PACAP in developing sensory and peripheral organs of the zebrafish, *Danio rerio*

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The anatomical distribution of PACAP-like immunoreactivity was investigated in sensory and peripheral organs of the zebrafish, *Danio rerio*, during the pharyngula, hatching and larval periods, by using indirect immunofluorescence methods. First PACAP-like immunoreactive (ir) elements appeared during the pharyngula period, at 24 hours post fertilization (hpf), within the most superficial layer of the retina and the dorsal aorta. At 48 hpf, additional ir cells were found in the olfactory placode and esophagus. At 72 hpf (hatching period), PACAP-like immunoreactivity was first detected in the ganglion cell layer of the retina, the otic sensory epithelium, pharyngeal arches, swim bladder and pancreatic progenitor cells. During day 5 of larval development, new groups of ir cells appeared in the liver, whereas no ir elements were observed in the olfactory placode. Subsequently, at day 13 of larval development, additional ir elements were found for the first time in some gut epithelial cells while those previously observed in the retina and otic sensory epithelium were absent. The transient expression of PACAP-like ir material in sensory organs suggests that the peptide could be implicated in neurotrophic activities and neurosensorial connections in the migration and/or differentiation processes. The appearance of PACAP-like ir elements in peripheral organs at different developmental stages, indicates that this peptide could be involved in the control of more specific functions as soon as these peripheral structures begin to operate.

Key words: neuropeptides; development; immunohistochemistry; zebrafish

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino acid amidated neuropeptide first isolated from the ovine hypothalamus on the basis of its ability to stimulate cyclic AMP formation in rat pituitary cells (Miyata *et al.,* 1989). Subsequently, an amidated proteolytic fragment of PACAP, corresponding to the (1-27) N-terminal sequence of the peptide was isolated in sheep (Miyata *et al.,* 1990). Structurally, PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide family that includes peptide histidine isoleucine, peptide histidine methionine, gastric inhibitory peptide, growth hormone-releasing hormone (GHRH), helospectin and helodermin (Campbell and Scanes 1992). The cDNA encoding the PACAP precursors has been cloned in humans (Ohkubo *et al.,* 1992), sheep (Kimura *et al.,* 1990) and rats (Ogi *et al.,* 1990). The analysis of the deduced amino acid sequences shows that the structure of PACAP38 has been fully maintained in these mammalian species (Kimura *et al.,* 1990). The sequence of PACAP has been remarkably well preserved throughout evolution (Vaudry *et al.,* 2000). In particular, in zebrafish, as well as in chicken and frog (Chartrel *et al.,* 1991, McRory *et al.,* 1997, Alexandre *et al.,* 2000), the structure of PACAP38 is strikingly similar to the one found in mammals (Miyata *et al.,* 1989; Fradinger and Sherwood, 2000). There has been great evolutionary pressure to maintain the sequence of the PACAP molecule, thus indicating that the peptide must play important physiological functions.

Soon after its isolation, PACAP was shown to be present not only in the hypothalamus but also in other brain areas and peripheral organs (Arimura and Shioda, 1995). *In vivo* and *in vitro* studies have shown that PACAP exerts multiple activities as a hypothalamic hormone, neurotransmitter, neuromodulator and neurotrophic factor. In mammalian peripheral tissues, it has been shown that PACAP

has a strong relaxant action on smooth muscle fibers of blood vessels, lung and gut, stimulates gastric acid and intestinal secretion, hormone/enzyme release from pancreas, and induces or inhibits neuroendocrine cell proliferation (Gonzalez *et al.,* 1998). Recent data also describes the effect of PACAP on hepatic bicarbonate secretion (Glad *et al.,* 2003) and its role as critical hormonal regulator of lipid and carbohydrate metabolism (Gray *et al.,* 2001). PACAP and/or PACAP receptors have also been found in certain sensorial organs in mammals, such as the adult and fetal retina (Nilsson *et al.,* 1994; Onali and Olianas, 1994; Wang *et al.,* 1995; Olianas *et al.,* 1997).

In fish, PACAP heavily stimulates the secretion of growth hormone (Parker *et al.,* 1997; Montero *et al.,* 1998; Wong *et al.,* 1998; Rousseau *et al.,* 2001; Wirachowsky *et al.,* 2000; Wong *et al.,* 2000) and gonadotropin (Chang *et al.,* 2001). Moreover, in fish it can control contractions in the intestine (Matsudaa *et al.,* 2000; Olsson *et al.,* 2000) and induce catecholamine secretion from chromaffin tissue (Montpetit and Perry, 2000).

