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Distribution of neuropeptide Y-like immunoreactivity in the brain of *Salmo salar* and *Gambusia affinis*

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Summary. Through the immunohistochemical PAP technique, the distribution of immune positive neurons and fibres for an antibody anti-NPY in the encephalon of salmon fixed in Bouin have been located and studied.

NPY-positive neurons are found forming three important nuclei: in the ventrolateral telencephalon; in the tegmentum mesencephali; and in the locus coeruleus. Neurons in the optic tectum, in the thalamic region and a few in the preoptic recess have also been located. The fibres were found throughout the brain, with the exception of the cerebellum, presenting a greater density in three regions: in the dorsal telencephalon; in the mesencephalon; and in the visceral lobes in the rhombencephalon.

With the aim of proving if this distribution is found in other groups of teleosts, we processed, with the same technique, the advanced teleost *Gambusia affinis*, in order to compare it with the primitive teleost *Salmo salar*. The results show that in both fish this neuropeptide has the same pattern of distribution.

The results also suggest that in fish this neuropeptide can be involved in several functions of the central nervous system, as has been demonstrated for mammals. The innervation of the visceral lobes and also the presence of NPY-fibres in the posterior hypothalamus are anatomical supports of the studies which suggest that NPY is related to the control of the food intake.

Key words: Neuropeptide, Immunocytochemistry, Teleost, Fish

Introduction

Neuropeptide Y (NPY) is the 36-aminoacid peptide that was isolated and characterized from porcine brain extracts by Tatemoto in 1982 (Tatemoto, 1982; Tatemoto et al., 1982). NPY is widely distributed in the central and periphral nervous systems of vertebrates, and is one of the most abundant brain peptides discovered so far (Gray and Morley, 1986; Potter, 1988). Several functions have been proposed for NPY in the central nervous system of mammals, including stimulation of feeding behaviour (Sahu et al., 1988), inhibition of sexual behaviour (Kalra et al., 1987), control of cardiovascular physiology (Gibbins and Morris, 1988; Martin et al., 1988), modulation of neuroendocrine secretion (Wahlestedt et al., 1987), and regulation of circadian rhythms (Albers and Ferris, 1984; Card and Moore, 1989).

Numerous immunohistochemical investigations have been conducted in mammals in order to determine the distribution of NPY-containing neurons in the central nervous system (Allen et al., 1983; Chronwall et al., 1985; De Quidt and Emson, 1986; Bons et al., 1990) but, in spite of the very important putative functions of NPY, few studies have been undertaken to examine the distribution of this peptide in the brain of lower vertebrates, and only three studies have been made in fish; one in the elasmobranch *Scyliorhinus canicula* (Vallarino et al., 1988) and the others in teleosts *Carassius auratus* (Pontet et al., 1989) and *Poecilia latipinna* (Batten et al., 1990). In addition, recently, Vecino and Ekstron (1990), described the distribution of several neuropeptides, NPY among others, in a small area of the *Salmo* brain, the optic tectum. Because these studies show important differences in the distribution of NPY and because the ray-finned fish, whose 20,000 species constitute the largest vertebrate radiation (David and Northcutt, 1983), more data is needed in this group in order to understand the evolution and diversity of the NPY system.

An immunohistochemical study of the distribution of NPY-immunoreactive cells in the whole brain of a primitive teleost, *Salmo salar,* (salmonidae), and in the advanced teleost *Gambusia affinis*, (poecilidae) is presented in this study.

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Materials and methods

Adult animals of *Salmo salar* and *Gambusia affinis* were used for the immunohistochemical localization of NPY neurons in the brain. They were anesthetized with tricaine methanesulphonate (Sigma) in a dilution of 1:10,000. Salmo were fixed by transcardiac perfusion with Bouin's fixative and gambusia specimens were fixed by immersion in the same liquid. Brains were dissected and postfixed for 12 hours at room temperature in the same fixative and then transferred to 0.1 M phosphate buffer, pH 7.4, overnight. After dehydration in graded alcohols they were embedded in paraffin and cut in serial 10 µm sections in sagittal and frontal planes or directly cut with a vibratome in 50 µm sections. They were mounted on gelatin-coated slides, and processed for the peroxidase-antiperoxidase (PAP) technique.

