Expression of pcp4a in Subpopulations of CNS Neurons in Zebrafish

MARINA MIONE,1,2* ZSOLT LELE,1,3 CAMILLA T. KWONG,1 MIGUEL L. CONCHA,4 AND JONATHAN D. CLARKE1

1Department of Anatomy and Developmental Biology, University College London, London WC1E 6BT, United Kingdom
2Istituto Fondazione Italiana Per La Ricerca Sul Cancro (FIRC) Di Oncologia Molecolare, FIRC Institute of Molecular Oncology, 20139 Milano, Italy
3Institute of Experimental Medicine, H-1083 Budapest, Hungary
4Anatomy and Developmental Biology Program, Faculty of Medicine, Institute of Biomedical Sciences, Universidad de Chile, Santiago Clasificador 7–Correo 7, Chile

ABSTRACT

The molecular organization of the zebrafish brain and its relation to neuroanatomical divisions are still largely unknown. In this study we have analyzed the expression of a small transcript encoding for the IQ containing polypeptide Pcp4a in developing and juvenile zebrafish. The transcript is exclusively expressed in neural structures with a pattern that is highly specific for restricted domains and cell populations throughout development, and it allows us to follow the development of these structures at different times. The expression of pcp4a characterizes the dorsocaudal telencephalon, dorsal habenula, pretectal nuclei, preglomerular complex, mammillary bodies, and deep layers of the optic tectum and is a hallmark of a subpopulation of reticulospinal neurons. In the telencephalon, comparison of the expression of pcp4a with other pallial markers showed a rostrocaudal gradient in the expression of these genes, which suggests that the dorsal telencephalon of zebrafish may be organized in distinct areas with different molecular natures. Pcp4 has been involved in modulating calcium signals and in binding to calmodulin, but its precise role in neuronal functions is not known. The analysis of pcp4a expression and localization in the zebrafish brain suggests that pcp4a may be a useful marker for sensory and some motor neuronal circuitries and for telencephalic areas processing sensory inputs.

Indexing terms: telencephalon; teleost; tbr1; reelin; pattern

The zebrafish has become the leading teleost model for studies of molecular genetics and embryology. Forward genetics through mutagenesis screens has yielded a large number of mutations in many developmental processes, and no doubt the list of mutated genes will soon be extended to genes affecting behavior or complex neuronal functions. However, knowledge of the organization, connectivity, and functions of zebrafish brain areas is disappointingly scanty. An understanding of how the zebrafish brain is patterned and wired and the molecular nature of its divisions will help to address issues of structure-function relationship.

One possible approach is to use molecular markers that in other species have proved helpful in identifying the major divisions of the vertebrate brain during development (Puelles et al., 2000; Bachy et al., 2002; Brox et al., 2004; Medina et al., 2004). The rationale behind this is the understanding that regulatory genes consistently expressed in the same or a homologous domain in different species play a fundamental role in the development of the specialized features of that area.

In parallel to the analysis of regulatory gene expression domains in the developing zebrafish brain, we have...
started a systematic search for transcripts specifically expressed during development, using both bioinformatic approaches and classical subtractive hybridization techniques. Those transcripts that show a specific pattern of expression and, based on sequence analysis, encode for functionally relevant products are used for detailed analysis of their expression. We hope that this approach will give insights into the functional divisions of the zebrafish brain during development and complement the molecular and neuroanatomical data. Moreover, the identification of transcripts with specific patterns of expression represents an indispensable tool aiding in the identification of mutated genes causing neuronal specific phenotypes.

Purkinje cell peptide 4 (Pcp4), also known as Pep19 (Ziai et al., 1986), is a small polypeptide carrying an IQ motif: a 23-amino acid domain that binds EF-hand proteins and shows high affinity for calcium-poor calmodulin (Ziai et al., 1986; Skene, 1989; Liu and Storm, 1990; Johanson et al., 2000; Bahler and Rhoads, 2002). The expression of pcp4 in subpopulations of mature neurons of mouse and rat central nervous system (CNS) has been correlated with their ability to undergo long-term potentiation (LTP) and long-term depression (LTD) and their resistance to apoptotic insults (Johanson et al., 2000). Other members of the IQ motif class of polypeptides, besides Pcp4, are neuromodulin or GAP-43 and neurogranin or RC3, all neuron-specific peptides (Slemmon et al., 2000) expressed both during development and in mature neurons.