It is now well known that, in the central nervous system, PACAP promotes cell proliferation (Matsumoto *et al.,* 1993; Lu and DiCicco-Bloom, 1997; Lu *et al.,* 1998), neurite outgrowth (Deutsch *et al.,* 1993; Gonzalez *et al.,* 1997) and protein synthesis (West *et al.,* 1995) suggesting its involvement in neurotrophic activities (Lindholm *et al.,* 1998; Vaudry *et al.,* 1999). PACAP and its receptors have already been described in the central and peripheral nervous system of the mammalian embryo (Arimura *et al.,* 1994; Lindholm *et al.,* 1998; Nielsen *et al.,* 1998; Sheward *et al.,* 1998; Skoglosa *et al.,* 1999; Zhou *et al.,* 1999; DiCicco-Bloom *et al.,* 2000) and the ontogeny of PACAP has been studied in detail in the CNS of the frog as well (Mathieu *et al.,* 2001). To our knowledge, studies in fish related to the developmental changes of PACAP expression have been performed in rainbow trout and zebrafish as well (Krueckl and Sherwood, 2001; Krueckl *et al.,* 2003). In particular, in zebrafish, the authors described the expression of *ghrh-pacap 1* transcript only during segmentation, gastrulation and first embryonal stages. In addition, we have recently investigated the distribution of PACAP immunoreactivity in the zebrafish brain throughout a longer developmental period, starting from embryonal up to juvenile stages, as well as in adult animals (work in press). However, there is no data available yet on the distribution of PACAP ir system in zebrafish peripheral and sensory organs during embryonal and larval development. Thus, we have decided to investigate the developmental changes of PACAP-like immunoreactivity in peripheral and sensory organs of the zebrafish, Danio rerio, starting from the pharyngula period as far as the late larval period. This study represents a first step towards the understanding of PACAP function during the ontogenesis of peripheral and sensorial structures in zebrafish. In particular, although the PACAP expression pattern has been already investigated during early zebrafish embryogenesis (Krueckl *et al.,* 2003), there still isn't sufficient information on the presence and/or function of PACAP system during later embryonal stages as well as at posthatching and larval periods. We chose *Danio rerio* because it's a common and simple model for ontogenetic studies.

Materials and Methods

Animals

Specimens of zebrafish, *Danio rerio*, at different stages of development during the pharyngula period (24 and 48 hpf), the hatching period (72 hpf) and the larval period (day 5, day 13), were sampled from different aquaria, at 25-28°C. At least 5 animals were used for each stage. The developmental stages were classified according to Kimmel *et al.,* (1995). The fishes were anesthetized with tricaine methane-sulfonate (MS 222, Sigma Chemical Co., MO), fixed in freshly prepared Bouin's fluid or in 4% paraformaldehyde in cold phosphate buffered saline (PBS) 0.2 M, pH 7.4, at room temperature for 4 h. Paraffin-embedded, 4 μm thick, serial sagittal, frontal or coronal sections were mounted on chrome alum/gelatin-coated glass slides.

Animal manipulations and experimental protocols were performed according to the recommendations of the Ethical Committee of our institution and under the supervision of authorized investigators.

Immunofluorescence procedure

The sections were rehydrated and processed using indirect immunofluorescence microscopy. Briefly, the sections were rinsed in cold phosphate-buffered saline, preincubated with normal swine serum (1:50) for 20 min to reduce non specific staining, and incubated in a dark moist chamber for 18 h at

Table 1. Distribution and relative density of PACAP-like ir cells and fibers in sensory and peripheral organs of Danio rerio at the pharyngula period (24 hpf and 48 hpf stages), the hatching period (72 hpf stage) and the larval period (day 5 and day 13).

	Pharyngula period				Hatching period		Larval period			
	24 hpf		48 hpf		72 hpf		Day 5		Day 13	
	cells	fibers	cells	fibers	cells	fibers	cells	fibers	cells	fibers
Dorsal aorta (Da)	++	-	++	-	++	-	++	-	++	-
Esophagus (E)	-	-	+++	-	+++	-	+++	-	+++	-
Ganglion cells layer of the retina (Gcl)	-	-	-	-	++	-	++	-	--	
Gut (I)	-	-	-	-	-	-	-	-	++	-
Intestine (I)	-	-	-	-	-	-	-	-	-	+++
Liver (L)	-	-	-	-	-	-	++	-	++	-
Olfactory placode (Op)	-	-	++	-	++	-	-	-	-	-
Otic sensory epithelium (Ose)	-	-	-	-	+++	-	+++	-	-	-
Pancreas (Pa)	-	-	-	-	+++	-	+++	-	+++	-
Pharyngeal arches (Pha)	-	-	-	-	++	-	++	-	++	-
Superficial layer of the retina (Slr)	++	-	++	-	++	-	++	-	-	-
Swim bladder (Sb)	-	-	-	-	+++	-	-	-	-	-

+, low density; ++, moderate density; +++, high density; -, absence of PACAP-like immunoreactivity; Cc: cranial cavity; Da: dorsal aorta; Di: diencephalon; E: esophagus; Ep: ethmoid plate; Exp: exocrine pancreas; G: gut; Gcl: ganglion cells layer of the retina; Hyv: hypothalamus, ventral part; Ipl: inner plexiform layer of the retina; L: liver; Le: lens; N: nothocord; On: optic nerve; Op: olfactory placode; Ose: otic sensory epithelium; Pa: pancreas; Pc: parachordal cartilage; Pd: pronephric duct; Ph: pharynx; Pha: pharyngeal archs; Ppd: pituitary pars distalis; Rc: rhodes and cones of the retina; Rh: rhombencephalon; Sb: swim bladder; Sc: spinal cord; Slr: superficial layer of the retina; Tb: trabeculae; Te: tectum of the mesencephalon; Tel: telencephalon; Tg: tegmentum of the mesencephalon; Y: yolk.

4°C with a polyclonal antiserum raised in rabbit against mammalian PACAP38 (Peninsula, Belmont, CA). The antiserum was diluted 1:200 in PBS, containing 1% BSA and 0.3% Triton X-100. Then, the sections were rinsed several times in PBS and incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated swine anti-rabbit gamma globulins (Dakopatts, Copenhaghen, Denmark), diluted 1:100 in PBS. Finally, the sections were rinsed twice in PBS, mounted in glycerol/PBS (1:5), and examined under a Zeiss epifluorescence microscope (Oberkochen, Germany). Nomenclature of zebrafish areas at the different stages of development was based on the work of Kimmel *et al.,* (1995).