After 3 x 10 minute rinses in phosphate buffer, endogenous peroxide within the tissue was blocked by a solution of 0.3% hydrogen peroxide in PBS at room temperature for 30 min. This was followed by 3 washes in PBS and incubation for 30 min at room temperature in a solution of normal pig serum 1/30 in PBS. Sections were treated with rabbit polyclonal antisera to NPY (Amersham), diluted 1/100 in PBS containing 2% normal pig serum and incubated for 48 hours at 4º C in a humid atmosphere. The slides were then rinsed in PBS and incubated with pig antirabbit immunoglobulin diluted 1/100 in PBS for 1 hour and then with rabbit PAP complex (Sigma) diluted 1/100 in PBS for 1 hour. The sites of peroxidase attachment were demonstrated by incubation in 0.005% 3,3'diaminobenzidine-tetrahydrochloride (DAB, Sigma) solution in tris-HCl buffer (0.05% M, pH 7.6) containing 0.025% hydrogen peroxide. Finally, the sections were rinsed in water, dehydrated and coverslipped.

To ensure method specificity, controls were performed by incubating parallel sections with normal non-immune serum replacing NPY antibody or by omitting one of the steps of the immunohistochemical procedure.

Results

The neuropeptide Y-immunoreactive structures (NPY-ir), neurons and fibres, were detected throughout the brain of *Salmo salar* and *Gambusia affinis* and they are schematically represented in figure 1. No labelling was observed when the NPY antiserum was substituted by normal serum or after omitting one of the steps of the immunohistochemical procedure. The pattern of distribution of NPY-ir in these two teleosts was very similar.

Distribution of Salmo salar

In the brain of *Salmo salar* several groups of cells were found that stained positively with NPY antisera.

The telencephalon showed the highest NPY-ir cell density. Numerous immunoreactive neurons were located in their ventral and lateral part at the level of the anterior commissure; in the area ventralis telencephali pars lateralis (lateral nucleus and commissural nucleus) (Figs. 2a, 2b). The ir neurons were located surrounding the anterior commissure in saggital sections (Fig. 2b), and some of them were scattered caudally toward the hypothalamus and dorsally in the area dorsalis telencephali pars centralis. These neurons were bipolar or multipolar. The NPY-ir nerve fibres in the telencephalon of *Salmo* were very abundant. It was found that ir-fibres were denser in dorsal and lateral areas (area dorsalis telencephali, pars dorsali and pars lateralis) than in the pars medialis and area ventralis telencephali.

A few stained perikarya were located in the preoptic recess in the hypothalamus, in their rostral and ventral side (Fig. 2c). The NPY-ir cells were close to the ventricle but never with processes towards this cavity. These small and monopolar ir-cells sent their projections ventrally. A few NPY-ir fibres were scattered throughout the hypothalamus, except around the lateral and posterior recess, where they were more abundant. No fibres were found in the sacus vasculosus but some were found in the neurointermediate lobe of the pituitary gland.

A few NPY-ir cell bodies and fibres were found in the thalamus. These ir-neurons were located laterally to the nucleus dorsomedialis thalami.

Ir-perikarya were seen in two regions of the mesencephalon. One group of approximately 20 NPY-ir neurons were found in the tegmentum mesencephali, medial to the torus semicircularis and rostral to the nucleus lateralis valvulae, in a subependimal position (Fig. 2d). These ir neurons were large and pear-shaped, and sent their projections ventrocaudally. Laterally to this main group, a few NPY-ir fusiform cells were found in the torus semicircularis. The other group of NPY-ir neurons in the mesencephalon was found in the optic tectum, in the stratum periventriculare. These were numerous and small pear-shaped cells with processes towards the stratum album centrale (Fig. 2e). Positive fibres were detected throughout all the layers of the optic tectum.

