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Localization of mRNA Encoding Thyrotropin-Releasing Hormone Precursor in the Brain of Sockeye Salmon

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ABSTRACT—The distribution of mRNA encoding thyrotropin-releasing hormone (TRH) precursor in the brain of sockeye salmon was studied by *in situ* hybridization histochemistry using digoxigenin-labeled riboprobes as a basis to investigate its physiological functions in the salmon brain. Since seasonal variation in TRH gene expression was expected in relation to smolting or maturation, fish were sampled in February and October. In both groups, TRH mRNA was widely distributed in discrete brain regions including the internal cellular layer (ICL) of the olfactory bulb, postcommissural nucleus of area ventralis telencephali (Vp), nucleus preopticus parvocellularis anterioris (PPa), nucleus preopticus magnocellularis, dorsal zone of periventricular hypothalamus (Hd), torus semicircularis, and also the motor nucleus of vagus nerve in the medulla oblongata. TRH neurons in ICL and Hd are round and small with diameters of 5-10 μm. In contrast, TRH neurons in the ventral telencephalon and the preoptic area are medium-sized (10-20 μm), and appear to have multiple processes. Most of these cells are restrictively localized along the lateral margin of the preoptic nuclei. The number of TRH neurons in Vp and PPa was smaller in February than in October, suggesting a seasonal change of TRH neurons in the preoptic area. In the medulla oblongata, a cluster of large oval-shaped cells (20-30 μm) showed signals for TRH mRNA. The present results suggest that TRH may function as a neurotransmitter or neuromodulator involved in olfactory activity and also autonomic motor integration, in addition to neurohormonal control of secretion of pituitary hormones.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a tripeptide neurohormone (pGlu-His-Pro-NH_2), which was first isolated from the mammalian hypothalami as a factor that stimulates secretion of thyroid-stimulating hormone (TSH) (Schally *et al*., 1969; Burgus *et al*., 1970). Stimulatory roles of TRH on the pituitary-thyroid axis have been well studied in mammals (Jackson 1982). TRH immunoreactivity has been shown in the hypothalamic tuberoinfundibular system and also in the extrahypothalamic brain regions by radioimmunoassay (RIA) (Jackson and Reichlin, 1974) and immunohistochemistry (Lechan and Jachson, 1982; Merchenthaler *et al*., 1988; Fliers *et al*., 1994). Extrahypothalamic TRH may function as a neurotransmitter or neuromodulator in the central nervous system as well as in the gastrointestinal tract (Jackson 1982). In nonmammalian vertebrates, however, the physiological functions and distribution of TRH have been controversial (Jackson and Reichlin, 1974; Ball, 1981).

TRH stimulated TSH release in amphibians (Denver, 1988) and reptiles (Preece and Licht, 1987), whereas earlier studies had shown no effects (see Ball, 1981). TRH also stimulated release of prolactin (PRL) and α-melanocyte-stimulating hormone (α-MSH) in amphibians (Toron *et al*., 1983; Seki and Kikuyama, 1986). In birds, TRH elevated plasma levels of thyroid hormones and growth hormone (GH) (Leung *et al*., 1984). Actions of TRH in fishes are more confusing (see Crim *et al*., 1978). Some studies showed that TRH stimulates pituitary-thyroid axis in various teleosts (Tsuneki and Fernholm, 1975; Eales and Himick, 1988), whereas other studies could not find any effects (Bromage *et al*., 1976). Furthermore, Bromage (1975) suggested an inhibitory role of TRH on TSH release in guppy. As in other vertebrate classes, TRH stimulated release of pituitary hormones other than TSH in teleosts, such as PRL (Barry and Grau, 1986), GH (Trudeau *et al*., 1992) and α-MSH (Lamers *et al*., 1994).

Localization of TRH immunoreactive (TRH-ir) cells in the fish brains, particularly in the preoptic area, was not consistent among several studies. Hamano *et al*. (1990) showed TRH-ir cell bodies only in the nucleus recessus lateralis (NRL) of carp. Batten *et al*. (1990) demonstrated a number of TRH-ir cells in the NRL, but only a few TRH-ir cells in the nucleus preopticus (PON) of sea bass. On the other hand, Matz and Takahashi (1994) showed the presence of TRH-ir cells in the olfactory bulb (OB), ventral telencephalon and the preoptic area in the brain of chinook salmon. The distribution of TRH-ir cells in the PON along with TRH-ir fibers in the hypothalamus and pituitary is consistent with the hypophysiotropic role of TRH. Recently, Ohno *et al*. (1995) demonstrated TRH-ir

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cells in the preoptic area of sockeye salmon. TRH-ir cells in the OB of chum salmon were reported by Hamano *et al.* (1996).

