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Immunohistochemical Localization of C-RFamide, a FMRF-related Peptide, in the Brain of the Goldfish, *Carassius auratus*

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ABSTRACT—Carassius RFamide (C-RFa) is a novel peptide found in the brain of the Japanese crucian carp. It has been demonstrated that mRNA of C-RFa is present in the telencephalon, optic tectum, medulla oblongata, and proximal half of the eyeball in abundance. Immunohistochemical methods were employed to elucidate the distribution of the peptide in the brain of the goldfish (*Carassius auratus*) in detail. C-RFa-immunoreactive perikarya were observed in the olfactory bulb, the area ventralis telencephali pars dorsalis and lateralis, nucleus preopticus, nucleus preopticus periventricularis, nucleus lateralis tuberis pars posterioris, nucleus posterioris periventricularis, nucleus ventromedialis thalami, nucleus posterioris thalami, nucleus anterior tuberis, the oculomotor nucleus, nucleus reticularis superior and inferior, facial lobe, and vagal lobe. C-RFa immunoreactive fibers and nerve endings were present in the olfactory bulb, olfactory tract, area dorsalis telencephali pars centralis and medialis, area ventralis telencephali, midbrain tegmentum, diencephalon, medulla oblongata and pituitary. However, in the optic tectum the immunopositive perikarya and fibers were less abundant. Based on these results, some possible functions of C-RFa in the nervous system were discussed.

INTRODUCTION

The tetrapeptide FMRFamide was first discovered as a cardio-excitatory peptide in molluscs (Price and Greenberg, 1977). Subsequently, many other bioactive peptides containing the consentaneous sequence -Arg-Phe-NH_2 (RFamide) at their C-terminus have been characterized in various animals. Several cardioactive RFamides have also been identified in other molluscs. In addition, FMRFamide and/or their related peptides have been localized and characterized by immunocytochemical analyses in the central nervous systems of both vertebrates and invertebrates (Bonn and König, 1989; Fischer *et al*., 1996; Stell *et al*., 1984). For example, LPLRFamide is present in the chicken brain (Dockray *et al*., 1983), and NPFF and NPAF in the bovine brain (Yang *et al*., 1985) (Table 1).

In the teleost, Fujimoto *et al.* (1998) isolated and characterized a novel RFamide (Carassius RFamide: C-RFa) from the brain of the Japanese crucian carp. It consisted of 20 amino acids with RFamide; the sequence is SPEIDPFWYVGRG-VRPIGRF-NH_2, which is significantly homologous to the molluscan neuropeptide ACEP-1 (Fujimoto *et al*., 1990) (Table 1). Recently the cDNA encoding a precursor of C-RFa was characterized (Satake *et al*., 1999). Southern blot analyses of RT-PCR products have demonstrated C-RFa mRNA to be abundant in the telencephalon (TE), optic tectum (OTec), medulla oblongata (MO) and proximal half eyeball (Satake *et al*., 1999). In the present study, the localization of C-RFa in the brain of goldfish was characterized by immunohistochemical methods and its possible functions in the nervous system were discussed.

MATERIALS AND METHODS

Twenty goldfish, *Carassius auratus*, (40–60 mm in total body length) were used. The experimental procedures in the present study were performed under the guidelines for the Care and Use of Animals in the Fields of Physiological Sciences approved by the Physiological Society of Japan. Fish were anesthetized by immersion in a solution containing 3-aminobenzoic acid ethyl ester (MS222) (150 mg/l). The brains were dissected rapidly from the skull, and then fixed in 4% paraformaldehyde in 0.2M phosphate buffer, pH 7.2 for 24 hr. After thorough rinses in 0.02M phosphate buffered saline (PBS, pH 7.2) and in PBS containing 1% Triton X-100 (PBS-Triton), the brains were

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Table 1. FMRFamide and several related peptides

Name	Sequence	Source
FMRFamide	FM RFa	Molluscs, Annelids
LPLRFamide	LPL RFa	Chicken
NPFF (F8Famide)	FLFQPQ RFa	Ox
NPAF (A18Famide)	AGEG LS S P FWSL AAPQ RFa	Ox
ACEP-1	SGQSWRPQG RFa	African giant snail
C-RFa (Carassius RFamide)	SPE I DPFWY VGRGV RP I G RFa	Crucian carp
Prolactin-releasing Peptide	TPD I NPAWY AGRG I RPVG RFa	Bovine

immersed in 30% sucrose in 0.2M phosphate buffer until they sank to the bottom of the container, usually overnight.

