

Expression Analysis of PAC1-R and PACAP Genes in Zebrafish Embryos

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Abstract This study describes the expression of the pituitary adenylate cyclase-activating polypeptide (PACAP1 and PACAP2) and PAC1 receptor genes (PAC1a-R and PAC1b-R) in the brain of zebrafish (*Danio rerio*) during development. In situ hybridization of the 24- and 48-hpf embryos revealed that PACAP genes were expressed in the telencephalon, the diencephalon, the rhombencephalon, and the neurons in the dorsal part of the spinal cord. PACAP2 mRNA appears to be the most abundant form during brain development. The two PAC1-R subtypes showed a similar expression pattern: mRNAs were detected in the forebrain, the thalamus, and the rhombencephalon. However, in the tectum, only PAC1b-R gene was detected. These results suggest that, in fish, PACAP may play a role in brain development.

Keywords Zebrafish · PACAP receptors · Ontogeny · Central nervous system

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was 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). PACAP exists in two amidated forms of 38 (PACAP38) or 27 (PACAP27) amino acid residues and is structurally related to be a member of the vasoactive intestinal polypeptide (VIP), glucagon, secretin, and growth hormone-releasing hormone superfamily. PACAP acts through three types of G protein-coupled receptors. Two of these receptors, VPAC1-R and VPAC2-R, bind PACAP and VIP with equal affinity, whereas the third receptor, PAC1-R, selectively binds PACAP with high affinity (Alexandre et al. 1999; Alexandre et al. 2002; Inagaki et al. 1994; Spengler et al. 1993; Sreedharan et al. 1995). The sequence of PACAP has been highly conserved during evolution; human PACAP38 peptide shares 97% homology with the frog PACAP38 (Alexandre et al. 2000) and 90% with the salmon counterpart (Parker et al. 1993). In some species like in zebrafish, two PACAP genes were identified and termed PACAP1 and PACAP2 (Fradinger and Sherwood 2000; Wang et al. 2003). PACAP1 and PACAP2 peptides share 82% of identities, and 84% and 92%, respectively, with the mammalian PACAP38 peptide. The presence of two copies of these genes in the genome is likely due to genome duplication during evolution (Vogel 1998). Moreover, two PAC1-receptors, namely PAC1a-R and PAC1b-R, were also identified in zebrafish (Fradinger et al. 2005).

The distribution of PACAP and PAC1R mRNAs has been investigated in the embryonic central nervous system

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of species such as mice (Waschek et al. 1998), xenopus (Hu et al. 2002), and zebrafish (Krueckl et al. 2003). In adult humans, PAC1-R gene is mainly expressed in the olfactory bulb, the cerebral cortex, the supraoptic nucleus, and the cerebellum (Vigh et al. 1991). PACAP is expressed in neurons of the cerebral cortex, the amygdala, and the thalamus, and is particularly abundant in the hypothalamic nucleus where PACAP acts as a hypophysiotropic factor on pituitary cells (Rawlings and Hezareh 1996). The expression patterns in the adult brain suggest that PACAP acts as a neurotransmitter and/or a neuromodulator. Indeed, PACAP also exerts numerous effects on adult brain, and is involved in circadian rhythms (Mertens et al. 2007) (Colwell et al. 2004) and learning and memory processes, as well as in the neurite outgrowth, cell differentiation, and proliferation (Vaudry et al. 2009). However, relatively little information is available concerning the functional roles of the PACAP during development. Thus, in the present study, we examined by using whole-mount in situ hybridization in the zebrafish embryos the expression patterns of genes encoding the two PACAP precursors (PACAP1 and PACAP2) and PAC1 receptors (PAC1Ra and PAC1Rb) during the early development.