Immunohistochemical localization of TRH in the brain has two major problems: (1) difficulty of raising potent and specific antibodies to TRH, and (2) loss of the peptide or alterations of its antigenic determinants during tissue fixation (Merchenthaler *et al.*, 1988). To overcome these difficulties, and more importantly to elucidate the mechanism of TRH biosynthesis, some investigators used an antiserum that recognize TRH prohormone (pro-TRH) (Lechan *et al.*, 1986; Merchenthaler *et al.*, 1989; Hamano *et al.*, 1996). In the rat brain, pro-TRH-ir cells were more widely distributed than TRH-ir cells, suggesting that TRH prohormone may be differentially processed in certain regions of the brain. *In situ* hybridization (ISH) of mRNAs for TRH precursor and also prohormone convertase (PC) demonstrated that PC genes were coexpressed in a subset of pro-TRH-expressing neurons in the rat (Segerson *et al.*, 1987; Pu *et al.*, 1996) and frog (Pu *et al.*, 1995).

It is, therefore, important to determine in the brain the distribution of cells containing TRH mRNA to address physiological roles of TRH and also of non-TRH peptides or extended forms of TRH peptides differentially processed from pro-TRH. Recently, we have isolated cDNAs encoding TRH precursors of sockeye salmon (Ohide *et al.*, 1996). In the present study, the distribution of TRH mRNA in the sockeye salmon brain was studied by ISH. In addition, we found a seasonal variation of TRH gene expression in the ventral telencephalon and anterior part of the preoptic area.

MATERIALS AND METHODS

Animals

Three-year-old sockeye salmon, *Oncorhynchus nerka*, of both sexes were obtained from the Toya Lake Station for Environmental Biology, Japan in October 1995 and February 1996. The fork length of the fish ranged from 20.6 to 23.5 cm, weighing from 82 to 139 g in the former fish, and from 21.2 to 24.4 cm, weighing from 107 to 164 g in the latter fish. Three males and three females of each month were used for ISH. They were not reproductively matured yet (GSI 0.03-0.11% for males, 0.50-0.89% for females) except for one male (GSI 3.5%) and two females (GSI 15 and 17%) in October.

Tissue preparation

Fish were lightly anaesthetized in 0.05% phenoxy ethanol. Immediately after decapitation, brains were dissected out and were fixed in 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7.3) at 4°C for 20 hr. After fixation, the tissues were washed in 70% ethanol at 4°C for 3 days, dehydrated through a series of ethanol solutions, and were embedded in paraplast. When embedded, the brains were placed ventral side up on the bottom of a paraffin cup, and were sectioned at 6 μm in either the sagittal or transverse plane at right angles to the plane parallel to the cranical bone (Fig. 1). They were separated into five groups, and were mounted on 3-amino-propyltriethoxysilane coated slides.

In situ hybridization (ISH)

ISH was performed as previously described (Zwingman *et al.*, 1994) with some modifications. Briefly, after rehydration, sections were treated with 0.3% Triton X-100 and 0.2 M HCl, immersed in acetic anhydrate (0.25% in triethanolamine 0.1 M, pH 8.0), and treated with 0.2% glycine. Digoxigenin-labelled riboprobes were synthesized with the sockeye salmon pro-TRH-A cDNA (Ohide *et al.*, 1996) cloned in pGEM-T (Promega, WI, USA). After linearization with *Spe* I, sense and antisense probes were transcribed *in vitro* with digoxigenin-UTP using a RNA labelling kit (Boehringer Mannheim) according to the manufacturer's instruction.

After prehybridization in 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 4.5) containing 50% formamide at room temperature for 30 min, hybridization was carried out at 42°C overnight in a hybridization buffer (50% formamide, 10% dextran sulfate, 5 × SSC, pH 4.5, 1 × Denhardt's solution, 0.1 mg/ml heparin, 0.1 mg/ml calf thymus DNA, 0.2 mg/ml yeast tRNA), containing digoxigenin-labelled riboprobes. Then, the sections were washed in 4 × SSC at 42°C for 20 min, treated with RNase A (0.02 mg/ml) in buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5) at 37°C for 1 hr, and washed sequentially with 2 × SSC and 0.1 × SSC at 50°C for 30 min twice. Hybridization signals were visualized using a nucleic acid detection kit (Boehringer Mannheim) with alkaline-phosphatase conjugated antidigoxigenin antibody and nitroblue tetrazolium (NBT), according to the manufacturer's instruction.

Data analysis

TRH mRNA-containing neurons in parasagittal and frontal sections, taken from different levels of the brain, were drawn under a microscope with a camera lucida. For histological identification of the localization of these neurons, one group of sections were stained with cresyl violet. The nomenclature for brain loci was taken from Northcutt and Davis (1983) for the telencephalon, Braford and Northcutt (1983) for the diencephalon and pretectum, and Nieuwenhuys and Pouwels (1983) for the brain stem. The abbreviations of brain loci are listed in Table 1.

