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Colocalization of neuropeptide Y (NPY)-like and FMRFamide-like immunoreactivities in the brain of the Atlantic salmon (*Salmo salar*)

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Summary. The colocalization of the peptides neuropeptide Y (NPY) and Phe–Met–Arg–Phe–NH_2 (FMRFamide) in the brain of the Atlantic salmon was investigated with double immunofluorescence labeling and peroxidase-antiperoxidase immunocytochemical techniques. Colocalization of NPY-like and FMRF amide-like immunoreactivities was observed in neuronal cell bodies and fibers in four brain regions: in the lateral and commissural nuclei of the area ventralis telencephali, in the nucleus ventromedialis thalami, in the laminar nucleus of the mesencephalic tegmentum, and in a group of small neurons situated among the large catecholaminergic neurons in the isthmal region of the brainstem. All cell bodies in these nuclei were immunoreactive to both NPY and FMRF. We consistently observed larger numbers of FMRF-immunoreactive than NPY-immunoreactive fibers. In the nucleus ventromedialis thalami NPY- and FMRFamide-like immunoreactivities were colocalized in cerebrospinal fluid (CSF)-contacting neurons. NPY-immunoreactive, but not FMRF-immunoreactive, neurons were found in the stratum periventriculare of the optic tectum, and at the ventral border of the nucleus habenularis (adjacent to the nucleus dorsolateralis thalami). Neurons belonging to the nucleus of the nervus terminalis were FMRF-immunoreactive but not NPY-immunoreactive. The differential labeling indicates, as do our cross-absorption experiments, that the NPY and FMRFamide antisera recognize different epitopes. Thus, it is probable that NPY-like and FMRF amide-like substances occur in the same neurons in some brain regions.

Key words: Neuropeptides – Neuropeptide Y – FMRF-amide – Immunocytochemistry – Telencephalon – Thalamus – *Salmo salar* (Teleostei)

It is generally accepted that neurons can produce, store and release more than one type of transmitter molecule. However, it is difficult to determine the functional role of such coexistence. The colocalization of two neuropeptides within the same neuron appears to be a widespread phenomenon in the nervous system of mammals (Watson et al. 1978; Hökfelt et al. 1983; Triepel and Grimmelikhuijzen 1984; Chronwall 1985; Sasek and Elde 1985; Sasek et al. 1990) and birds (Erichsen et al. 1982). It has also been described in certain regions of the central nervous system (CNS) of fish (Stell et al. 1984; Yulis and Lederis 1987; Olivereau and Olivereau 1988).

The immunohistochemical relationship of neuropeptide Y (NPY) and Phe–Met–Arg–Phe–NH_2 (FMRFamide) has been the subject of several investigations (Triepel and Grimmelikhuijzen 1984; Sasek and Elde 1985; Muske et al. 1987). In rat and guinea pig, the peptides have been found colocalized in neurons of the hypothalamic arcuate nucleus, medulla oblongata, and the gray commissure of the spinal cord (Hökfelt et al. 1983; Triepel and Grimmelikhuijzen 1984; Chronwall 1985; Sasek and Elde 1985). However, the functional significance of this colocalization remains unknown.

By using high-performance liquid chromatography and radioimmunoassay, Muske et al. (1987) and Pontet et al. (1989) have shown that NPY and FMRFamide are present in the CNS of teleosts. Kimmel et al. (1986) have isolated, from coho salmon endocrine pancreas, a peptide that has 83% homology with porcine NPY. Larhammar et al. (1990) have cloned the NPY gene from different vertebrates and have found a strong similarity between the NPY gene in shark, goldfish, and man.

The distribution of NPY-like immunoreactivity (Vallarino et al. 1988; Brodin et al. 1989; Pontet et al. 1989; Vecino et al. 1989; Vecino 1989) and FMRFamide-like immunoreactivity (Boer et al. 1980; Bonn and König 1988; Ohtomi et al. 1989; Östholm et al. 1990) has been investigated in the CNS of fishes. FMRFamide has been found to be colocalized with luteinizing hormone-releasing hormone in the olfactoretinal pathway of the goldfish brain (Stell et al. 1984). NPY is colocalized with tyrosine hydroxylase in amacrine cells in the goldfish retina (Muske et al. 1987). In vitro experiments have demonstrated that NPY modulates the release of gona-

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dotropin from the pituitary in goldfish (Kah et al. 1989) and rainbow trout (Breton et al. 1989). To date, there have been no reports on the colocalization of NPY and FMRFamide in the CNS of fishes.