Specificity of the immunoreaction

The specificity of the immunoreaction was verified by (1) substitution of the primary antiserum with PBS; (2) replacement of the primary antiserum with nonimmune rabbit serum diluted 1:200; preincubation of the PACAP antiserum with synthetic PACAP38, PACAP27, VIP or CRF (10-7 M each).

Results

The distribution of PACAP-like ir elements in the sensory and peripheral organs of the zebrafish, *Danio rerio*, was investigated in animals at stages ranging from the pharyngula period to the larval period. Incubation of sections with the PACAP antiserum revealed the presence of positive elements during the pharyngula period (24 and 48 hpf stages), hatching period (72 hpf stage) and larval period (day 5 and day 13). No differences were found between Bouin-fixed and paraformaldehyde-fixed tissues. The anatomical distribution and relative density of PACAP-like ir material in sensory and peripheral organs of Danio rerio during the different stages of development is schematically illustrated in Figure 1. For abbreviations see the list and Table 1.

Pharyngula period

24 hpf stage

During the 24 hpf stage, ir elements first appeared in some sensory and peripheral organs. In particular, a group of positive cells was found in the most superficial layer of the retina (Figure 2A, level 1a in Figure 1). A second group of ir cells showing a bright fluorescence was first detected in the dorsal aorta (Figure 2B, level 2a in Figure 1).

48 hpf stage

At the 48 hpf stage, new ir elements appeared in both sensory and peripheral organs. In particular, in sensory organs, positive cells were first observed in the caudal portion of the olfactory placode (Figure 2C, level 1b in Figure 1). Ir elements were still pres-

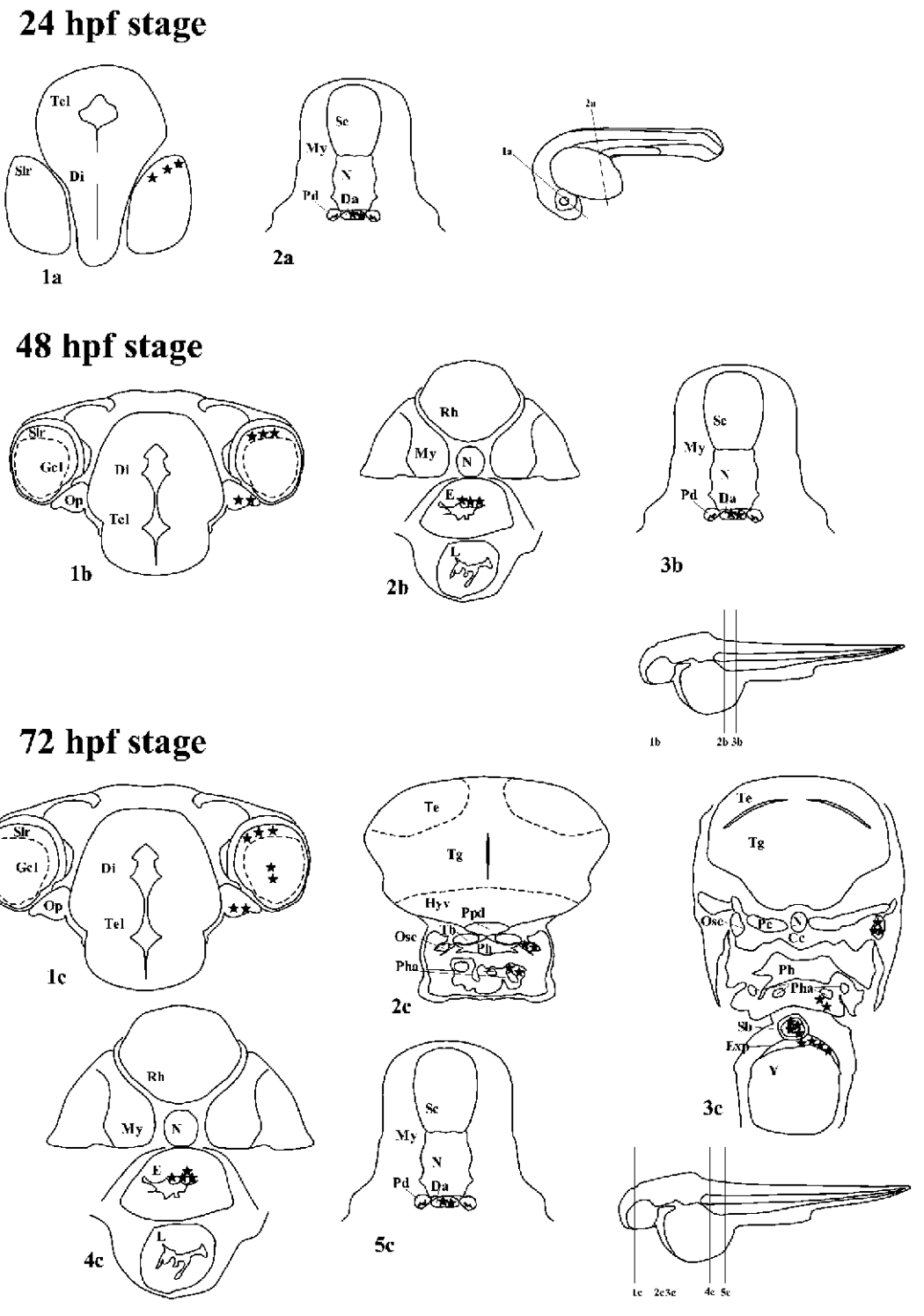

Figure 1. Schematic drawings illustrating the distribution of PACAP-like ir elements in sensory and peripheral organs of the zebrafish, *Danio rerio*, at the pharyngula period (24 hpf and 48 hpf stages), hatching period (72 hpf stages) and larval period (day 5 and day 13). The ir cells are represented by stars.

