send axons centrally to the ventral telencephalon through the MOT [1, 21], and (2) electrical stimulation of the optic nerve evokes sperm release in reproductively active male goldfish, which is claimed to be due to the stimulation of axon collateral of the nervus terminalis [1]. Furthermore, recent brain-lesion and/or -stimulation studies in goldfish and some other teleosts have suggested that both ventral telencephalon (particularly Vv and Vs) and preoptic area are also involved in the facilitation of male sexual behavior (goldfish [22–24], kokanee [25]). As shown in the present study, the distributional area of LHRH-positive fibers of the nervus terminalis system in the Japanese eel overlaps largely with the above-mentioned areas. Since LHRH is the neuropeptide most closely associated with reproductive function, LHRH-like substance in the nervus terminalis system may play a role in reproductive responses toward sex pheromones. Supporting this idea, in rodents it has been sug-

gested that LHRH acts directly on the brain and facilitates the reproductive behavior [26].

The nucleus lateralis tuberis (NLT) of teleosts has been suggested as the hypothalamic center for pituitary gonadotropin secretion and possibly may be the origin of the gonadotropin-releasing hormone [27–29]. Supporting the latter idea, LHRH-positive perikarya were demonstrated in the NLT of the goldfish [6, 21] and platyfish [3, 16]. However, in our study using the Japanese eel, LHRH-positive fibers of the neurohypophysis originated from the ventral telencephalon and preoptic area, but not from the NLT. There are two possible (mutually exclusive) reasons for this: (1) LHRH-neurons may be present in the NLT, but cannot be observed by immunocytochemical techniques (perhaps owing to an inadequate amount of LHRH in the perikarya), and (2) possibly LHRH-neurons may not be present in the NLT of the Japanese eel. In the rat, although the arcuate nucleus is generally considered as the hypothalamic center for tonic gonadotropin secretion, LHRH-neurons are not detectable there [30–32]. Clarification of this matter will require further research.

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