Antiserum (anti-C-RFa/rabbit) was raised in the Yanaihara Inst. Inc. (Shizuoka, Japan) against C-terminal C-RFa decapeptides, $[Cys]^{10}$-C-$RF^{11–20}$ (GRGVRPIGRF). The specificity of the antibody to FMRFa was evaluated by a competitive enzyme-linked immunosorbent assay (ELISA). Different concentrations, 0.01–10000 pmol/well, of C-RFa and FMRFa were used as competitors. The antiserum reacted with C-RFa less than a picomole of C-RFa, but not with FMRFa. Transverse, horizontal and sagittal sections (10 μm in thickness) were cut on a cryostat. Following three 10 min washes with 0.02 M PBS, sections were stained with anti-C-RFa and a commercial Kit (VECTASTAIN elite ABC kit, Vector Laboratories, Burlingame, USA).

To reduce the endogenous peroxidase activity, the sections were treated with 2% H_2O_2, rinsed in 0.02M PBS and subsequently incubated with diluted normal goat serum for one hour to minimize background staining. Subsequently the sections were incubated in the primary antiserum (anti-C-RFa) diluted 1:1000 in 0.02M PBS containing 1% bovine serum albumin (BSA) at 4°C overnight. The BSA was also added to the second antiserum to reduce further the non-specific background staining.

The sections were rinsed in PBS and then incubated in the secondary antiserum (goat anti-rabbit IgG) for one hour at room temperature. The slides were washed with 0.02M PBS, and then incubated in avidin-biotin-HRP complex for one hour at room temperature. After a rinse in PBS, the preparations were reacted with 3.3'-diamino benzidine tetrahydrochloride, nickel chloride hexahydrate and 0. 05% H_2O_2 in 0.1M Tris-HCl (PH7.2) for 10 min. The slides were then washed and covered with a mounting medium (Crystal/Mount, Biomeda Corp., USA).

Instead of the primary antibody normal rabbit serum was used to test the specificity of the antisera. All of the positive immunoreactions reported below were absent when normal rabbit serum was used instead of anti-C-RFa. A preabsorption control experiment was also done and all labeling of cells in the brain was abolished. Therefore, the immunoreactions were specific within the parameters used.

We followed the terminology of Peter and Gill (1975) for the forebrain, and Nieuwenhuys and Pouwels (1983) for the brainstem.