Abbreviations of Figure 1 and figure 2 (next page)
Cc: cranial cavity; Da: dorsal aorta; Di: diencephalon; E: esophagus; Ep: ethmoid plate; Exp: exocrine pancreas; G: gut; Gcl: ganglion cells layer of the retina; Hyv: hypothalamus, ventral part; Ipl: inner plexiform layer of the retina; L: liver; Le: lens; N: nothocord; On: optic nerve; Op: olfactory placode; Ose: otic sensory epithelium; Pa: pancreas; Pc: parachordal cartilage; Pd: pronephric duct; Ph: pharynx; Pha: pharyngeal archs; Ppd: pituitary pars distalis; Rc: rhodes and cones of the retina; Rh: rhombencephalon; Sb: swim bladder; Sc: spinal cord; Slr: superficial layer of the retina; Tb: trabeculae; Te: tectum of the mesencephalon; Tel: telencephalon; Tg: tegmentum of the mesencephalon; Y: yolk.

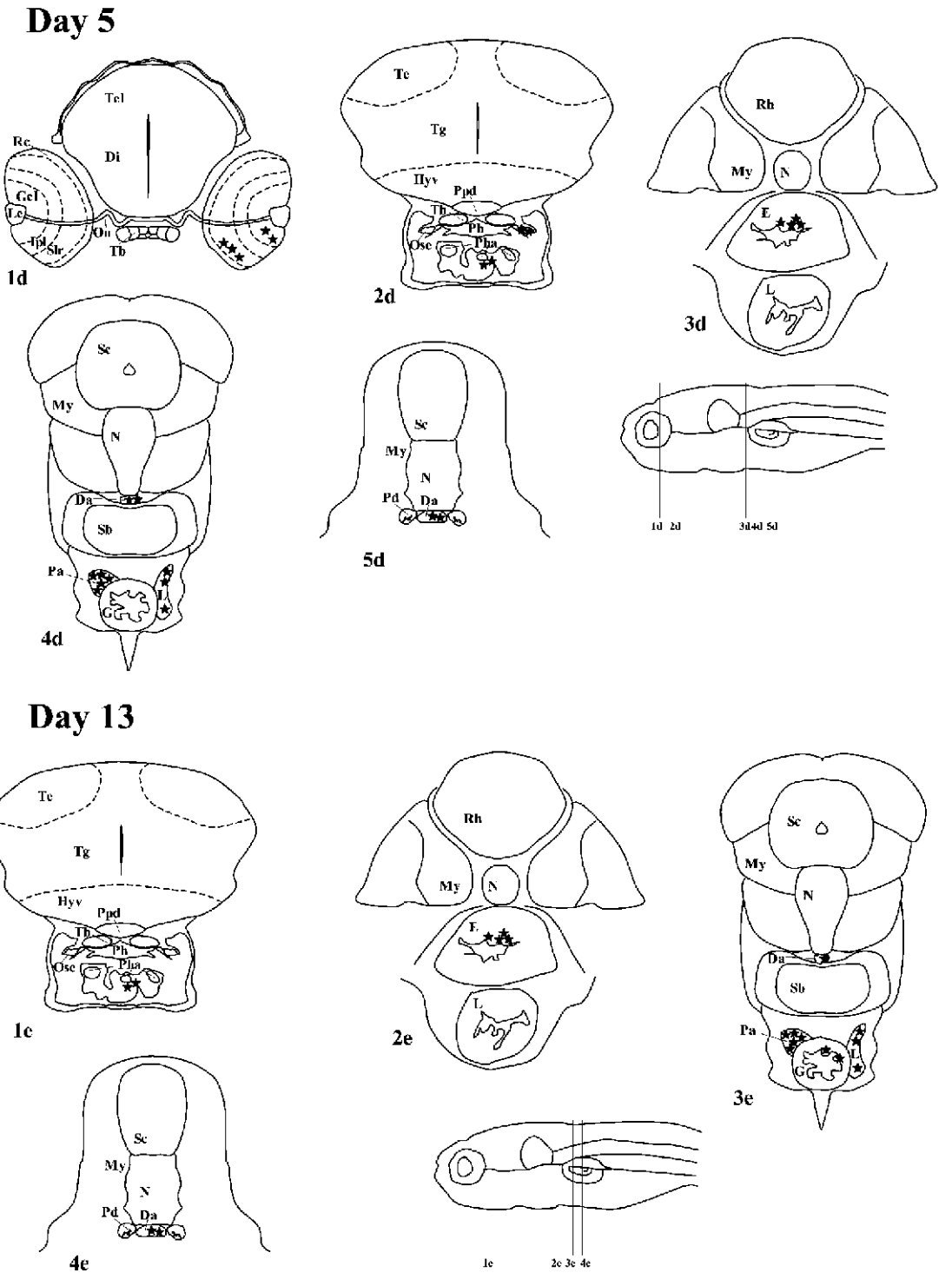

Figure 2. Immunofluorescence photographs showing the distribution of PACAP-like immunoreactivity in the sensory and peripheral organs of *Danio rerio* at the pharyngula period (24 hpf and 48 hpf stages) and hatching period (72 hpf stage). A: Coronal section showing a group of PACAP-like positive cells (arrows) located in the most superficial layer of the retina (Slr; level 1a in Figure 1). 24 hpf stage. Bouin-fixed tissue. B: Coronal section showing bright fluorescent ir cells (arrows) in the dorsal aorta (level 2a in Figure 1). Da, dorsal aorta; My, myotome; N, notochord; Pd, pronephric ducts. 24 hpf stage. Bouin-fixed tissue. C: A small group of bright fluorescent positive cells (arrows) located in the caudal portion of the olfactory placode (level 1b in Figure 1). Did, dorsal diencephalon; Op, olfactory placode; Telm, medial telencephalon. 48 hpf stage. Coronal section of a bouin-fixed tissue. D: Numerous bright stained positive cells (arrows) located adjacent to the lumen (Lu) of the developing esophagus (level 2b in Figure 1). E, esophagus. 48 hpf stage. Coronal section of a bouin-fixed tissue. E: Bright fluorescent immunopositive cells (arrows) in the granular cells layer of the retina (Gcl; level 1c in Figure 1). 72 hpf stage. Coronal section of a paraformaldehyde-fixed tissue. F: Coronal section showing two groups of ir cells (arrows) at level of the otic sensorial epithelium (Ose; levels 2c and 3c in Figure 1). Cc, cranial cavity; N, notochord; Pc, parachordal cartilage; Pha, pharyngeal arches. 72 hpf stage. Paraformaldehyde-fixed tissue. Scale bars: 200 μm.

ent in the most superficial layer of the retina (level 1b in Figure 1). In peripheral organs, a high number of bright fluorescent ir cells was first detected in the developing esophageal epithelium (Figure 2D, level 2b in Figure 1). As observed at 24 hpf stage, ir cells were present in the dorsal aorta (level 3b in Figure 1).

Hatching period

72 hpf stage

During the 72 hpf stage, a moderate number of bright fluorescent immunopositive cells first appeared in the ganglion cell layer of the retina (Figure 2E, level 1c in Figure 1) and numerous PACAP-like ir cells were first observed in the otic sensory epithelium (Figure 2F, levels 2c and 3c in Figure 1). In peripheral organs, new ir elements were present in the epithelium of the developing pharyngeal arches (Figure 3A-B, levels 2c and 3c in Figure 1). A bright fluorescent immunoreactivity was found for the first time in numerous cells of the swim bladder and in the exocrine pancreas progenitor cells adjacent to the yolk (Figure 3B, level 3c in Fig. 1). The distribution of PACAP-like ir material in the dorsal aorta and developing esophagus was similar to that observed at 48 hpf stage of pharyngula period (levels 4c and 5c in Figure 1).