In addition, a group of approximately 20 immunoreactive cell bodies was seen in the isthmo-locus coeruleus area,at the same level as the entrance of nerve VIII. These neurons, with a elongated shape, were near the ventricular surface, but no projections were seen towards the ventricular space. They were provided with a long ventrolateral process (Fig. 2f) towards the area of the nucleus isthmus where a dense fibres network were found.

Distribution in Gambusia affinis

In the brain of this advanced teleost four

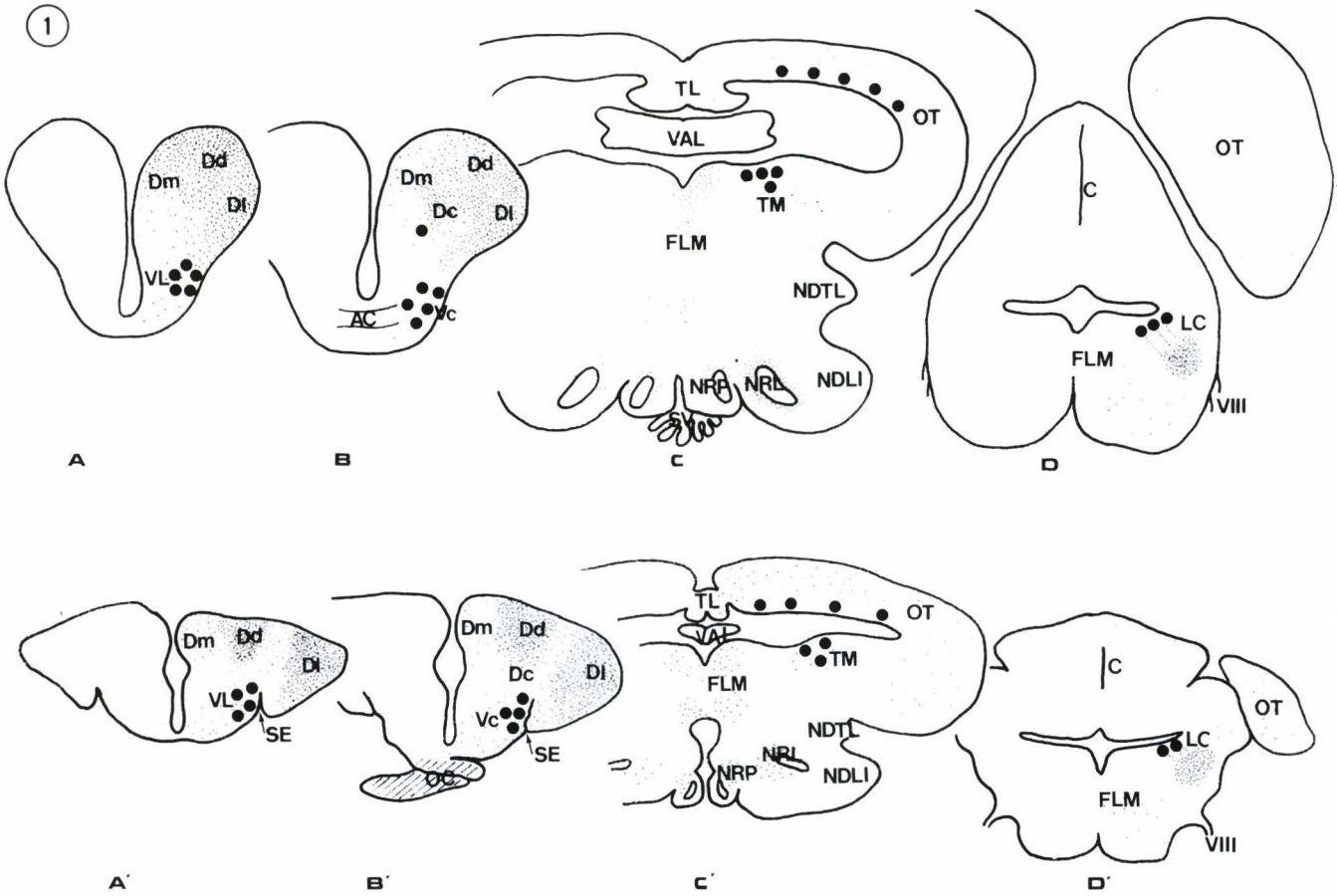

Fig. 1. Schematic frontal sections through the *Salmo* brain **(A,B,C,D)** and *Gambusia* brain **(A',B',C',D')**, showing the most representative areas with the distribution of NPY-immunoreactive perikarya (full circles) and neuronal processes (dots). AC, anterior commissure; C, cerebellum; Dc, area dorsalis telencephali, pars centralis; Dd, area dorsalis telencephali, pars dorsalis; Dl, area dorsalis telencephali, pars lateralis; Dm, area dorsalis telencephali, pars medialis; FLM, fasciculus longitudinalis medialis; LC, locus coeruleus; NDLI, nucleus diffusus lobi inferioris; NDTL, nucleus diffusus tori lateralis; NRP, nucleus recessus posterioris; NRL, nucleus recessus lateralis; OC, optic chiasm; OT, optic tectum; SE, external sulcus; SV, saccus vasculosus: TL, torus longitudinalis; TM, tegmentum mesencephali; VAL valvula cerebelli; Vc, area ventrlalis telencephali, commissural nucleus; VL, area ventralis telencephali, lateral nucleus; VIII, entrance of the eighth nerve.

areas containing NPY immunoreactive cell bodies could be demonstrated.