In the present study, we report the colocalization of NPY and FMRFamide in the CNS of salmon. The results are discussed on the basis of the phylogenetic preservation of the coexistence of these two peptides and their possible roles in the fish CNS.

Materials and methods

Seven Atlantic salmon (*Salmo salar* L.), from one to two years old, were used. The animals were purchased from a commercial hatchery and kept in fresh water at 8° C under a LD 12:12 cycle until used.

The animals were anesthetized with tricaine methane sulfonate (MS-222) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were postfixed in the same fixative for 2 h, rinsed in PB and then placed in 25% sucrose in PB until they sank. The brains were then frozen on a metal plate cooled by liquid nitrogen, and cut in the frontal or sagittal plane on a cryostat. Alternate 10-µm-thick sections were mounted on four sets of chrome alum-gelatine-subbed slides. In this way, four comparable series of adjacent sections were obtained from each brain.

Immunocytochemistry

From three brains, the first and third sets of sections were incubated with rabbit anti-FMRFamide (Immunonuclear, Stillwater, USA: lot # 8630014) diluted 1:3000. The second set was incubated with rabbit anti-NPY (1–36) (Cambridge Research Biochemicals/Diagnostika, Falkenberg, Sweden: lot # 00317/1098) diluted 1:2000. They were then processed according to the peroxidase-antiperoxidase method (Sternberger 1979; Vecino and Ekström 1990). All antibodies were diluted in 0.01 M phosphate-buffered saline (PBS) containing 0.25% Triton-X100 and 1% bovine serum albumin. The fourth set was Nissl stained with cresyl violet.

Control experiments

The specificity of the immunostaining for NPY and FMRFamide was tested as follows: (1) replacement of the primary antisera by immune rabbit serum alone; (2) pre-incubation with the respective antigen of the antisera diluted to the concentration used in the immunocytochemical protocol; (3) cross-preabsorption of the NPY-antiserum with synthetic FMRFamide, and of the FMRF amide-antiserum with synthetic NPY.

For the cross-reactivity test, four sets of alternate transverse 10 µm-thick-sections from one brain were used. Excess synthetic FMRFamide (100 µg/ml) and NPY (10 nM/ml) were pre-incubated overnight with antisera against NPY and FMRFamide, respectively, diluted to the concentrations used in the immunocytochemical protocol. The concentration of the antigens used for preabsorption was chosen according to the suggestions of the manufacturers of the antibodies. The four sets of alternating sections were then incubated overnight as follows: the first set with NPY antiserum, the second with the mixture of NPY-antiserum and synthetic FMRFamide, the third with FMRFamide-antiserum, and the fourth set with the mixture of FMRFamide-antiserum and synthetic NPY. The sections were then processed for the peroxidase-antiperoxidase method according to the protocol described above.

The control experiments (1) and (2) resulted in the elimination of all immunostaining. After the cross-reactivity test (3), no differences were observed in the immunoreactivity pattern between the series in which the NPY antiserum was pre-incubated with FMRFamide, and the series incubated with the NPY antiserum. Furthermore, preabsorption of the FMRFamide antiserum with NPY did not reduce the immunoreactivity. Thus, the NPY antibodies did not appear to cross-react with FMRFamide, or vice versa.

The nomenclature for the telencephalon followed Northcutt and Davis (1983), that for the diencephalon followed Billard and Peter (1982), and that for the brainstem was after Nieuwenhuys and Pouwels (1983).

Results

The distribution of FMRFamide-like and NPY-like immunoreactivity in the brain of *Salmo salar* was analyzed in frontal and sagittal sections. However, for brevity we will, when relating to the possible colocalization of these immunoreactivities, refer to them as "FMRFamide and NPY" or "the two peptides".

We found colocalization of NPY and FMRFamide in neuronal cell bodies situated in circumscribed nuclei in the telencephalon, diencephalon, mesencephalic tegmentum and the isthmal region of the brainstem (Fig. 1 A–D). We also observed brain areas where we found neurons that were immunoreactive to only one of the antisera.

Telencephalon

A group of NPY-like immunoreactive (NPYir) and FMRFamide-like immunoreactive (FMRFir) neurons was located in the lateral nucleus of the area ventralis telencephali and in the commissural nucleus of the area ventralis telencephali (Fig. 1 A). We found NPYir and FMRFir neurons on both sides of the anterior commissure. This commissure, which connects the telencephalic hemispheres, did not show any immunoreactivity to any of the two peptide studied.