Larval period

Day 5

During day 5 of larval period, the distribution of PACAP-like immunoreactivity in sensory organs was similar to that described at previous developmental stages. In particular, ir elements were still found in the retina (level 1d in Figure 1) as well as in the otic sensory epithelium (level 2d in Figure 1). On the other hand, no ir elements were observed in the olfactory placode. In peripheral organs, in addition to bright fluorescent positive cells located in the exocrine portion of the pancreas, moderate concentrations of ir cells were also observed for the first time in the liver (Figure 3C, level 4D in Figure 1). No immunopositive elements were observed in the swim bladder. By contrast, the distribution of PACAP-like ir elements in the pharyngeal arches, esophagus and dorsal aorta was similar to that described at the hatching period (levels 2d-5d in Figure 1).

Day 13

At day 13 of larval development, the distribution of PACAP-like ir material in sensory and peripheral organs showed some differences when compared to the observations of day 5 of larval stage. In sensory organs, the ir elements previously observed in the retina and otic sensory epithelium disappeared. By contrast, in peripheral organs, a moderate number of immunopositive cells was first detected in the gut epithelium (Figure 3D, level 3e in Figure 1). High concentrations of moderate fluorescent ir fibers were also present in the smooth muscolar wall layer of the intestine (Figure 3E). The distribution of PACAP-like ir system in the pharyngeal arches, esophagus, liver, pancreas and dorsal aorta was similar to that described at day 5 of larval stage (levels 1e-4e in Figure 1).

Specificity of the immunoreaction

Preincubation of the PACAP38 antiserum with 10^{-7} M synthetic PACAP38 resulted in complete loss of the immunoreaction (Figure 3E-F). On the other hand, preincubation of the PACAP38 antiserum with 10^{-7} M synthetic PACAP27, VIP or CRF did not affect the intensity of the immunostaining (Figure 4A-F). When the primary antiserum was substituted with either nonimmune rabbit serum or PBS, no immunofluorescence was observed.

Discussion

The present study provides the first anatomical description of PACAP containing elements in peripheral and sensory organs of the zebrafish *Danio rerio* during embryonal and larval development. The antiserum used to identify PACAP-like ir structures in the developing zebrafish was raised against mammalian PACAP38. In fact, the primary structure of PACAP38 is very similar in mammals (Miyata *et al.,* 1989) and zebrafish (Fradinger and Sherwood, 2000). Preabsorption tests showed that the PACAP antiserum specifically recognizes PACAP38 and did not cross-react with synthetic PACAP27, VIP or CRF. Previously, it was shown that PACAP38 is the main molecular form occurring in fish brain (Montero *et al.,* 1998). However, whether PACAP38 is also the predominant molecular form in fish extraencephalic regions, remains to be established. The antiserum employed in this study has also been successfully used to localize PACAP-like immunoreactivity in the zebrafish brain (Mathieu *et al.,* work in press), tadpole brain