Materials and Methods

Whole-Mount in Situ Hybridization

Zebrafish embryos were collected during natural spawning periods and were cultured in egg water. Embryonic stages are represented as the unit hour post-fertilization (hpf) at 24 and 48 hpf. Full-length cDNAs encoding PACAPs and their receptors were amplified using the total RNA from the 48-hpf zebrafish embryos and were cloned in pCR4-TOPO vector for in vitro transcription using T3 and T7 RNA polymerases to make sense and antisense digoxigenin (DIG)-labeled riboprobes (Roche Applied Science, France).

For the whole-mount in situ hybridization experiments, about ten to 15 zebrafish embryos were fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS) containing 0.2% Tween 20, and then incubated in hybridization buffer (50% formamide, 5X SSC, 0.5 mg/L ribosomal RNA, 0.1% Tween 20) with DIG-labeled RNA probe at 65°C overnight. Embryos were washed in PBS–Tween buffer and incubated with an anti-DIG antibody conjugated to alkaline phosphatase. The hybridization signals were detected using the NBT/BCIP colorimetric system according to the manufacturer's instructions (Roche Applied Science, France). Each in situ hybridization experiment was performed two times.

Gene Quantification

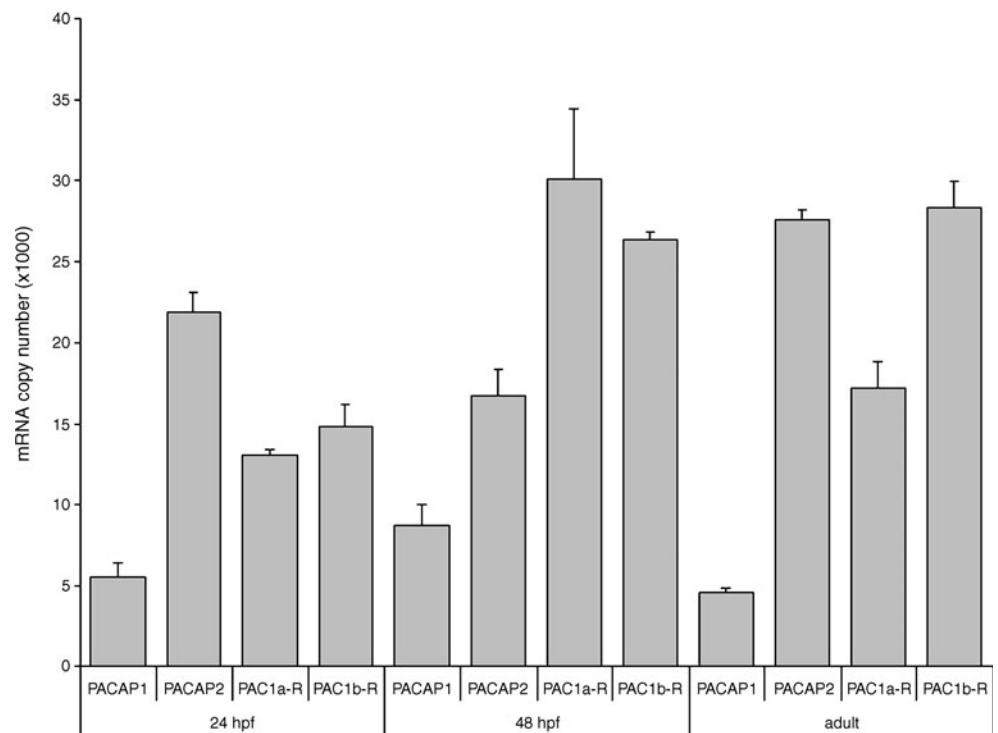
To determine the expression levels of the PACAP and PAC1-R genes, quantitative polymerase chain reaction (Q-PCR) was performed on the 24- and 48-hpf embryos. Total RNA was isolated from 20 embryos per stage using TRIzol reagent (Invitrogen, France) and quantified by UV absorbance, and 1 µg of RNA was reverse transcribed into cDNA prior to PCR with the ImProm-II reverse transcription system (Promega, France). PCR primers for PACAP1 were: forward, 5' AGGCACGCTGACGGGATGTT3' and reverse, 5' TGTCATCTTCTGTTGTGCTCCC3', and correspond to nucleotides 706–725 and 808–829 of the mRNA (GenBank accession number NM_152885.1), respectively. PCR primers for PACAP2 were: forward, 5' CACCCGAGTCGCATC CAACA3' and reverse, 5' CGTGCAGTAGGCGCTG TAGTG3', and correspond to nucleotides 235–254 and 338–358 of the mRNA (GenBank accession number NM_214715.1). PCR primers for PAC1a-R were: forward, 5' CCATCATCGGCTGGGGAACGC3' and reverse, 5' TCACCCACCAGAGGGCAGTGT3', and correspond to nucleotides 932–952 and 1028–1048 of the mRNA (GenBank accession number NM_001142925.1). PCR primers for PAC1b-R were: forward, 5' GATGATCCCAACAGT GAACCG3' and reverse, 5' ACAGGGCATCCAGA CAACTTGA3', and correspond to nucleotides 130–150 and 210–231 of the mRNA (GenBank accession number XM_677888.3). Elongation factor 1-alpha (EF1-α) primers, used as an external control, were: forward, 5' CTGGAGGC CAGCTCAAACAT3' and reverse, 5' ATCAAGAAGAG TAGTACCGCTAGCATTAC3', and correspond to nucleotides 678–697 and 736–764 of the mRNA (GenBank accession number NM_131263.1). Q-PCR was performed using 6.5 µL of 2X SYBR Green PCR Master Mix (Applied Biosystems, France), 300 nM of each primers, and 3 µL of 1:20 diluted template for standard reactions or 3 µL of nuclease-free water for negative controls. Real-time PCR reaction cycles were: 2 min at 94°C, then 45 cycles of 15 s at 94°C, 25 s at 60°C, and 20 s at 72°C, with a dissociation stage at the end of the amplification by using an ABI Prism 9700HT Fast Real-Time PCR System (Applied Biosystems, France).