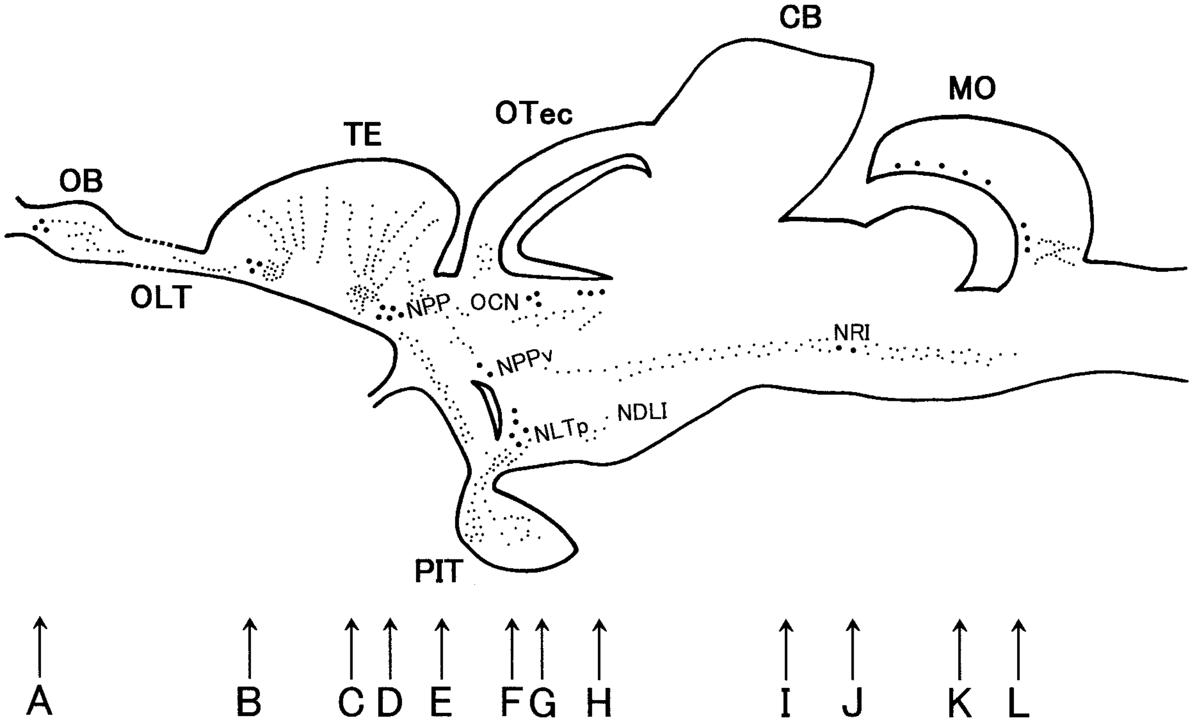

Fig. 1. Schematic illustration of the distribution of C-RFa-immunoreactive perikarya (filled circles) and fibers (dotted lines) in the paramedian sagittal section of the goldfish brain and pituitary. Arrows (B-L) at the bottom of the figure indicate the transverse sections depicted in Fig. 2 (B-L). Abbreviations: CB, cerebellum; MO, medulla oblongata; NDLI, nucleus diffusus lobi inferioris; NLTp, nucleus lateralis tuberis pars posterioris; NPP, nucleus preopticus periventricularis; NPPv, nucleus posterioris periventricularis; NRI, nucleus reticularis inferior; OB, olfactory bulb; OCN, oculomotor nucleus; OLT, olfactory tract; OTec, optic tectum; PIT, pituitary; TE, telencephalon

RESULTS

A schematic representation of perikarya and fibers showing C-RFa-immunoreactivity projected on the paramedian sagittal plane of the goldfish brain and pituitary is shown in Figure 1.

1. **Olfactory Bulb** (Fig. 2A)

A group of weakly stained, small C-RFa-immunoreactive perikarya was present in the rostroventral region of the olfactory bulb (OB). Some fibers were stained in the medial margin of the rostroventral parts of the OB and olfactory tract (OLT). A few fibers passed through the lateral OLT and entered the rostral TE.

2. Telencephalon

There were two groups of intensely C-RFa-immunoreactive perikarya in the area ventralis telencephali pars dorsalis (Vd) and pars ventralis (Vv) (Fig. 2B, Fig. 3A). A large number of nerve endings and fibers in the area dorsalis telencephali pars medialis (Dm), centralis (Dc) and dorsalis (Dd) were stained.

3. Diencephalon

[Perikarya]

At the level of the optic chiasm, there were numerous intensively C-RFa immunoreactive perikarya in the NPO and NPP (Fig. 2D, Fig. 3B). At the level of the postoptic commissure (PC), a number of C-RFa-immunoreactive perikarya were present in the NAPv, NPO and NPP (Fig. 2E) which were located subependymally and laterally to the third ventricle. The fibers of these neurons ran to the nucleus recessus lateralis (NRL). At the level of the posterior commissure, there were some scattered C-RFa-immunoreactive perikarya in the nucleus ventromedialis thalami (NVM), which continued caudally to the nucleus posterioris periventricularis (NPPv) and the nucleus anterioris tuberis (NAT). In the nucleus lateralis tuberis pars posterioris (NLTp), weakly stained C-RFa-immunoreactive perikarya were found among the dense arrays of immunoreactive fibers (Fig. 2F, Fig. 3C, D). There were numerous C-RFa-immunoreactive perikarya in the nucleus posterioris thalami (NTP) at the level of the nucleus cerebellosus hypothalami (Fig. 2G, Fig. 3E).