The telencephalon of *Gambusia affinis* contained the greatest number of NPY-ir neurons and was similar to that of *Salmo*. They were found in the pars lateralis of the area ventralis telencephali, in the lateral and commissural nuclei, near the external sulcus (Fig. 3a). The distribution of NPY-positive fibres in the telencephalon of this teleost showed the same pattern as that of *Salmo*. They were abundant in the dorsal and lateral zones, with some zones free of fibres such as those which are found between the dorsal and the lateral areas of the telencephalon.

Unlike that observed in *Salmo* no NPY-positive cells in the anterior hypothalamus were seen.

NPY ir fibres were located in the hypophysis of *Gambusia*.

In the mesencephalon ir neurons were observed in the tegmentum mesencephali, medial to the torus semicircularis (Fig. 3b). This nucleus seen in *Gambusia* could be homologus to that of the same zone of *Salmo*, the number of cells being around 20, forming a compact nucleus, with none of them being scattered toward the torus semicircularis. These cells had a piriform morphology, with a process leading toward the ventrocaudal zone of the mesencephalon. In the optic tectum of *Gambusia* small positive cells were seen in the periventricular layer with the same morphology and position as in *Salmo*.

Finally, a nucleus of approximately four NPY-ir neurons was located in a position near the fourth ventricle at the level of the entrance of the eighth cranial nerve, in the zone of the isthmo-locus coeruleus. These positive neurons possessed a piriform soma and a