Results

PAC1-R and PACAP Gene Expressions

Q-PCR was performed on the 24- and 48-hpf embryos, as well as in the adult zebrafish brain (Fig. 1). During development, the two PAC1-Rs showed similar level of detection, whereas in adult the PAC1b-R is detected at 50% higher level than the PAC1a receptor. Concerning the

Figure 1 Quantifications of PAC1a-R, PAC1b-R, PACAP1, and PACAP2 mRNA during the early development in the 24- and 48-hpf embryos and adult zebrafish. Elongation factor-1 gene was used as internal reference to normalize mRNA quantification. Data are means \pm SE of three experiments



PACAP genes, PACAP2 mRNA appeared as the predominant form in embryos and in adult. PACAP2 gene expression was detected at four-, two-, and six-fold levels than that of PACAP1 in 24 hpf, 48 hpf, and adult zebrafish, respectively (Fig. 1). Normalization of the quantitative PCR results with the housekeeping gene *EF1- α* , which is known to be constant during embryogenesis, revealed that PAC1 receptor mRNA level was two times higher at 48 hpf than at 24 hpf, whereas the expression of PACAP genes did not change. In fact, PACAP1 gene expression increased and the PACAP2 gene expression decreased between 24 and 48 hpf.

Gene Expression Patterns

The expressions of PACAP and PAC1-R genes were investigated by using in situ hybridization with specific DIG-labeled probes in the early development on the 24- and 48-hpf zebrafish embryos. Figure 2 showed that PACAP1 gene is mainly expressed in the neural crest, the floor plate, and the dorsal neurons of the spinal cord. PACAP2 messenger RNAs were first detected in the spinal cord, the rhombencephalon, and the telencephalon (Fig. 3a, b). Then, in the 48-hpf embryos, the hybridization signal revealed the presence of mRNA in several areas identified

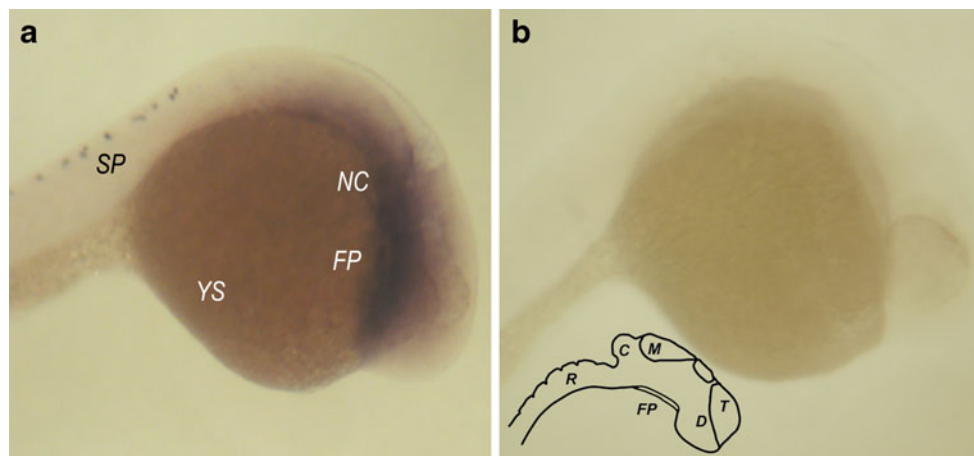
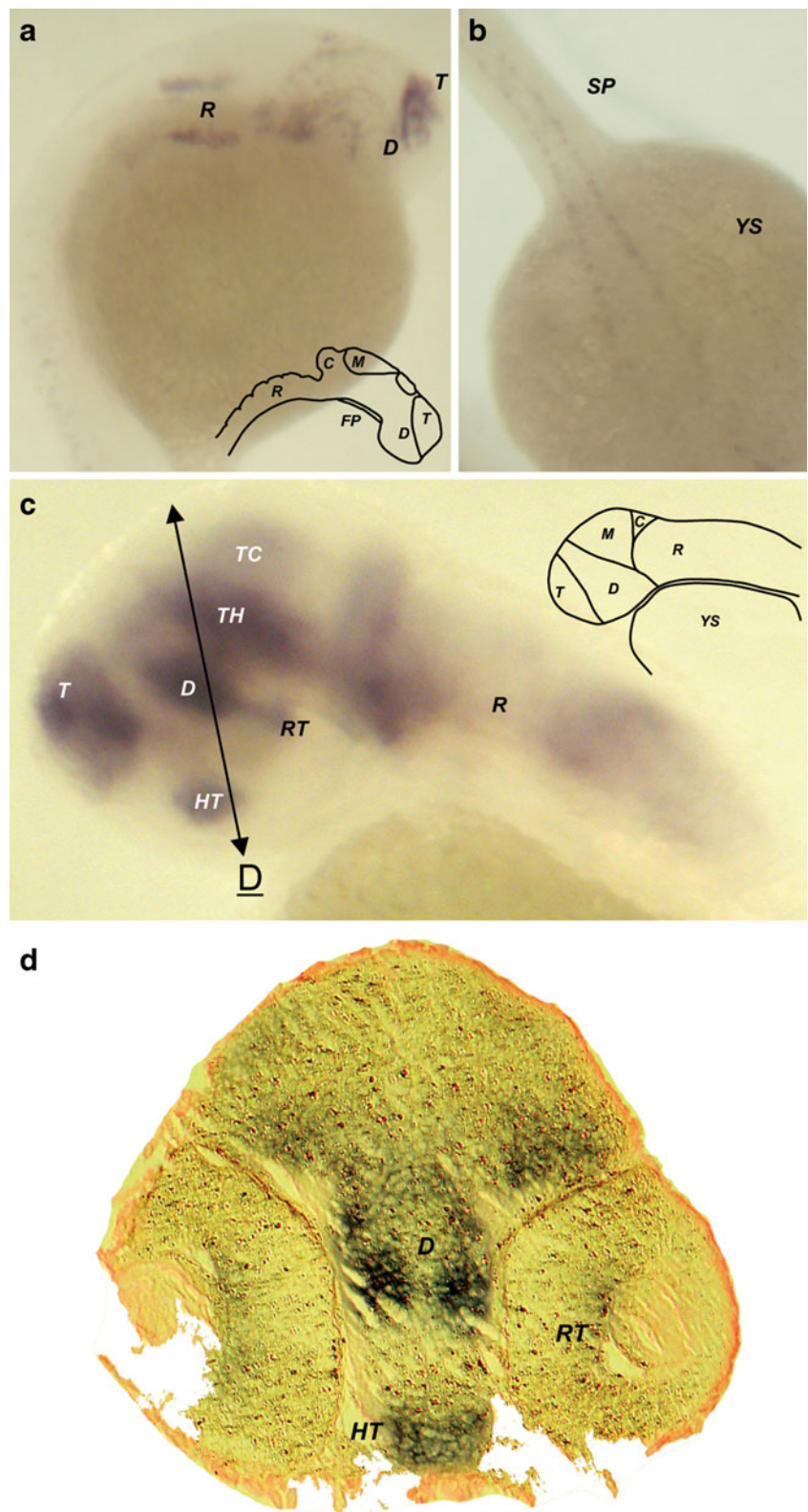