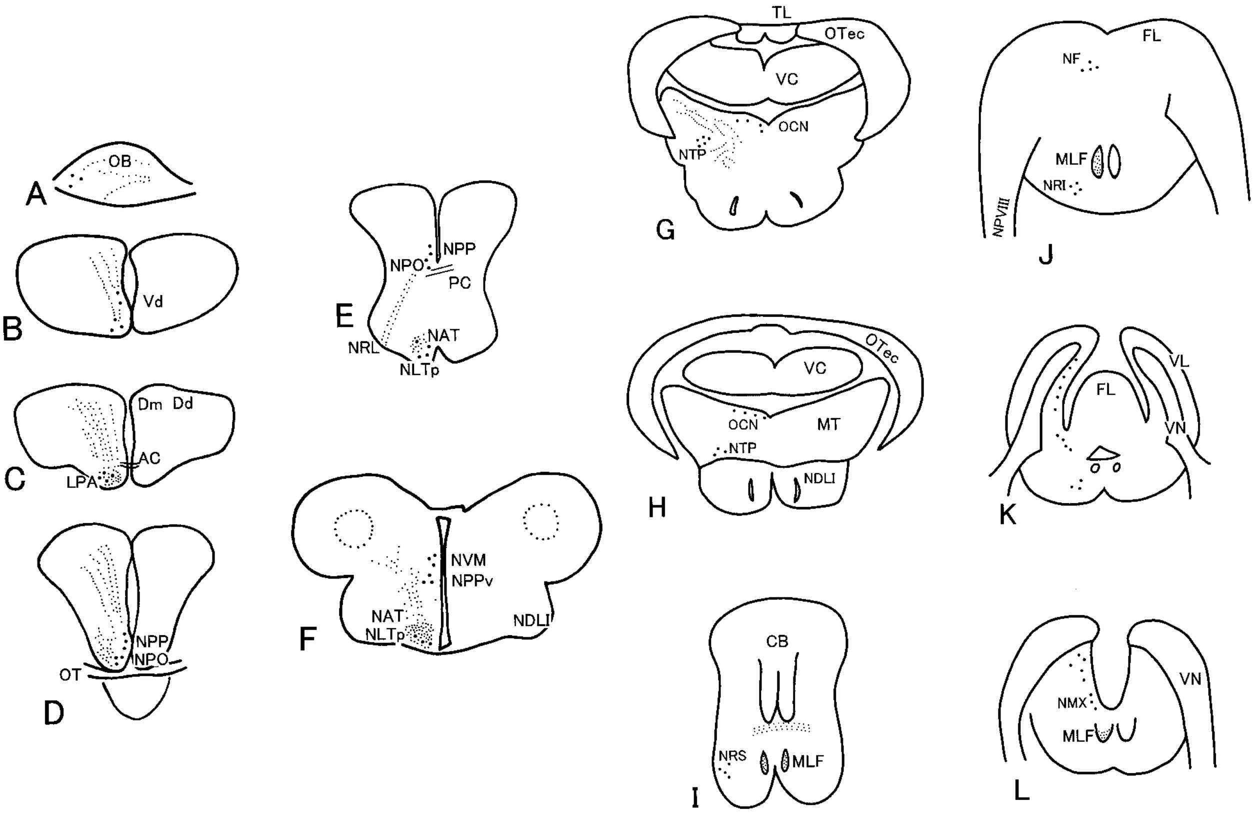

Fig. 2. Schematic illustration of the distribution of C-RFa-immunoreactive perikarya (filled circles) and fibers (dots or lines) in the paramedian sagittal section of the olfactory bulb (A) and in the transverse brain sections cut at the levels indicated by B-L in Fig. 1.

Abbreviations: AC, anterior commissure; CB, cerebellum; Dd, area dorsalis telencephali pars dorsalis; Dm, area dorsalis telencephali pars medialis; FL, facial lobe; LPA, lateral preoptic area; MLF, medial longitudinal fascicle; MT, midbrain tegmentum; NAT, nucleus anterioris tuberis; NDLI, nucleus diffusus lobi inferioris; NDTL, nucleus diffusus tori lateralis; NF, nucleus facies; NLTp, nucleus lateralis tuberis pars posterioris; NMX, nucleus motorius nervi vagi; NPO, nucleus preopticus; NPP, nucleus preopticus periventricularis; NPPv, nucleus posterioris periventricularis; NPVIII, posterior branch of the eighth nerve; NRI, nucleus reticularis inferior; NRL, nucleus recessus lateralis; NRS, nucleus reticularis superior; NTP, nucleus posterioris thalami; NVM, nucleus ventromedialis thalami; OB, olfactory bulb; OCN, oculomotor nucleus; OT, optic tract; OTec, optic tectum; PC, postoptic commissure; TL, torus longitudinalis; VC, valvula cerebelli; Vd, area ventralis telencephali pars dorsalis; VL, vagal lobe; VN, vagal nerve; Vv, area ventralis telencephali pars ventralis