Recently, the expression patterns of Pcp4 (Bulfone et al., 2004; Thomas et al., 2003) and those of a related peptide, Pcp4-like1 (pcp4L1; Bulfone et al., 2004) have been reported in the developing mouse embryo, demonstrating that the expression of these genes is not confined to mature CNS structures. We isolated a cDNA clone (pcp4a) encoding for a zebrafish pcp4 polypeptide through the screening of an adult zebrafish brain library with a complex probe representing transcripts preferentially expressed in the embryonic brain. A second transcript (pcp4b) encoding for a highly similar peptide and one encoding for a Pcp4-like1 peptide were identified through a BLAST search of the expressed sequence tag (EST) database at the National Center for Biotechnology Information (NCBI). Their expression patterns differ slightly from that of pcp4a and will be reported separately. The identification of the transcript for pcp4a in an adult brain library screened with a probe enriched for developmentally regulated transcripts indicated that the gene was expressed throughout development, and the analysis of its expression pattern suggested a highly specific expression in neuronal groups.

Here we report the expression of pcp4a throughout development: a comparison with the expression of other genes marking the same or adjacent population of neurons allows us to consider pcp4a as a novel marker for cell groups that so far are not known to be specifically marked by other gene expression. Moreover, the analysis of pcp4a expression in the dorsal telencephalon suggests the existence of areal divisions that may be related to the functional organization of the telencephalon in zebrafish.

**MATERIALS AND METHODS**

**Cloning of the zebrafish pcp4a gene**

Hybridization of a microarrayed adult brain zebrafish library distributed by RZPD (Berlin, Germany; library no. 2000; Bahler and Rhoads, 2002). The expression of **pcp4** in subpopulations of mature neurons of mouse and rat central nervous system (CNS) has been correlated with their ability to undergo long-term potentiation (LTP) and long-term depression (LTD) and their resistance to apoptotic insults (Johanson et al., 2000). Other members of the IQ motif class of polypeptides, besides Pcp4, are neuromodulin or GAP-43 and neurogranin or RC3, all neuron-specific peptides (Slemmon et al., 2000) expressed both during development and in mature neurons.

Recently, the expression patterns of Pcp4 (Bulfone et al., 2004; Thomas et al., 2003) and those of a related peptide, Pcp4-like1 (pcp4L1; Bulfone et al., 2004) have been reported in the developing mouse embryo, demonstrating that the expression of these genes is not confined to mature CNS structures. We isolated a cDNA clone (pcp4a) encoding for a zebrafish pcp4 polypeptide through the screening of an adult zebrafish brain library with a complex probe representing transcripts preferentially expressed in the embryonic brain. A second transcript (pcp4b) encoding for a highly similar peptide and one encoding for a Pcp4-like1 peptide were identified through a BLAST search of the expressed sequence tag (EST) database at the National Center for Biotechnology Information (NCBI). Their expression patterns differ slightly from that of pcp4a and will be reported separately. The identification of the transcript for pcp4a in an adult brain library screened with a probe enriched for developmentally regulated transcripts indicated that the gene was expressed throughout development, and the analysis of its expression pattern suggested a highly specific expression in neuronal groups.

Here we report the expression of pcp4a throughout development: a comparison with the expression of other genes marking the same or adjacent population of neurons allows us to consider pcp4a as a novel marker for cell groups that so far are not known to be specifically marked by other gene expression. Moreover, the analysis of pcp4a expression in the dorsal telencephalon suggests the existence of areal divisions that may be related to the functional organization of the telencephalon in zebrafish.

**MATERIALS AND METHODS**

**Cloning of the zebrafish pcp4a gene**

Hybridization of a microarrayed adult brain zebrafish library distributed by RZPD (Berlin, Germany; library no.
Fig. 1. Alignment of pcp4 sequences. A: Optimal alignment of amino acid sequences of human, mouse, rat, pig, cow, chick, Xenopus, and zebrafish Pcp4 sequences was obtained with the Clustal-X program and checked manually. The consensus amino acid sequence is at the top of each group, and the IQ motif is underlined at the bottom.