The ir neurons in the telencephalon were small (8–10 × 10–14 µm) with fusiform cell bodies. In sagittal sections, we observed that most of the ir neurons were fusiform with ventro-caudally oriented long axes. Thus, in frontal sections, the cell bodies appeared to be round. NPYir and FMRFir neurons were of similar morphology. Moreover, their distribution and relative density within the telencephalic nuclei were almost identical.

Figs. 1–8. Abbreviations used in figures. *AC* Anterior commissure; *CCb* corpus cerebelli; *Dd* dorsal zone of area dorsalis telencephali; *Dm* medial zone of the area dorsalis telencephali; *flm* fasciculus longitudinalis medialis; *LA* laminar nucleus; *LC* locus coeruleus; *mcba* tractus mesencephalo-cerebellaris anterior; *NAT* nucleus anterior tuberis; *NDLI* nucleus diffusus lobi inferioris; *NDTL* nucleus diffusus tori lateralis; *NLT* nucleus lateralis tuberis; *NPPV* nucleus posterior periventricularis; *ntdl* nucleus tegmenti dorsalis lateralis; *NVM* nucleus ventromedialis thalami; *OT* optic tectum; *Pit* pituitary; *TL* torus longitudinalis; *TS* torus semicircularis; *v* ventricle; *Vc* commissural nucleus of area ventralis telencephali; *VCb* valvula cerebelli; *Vl* lateral nucleus of area ventralis telencephali; *Vs* supracommissural nucleus of area ventralis telencephali; *Vv* ventral nucleus of area ventralis telencephali