Figure 2 **a** Representative photomicrograph showing the expression of PACAP1 messenger RNA in the 24-hpf embryo. **b** No significant hybridization signal was seen with a PACAP1 sense probe. Cerebellum

C, diencephalon *D*, floor plate *FP*, mesencephalon *M*, neural crest *NC*, telencephalon *T*, rhombencephalon *R*, spinal cord *SP*, and yolk sac *YS*

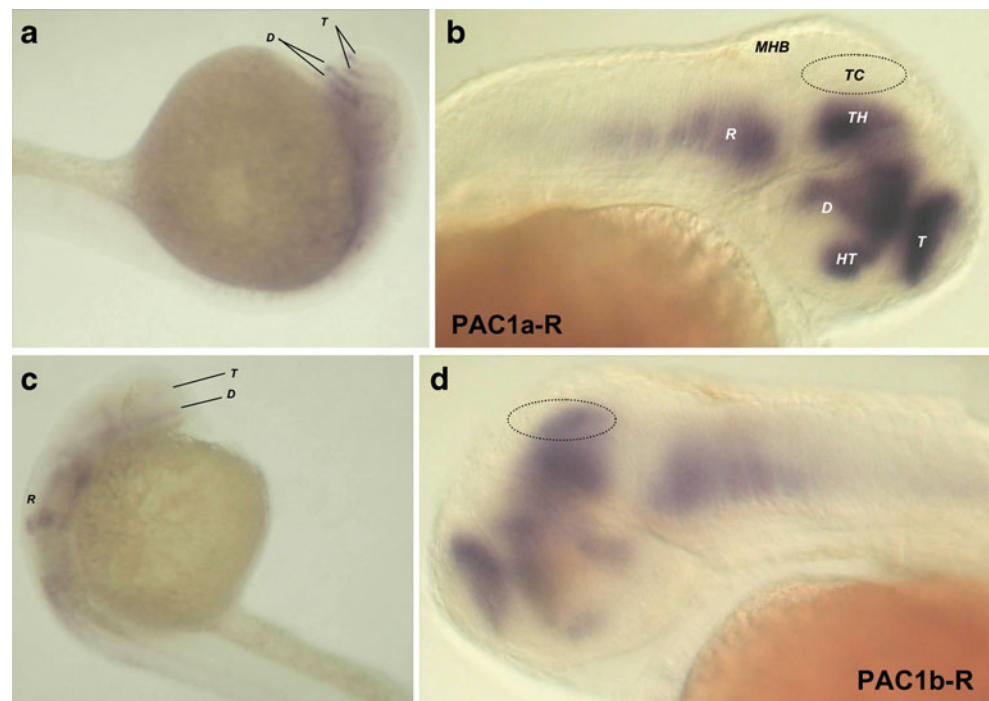
Figure 3 Photomicrographs showing the expression of PACAP2 mRNA in the 24-hpf (a, b) and 48-hpf (c, d) embryos. Cerebellum *C*, diencephalon *D*, floor plate *FP*, hypothalamus *HT*, mesencephalon *M*, telencephalon *T*, thalamus *TH*, retina *RT*, rhombencephalon *R*, spinal cord *SP*, tectum *TC*, and yolk sac *YS*