B: A distance tree of the Pcp4 proteins isolated from different vertebrate species. The distance tree was drawn with the Neighbor-joining program from the Phylip package. As the tree indicates, zebrafish has 2 pcP4 genes (named pcP4a and pcP4b) and only one pcP4L1 gene. Numbers indicate the bootstrap values.
Fig. 2. pcp4a expression in the brain of 24-hpf zebrafish. A–H: Lateral (A,E), dorsal (B,D,F,H), and anterior (G) views of pcp4a expression in whole embryos/brains. A,C: Double staining with pax2.1 and pcp4a probes (colors as indicated in the picture). Arrowheads point to pcp4a expression in the RS neuron precursors. B: Double staining with krox20 and pcp4a probes. Arabic numbers indicate rhombomeres. D: Double staining with val and pcp4a probes. Arrow indicates double staining in RS cells in rhombomere 5. E: Expression of pcp4a in the epiphysis. E′ Inset shows a dorsal view of the diencephalon with the expression of pcp4a at the two lateral poles of the epiphysis. E″: Inset shows pcp4a expression (blue) in two RS neurons (Mid3cm) retrogradely labeled with biotin dextran (brown). F: Double staining with islet1 and pcp4a probes. Black arrows point to pcp4a-expressing RS neuron precursors; white arrowhead points to the facial motor nucleus, and white arrow indicates the octaval nucleus, both expressing islet1. G: Double staining with GFP antibody and a pcp4a probe in an embryo of the tg(foxD3-GFP) line. Arrows point to pcp4a-expressing cells and their axons. H: pcp4a expression in the spinal cord at 3 dpf. I: Double staining with GFP antibody (brown) and a pcp4a probe (blue) in an embryo of the tg(islet1-GFP) line. Arrowheads point to double-stained neurons. For abbreviations, see list. Scale bars = 100 μm in A–I. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)
611) with a complex probe generated through subtractive hybridization (Mione, unpublished data) yielded a number of positive clones, which were analyzed for their sequence and expression pattern. The complex probe was generated with the ClonTech (Palo Alto, CA) PCR Select Kit, which is based on the RDA method (Lisitsyn et al., 1993), following subtraction of mRNAs common to adult zebrafish brain and 24-hpf whole zebrafish embryos. The remaining mRNAs (representing genes expressed predominantly in developing embryos) were used to synthesize a cDNA probe in the presence of $^{32}$P-dCTP. With this complex (so-called as it represents several mRNAs) probe we screened an arrayed cDNA library generated from adult zebrafish brain by John Ngai (University of California at Berkeley, CA) and distributed by RZPD (www.rzpd.de). The goal of this strategy was double: 1) to identify full-length clones, corresponding to the 300-nt polymerase chain reaction (PCR) fragments generated by the subtraction approach, in order to facilitate the identification of the clones through sequencing; and 2) to restrict further work to clones expressed exclusively in the CNS.

In situ hybridization for two of these clones with identical sequence gave a neuronal specific pattern. Conceptual translation yielded an open reading frame of 62 amino acids, highly similar to that of mouse and human Pcp4.

**Fish strains**

*Danio rerio* of wild-type and transgenic lines were from the UCL Zebrafish Facility. The *tg* (islet-1-GFP) line was donated by H. Okamoto (Higashijima et al., 2000). The *tg* (foxD3-GFP) line was donated by C. Nusslein-Volhard (Gilmour et al., 2002). Backfilling experiments were carried out according to European Experimental Animal Care guidelines.

**In situ hybridization**

Antisense probes for pcp4a, reelin (Costagli et al., 2002), and tbr1 (Mione et al., 2001) were generated by linearization with *SalI* and in vitro transcription with SP6 in the presence of digoxigenin (dig)-labeled ribonucleotides (Roche, Indianapolis, IN). Other plasmids used in this study were: *pax2.1* (Macdonald et al., 1997); *krox20* (Oxtoby and Jowett, 1993); *islet1* (Korzh et al., 1993); *val* (Moens et al., 1996); *emx1*, *emx2*, and *emx3* (Morita et al., 1995); and *pax6.1* and -6.2 (Nornes et al., 1998). Single and double in situ hybridization with two color substrates was performed according to Hauptmann and colleagues (2002). Substrates used in this study are 5-bromo-4-chloro-3-indoloyl phosphate (BCIP)/NBT for blue color and INT/BCIP for red color, both from Roche.