Cell measurement and statistics

The number of TRH mRNA-containing cells in the preoptic area was counted by examining every section at 30 μm intervals to estimate the total number of TRH neurons contained in each brain. Differences in the number of TRH neurons in the Vp and PPa regions between the fish in February and October were tested by Mann-Whitney U-test.

RESULTS

Localization and morphology of TRH mRNA-containing cells

ISH for TRH mRNA demonstrated the presence of hybridization positive cells in several regions of the forebrain and the brain stem. An overview of the localization of these cells is shown in Fig. 1.

Olfactory bulb: TRH mRNA-containing cells were scattered in the internal cellular layer (ICL), whereas no hybridization signals were observed in the external cellular layer (ECL) (Fig. 2a-c). TRH cells in the ICL were dispersed among non-TRH neurons. They were round and small with diameters of 5-10 μm (Fig. 3a). Some TRH cells were located in the neighboring region of the ventral nucleus of area ventralis telencephali (Vv) (Fig. 2c).

Telencephalon: TRH cells were located in the postcommissural nucleus of area ventralis telencephali (Vp) (Fig. 2d-f). Most of TRH cells were scattered in the lateral region of Vp. The diameter of round somata ranged 5-10 μm. No hybridation positive cells were observed in the anterior region to the anterior commissure (AC) in the telencephalon.

Table 1. Abbreviations

AC	anterior commissure	PMg	nucleus preopticus magnocellularis, pars gigantocellularis
C	cerebellum		
D	area dorsalis telencephali	PMm	nucleus preopticus magnocellularis, pars magnocellularis
DF	nucleus diffusus of the inferior lobe		
Dc	central zone of D	PMp	nucleus preopticus magnocellularis, pars parvocellularis
Dd	dosal zone of D		
Dld	dorsal part of lateral zone of D	PON	nucleus preopticus
Dlv	ventral part of lateral zone of D	PPa	nucleus preopticus parvocellularis anterioris
Dm	medial zone of D	PPp	nucleus preopticus parvocellularis posterioris
Dp	posterior zone of D	Psm	nucleus pretectalis superficialis, pars magnocellularis
E	entopeduncular nucleus		
ECL	external cellular layer of olfactory bulb	SC	nucleus suprachiasmaticus
G	nucleus glomerulosus	SV	saccus vasculosus
HA	habenula	T	telencephalon
HT	hypothalamus	TL	nucleus tuberis lateralis
Hd	dorsal zone of periventricular hypothalamus	TLo	torus longitudinalis
Hv	ventral zone of periventricular hypothalamus	TP	nucleus tuberis posterior
ICL	internal cellular layer of olfactory bulb	TS	torus semicircularis
LH	nucleus lateralis hypothalami	V	area ventralis telencephali
LV	nucleus lateralis valvulae	VCg	granular layer of the valvula cerebelli
M	medulla oblongata	Vcm	molecular layer of the valvula cerebelli
nMLF	nucleus of the medial longitudinal fasciculus	VL	nucleus ventrolateralis
OB	olfactory bulb	VM	nucleus ventromedialis
OC	optic chiasm	Vd	dorsal nucleus of V
ON	optic nerve	Vp	postcommissural nucleus of V
OT	optic tectum	Vv	ventral nucleus of V
PIT	pituitary		

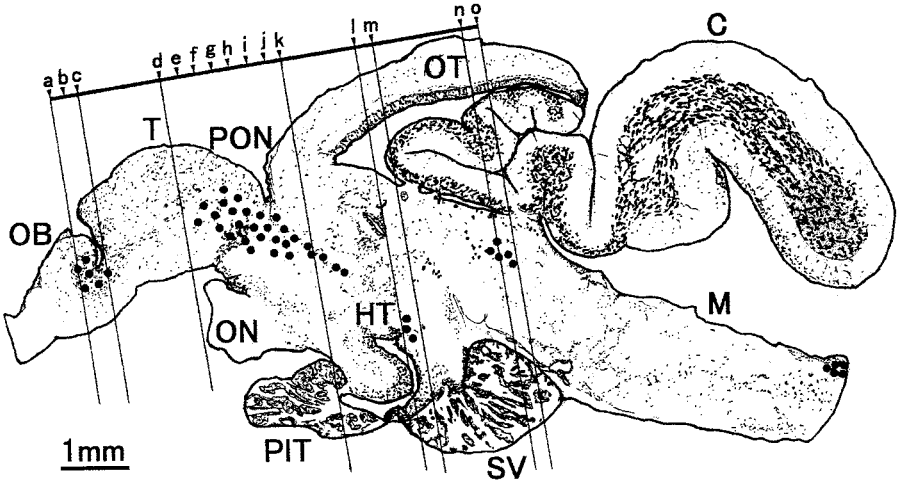


Fig. 1. Schematic illustration of the distribution of TRH mRNA-containing cells in the brain of sockeye salmon. Large dots represent TRH-expressing perikarya, and small dots indicate the Nissl-stained neurons. Planes of section for Fig. 2a-o are indicated. These planes are vertical to the plane parallel to the cranial bone.