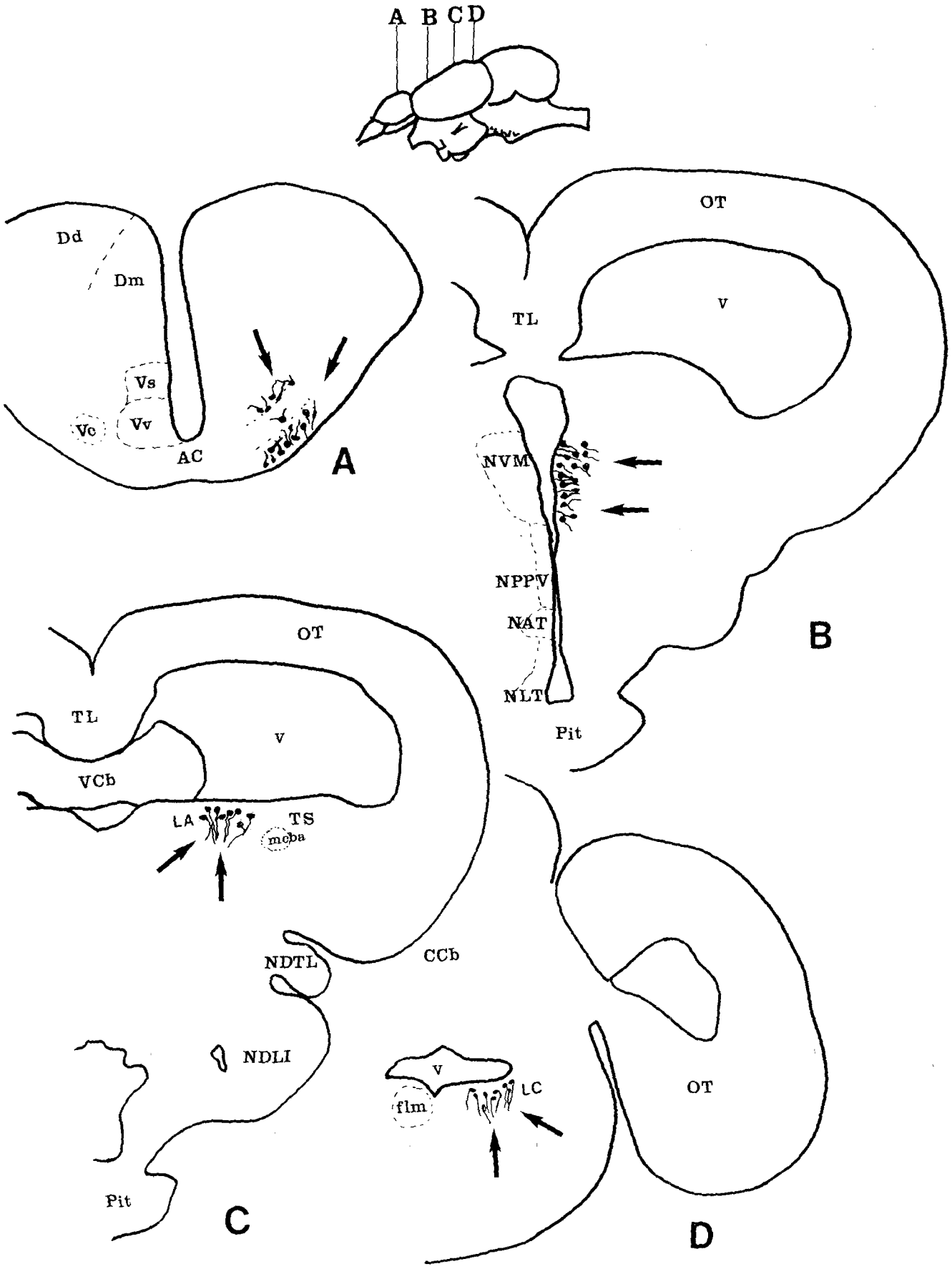
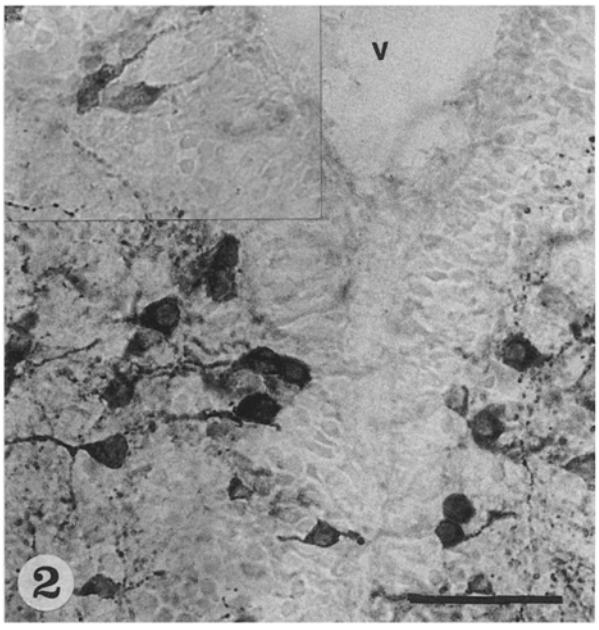
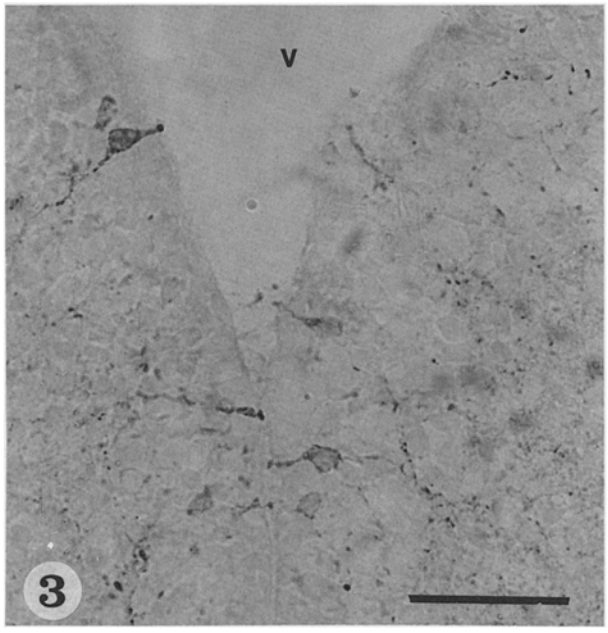


Fig. 1A–D. Transverse sections showing brain areas containing cell bodies in which NPY-like and FMRFamide-like immunoreactivities were colocalized (*arrows*). The levels *A–D* are represented in the *upper* drawing. **A** Cell bodies located in the *Vc*. **B** CSF-contacting and other neurons in the nucleus ventromedialis thalami. **C** Cell bodies in the *LA*. **D** Cell bodies in the *LC* of the isthmal region





Figs. 2, 3. FMRFir (Fig. 2) and NPYir (Fig. 3) CSF-contacting neurons in the nucleus ventromedialis thalami, located in and below the ependyma lining the third ventricle. Photomicrographs taken at the same level as in Figs. 4 and 5. *Scale bars:* 50 μm

NPYir and FMRFir fibers also showed similar distribution. Unfortunately, the small size of the neurons precluded conclusive demonstration of colocalization of NPY and FMRFamide by means of comparison of adjacent sections.