**Backfilling**

We performed three types of backfilling experiments. First, to compare the pattern of expression of pcp4a with the localization of reticulospinal neurons in wholemount preparations of the hindbrain, we injected fluorescein dextran (molecular weight 10,000; Molecular Probes, Eugene, OR) into the upper spinal cord of 4-dpf zebrafish as described (Alexandre et al., 1996) to label the reticulospinal (RS) neurons retrogradely. Survival time after this injection was 12–18 hours. Fluorescein dextran was revealed with a dig-labeled anti-fluorescein antiserum (Roche) used at a concentration of 1:10,000 in paraformaldehyde-fixed larvae and revealed by using BCIP/NBT as the substrate, as described previously (Costagli et al., 2002). In a second set of backfilling experiments, designed to reveal expression of pcp4a in backfilled RS neurons, we used biotinylated dextran as the tracer (Molecular Probes) and the same labeling/survival parameters used in the previous backfilling experiment. To reveal the co-existence of pcp4a transcripts and biotin dextran, we sectioned the hindbrains of injected larvae with a cryostat and subjected the serial sections to in situ hybridization for pcp4a, as described above. Following the detection of the pcp4a transcripts with BCIP/NBT as the substrate, we incubated the sections with a streptavidin-horseradish peroxidase complex (ABC, Vector, Burlingame, CA) following the manufacturer’s instructions and revealed the biotinylated dextran with a diaminobenzidine (DAB) and H$_2$O$_2$ substrate.
Fig. 4. Distribution of \textit{pcp4a} mRNA transcripts. Drawings of a series of transverse sections through the zebrafish brain, depicting the distribution of \textit{pcp4a} transcripts. \textbf{A}: Schematic drawing of a lateral view of an adult zebrafish brain, to show the levels and orientation of the sections in \textbf{B–M}. \textit{pcp4a} transcripts are represented by black dots on the right side of the sections. For abbreviations, see list.
as described in the double in situ/immunocytochemistry protocol (Costagli et al., 2002).

A third set of backfilling experiments was performed to analyze spinal cord intersegmental and long projection neurons and their relation to pcp4a expression. We labeled subpopulations of spinal cord neurons with intraspinal injections (at the level of the yolk extension) of a mixture of biotinylated dextran and fluorescein dextran at 5 dpf as described (Higashijima et al., 2004). The day after, larvae were examined for the distribution of fluorescent labeling and fixed with 4% paraformaldehyde (PFA; in phosphate-buffered saline [PBS], pH 7.4). Labeled regions of spinal cords were processed for pcp4a in situ hybridization on cryostat sections as described above, reacted with horseradish peroxidase (HRP)-conjugated streptavidin (Vector ABC), and revealed with DAB and H$_2$O$_2$ substrate incubation.
Figure 5.
pcp4α IN ZEBRAFISH NEURONS

Photography
Images were captured with a Jenopick digital camera (Improvision, Coventry, UK) connected to a Nikon Eclipse 1000 microscope, using ×4, ×20, and ×40 Plan-apo lenses and Open Lab software (Improvision). Digital images were stored as 1,600 × 1,200 pixels at a resolution of 300 dpi and manually arranged to form composite pictures by using Adobe Photoshop 7 (San Jose, CA).

RESULTS
During a screening of an adult zebrafish brain cDNA library with a complex probe representing transcripts preferentially expressed in embryonic brains, two identical clones were identified, which represented the same full-length cDNA encoding for a putative pcp4 peptide (pcp4a; Fig. 1A, accession number: DQ122930). A search of zebrafish and Xenopus databases revealed several EST clones that, when conceptually translated, gave the sequences reported in Figure 1A. The zebrafish EST sequences corresponded to two different transcripts, one identical to pcp4a and another slightly divergent, which we called pcp4b. Differences in the sequences suggested that the two transcripts were those of two different genes that may have resulted from the well-documented teleost-specific gene duplication event that took place about 350–400 million years ago (Amores et al., 1998), rather than alternatively spliced transcripts of the same gene. This is further confirmed by the different chromosomal location of two Ensemble transcripts corresponding to pcp4a and pcp4b (data not shown). Thus pcp4a and pcp4b are very likely to be paralogues. A third class of transcripts encoding for another IQ-containing peptide highly similar to pcp4a and -b was identified through a Blast search. These transcripts encode for the zebrafish orthologue of pcp4L1 (Bulfone et al., 2004), as suggested by phylogenetic analysis of sequences from different species (Fig. 1B).

High similarity is found among all vertebrate sequences, with the Xenopus clone being the most divergent (Fig. 1B). The IQ motif was identified in all sequences (underlined in Fig. 1A).

Expression of pcp4α during embryonic development
The expression of pcp4a in zebrafish starts around 22 hpf (26-somite stage) in two bilateral cellular groups located in the lateral region of the hindbrain (Fig. 2A,B).