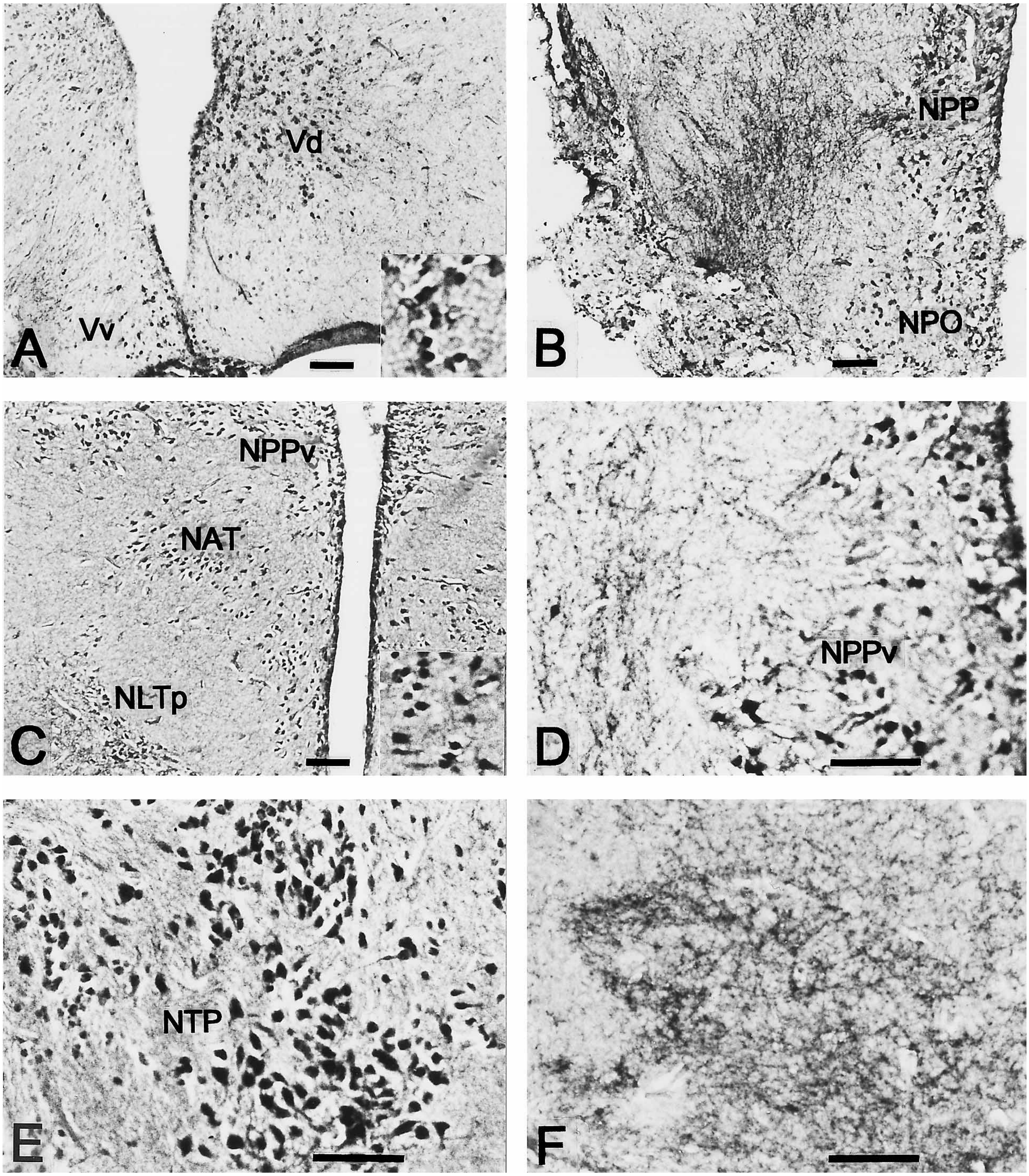

Fig. 3. Transverse sections of the brain. A: Section through the rostral telencephalon (TE). Numerous immunoreactive perikarya can be observed in the area ventralis telencephali pars ventralis (Vv) and dorsalis (Vd). The inset in the lower right corner shows the Vd cells magnified. B: Section through the TE and rostral thalamus at the level of optic chiasm. Immunoreactive perikarya are found in the nucleus preopticus (NPO) and nucleus preopticus perivertricularis (NPP). Numerous immunoreactive fibers can be observed in the lateral region of the NPP. C: Section through the optic tectum (OTec) and hypothalamus at the level of posterior commissure (PC). In the nucleus lateralis tuberis pars posterioris (NLTp), perikarya showing weak C-RFa-immunoreactivity can be found among the dense arrays of C-RFa-immunoreactive fibers. Intensely C-RFa-immunoreactive perikarya can be found in the nucleus posterioris periventricularis (NPPv) and the nucleus anterior tuberis (NAT). D: Section through the OTec and hypothalamus in back of the PC. Immunoreactive perikarya are found in the NPPv. The inset shows the NAT magnified. E: Section through the midbrain tegmentum at the level of the oculomotor nucleus showing immunoreactive perikarya in the nucleus posteriors thalami (NTP). F: C-RFa-immunoreactive fibers which form varicosities crowded in the lateral preoptic area. Scale bar: 50 μm.

[Fibers]

At the level of the anterior commissure (AC), there were abundant C-RFa fibers in the lateral preoptic area (LPA) showing varicosities (Fig. 2C, Fig. 3F). A large number of C-RFa-immunoreactive fibers and nerve endings were found below the nuclei in the ventrorostral diencephalon. At the level of the PC, there was a C-RFa-immunoreactive fiber tract which went from the NAPv, NPO and NPP downward to the NRL of the hypothalamus (Fig. 2E). Many C-RFa-immunoreactive fibers were found close to and within the NAT, and in the whole ventral diencephalon. In all cases most of the C-RFa fibers were observed in the surrounding structures of the thalamic and hypothalamic nuclei, next to the NVM, NPPv and NLTp. There was an increase in the number of fibers with C-RFamide varicosities around the NLTp. There were a few C-RFa-immunoreactive fibers in the nucleus habenularis.

4. Pituitary

Many C-RFa-immunoreactive fibers and nerve endings, which may originate from NLTp, traversed the pituitary stalk, and connected to the neurohypophysis (Fig. 4A). In the pituitary, some fibers and nerve endings were stained in the proximal pars distalis and the rostral pars distalis of the adenohypophysis (Fig. 4B).