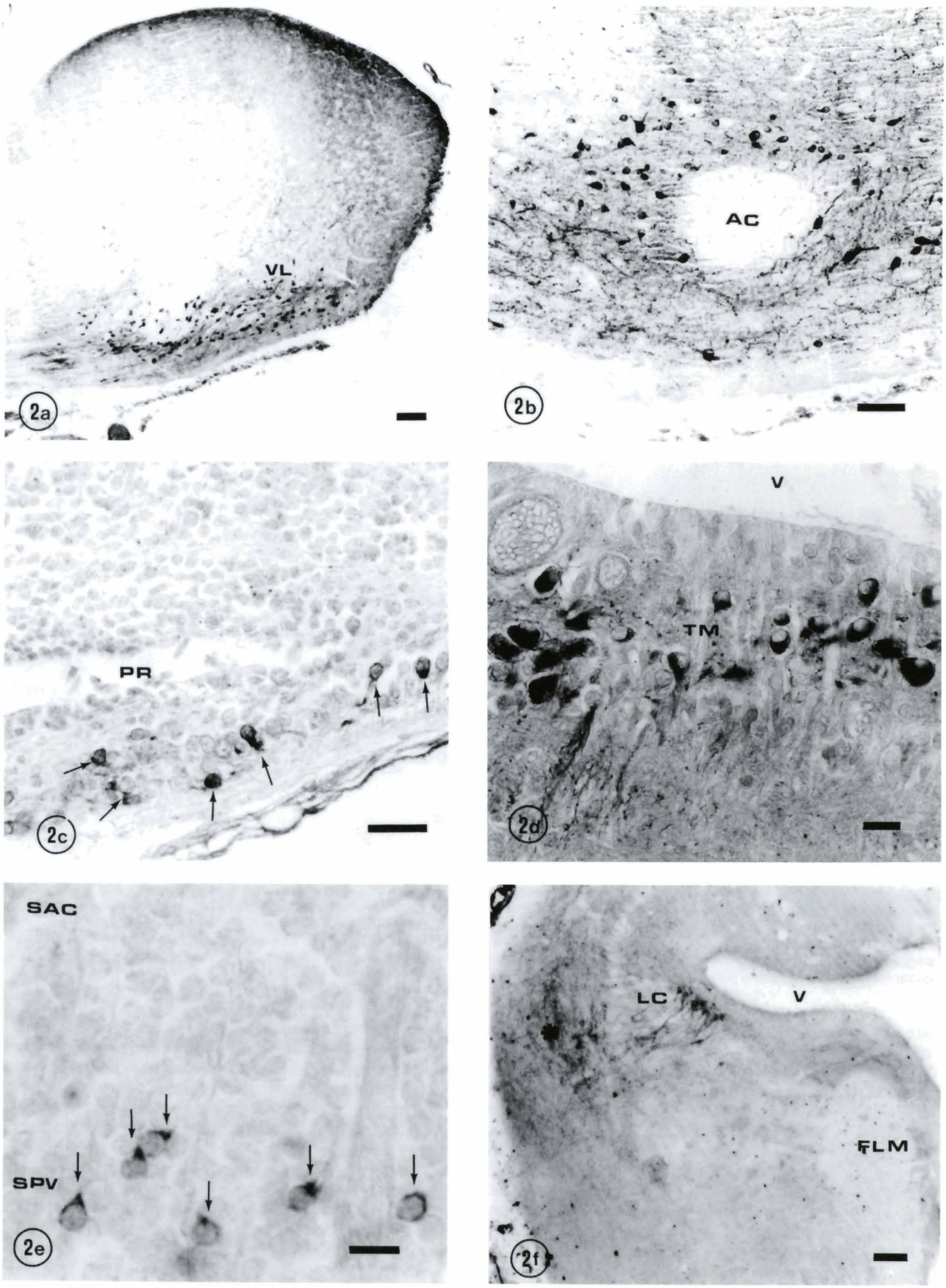

Fig. 2. Photomicrographs illustrating the location of NPY immunoreactive perikarya in the *Salmo brain.* **2a,** sagittal section through the telencephalon showing the NPY cell group in the ventral area (VL). Bar. 100 μm. **2b,** higher magnification of a sagittal section through the basal telencephalon showing NPY cell bodies and fibres placed around the anterior commissure (AC). Bar. 50 μm. **2c,** sagittal section through the hypothalamus showing immunoreactive cell bodies (arrows) located ventrally to the preoptic recess (PR). Bar. 25 μm. **2d.** sagittal section through the mesencephalon, numerous NPY immunoreactive perikarya are observed in the tegmentum mesencephali (TM) in a periventricular position (V, ventricle). Bar. 25 μm. **2e,** transversal section through the optic tectum, a few NPY-ir cells are visible (arrows) in the stratum periventriculare (SPV) projecting towards the stratum album centrale (SAC). Bar. 10 μm. **2f,** transversal section through the brainstem showing NPY-immunoreactivity in the locus coeruleus (LC) and many fibres laterally situated (asterisk). FLM, fasciculus longitudinalis medialis; V, ventricle. Bar. 100 μm.