Diencephalon

NPYir and FMRFir neurons were located in the nucleus ventromedialis thalami (Figs. 1 B, 2, 3). All ir neurons were small (8–10 × 10–14 μm) and monopolar, with round or pyriform cell bodies. We classified the ir cells according to their position as (1) neurons that sent the main neurite away from the ventricle and that were not in contact with the cerebrospinal fluid (CSF), and (2) CSF-contacting neurons. Some of the CSF-contacting neurons were located in the ependymal layer, whereas others were found in a subependymal position and emitted their main dendritic processes through the ependymal layer into the third ventricle (Figs. 2, 3). Both classes of neurons were NPYir and FMRFir (not shown).

As in the telencephalon, NPYir and FMRFir neurons were of similar morphology, and showed similar distribution. However, NPYir neurons were generally more weakly immunoreactive, and the number of NPYir in neurons appeared more variable between specimens than NPYir neurons. As in the telencephalon, the small size of the ir neurons precluded conclusive demonstration of colocalization of NPY and FMRFamide.

Differences in the distribution of ir neurons were also noted. NPYir CSF-contacting neurons were found ventral to the nucleus ventromedialis thalmi, and at the ventral border of the nucleus habenularis, adjacent to the nucleus dorsolateralis thalami. FMRFir neurons were never observed in these locations.

Mesencephalic tegmentum

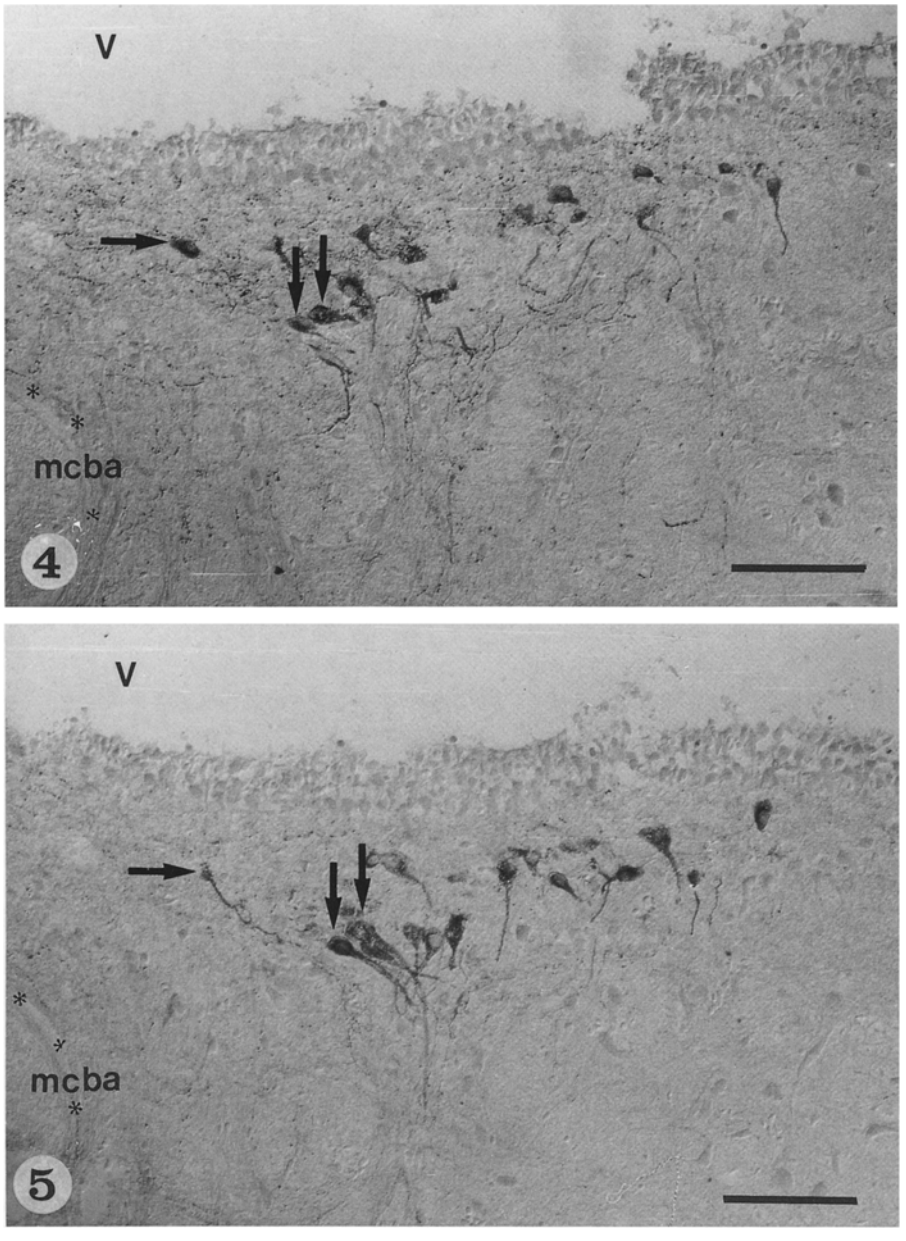
Colocalization of NPY and FMRFamide was observed in a group of neurons in the dorsal mesencephalic tegmentum. This group of neurons was located close to the ependymal layer of the third ventricle, lateral to the point where the ependymal lining of the valvula cerebelli unites with that of the tegmentum, and dorsomedial to the tractus mesencephalo-cerebellaris anterior (Fig. 1 C). These neurons were monopolar and of variable size (8–10 × 14–20 μm). All their processes extended away from the ventricle, toward the ventral tegmentum (Figs. 4, 5). It appeared that all the ir cell bodies of this nucleus were both NPYir and FMRFir, whereas FMRFir fibers were more abundant than NPYir ones.