Diencephalon: The largest number of cells containing TRH mRNA was observed in the hypothalamus. In the preoptic area, they were located in the nucleus preopticus parvocellularis anterioris (PPa) and nucleus preopticus magnocellularis (PM) (Fig. 2e-k). TRH cells in the PPa were mostly dispersed in the lateral margin of the PPa region (Figs. 2e-g, 3b), and their diameter ranged 10-15 μm. On the other hand, slightly larger cells (10-20 μm in diameter) showing intense hybridization signals were observed in the nucleus preopticus magnocellularis pars magnocellularis (PMm) and the nucleus preopticus magnocellularis pars gigantocellularis (PMg) (Fig. 2h-k). They were confined to the lateral border of the PM region, and appeared to have multiple processes (Fig. 3c and d). Some cells formed clusters, whereas the others were scattered among other neurons (Fig. 3d). In the ventral hypothalamus, weakly hybridization positive cells were located in the dorsal zone of periventricular hypothalamus (Hd) (Fig. 2l and m). They were round and small with diameters of

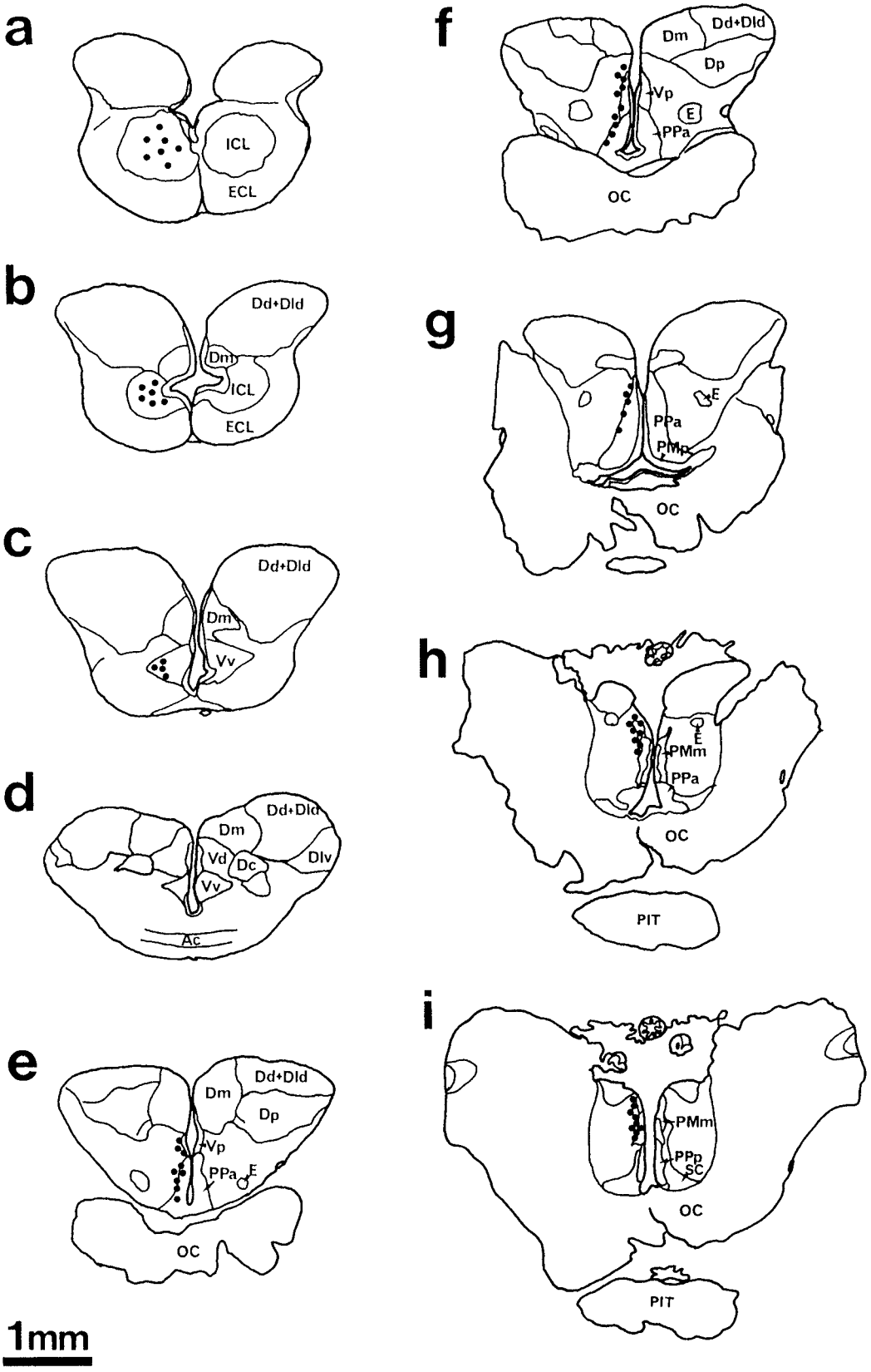
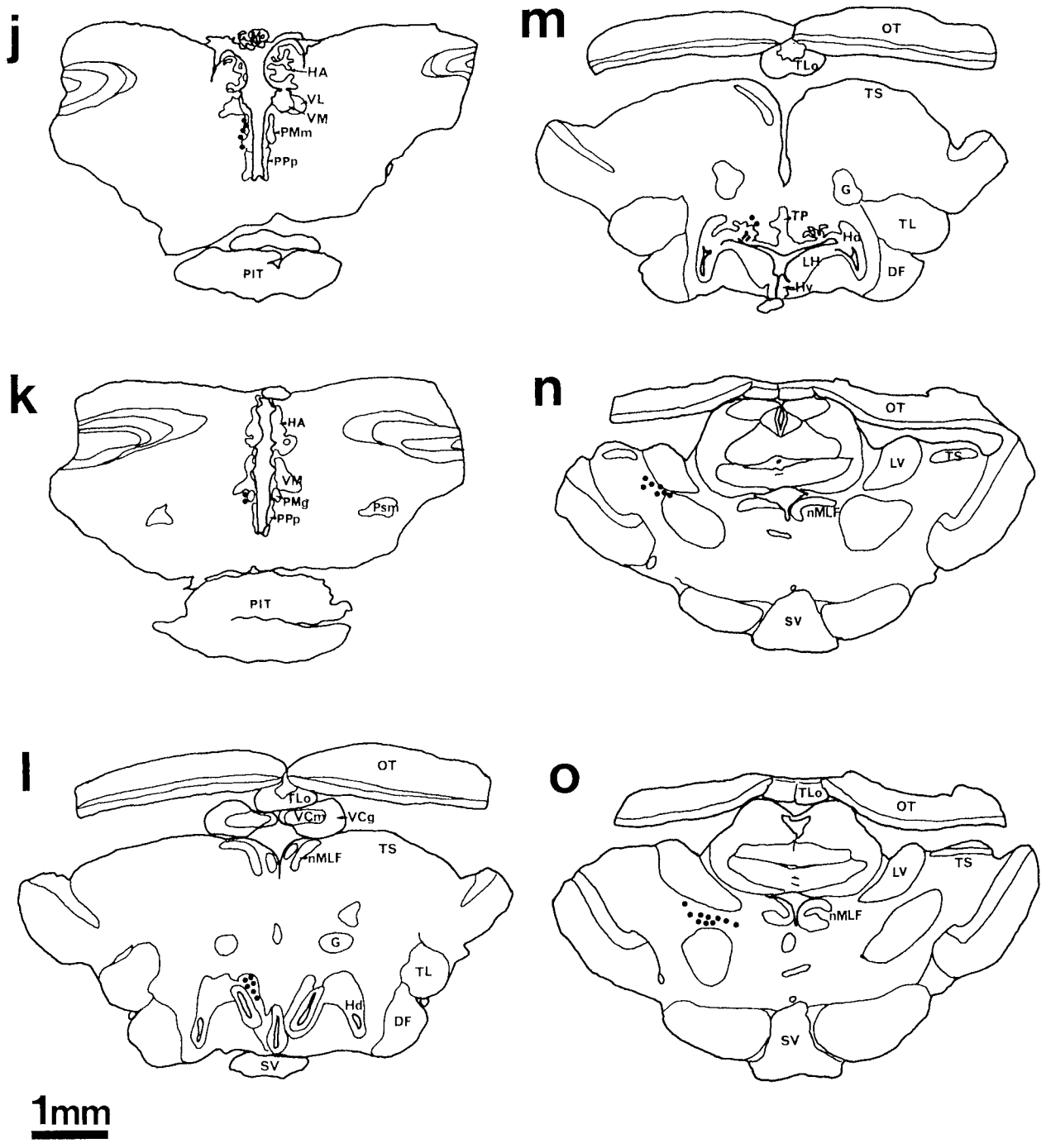


Fig. 2. (**a**-**o**) Schematic representation of TRH mRNA-containing perikarya (dots) in the salmon brain in frontal sections. Abbreviations are shown in Table 1.



5-10 μm.

Mesencephalon: Weakly hybridizing cells were distributed in the region lateral to the torus semicircularis (TS) in the midbrain tegmentum (Figs. 2n and o, 3e).