Double labeling with a pax2.1 probe that marks the otic capsule shows that pcp4a-expressing cells are located medial to it (Fig. 2A,C). Double labeling with a krox20 cRNA probe at 22 hpf to mark rhombomeres 3 and 5 shows that pcp4a-expressing cells are located at the level of rhombomere 5 and 6 (Fig. 2B). To gain insights into the identity of these groups of cells expressing pcp4a we performed double in situ hybridization with a val probe, which labels Mauthner neurons and other reticulospinal neurons (Moens et al., 1996). The paired group of cells expressing pcp4a located in rhombomere 5 co-expresses val, suggesting that they maybe reticulospinal neurons (Fig. 2D). This has been shown by double labeling of reticulospinal neurons using retrograde back filling and pcp4a in situ hybridization at 5 dpf (Figs. 2E’–’F, 8).

By 30 hpf the presumptive reticulospinal neurons labeled by the expression of pcp4a are located in a more medial position. Double labeling with a islet-1 probe to label cranial nuclei (Fig. 2F) shows that the upper group of pcp4a-expressing cells in the hindbrain is next to neurons of the octaval nerve nuclear complex, whereas the lower group is located just lateral to neurons of the facial (VII) nerve nucleus. Another domain of expression appears in the epithalamus at 50 hpf, where pcp4a marks two groups of cells in the lateral poles of the epithysis (Fig. 2E,E’). Analysis of pcp4a expression at 48 hpf in a transgenic line expressing GFP specifically in pineal neurons and their projections, tgf(islet-D3-GFP), allowed us to identify the pineal cells expressing pcp4a tentatively as projection neurons, because they give rise to GFP+ axons projecting out of the epiphysis into the lateral forebrain bundle (Fig. 2G and data not shown). Expression of pcp4a is also detected in a chain of dorsal spinal cord neurons, some of which are GFP+ in the tgf(islet1-GFP) line, suggesting that they may be Rohon Beard neurons (Fig. 2H). However, not all cells express both markers (Fig. 2I).

At 48 hpf, analysis of pcp4a expression reveals additional domains (Fig. 3A–C). These include the caudal telencephalon, the preoptic area, the pretectal nuclei, several diencephalic nuclei in the mesencephalic tegmental area, restricted cells in the optic tectum, and cells at the mid-hindbrain boundary, in the hindbrain, and throughout the ventral region of the spinal cord, plus cells in the central portion of the ganglion cell layer in the retina.

Thus, by 48 hpf, expression of pcp4a has spread to a large number of neuronal cell populations likely to have just become functional active.

Expression of pcp4α at 4 days, 2 weeks, and 1 month post fertilization
The expression of pcp4a was analyzed at 4–5 dpf, 2 weeks, and 1 month post fertilization by using cryostat sections to identify the areas and cell groups that express pcp4a during development. The pattern of expression of pcp4a is similar at the three age end-points, with a few exceptions (i.e., in the retina); therefore the description of the expression pattern of pcp4a will be carried out in parallel for all the age points, and examples of the staining at the different ages will be provided. Because morphogenesis of many brain structures is still ongoing between 4 dpf and 1 month, the comparison of pcp4a expression between these two stages reveals how morphogenetic movements and restricted proliferation influence the location and size of brain nuclei expressing pcp4a. An overview of the expression pattern of pcp4a in the brain of...
Fig. 6. *pcp4a* expression in the diencephalon and mesencephalon. A: Sagittal and (B–E) transverse sections showing *pcp4a* expression in the mesencephalon at 1 month (A,E,F), 2 weeks (C), and 4 dpf (B). Black lines with letters in A indicate the levels of the corresponding transverse sections. F: Section through the trigeminal ganglion at 1 month. For abbreviations, see list. Scale bars = 70 μm in A–F.
Figure 7.
1-month-old zebrafish is presented schematically in Figure 4.

**pcp4a expression in the telencephalon.** At 5 dpf and 2 weeks, pcp4a transcripts were detected in the lateromedial region of the dorsal telencephalon (specifically in the DL and Dp; Fig. 5A,B). However, by 1 month, pcp4a is expressed mainly in the caudal third of the telencephalon in both the dorsal and ventral telencephalon (Fig. 5C,D). In the ventral telencephalon, pcp4a is expressed in the entopeduncular nucleus (En; Fig. 5A,E) and in the parvocellular preoptic nucleus (PPn; Fig. 5D) caudal to the anterior commissure (Fig. 5A). There is no expression in the olfactory bulb.