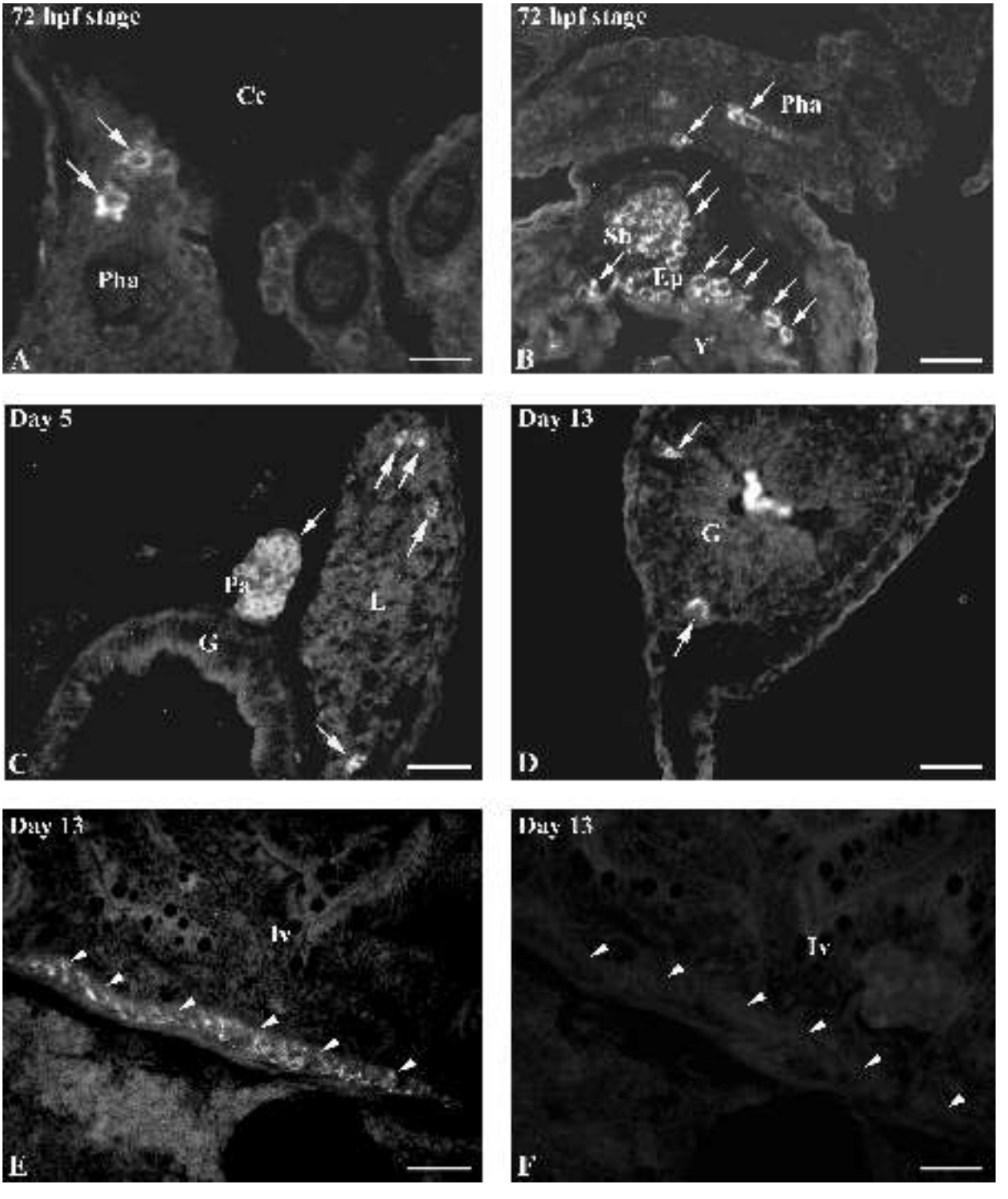

Figure 3. Immunofluorescence photographs showing the distribution of PACAP-like immunoreactivity in the sensory and peripheral organs of *Danio rerio* at the hatching period (72 hpf stage) and larval period (day 5 and day 13). A: Coronal section showing PACAP-like immunopositive cells (arrows) located in the dorsal epithelium of the developing pharyngeal arches (level 2c in Figure 1). Cc, cranial cavity; Pha, pharyngeal arches. 72 hpf stage. Bouin-fixed tissue. B: Coronal section showing numerous bright fluorescent ir cells (arrows) in the swim bladder (Sb). Dorsally to the swim bladder are present some ir cells located in the ventral epithelium of the developing pharyngeal arches (Pha). A higly fluorescent immunoreactivity is also present in numerous exocrine pancreas (Ep) progenitor cells located adjacent to the yolk (level 3c in Fig. 1). Y, yolk. 72 hpf stage. Bouin-fixed tissue. C: Coronal section showing bright fluorescent ir cells (arrows) in the pancreas and liver (level 4d in Figure 1). G, gut; L, liver; Pa, pancreas. Day 5 stage. Paraformaldehyde-fixed tissue. D: Coronal section showing two PACAP-like immunopositive cells (arrows) located in the gut (G; level 3e in Figure 1). Day 13 stage. Bouin-fixed tissue. E: Sagittal section showing numerous moderate fluorescent ir fibers (heads of arrow) located in the smooth muscolar wall layer of the intestine around the intestinal villus (Iv). Day 13 stage. Bouin-fixed tissue. F: Adjacent section of E showing that no immunoreaction is present after preincubation of the primary antiserum with synthetic PACAP38. The heads of arrow show the not stained intestinal regions that in E are labeled. Iv, intestinal villus. Day 13 stage. Bouin-fixed tissue. Scale bars: 200 μm.