5. Mesencephalon

[Perikarya]

At the level of the rostral medial longitudinal fascicle (MLF), intensely C-RFa-immunoreactive perikarya were found in the nucleus medialis fasciculi longitudinalis. A few large labeled somata were localized in the oculomotor nucleus (OCN) (Fig. 2G, H).

[Fibers]

In the OTec there were a few C-RFamide-immunoreactive fibers in the whole tectal area. In most sections C-RFa-immunoreactive fibers were present in the stratum fibrosum et griseum centrale, with fewer reactive fibers in the stratum album centrale. Exquisitely stained fibers were present in the

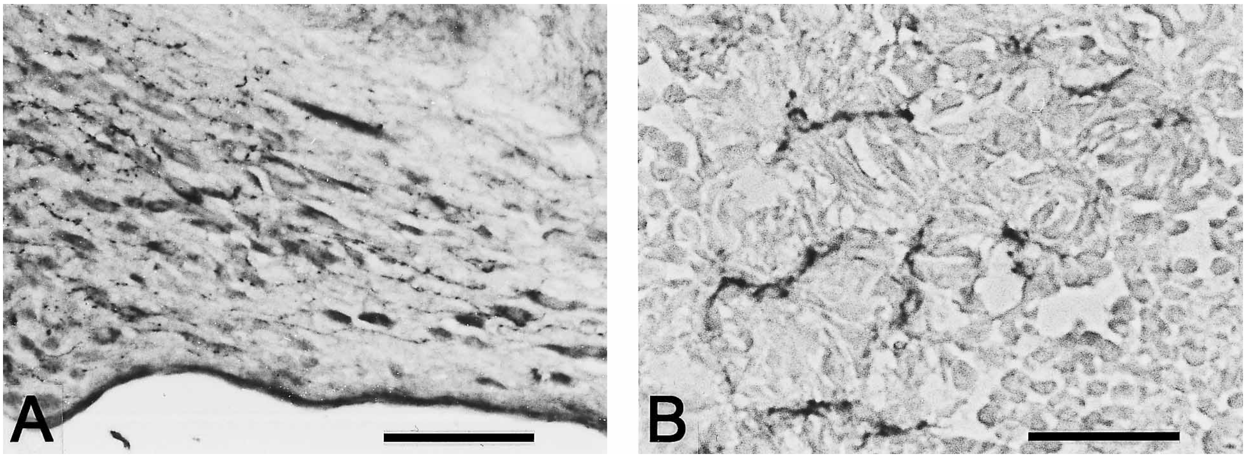

Fig. 4. Parasagittal section of the pituitary stalk (A) and the pars distalis of the adenohypophysis (B) showing C-RFa-immunoreactive fibers and nerve endings . Scale bar: 50 µm.

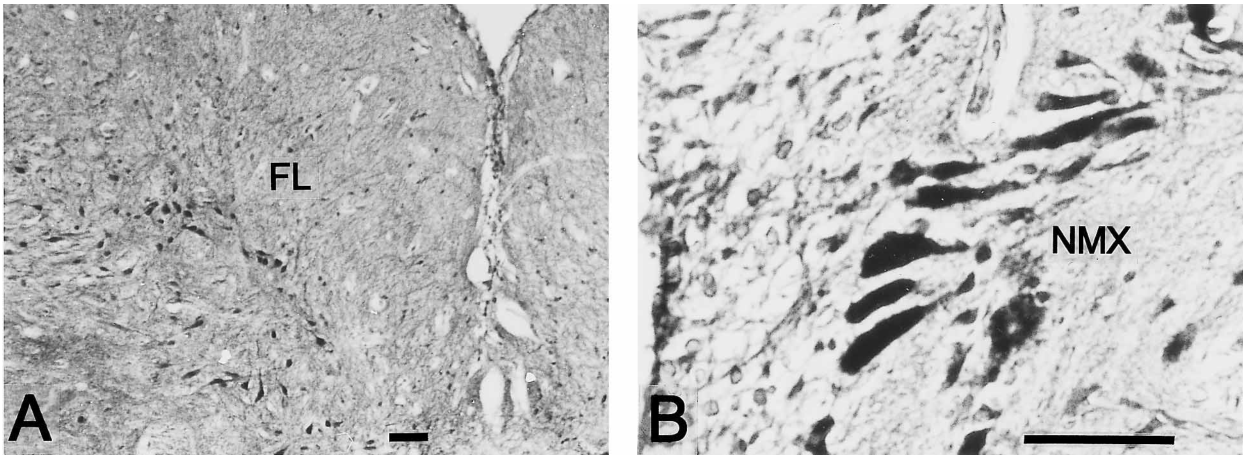

Fig. 5. A: Transverse section through the medulla oblongata at the level of facial lobe (FL). Two groups of weakly immunoreactive perikarya can be observed in the facial nucleus. B: Transverse section through the dorsal medulla oblongata showing the nucleus motorius nervi vagi (NMX). Scale bar: 50 µm.

torus longitudinalis (TL).