**pcp4a expression in the diencephalon.** In the diencephalon, pcp4a is strongly expressed in the dorsal habenular nucleus (stronger in the lateral than in the medial portion; Fig. 5E,F), in the presumptive eminentia thalami (ET; Fig. 5E), in the migrated pretectal nuclear complex (only present at 1 month; Fig. 5G,H; present in both their periventricular and migrated groups at 5 dpf, Fig. 6A,B), and in the central nucleus of the dorsal thalamus (CP; Fig. 6C,D). Low levels of expression of pcp4a were also detected in the nuclei of the posterior tuberculum (TPp; Fig. 6D). In addition, large cells in the nucleus of the medial longitudinal fascicle (nMLF) express pcp4a (Fig. 6E), and finally, the nucleus subglomerulosus (SG) and the torus lateralis (TLA) are among of the most intensely labeled areas (Fig. 6A,E). The preglomerular complex (PGC) expresses the highest levels of pcp4a transcripts in the diencephalon (Fig. 6A,C,D).

**pcp4a expression in the hypothalamic region.** Besides a faint expression of pcp4a in the anterior periventricular region (Fig. 6C), the caudal hypothalamus expresses the highest levels of pcp4a in its paired mammillary bodies (Fig. 7A,C); a low expression of pcp4a is also present in the diffuse nucleus of the inferior lobe (DIL; Fig. 6E). Note that at 5 dpf, the mammillary bodies are located in a lateral position in the hypothalamic alar region (Fig. 7B, arrowheads) whereas at 1 month they occupy the medioventral region of the caudal hypothalamus (Fig. 7C).

**pcp4a expression in the mesencephalon.** Expression of pcp4a can be detected in the periventricular gray zone (PGZ) of the optic tectum and in sparse cells of the stratum griseum centrale (Figs. 6A–F, 7A,B). Large cells at the ventral side of the torus longitudinalis (TL) express pcp4a, throughout its rostrocaudal extension (Fig. 7D,E), whereas the majority of the granule-like cells of TL express reelin (cf. Fig. 4b in Costagli et al., 2002). In the torus semicircularis (TS), two groups of cells express pcp4a transcripts (Figs. 6A,E, 7A,B). One group is composed of medium cells found in the lateral torus semicircularis, close to the tectal ventricle. The other group consists of large cells in the ventral part (layer 1, Ito, 1974). At a more caudal level, the secondary gustatory nucleus (SGN; Wullimann et al., 1996) expresses low levels of pcp4a (not shown).

**pcp4a expression in the rhombencephalon and spinal cord.** Expression of pcp4a can be detected at low levels in the Purkinje cells of the valvula and crista cerebelli (Fig. 7D–F) but not in the corpus cerebelli. A similar relationship between reelin + granule cells and pcp4a + large neurons is found in the TL (see above), valvula, and crista cerebellaris (Fig. 7D,E), suggesting that these structures may share similar molecular identities. Note that the Purkinje cells of the corpus cerebelli do not express pcp4a (Fig. 7D, asterisks). Cells in deep layers of lobus VII and in the superficial layer of lobus X (sensory relay nuclei for the facial and vagal nerves, respectively) express pcp4a (Fig. 8D).

pcp4a continues to be expressed by reticulospinal neurons at 5 days (Fig. 8B,C,G) and 1 month post fertilization, when most, but not all, reticulospinal neurons labeled by backfilling with fluorescein dextran (Fig. 8A) appear to express pcp4a (Fig. 8B,C). Notably, Mauthner cells do not express pcp4a (Fig. 8A–C), whereas their homologues in r5, Mid3cm, do express pcp4a throughout all developmental stages studied (Figs. 2, 8C,G,H,K). pcp4a transcripts could also be detected in the descending axons of some reticulospinal neurons (Fig. 8E, arrow).

In the spinal cord, neurons of different sizes, including visceral motor neurons expressing reelin (Costagli et al., 2002), express pcp4a throughout the length of the spinal cord (Fig. 9B,E). For visceral motor neurons and primary sensory neurons in the dorsal root ganglia (which do not express reelin; Fig. 9A,C, arrow), compare reelin and pcp4a expression in adjacent sections (Fig. 9A,C). Besides the visceral motor neurons, a large number and variety of spinal cord cells express pcp4a. In order to assess whether the cells that express pcp4a in the spinal cord include motor neurons or interneurons, we performed intraspinal injections of biotinylated dextran to mark the interneuron population and assessed the expression of pcp4a in the labeled cells. Most classes of interneurons, including the C1d, CoLa, CoBl, and UCoD neurons (as defined by Hale et al., 2001 and Higashijima et al., 2004) labeled at 5 dpf by this procedure express pcp4a (Fig. 9E–G).