Myelencephalon: A cluster of large oval-shaped cells (20-30 μm in diameter) showed TRH mRNA signals (Fig. 3f). They were located in the the motor nucleus of vagus nerve in the medulla oblongata (Fig. 1).

Controls: In contrast to the specific hybridization with the antisense probe, parallel *in situ* hybridization studies with the sense riboprobe did not show any hybridization signals in all regions of the brain studied.

Seasonal variation in the number of TRH cells in the PPa and Vp regions

The number of TRH mRNA-containing cells in the preoptic area was examined in February (prior to smolting season) and October (spawning season). The number in the PPa and Vp regions was significantly smaller in the fish in February than in October, whereas no difference was observed in the

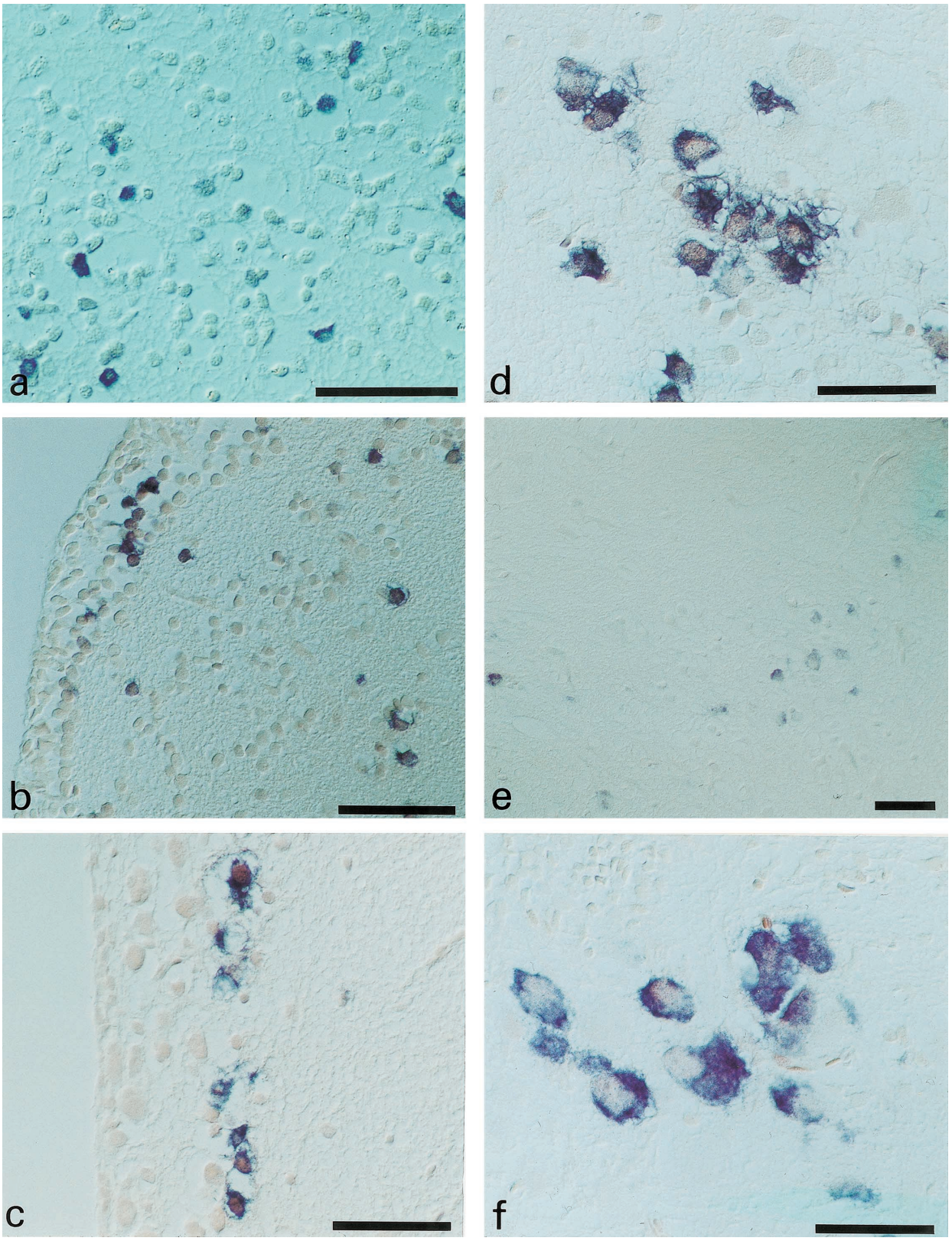


Fig. 3. TRH mRNA-containing perikarya in the salmon brain. (**a**) Coronal section through the internal cellular layer (ICL) of the olfactory bulb. (**b**) Coronal section through the nucleus preopticus parvocellularis anterioris (PPa). (**c**) Coronal section through the nucleus preopticus magnocellularis (PM). Note that TRH cells are confined to the lateral margin of the PM region. (**d**) Sagittal section through the PM. A cluster of TRH cells was observed. (**e**) Coronal section through the torus semicircularis (TS) in the mesencephalon. TRH cells were dispersed laterally. (**f**) Sagittal section through the motor nucleus of vagus nerve in the medulla oblongata. A cluster of large oval-shaped cells (20-30 μm in diameter) showed TRH mRNA signals. Scale bar, 50 μm.