from the entire embryo and frontal sections. A high to moderate expression of PACAP2 gene was found in the telencephalon, the diencephalon, the thalamus, the hypothalamus, the rhombencephalon, the retina, and the tectum

(Fig. 3c, d). Concerning the PAC1 receptors, PAC1a-R antisense probe revealed a hybridization signal mainly in the diencephalon and the telencephalon in the 24-hpf embryos (Fig. 4a). At the same developmental stage,

Figure 4 PAC1-R gene expression in the 24-hpf (a–c) and 48-hpf (b–d) zebrafish embryos. Whole-mount in situ hybridization by using a DIG-labeled PAC1a-R antisense probe (a, b) or a PAC1b-R antisense probe (c, d). Diencephalon *D*, hypothalamus *HT*, midbrain–hindbrain boundary *MHB*, rhombencephalon *R*, telencephalon *T*, thalamus *TH*, and tectum *TC*



PAC1b-R is expressed in the diencephalon and the rhombencephalon, whereas only faint expression was seen in the telencephalon (Fig. 4c). At later stage, in the 48-hpf zebrafish embryos, both PAC1 receptors showed similar expression profiles with a strong labeling in the telencephalon, the thalamus, and the diencephalon (Fig. 4b, d). A moderate signal was found in the rhombencephalon, whereas the metencephalon and the retina were devoid of hybridization signals of the two PAC1 receptors. In the hypothalamus, PAC1b-R is expressed at low levels compared to the PAC1a-R. The main difference between the two PAC1 receptor expressions was found in the tectum area in which only the PAC1b-R is expressed, whereas no signal was detected with the PAC1a-R probe in this region (Fig. 4b, d).

Discussion

In this study, the expression of the PACAPergic system was examined during the development of the zebrafish *Danio rerio* in the pharyngula period corresponding to the 24- to 48-hpf embryos. Kimmel and collaborators described this period as an important time of development corresponding to the establishment of rudiment organs, for example, a functional heart and circulation, and pigmented retinal epithelium that differentiates and surrounds the lens. Embryos at 24 hpf have 30 somites. Rapid morphogenesis then sculpts the nervous system, which expands anteriorly and results into a brain with five lobes and ventricles. This is the time of development when one can most readily

compare the morphologies of embryos of diverse vertebrates (Kimmel et al. 1995).

Then, the expression levels of PACAP1, PACAP2, PAC1a-R, and PAC1b-R genes were investigated by using real-time PCR technique on reverse transcribed mRNA from the 24- and 48-hpf embryos and adult zebrafish. The EF1- α was used as a housekeeping gene since its expression was shown to be constant during the development of zebrafish (Tang et al. 2007). PACAP2 mRNA was the principal form detected in embryos in comparison to the expression level of PACAP1 gene (Fig. 1). This result is consistent with the 24-h staining period needed to detect the PACAP1 DIG-labeled probe versus the 30–40-min staining period with the PACAP2 probe in whole-mount in situ hybridization (Figs. 2 and 3). In adult, PACAP2 gene expression was detected at six-fold high levels than the PACAP1 gene, indicating that PACAP1 might play the predominant role during the embryogenesis. Moreover, PACAP1 mRNA is mainly expressed in the neural tube in all species examined, suggesting an essential role in the early neural patterning, in part through protein kinase A-mediated antagonisms of sonic hedgehog signaling (Lelievre et al. 2008).