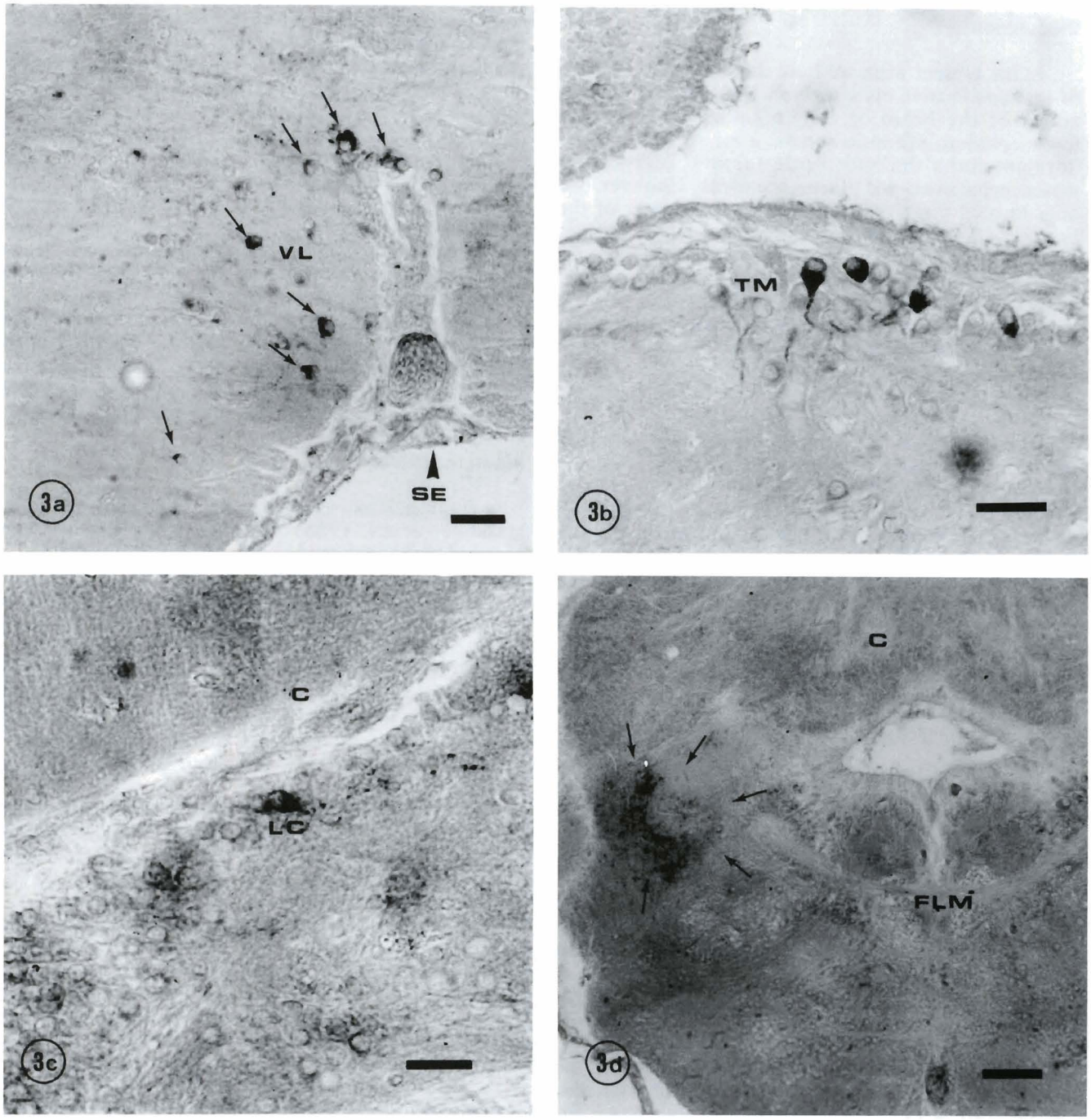

Fig. 3. Photomicrographs showing the location of NPY immunoreactive perikarya and fibres through the *Gambusia* brain; **3a,** transversal section of the telencephalon showing NPY-like immunoreactive neurons (arrows) in the area ventralis telencephali, lateral nucleus (VL). SE, external sulcus. Bar. 25 μm. **3b,** transversal section of the mesencephalon showing the NPY-positive nucleus in the tegmentum (TM). Bar. 25 μm. **3c,** transversal section of the brainstem showing one NPY-like immunoreactive neuron in the locus coeruleus (LC). c, cerebellum. Bar. 25 μm. **3d,** transversal section of the rombencephalon showing numerous NPY-like immunoreactive fibres in the visceral areas (delimited by arrows). C, cerebellum; FLM, fasciculus longitudinalis medialis. Bar. 50 μm.

ventrolateral process (Fig. 3c). In spite of their close proximity to the ventricle no contact with this cavity was seen. Numerous NPY-like immunoreactive fibres were found in the visceral areas of the rombencephalon (Fig. 3d).

The system of NPY-ir fibres in Gambusia followed the same pattern of distribution as that in *Salmo*, although the immunostaining was always less intense.

Discussion

In the present work we have described the presence of immunoreactive cells with an NPY antibody in six zones of the brain of *Salmo salar*: ventrolateral telencephalon; preoptic recess; zone of the nucleus dorsomedialis thalami; optic tectum; tegmentum mesencephalicum; and locus coeruleus. However, we only located positive NPY cells in four