The NPYir and FMRFir neurons formed a fusiform nucleus, with its long axis oriented rostrocaudally. Thus, in the most rostral and caudal sections of the nucleus, we found only one or two ir neurons per section, whereas in the middle of the nucleus, we observed 14–16 neurons per section. The ir neurons were linearly arranged and were, at all levels, situated approximately at the same distance from the mesencephalic ventricle. The dorsocaudal length of this nucleus was approximately 300 μm.

Brainstem

Colocalization of FMRFamide and NPY immunoreactivities was found in a group of neurons situated among the large catecholaminergic neurons of the isthmus region in the brainstem (Figs. 1 D, 6 "locus coeruleus").

The ir neurons were monopolar and of medium size (8–10 × 12–16 μm). The cell bodies were oriented toward the ventricle, with their ir processes projecting away from it. Adjacent series processed for FMRFamide or NPY showed the same cells sectioned at different levels



Figs. 4, 5. Photomicrographs of adjacent transverse 10-μm-thick sections of the laminar nucleus immunostained for FMRFamide (**Fig. 4**) or NPY (**Fig. 5**). Note that the same neurons were sectioned at different levels and stained for both peptides (*arrows*). *Asterisks* delineate the limit of the mcba. *Scale bars:* 100 μm

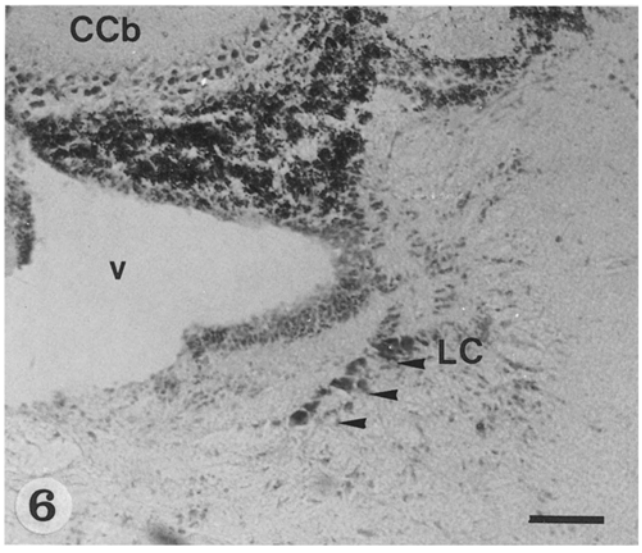
and labeled for both substances (Figs. 7, 8). More FMRFir than NPYir fibers were seen surrounding this nucleus.