There were few scattered C-RFa fibers in the rostro-lateral midbrain tegmentum (MT). The torus semicircularis also contained C-RFa-immunoreactive fibers at its margin.

6. Rhombencephalon

[Perikarya]

At the level of the corpus cerebelli, there were many intensely labeled somata confined to the nucleus reticularis superior (NRS) (Fig. 2I). Small, weakly labeled somata were confined to a dorsomedial part of the facial lobe (FL) (Fig. 2J, Fig 5A). At the level of the posterior branch of the eighth nerve, there were intensely labeled C-RFa-immunoreactive perikarya in the nucleus reticularis inferior (NRI) (Fig. 2J). At the level of vagal lobe (VL), numerous small and some large C-RFa-immunoreactive perikarya were observed in the VL and nucleus motorius nervi vagus (NMX)(Fig. 2K, L, Fig. 5B).

[Fibers]

In the whole goldfish brain, there were no C-RFa fibers in the valvula cerebelli (VC) and corpus cerebellum (CB). Two groups of C-RFa-immunoreactive fibers were found in the MO. The first group was localized in the MLF which connects the rostral brainstem with the caudal MO (Fig. 2I); the second was located ventrolaterally beneath the MLF. In the caudoventral VL, there were a few C-RFa fibers in the caudal base of the VL (Fig. 1).

DISCUSSION

C-RFa is the first RFamide found in the fish brain (Fujimoto *et al.*, 1998). Therefore it was of interest to investigate its distribution to understand its physiological function. C-RFa may have several functions in the nervous system since it has an RFamide residue at the C-terminus as does FMRFamide. Furthermore, the primary sequence of C-RFa is quite homologous to that of mammalian prolactin-releasing peptide (PrRP) (Hinuma *et al.*, 1998). A comparative study has revealed that several sequences conserved in preproPrRPs are also found in the C-RFa precursor (Satake *et al.*, 1999). This suggests that C-RFa and PrRPs may share analogous physiological functions in fish and mammals.

In the previous study, the expression of the C-RFa mRNA was examined in five divisions of the brain. The levels of C-RFa transcript were high in the TE, OTec and MO, and rather low in the CB and very low in the OB (Satake *et al.*, 1999). In this study, we have detected C-RFa-immunoreactive perikarya in the TE and MO as in the previous study. Abundant perikarya were also found in the diencephalon and there were a few perikarya in the MT and OB. On the other hand, C-RFa-immunoreactive fibers were widely distributed in the OB, TE, pituitary, diencephalon, OTec, MT, and MO. However, there were no C-RFa-immunoreactive perikarya and fibers in the CB.

These discrepancies are probably due partly to the broad division of the brain in the previous work. In consideration of the anatomy of the goldfish brain, the preparations of the OTec used in the previous study might have contained some portions of the diencephalon, pituitary and MT. The preparation of CB might also have contained a part of the brainstem. Employment of in situ hybridization is needed to examine the coincidence of localization of immunopositive cells and its gene expression.

It has been reported that the perikarya of the terminal nerve send their fibers to the TE, retina and other several regions of the brain (Demski and Northcutt, 1983; Uchiyama, 1990; Yamamoto *et al.*, 1995). Stell *et al.* (1984) demonstrated that some retinopetal fibers in the goldfish were immunoreactive to antibodies to LHRH and FMRFamide. In the present study, C-RFa-immunoreactive cells were found to be present in the rostroventral part of the OB. However, in the retina, the antibody labeled some cells in the inner nuclear layer (amacrine cell layer), which were not FMRFa-immunoreactive (Wang, *et al.*, 2000). It seems that the C-RFa-immunoreactive cells and fibers are different from the FMRFa-immunoreactive cells and fibers reported by Stell *et al.* (1984).

We observed dense arrays of C-RFa immunoreactive fibers distributed in the ventral TE and the LPA of the goldfish. Koyama *et al.* (1984) have shown by electrolytic lesions that the area ventralis telencephali pars supracommissuralis and/or the posterior portion of the Vv, and NPP play an important role in the sexual behavior of male goldfish. Therefore, there may be some relationship between sexual behavior and the C-RFamide peptide, which would reflect the homology between C-RFa and mammalian PrRP.