In all species examined, including zebrafish, PACAP and PAC1R genes are expressed in at particularly high levels in the posterior part of the embryonic nervous system, corresponding to the hindbrain and the spinal cord. At 48 hpf, PACAP and PAC1-R mRNA are widely detected in the central nervous system of zebrafish from the telencephalon to the rhombencephalon through the diencephalon and the mesencephalon. Only the metencephalon was devoid of

mRNA encoding PACAP and its receptors (Figs. 2, 3, and 4). Another group detected expression of the PACAP1 gene in the zebrafish cerebellar primordium at 24 hpf, perhaps owing to a higher level of assay sensitivity (Krueckl et al. 2003).

A comparison of distribution of PACAP and PAC1-R mRNA in the embryos of different species reveals slight differences. At the same developmental stage, corresponding to E10–15 in mice (Sheward et al. 1998; Waschek et al. 1998), stage 25–35 in *Xenopus laevis* (Hu et al. 2001), and pharyngula stage in zebrafish (Krueckl et al. 2003, our study), the expression patterns of PACAP and PAC1-R in xenopus appeared more restricted than in mice. Indeed, the PAC1-R gene was not expressed in the telencephalon and the metencephalon, and PACAP gene was expressed only in the hindbrain at the rhombencephalon level and in the spinal cord. In mice, peptide and receptor mRNA were identified in all different parts of the brain suggesting that fish showed a gene expression pattern closer to the mice than to the xenopus. These apparent differences in mice/fish and xenopus are probably due to differing temporal patterns of gene expression in brain sub-regions in these species.

In situ hybridization analysis revealed a similar expression profile of the two zebrafish PAC1-R genes except in the tectum. In the mesencephalon, only PAC1b-R mRNA was detected in the tectum area. In any case, the expression patterns suggest that PACAP peptide could be implicated in the visual information analysis and/or circadian rhythm regulation (Hannibal and Fahrenkrug 2004; Yanez et al. 2009).

To investigate the function of PACAP during the early development, we injected mRNA encoding PACAP in fish embryos and tried to knockdown PACAP gene expression by using morpholinos. Using these approaches, we could not see any obvious morphological changes in fish embryos (data not shown). However, Sherwood's group reported a severe phenotype consisting of a reduction of brain size, a modification of the midbrain–hindbrain boundary and smaller eyes, after injection of morpholinos against PACAP1 and PACAP2 genes (Wu et al. 2006). This difference of results between our teams may be due to the specific morpholinos used and/or the quantity of morpholinos injected. Finally, Blechman and coworkers did not see any morphological changes after morpholino injection, but demonstrated that PACAP and PAC1R are highly expressed in hypothalamus and are involved in the differentiation of neuron precursors into dopaminergic and oxytocinergic neurons (Blechman et al. 2007). In mice, targeted deletion of PACAP did not result in any obvious phenotype. However, PACAP gene deletion revealed the importance of this neuropeptide in many functions, such as the regulation of circadian rhythms (Colwell et al. 2004;

Hannibal et al. 2008), ontogeny of the cerebellum (Allais et al. 2007), testicular aging (Lacombe et al. 2006), and energy metabolism (Sherwood et al. 2007; Tomimoto et al. 2008).

In the present study, we have described the presence of PACAP and its cognate receptors mRNA in different structures of the developing nervous system, suggesting an important function for this peptide during the brain development in fish, as well as in higher vertebrates.

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