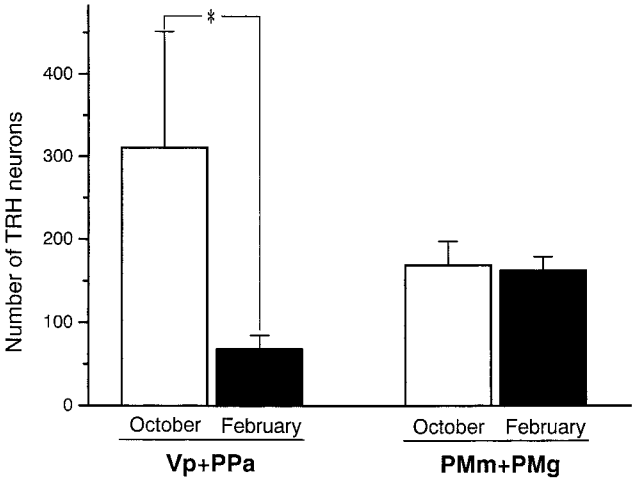


Fig. 4. The numbers of TRH mRNA-containing cells in the postcommissural nucleus of area ventralis telencephali (Vp) and nucleus preopticus parvocellularis anterioris (PPa) regions, and the nucleus preopticus magnocellularis pars magnocellularis (PMm) and nucleus preopticus magnocellularis pars gigantocellularis (PMg) regions of the salmon brain in October (open column) and February (solid column). *P < 0.05.

PMm and PMg regions (Fig. 4). Other regions containing TRH cells also did not show any apparent difference in the number of hybridization positive cells between February and October.

DISCUSSION

The present study demonstrated the distribution of TRH mRNA-containing cells for the first time in fish brain. ISH clearly showed TRH mRNA distribution in various regions of the salmon brain. We used riboprobes which were synthesized with the sockeye salmon TRH-A cDNA (Ohide *et al*., 1996). Ohide *et al*. (1996) showed that in sockeye salmon there are at least two types of TRH encoding genes (TRH-A and -B), which are highly homologous with each other (90% in a 242 bp region). Therefore, the ISH in this study may reveal distribution of both types of TRH mRNAs in the sockeye salmon brain. Differences in the distribution and amount of these mRNAs are unknown at present and should be determined in future using specific probes.

TRH mRNA-containing cells were distributed in several regions of the forebrain and the brain stem. Most prominent hybridization signals were observed in cells of the preoptic area. TRH cells were located in the nucleus preopticus parvocellularis anterioris (PPa) and nucleus preopticus magnocellularis (PM). Matz and Takahashi (1994) have demonstrated the presence of TRH-ir cell bodies in the PM but not in the PPa in the chinook salmon. Lack of TRH-ir cell bodies in the PPa is possibly because they used the fish in March and April when the level of expression of TRH encoding gene may be low in the PPa region as shown in the sockeye salmon in February (Fig. 4). It is also possible that in the PPa region the pro-TRH might be differentially procecced to generate non-TRH peptides or extended forms of TRH peptides as proposed in the rat brain (Pu *et al*., 1996). Otherwise, it may be because of the differences in species or stage. Ohno *et al*. (1995) have shown TRH-ir cells in the preoptic area of the sockeye salmon, but the exact location of the TRH-ir cell bodies was not specified. The results of the immunohistochemical and present ISH studies clearly demonstrate the presence of TRH cells in the preoptic area in salmon as reported in other vertebrates (rat, Lechan and Jackson, 1982; mallard, Jozsa *et al*., 1988; bullfrog, Seki *et al*., 1983), in concordance with the hypophysiotropic role of TRH. In the earlier studies, however, TRH-ir cell bodies could not be localized in the preoptic area in the carp (Hamano *et al*., 1990) and sea bass (Batten *et al*., 1990). This discrepancy may be because of the difficulty of immunohistochemisry of TRH peptide (Merchenthaler *et al*., 1988).

TRH cells in the preoptic area were frequently seen in the lateral marginal part of the nuclei (Fig. 3b and c). This feature is most apparent in the PMm. Because TRH-ir cells were shown to be dispersed within the preoptic area in the other classes of vertebrates (Lechan and Jackson, 1982; Seki *et al*., 1983; Jozsa *et al*., 1988), the distribution of TRH cells in the lateral border of the preoptic area may be unique to the salmon brain. In chinook salmon such tendency in the localization of TRH-ir cells in the PM was not mentioned (Matz and Takahashi, 1994).