zones of the brain of *Gambusia affinis*: ventrolateral telencephalon; optic tectum; tegmentum mesencephalicum; and locus coeruleus. Positive cells were not seen for this antibody in the olfactory bulbs of either of the teleosts studied, although they have been seen in goldfish (Pontet et al., 1989) and in rats (Chronwall et al., 1985).

Telencephalon

In the ventrolateral telencephalon we have described the most important NPY-positive nucleus, with the greatest number of cells, both in *Salmo* and *Gambusia*. Our description is in agreement with that carrried out in *Carassius* by Pontet et al. (1989) and in *Poecilia* by Batten et al. (1990). The latter authors suggested a coexistance with somatostatin in the telencephalic NPY immunopositive cell bodies.

Likewise, the system of NPY-ir fibres in the telencephalon was very abundant in the two teleosts studied and much greater in the dorsal zones than the ventral ones, which agrees with that described by the above authors.

According to Northcutt and Bradford (1980), the ventrolateral areas of the telencephalon are homologous to the olfactory bulbs of higher vertebrates. These ventrolateral zones, with the lateral nucleus of the area ventralis telencephali, according to Northcutt (1981), is a possible migrated pallial area. NPY-ir cells have also been described in these areas in the frog brain (Danger et al., 1985) and in the elasmobrach fish *Scyliorhinus canicula* (Vallarino et al., 1988). Possibly, the NPY-positive cells are present in this zone throughout the whole vertebrate groups.