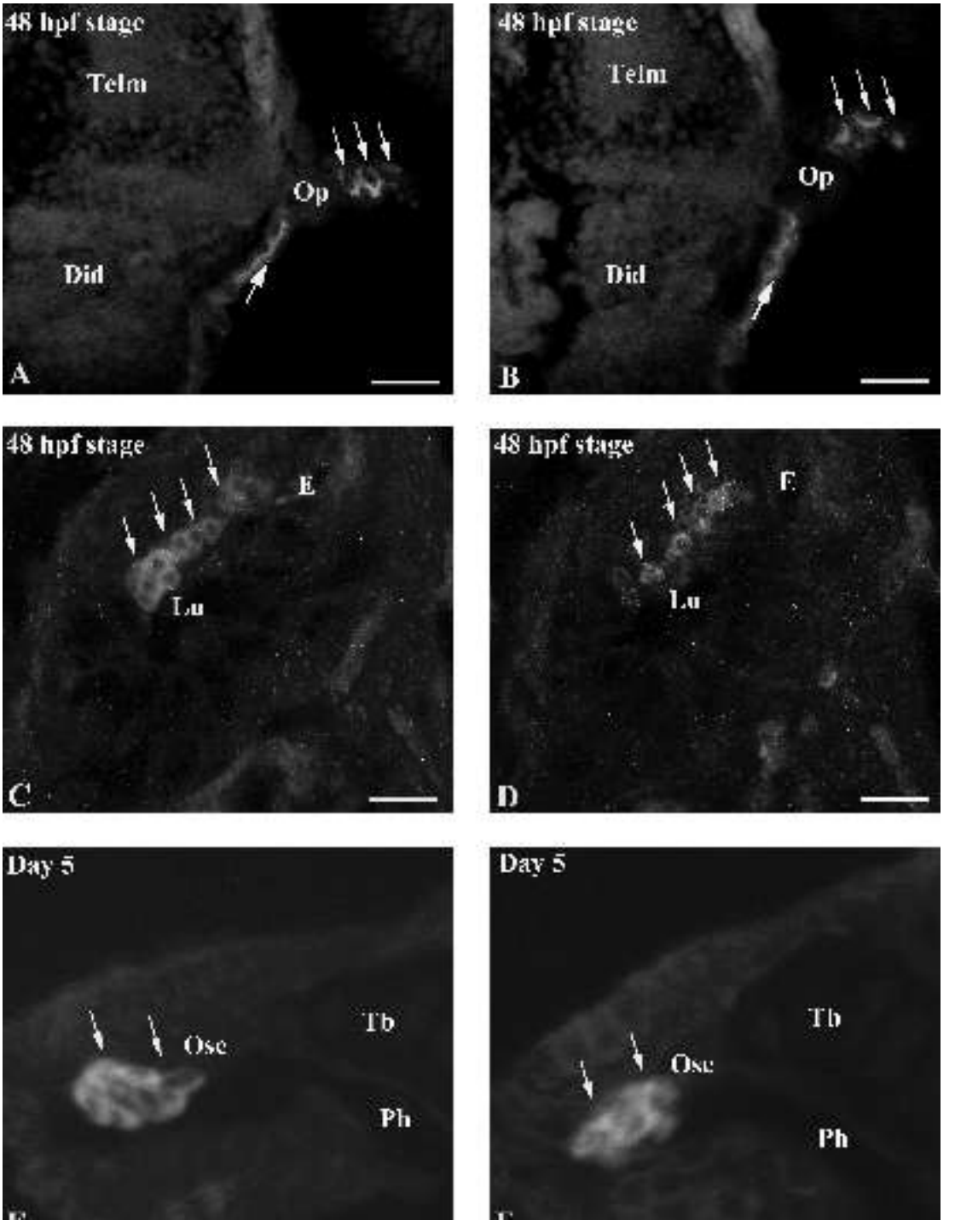

Figure 4. Immunofluorescence photographs showing the specificity of PACAP-like immunoreactivity in the sensory (A-B, E-F) and peripheral organs (C-D) of *Danio rerio* at the pharyngula period (A-D) and larval period (E-F). A: Coronal section (level 1b in Figure 1), showing PACAP-like immunopositive cells (arrows) located in the olfactory placode (Op) of 48 hpf old embryos. Did, dorsal diencephalon; Telm, medial telencephalon. Bouin-fixed tissue. B: Adjacent section of A showing that no changes in the intensity of the immunoreaction (arrows) are present after preincubation of the primary antiserum with 10^{-7} M synthetic PACAP27. Did, dorsal diencephalon; Op, olfactory placode; Telm, medial telencephalon. Bouin-fixed tissue. C: Coronal section showing numerous bright stained positive cells (arrows) located adjacent to the lumen (Lu) of the developing esophagus (level 2b in Figure 1). E, esophagus. 48 hpf stage. Bouin-fixed tissue. D: Adjacent section of C showing that no changes in the intensity of the immunoreaction (arrows) are present after preincubation of the primary antiserum with 10^{-7} M synthetic PACAP27. E, esophagus; Lu, lumen of the developing esophagus. Bouin-fixed tissue. E: Coronal section showing bright fluorescent ir cells (arrows) in the otic sensory epithelium (level 2d in Figure 1). Ph, pharynx. Ose, otic sensory epithelium. Tb, trabeculae. Day 5 stage. Paraformaldehyde-fixed tissue. F: Adjacent section of E showing that no changes in the intensity of the immunoreaction (arrows) are present after preincubation of the primary antiserum with 10^{-7} M synthetic PACAP27. Ph, pharynx. Ose, otic sensory epithelium. Tb, trabeculae. Paraformaldehyde-fixed tissue. Scale bars: 200 μm.