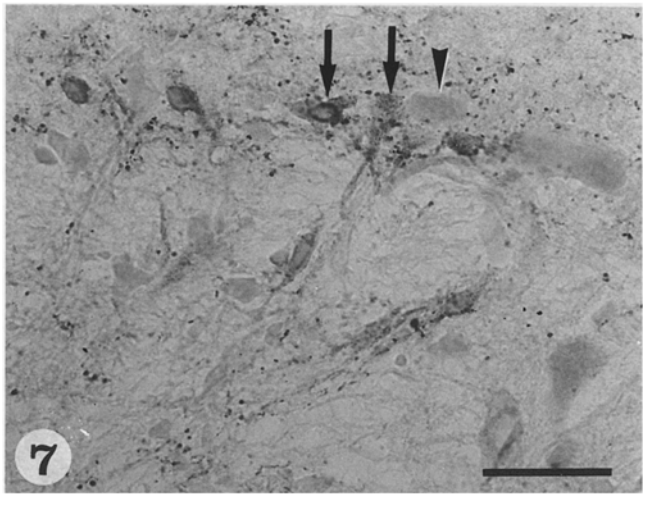
Discussion

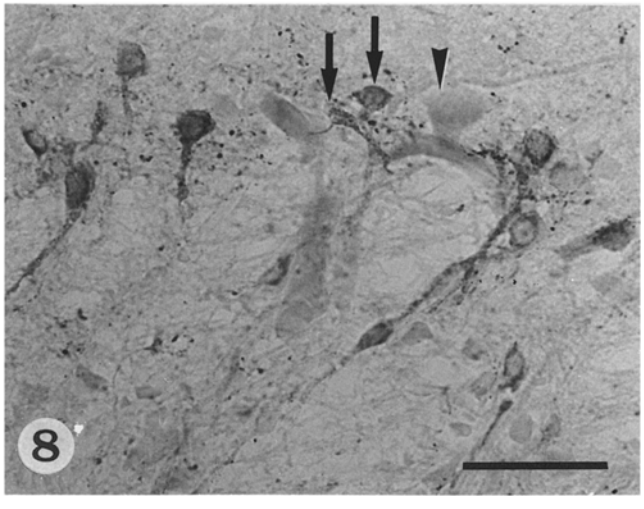
FMRFamide, a molluscan cardioexcitatory tetrapeptide (Price and Greenberg 1977), and NPY, a member of the pancreatic polypeptide family (Tatemoto et al. 1982), have functions in the endocrine system (O'Donohue et al. 1985; Potter 1988; Chen et al. 1989; Pontet et al. 1989). FMRFamide has also been reported to have an excitatory effect on brainstem neurons (Gayton 1982), playing a role in the modulation of pain (Sasek et al. 1982; Sasek and Elde 1985). Thus, intrathecal injection of IgG purified from antiserum directed against FMRFamide induces long-lasting moderate analgesia in rat, and also attenuates morphine tolerance (Tang et al. 1984). NPY, on the other hand, produces analgesia by blocking the release of substance P (personal communication, T. Yask). The presence of NPYir cell bodies in the hypothalamus and preoptic nucleus suggests that this peptide may be involved in neuroendocrine regulation (Danger et al. 1985; Tillet et al. 1989).

The distribution of NPYir and FMRFir neurons in the telencephalon and in the hypothalamus of the salmon brain is similar to that of NPYir and FMRFir neurons in other fish species (Boer et al. 1980; Vallarino et al. 1988; Bonn and König 1988; Brodin et al. 1989; Ohtomi et al. 1989; Pontet et al. 1989; Östholm et al. 1990; Vecino 1989), amphibians (Danger et al. 1985; Calliez et al. 1987), and mammals (Adrian et al. 1983; Allen et al. 1983; Pelletier et al. 1984; Triepel and Grimmelikhuijzen 1984; Chronwall 1985).

However, this study provides the first evidence for the colocalization of NPY-like and FMRFamide-like substances in the CNS of a teleost fish. (Again, for brevi-







Figs. 6, 8. Photomicrograph of a Nissl-stained transverse section of the brainstem showing the *LC* neurons (**Fig. 6**). Adjacent sections immunostained for FMRFamide (**Fig. 7**) and NPY (**Fig. 8**). The same cells sectioned at different levels are immunostained for both peptides (*arrows*). The large (noradrenergic) neurons of the *LC* can be discerned (*arrowheads*) among the immunoreactive cells. *Scale bars:* 50 μm

ty, we will refer to them as NPY and FMRFamide in this section). Colocalization of FMRFamide and NPY can be observed in neurons of the laminar nucleus of the mesencephalic tegmentum, and in the isthmus region. Colocalization was demonstrated by processing adjacent sections with antisera against FMRFamide and NPY. We could clearly see the same neurons labeled by the different antisera in adjacent sections. Moreover, NPY-like and FMRFamide-like immunoreactivity may be colocalized in neurons of the lateral and commissural nuclei of the area ventralis telencephali, and in the nucleus ventromedialis thalami, although this remains to be proven with other techniques.