Diencephalon

We observed in *Salmo* some NPY-ir cells in the recessus preopticus and very few in the thalamus near the nucleus dorsomedialis. However, we have not located them in the corresponding areas of *Gambusia*, and Batten et al. (1990) did not describe NPY cells in the hypothalamus of the green molly. These differences might be attributed to differences between species of teleosts. It is interesting to note that these positive cells of *Salmo* are not liquor contacting neurons. The co-localisation of NPY with monoamines in the same neuron is well known (Everitt et al., 1984). Some authors, such as Lefranc et al. (1970), Ekengren (1975) and Kah and Chambolle (1983), have found monoaminergic neurons in the preoptic recess of different species of teleosts, although others have failed in their preoptic localization and have only been described them in the medial and posterior hypothalamus (Gómez-Segade et al., 1989). All these aminergic nuclei possess liquor containing neurons. In *Scyliorhinus canicula* Vallarino et al. (1988), described numerous NPY-positive cells in the hypothalamic lobes, which are catecholaminergic zones, but we doubt that at least in the species studied in the present work this co-localization exists in the hypothalamus, although it is necessary to say that in our experiments we have not used colchicine, for which reason we cannot know the results after treatment with this drug.

The high NPY innervation of the nucleus recessus lateralis of the hypothalamus, site of the control of the feeding behaviour (Demsky, 1983), is the anatomical support of the studies which suggest that this neuropeptide is related to the control of the food intake.

Mesencephalon

As in goldfish (Pontet et al., 1989) and frog brains (Danger et al., 1985) numerous NPY-ir cells were located in the periventricular layer of the optic tectum of *Salmo* and *Gambusia*, which had the same characteristics as those described for *Carassius*, while in amphibians they have not only been observed in the periventricular layer but also in more superficial layers. However, they have not been described in *Scyliorhinus canicula* nor in *Poecilia latipinna*. Batten et al. (1990) described scattered beaded NPY-ir fibres in the optic tectum of *Poecilia latipinna*. Our results agree with a recent work on the optic tectum of *Salmo* (Vecino and Ekstrom, 1990).

In the tegmentum mesencephali medial to the torus semicircularis, and rostral to the nucleus lateralis valvulae, we have located a NPY-ir nucleus in the two teleosts studied. This nucleus was the same as that described by Batten et al. (1990) and Vecino and Ekstrom (1990), but we do not agree with these latter authors in that it forms part of the torus semicircularis, although a few club-shaped positive neurons were displaced toward this area, which is well known for its laminar structure. In *Gambusia* the positive cells have only been seen in the tegmentum and not in the torus semicircularis. These cells can be FMRF positive (Vecino and Ekstrom, 1990) and some of them can also be positive for LHRH-like peptide (Batten et al., 1990), but the most intriguing is the absence of this nucleus in goldfish (Pontet et al., 1989). These authors, studying this same zone of the torus semicircularis in the same fish, described NPY-positive fibres with a high density, but no positive neurons. In our results we observed very few fibres in this area. Finally, these cells are not liquor contacting either.

Brainstem

We located NPY ir cells in the area of the locus

coeruleus of *Salmo* and *Gambusia*. In a comparable location, Pontet et al. (1989) described in *Carassius* NPY-ir cell bodies with a similar appearance and they identified this place as the locus coeruleus. We do not believe that this corresponds with area number 6 of trout described by Frankenhuis van den Heuvel and Nieuwenhuys (1984) in their study of the distribution of serotonin. In spite of this, they say that it is homologous to the locus coeruleus of higher vertebrates, because this nucleus is found in a more rostral position than that identified by Pontet et al. (1989) and by ourselves in the present work.

It is known that the locus coeruleus is catecholaminergic, as indicated by Parent et al. (1978) in the sunfish *Lepomis gibbosus*. In mammals, NPY coexists with noradrenaline, both in the central and peripheral nervous system (Everitt et al., 1984).

Thus, the putative involvement of NPY-like peptide in several sensory and motor areas and pathways of the fish brain can be deduced from our results, but the real functions of this neuropeptide in the positive nuclei in fish is still unknown.

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