In the preoptic area, vasotocin (VT) and isotocin (IT) neurons are densely localized in the PM. IT neurons are further distributed in the PPa (Ota *et al*., 1996). Moreover, in the preoptic area, distributed are neurons containing hypothalamic releasing hormones, such as gonadotropin-releasing hormone (GnRH) (Amano *et al*., 1991) and corticotropin-releasing hormone (CRH) (Ando *et al*., 1999). Meister *et al*. (1990) have demonstrated a small number of both vasopressin (VP) and oxytocin (OT) neurons colocalized with TRH in the rat paraventricular nucleus. VP has been shown to elicit release of TSH (Lumpin *et al*., 1987), indicating that this colocalization may be functional. In salmon brain, although the colocalizations of TRH and VT, IT and/or CRH remain to be determined, TRH cells are likely to interact with these neurosecretory neurons and control pituitary functions.

It is of interest to note that the number of TRH cells in the preoptic area showed seasonal variation (Fig. 4). Seasonal variations in neurosecretory neurons are deeply involved in control of seasonal changes in physiological activities (Jokura and Urano, 1985; Hofman and Swaab, 1992). In the present study, the number of TRH cells in the PPa and Vp region was significantly different between February and October, whereas that in the PM region was not. This suggests that physiological functions of the TRH cells in the preoptic area are different between the PPa and PM nuclei. In mammals, the magnocellular neurons contain VP and OT, while the parvocellular neurons contain the hypothalamic releasing hormones. However, such anatomical distinctions have not been observed in salmon brain; IT (Ota *et al*., 1996) and GnRH (Amano *et al*., 1991) neurons are distributed in both PPa and PM nuclei. Therefore, it is difficult to correlate the location of

TRH cells with their function at present. Precise studies on colocalization of TRH and other neurohormones and also studies to determine to which types of pituitary cells the preoptic TRH neurons project their axons are necessary to clarify the physiological significance of the seasonal variation of TRH cells in the preoptic area.

Localization of TRH mRNA-containing cells in the ICL of the olfactory bulb (Fig. 3a) corresponds to the distribution of TRH-ir cell bodies (Matz and Takahashi, 1994). TRH-ir cell bodies in the ICL have been suggested to contribute TRH fibers in the olfactory tracts, and to project to olfactory targets in the ventral telencephalon, the preoptic area and the hypothalamic regions, all of which are known to receive olfactory projections in teleosts (Matz and Takahashi, 1994). Therefore, TRH is considered to play a role in the olfactory system, may act as a transmitter or neuromodulator in the salmon brain. Hamano *et al*. (1996) showed proTRH-ir neurons in the ECL, but not in the ICL in the chum salmon. This discrepancy might be due to the difference of animal stock. Hamano *et al*. (1996) used a wild stock of the chum salmon during the spawning migration, while we and Matz and Takahashi (1994) used salmons fed in the hatchery. In homing salmon, activities and functions of TRH neurons in the olfactory bulb might be different from those in the salmon fed in hatchery.

The present study showed the presence of TRH mRNA in the motor nucleus of vagus nerve in the medulla oblongata. Here the TRH cells are larger (20-30 μm) than those in other brain regions. The presence of TRH mRNA and TRH-ir in the dorsal motor nucleus of the vagus were reported in the rat (Lechan *et al*., 1986; Segerson *et al*., 1987). It was suggested that TRH in the dorsal vagal complex may function in the control of gastric secretion and motility in the rat (Rinaman *et al*., 1989). Furthermore, TRH in the rat medulla was involved in modulation of respiratory function (McCown *et al*., 1986). In teleost medulla, the motor nucleus of vagus nerve was suggested to be involved in the control of rhythmic breathing movements (Shelton, 1970). Therefore, TRH in the motor nucleus of vagus nerve may function as a neurotransmitter or neuromodulator involved in the autonomic control of breathing also in salmon.

The present study demonstrates a precise distribution of the TRH mRNA-containing neurons in the salmon brain. Based upon the comparison with the distribution of TRH-ir cells, the TRH mRNA-positive cells are more widely distributed than TRH-ir cells. Particualry, the possibility of differential splicing of pro-TRH was suggested in the PPa region. Interestingly, in this region the number of TRH cells was seasonally variable. It should be of considerable interest and importance to determine the physiological roles of TRH cells in the preoptic area in salmon brain. The present study also suggests the multiple TRH neuronal systems; TRH may function in the extrahypothalamic neuromodulatory systems involved in olfactory and respiratory functions as well as the conventional hypophysiotropic system.

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