**pcp4a expression in the retina and trigeminal sensory ganglion.** From 48 hpf pcp4a is expressed in the ganglion cell layer of the developing retina (Fig. 3). The expression is initially confined to the medial sector and then spreads to the rest of the ganglion cell layer by 4 dpf (Fig. 6B). Sparse cells in the inner nuclear layer also express pcp4a (Fig. 6B). No expression of pcp4a is detectable in the retina of 1-month-old zebrafish (data not shown). By 4 dpf, expression of pcp4 is also found in a few large neurons of the trigeminal sensory ganglion; this expression is still detectable at 1 month (Fig. 6E, arrowhead, Fig. 6F).

**Comparison of the expression domains of tbr1, reelin, and pcp4 in the telencephalon.** In mammals, pcp4 is strongly expressed in the dorsal telencephalon at late embryonic stages through adulthood, and its transcripts localize predominantly to the hippocampal region...
Figure 8.
Our study of the distribution of the transcripts of the small peptide pcp4a in brain areas and cell groups during zebrafish development identifies a new marker for specific neuronal populations through morphogenesis and migration until adulthood. Another outcome of this study is that the expression domains of pcp4a in the zebrafish brain are largely similar to those observed in the developing mouse brain for an orthologue gene, pcp4, thus allowing us to consider the expression of this peptide as a marker of brain areas or neuronal populations in two different vertebrates whose lineages diverged more than 400 million years ago (Carroll, 1988).

As a result of the observation that pcp4a expression highlights conserved domains in the fish and mammalian brain, we have uncovered a graded distribution of three dorsal-pallial transcripts that in zebrafish allow us to distinguish three different areas of the dorsal pallium: an anterior medial domain, a dorsal domain, and a caudal lateral domain. The homology of these domains with known divisions of the mammalian isocortex will be discussed.

**pcp4a is a marker of reticulospinal neurons throughout development**

The first groups of neurons to express pcp4a are the precursors of the reticulospinal neurons that develop in the laterodorsal region of the hindbrain, in r5 and r6, between 8 and 26 hpf. The RS neurons are among the first neuronal units to become active in the zebrafish larvae, and they mediate the escape response (Liu and Fetcho, 1999), the first coordinated neuronal reflex. There are very few molecular markers that allow us to identify these cells prior to the extension of their axons into the spinal cord, *val* being the best characterized, but it labels only the Mauthner neurons and another pair of RS neurons in r5 (Moens et al., 1996). Traditionally the RS neurons are identified through retrograde labeling. Thus the first indication of the origin and location of these neurons relies on our ability to label them retrogradely. Detailed studies of their number, location, and projections between 20 hpf and 5 dpf have been carried out (Mendelson, 1986a; Mendelson, 1986b; Metcalfe et al., 1986).

With the identification of *pcp4a* as an early marker of reticulospinal neurons and the study of its expression in RS neurons at various stages, new insights into the origin of these cells were obtained. It appears that these neurons are born/specified in a more lateral position than where they will eventually reside. The lateral origin of these cells was suggested by Mendelson (1986b) in his time course study using HRP to label RS neurons retrogradely as early as 24 hpf. A dorsolateral origin of hindbrain projection neurons, followed by a ventral migration, similar to that seemingly undertaken by zebrafish RS neurons, has been described in chick (Clarke et al., 1998). In addition, from the analysis of *pcp4a* expression, it appears that some RS neurons are present at early stages as clusters of pcp4a-expressing cells, which subsequently...
Figure 9.
delaminate in single neurons that acquire different positions and shape within the hindbrain. Whether this is really what happens requires time lapse analysis and awaits the generation of a transgenic line in which the RS neurons are marked by GFP expression from their first appearance. The pattern of location, shape, and projection of the RS neurons is invariant and is acquired in a relatively short time, thus suggesting that the mechanisms that regulate their specification, number, migration, and projections are very robust. Studies aimed at clarifying the inductive signals in the hindbrain have uncovered a role for fgf signaling secreted from rhombomere 1 and 4 in patterning the adjacent rhombomeres and in controlling the number and identity of the RS neurons (Maves et al., 2002). In addition, a box gene code controls the identity of neurons developing in the different rhombomeres, including the RS neurons (Moens et al., 1996; Prince et al., 1998). The ability to identify the precursors of RS neurons by their expression of \textit{pcp4a} at earlier stages than with any other methods and throughout their life allows us to address questions of their specification and migration. It also suggests that a transgenic line expressing GFP under the control of \textit{pcp4a} regulatory elements would provide an ideal tool to study the development of RS neurons in live embryos and larvae.