Some neurons in different locations of the brain were labeled with only one antiserum. Thus, NPYir neurons were found in the stratum periventriculare of the optic tectum (Vecino and Ekström 1990), among CSF-contacting neurons situated ventral to the nucleus ventromedialis thalami, and in a group of neurons at the ventral border of the nucleus habenularis, adjacent to the nucleus dorsolateralis thalami. FMRFir neurons were never observed in these locations. On the other hand, FMRFir neurons have been described in the nucleus of the nervus terminalis (Ekström et al. 1988) where no NPYir cell bodies are located (Vecino 1989; present study). These findings, together with the observation that FMRFir fibers generally occur in larger numbers than NPYir fibers, indicate that the distribution of NPY and FMRFamide is not the same in all areas of the salmon brain. However, there are four areas in the salmon brain that contain neurons in which both peptides appear to be present (see above).

As mentioned above, FMRFir fibers generally occur in larger numbers than NPYir fibers. Nevertheless, strongly NPYir neurons have been observed in several areas. Whereas NPYir fibers may have a more restricted distribution in the salmon brain than FMRFir fibers, the NPY recognized by the antiserum in the cell bodies may be further processed in the axons and will thus not be recognized by the antiserum.

Several studies of mammals have demonstrated that both NPY and FMRFamide immunoreactivities are found in the same neurons in the arcuate nucleus, in the medulla oblongata, and in the gray commissure of the spinal cord (Hökfelt et al. 1983; Chronwall 1985; Sasek and Elde 1985). Our results indicate that these two peptides may also be colocalized in some neurons in the fish brain. Bearing in mind the limitations of immunocytochemistry this suggests that, during evolution, peptides have preserved not only their chemical structure but also their colocalization with other substances. The native peptides have to be isolated and sequenced to finally resolve this issue.

In mammals, the amygdala and hippocampus have the highest concentrations of NPY in the forebrain (Chang et al. 1985). In these telencephalic areas, NPY has been demonstrated to modulate the processing of memory (Flood et al. 1987, 1989). Homology has been suggested to exist between the mammalian amygdala and the teleostean commissural nucleus of the area ventralis telencephali (Northcutt and Braford 1980), where we have observed FMRFir and NPYir in some neurons.

Moreover, the goldfish telencephalon has been implicated in food-reinforced color-discrimination-learning functions (Ohnishi 1989). It is thus possible that the ir cell bodies that we have described in the salmon telencephalon could take part in modulation of memory and learning processes.

The location of NPY and FMRFamide neurons in the nucleus ventromedialis thalami corresponds with the location of serotoninergic cell bodies in the hypothalamus of salmonids (Frankenhuis-van den Heuvel and Nieuwenhuys 1984; Ekström and Ebbesson 1989). NPY and FMRFamide have been described in CSF-contacting neurons of the periventricular hypothalamus in mammals (Sabatino et al. 1987; McDonald et al. 1988; Chen 1989) and in fish (Vallarino et al. 1988; Östholm et al. 1990; Vecino 1989). It is possible that both peptides could be released from these cells into the CSF. Moreover, in vitro experiments have demonstrated that NPY modulates the release of gonadotropin in goldfish (Kah et al. 1989) and in rainbow trout (Breton et al. 1989). Thus, the two peptides might interact in the neural control of hormone release from the pituitary.

In the mesencephalic tegmentum, we have found a large number of ir cell bodies in a nucleus that corresponds to the laminar nucleus (Östholm et al. 1990). Although continuous with the torus semicircularis, the laminar nucleus shows different immunocytochemical features compared with this structure, and appears to be a distinct nucleus. The laminar nucleus of the salmon is not continuous with the deep (periventricular) layer of the optic tectum, as is the case for the laminar nucleus of the mesencephalic tegmentum in the lizard (Ebbesson 1967). Thus, we use the term laminar nucleus to describe the morphology of this sheet-like cell mass, rather than to imply a homology with its reptilian namesake.

The most caudal group of neurons that shows coexistence of FMRFamide and NPY is located in the isthmus region of the brainstem, close to the catecholaminergic neurons that are usually termed the "locus coeruleus" (for discussion, see Ekström et al. 1986; Ekström and Ebbesson 1989). The noradrenergic neurons in the locus coeruleus of mammals are known to costore other transmitters, such as the neuropeptides NPY and galanin (Holets et al. 1988), enkephalins (Charnay et al. 1982) and somatostatin (Palkovits et al. 1982), and serotonin (Steinbusch 1981). Therefore, it is striking that the NPYir/FMRFir neurons in the isthmus region of the salmon are small and clearly distinct from the large noradrenergic neurons (Ekström et al. 1989).

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