It has been demonstrated in sea bass that hypothalamic hormones and neuropeptides are carried by fibers through the neurohypophysis to the adenohypophysis (Moons *et al.*, 1989). We found that C-RFa-immunoreactive perikarya were localized characteristically in the NPO, NAPv, NPPv and the lateral part of the NLTp. A number of C-RFa-immunoreactive fibers, which may originate from these nuclei, were distributed throughout the hypothalamus and continued to the pituitary as described for FMRFa-like immunoreactivity by Fujii and Kobayashi (1992). There were especially dense arrays of C-RFa immunoreactive fibers around the NLTp. Since it has been suggested that the NLTp is involved in the control of pituitary function in teleosts (Ball, 1981), this finding suggests that the C-RFa immunoreactive fibers in the lateral part of NLTp and in the pituitary may modulate pituitary function. From the close similarity of the primary sequence of C-RFa to that of mammalian PrRP, C-RFa might function in fish to facilitate the release of prolactin from the pituitary. A preliminary experiment showed that C-RFa facilitated the release of prolactin from the pituitary of tilapia (in preparation).

In the present study, C-RFa-immunoreactive neurons were seen in the OCN, NRS, NRI and nucleus of MLF. The distribution of C-RFa immunoreactive perikarya in the OCN in the region of the MT and brainstem resembles that of substance P (Sharma *et al.*, 1989). The areas afferent to OCN have been investigated by Torres *et al.* (1992) and by Graf and McGurk (1985). According to their studies, the NRS, NRI, and nucleus of MLF are afferent to the OCN. In the goldfish stimulation of MLF and the areas related to the oculomotor nerve near the trochlear nerve roots elicits convergence or unilateral vergence responses (Demski and Bauer, 1975). Therefore it is intriguing to speculate that C-RFa peptide might regulate eye movement.

The present results also demonstrated the presence of C-RFamide-immunoreactive perikarya and fibers in the NTP, FL, VL and NMX. The VL in goldfish is a laminated structure in midmedulla which is responsible for processing the vagal gustatory input from the oropharynx (Smeraski *et al.*, 1998). It has been proposed that the taste system of the goldfish is divisible into two subsystems, the external and internal taste systems (see review by Finger, 1997). The former, which receives input from the facial nerve, is utilized to locate food in the environment, and the latter, which receives input from the vagus nerve, is crucial for triggering swallowing. Each of these inputs connects to its own portion of the medullary viscerosensory column. Rink and Wullimann (1998) have mapped the lateral torus and inferior lobe topographically onto the vagal-related and the facial-related portions, respectively. They are the tertiary gustatory nuclei. The NTP is reciprocally connected with the lateral torus and inferior lobe. The lateral torus seems to be involved exclusively with gustatory and general visceral systems.

In the present study we have shown that NMX neurons are C-RFa immunoreactive. The VL receives inputs from other sensory systems, and therefore, likely represents a multisensory integration center. We have found that a few C-RFa immunoreactive fibers or nerve endings were distributed in the area of the myenteric plexus and the submucosal plexus of intestine where the terminals of some vagus nerve fibers synapse onto neurons of these plexuses; these inputs mediate visceral action (Wang, unpublished). Furthermore, a dose-dependent excitatory effect of C-RFa on the intestine of *Zacco temminckii* was reported by Fujimoto *et al.* (1998). Therefore we speculate that the C-RFa peptide may be a neurotransmitter which integrates visceral actions.

We are now investigating the functions of C-RFa peptide from various viewpoints. Further physiological and ultrastructural studies with brain lesions will help to elucidate the physiological roles of C-RFa peptide in fish.

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Abbreviations

AC:	anterior commissure;
CB:	cerebellum;
Dc:	area dorsalis telencephali pars centralis;
Dd:	area dorsalis telencephali pars dorsalis;
Dm:	area dorsalis telencephali pars medialis;
FL:	facial lobe;
LPA:	lateral preoptic area;
MLF:	medial longitudinal fascicle;
MO:	medulla oblongata;
MT:	midbrain tegmentum;
NAPv:	nucleus anterioris periventricularis;
NAT:	nucleus anterioris tuberis;
NDLI:	nucleus diffusus lobi inferioris;
NDTL:	nucleus diffusus tori lateralis;
NF:	nucleus facies;
NLTp:	nucleus lateralis tuberis pars posterioris;
NMX:	nucleus motorius nervi vagi;
NPO:	nucleus preopticus;
NPP:	nucleus preopticus periventricularis;
NPPv:	nucleus posterioris periventricularis;
NRI:	nucleus reticularis inferior;
NRL:	nucleus recessus lateralis;
NRS:	nucleus reticularis superior;
NTP:	nucleus posterioris thalami;
NVM:	nucleus ventromedialis thalami;
OB:	olfactory bulb;
OCN:	oculomotor nucleus;
OLT:	olfactory tract;
OT:	optic tract;
OTec:	optic tectum;
PC:	postoptic commissure;
TE:	telencephalon;
TL:	torus longitudinalis;
VC:	valvula cerebelli;
Vd:	area ventralis telencephali pars dorsalis;
VL:	vagal lobe;
VN:	vagal nerve;
Vv:	area ventralis telencephali pars ventralis