(Mathieu *et al.,* 2001) as well as in the frog brain and adrenal gland (Yon *et al.,* 2001).

A few studies have been carried out on the ontogeny of PACAP-like immunoreactivity in fish. A recent report (Krueckl *et al.,* 2003) describes the developmental changes in the PACAP expression in zebrafish by RT-PCR and in situ hybridization. However, the authors have focused their attention on early developmental periods, from the blastula to the pharyngula period.

This study shows that PACAP-like ir elements appear at the pharyngula period in both sensory and peripheral organs and that most of the positive elements are transiently expressed. In particular, in sensory organs, the presence of PACAP in the retina and otic sensory epithelium is limited to the embryonal and early larval periods whereas in the olfactory placode PACAP ir material is present only at pharyngula and hatching periods, suggesting that the peptide could be implicated in the cellular migration and/or differentiation at the level of these sensory structures. The presence of PACAP immunoreactivity in developing sensory organs of fish has never been investigated. Krueckl and collaborators (2003) have described PACAP mRNA expression in zebrafish retina from gastrula period to pharyngula period. In addition, the presence of other neuropeptides, such as the neuropeptide Y (NPY) has been observed in the zebrafish developing retina (Mathieu *et al.,* 2002). In humans, it was previously shown that PACAP is synthesized in the fetal retina, indicating that the PACAP may act on retinal cells by stimulating PACAP type I receptors coupled to cAMP formation (Olianas *et al.,* 1997). Also in rat, it has been shown that PACAP has neuroprotective effects in developing retina through intracellular cAMP-dependent protein kinase pathway (Silveira *et al.,* 2002).

Our results showed that from 24 hpf stage onward, PACAP-like immunoreactivity is present in the cells forming the dorsal aorta, suggesting that the peptide could be implicated in the control of vasculogenesis. A number of studies have recently examined the roles of several molecules in pathways that lead to the development of blood and vessels in zebrafish, and have provided insights into the regulation of these processes (Ahn *et al.,* 2000; Lawson *et al.,* 2001; Childs *et al.,* 2002; Crosier *et al.,* 2002; Szeto *et al.,* 2002; Jang *et al.,* 2003). However, the correlation between PACAP expression and the generation of vascular patterns in zebrafish is still unknown. In adult rat, it has been shown that PACAP has an antiproliferative effect on aortic smooth muscle cells through cAMP production (Oiso *et al.,* 1993). In addition, the importance of PACAP38 in vascular relaxation of adult rabbit aorta has been demonstrated (Wilson and Warren, 1993).

The occurrence of PACAP-like immunoreactivity in the pharyngeal arches from 72 hpf stages onward conforms with the finding of Krueckl and coworkers (2003) who described the presence of PACAP messenger expression in a region of the pharyngeal arches from which, later in zebrafish development, the jaw originates, suggesting a role of the peptide in stimulating withdrawal from the cell cycle prior to the differentiation and morhogenesis of the jaw.

Our results showed the presence and different temporal appearance of PACAP immunoreactivity in several peripheral organs of the zebrafish gastrointestinal system. In particular, in the esophagus, positive elements appeared from 48 hpf stage, in the exocrine pancreas from 72 hpf stage, in the liver from day 5 and in the gut only at day 13. To our knowledge, there is no data concerning the ontogeny of PACAP in either the gastrointestinal tract or glands of fish. Previously, the presence of PACAP immunoreactivity in the pancreas had been observed in 18- and 20-week-old human fetuses (Vincze *et al.,* 2001). These authors as well described the location of PACAP in the exocrine portion of the gland, indicating a role of the peptide in cell proliferation and differentiation of the epithelial foregut structures during fetal development. Recently, it has been demonstrated that rat immature embryonic pancreatic cells are sensitive to VIP and PACAP and express VPAC2 receptor between embryonic days 12 and 16, suggesting that these peptides are implicated in the control of survival and proliferation (Rachdi *et al.,* 2003).

The physiological significance of PACAP expression in developing liver cells of zebrafish and other species of fishes is unknown. However, it was shown previously that PACAP induces expression of corticosteroid-binding globulin in cultured fetal rat hepatocytes, acting through type II receptor isoforms, indicating that it could participate in the regulation of gluconeogenesis (El Fahime *et al.,* 1996). In the adult rat liver, PACAP stimulates glucose output from perfused tissue, although less strongly than glucagon (Inagaki *et al.,* 1994;

Yokota *et al.,* 1995). In addition, recent studies in rat have demonstrated that targeted disruption of the PACAP gene results in early postnatal death associated with dysfunction of lipid and carbohydrate metabolism (Gray *et al.,* 2001).

Our results showed the presence of PACAP-like immunoreactivity in the gut epithelial cells and intestinal fibers at late larval period of zebrafish development, suggesting a correlation between PACAP and the beginning of the digestive function. Previously, PACAP ir cells were observed in the growing end of the developing gastric and pyloric glands of 18-week-old rat fetuses, suggesting a protective and proliferative role of the peptide in the gastrointestinal mucosa (Vincze *et al.,* 2001). However, the involvement of PACAP in these proliferation and differentiation processes remains to be determined, although the protective role of several bioactive polypeptides, among which gastrin, is well known (Brown, 1993).

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