\textbf{pcp4a expression in neuronal functional systems}

The expression of \textit{pcp4a} in different regions of the developing zebrafish brain prompted us to examine whether these different regions and nuclei shared similar properties or were part of the same sensory or motor circuitries. For example, the expression of \textit{pcp4a} can be detected all along the relay nuclei of the gustatory system, which has been studied extensively by several authors in other teleosts (Rink and Wullimann, 1998; Ahrens and Wullimann, 2002; Folgueira et al., 2003). The gustatory inputs are collected by the facial, glossopharyngeal, and vagal sensory roots and are conveyed to the first sensory relay neurons in the facial and vagal lobes (which express \textit{pcp4a}). Both extra-oral gustatory input (via facial sensory nerve) and intra-oral (via glossopharyngeal and vagal nerves) gustatory stimuli converge on the secondary gustatory nucleus in the rhombencephalon (which expresses \textit{pcp4a}). Then gustatory inputs are received by the tertiary gustatory nuclei in the diencephalon. These include the TGN, the TLA, the SG, and the inferior lobe. The mammillary body or corpus mamillare (CM) is the most prominent paired nucleus in the inferior lobe of the teleostean hypothalamus and expresses \textit{pcp4a} at very high levels, as do the TGN, TLA, and SG. Also, the mammillary bodies are hypothesized to relay gustatory information to multisensory areas (Rink and Wullimann, 1998).

All these areas project to (and are interconnected with) the area dorsalis pars medialis (Dm) of the telencephalon (Rink and Wullimann, 1998; Sawai et al., 2000; Folgueira et al., 2003; Wullimann and Mueller, 2004), which expresses \textit{pcp4a}. Thus the expression of \textit{pcp4a} is a hallmark of the nuclei related to the gustatory system in zebrafish, which use taste discrimination not only for feeding purposes but also to orient and navigate toward a food source (Bardach et al., 1967; Kanwal and Finger, 1992). \textit{pcp4a} expression seems to be associated with some, but not all, relay nuclei of the somatic sensory system: for example, it is expressed in some primary sensory neurons, starting from early stages (Rohon-Beard cells) through adulthood (neurons in spinal sensory ganglia and trigeminal ganglion). Many interneurons in the spinal cord express \textit{pcp4a}, probably including both inhibitory (CoLA) and excitatory (CiD, McoD, UcoD; Higashijima et al., 2004) interneurons, which modulate the activity of segmental motor neurons in response to local or general sensory stimulation.

Similarly, the visual sensory system also expresses \textit{pcp4a} in several relay stations, starting from the GCL in the retina, the optic tectum, the proglomerular complex, and the area dorsalis pars lateralis (Dl), which is known to process visual stimuli (Nieuwenhuys et al., 1998). Because not all the connections and relay centers of the visual system in teleosts have been identified, the expression of \textit{pcp4a} may direct researchers to test whether \textit{pcp4a}-positive areas may be involved with translating visual or other sensory stimuli, given its preferential expression in sensory-related structures. Expression of \textit{pcp4a} is not only confined to the sensory system; many neurons belonging to the motor system, either as effectors (reticulospinal neurons) or as modulators (cerebellar neurons, neurons in the area ventralis [V] of the telencephalon) also express \textit{pcp4a}. Although we do not know the functions of this peptide in sensory and motor neurons, it is highly possible that they are related to its ability to bind calcium-free calmodulin and to modulate synaptic activity.

\textbf{Differential expression of \textit{pcp4a} in telencephalic divisions}

The homology of the divisions of the teleostean dorsal telencephalon with those of the mammalian pallium is still debated (Wullimann and Mueller, 2004; Mueller et al., 2004). This is due to differences in morphogenesis (eversion in teleosts versus evagination in mammals) and to differences in function (most of the teleostean dorsal telencephalic areas are involved in multimodal sensory processing, whereas several mammalian pallial areas are selectively specialized in motor or sensory [visual, auditory, olfactory, somatosensory] functions, in addition to associative functions). Despite these difficulties, expression data may help to find homologies in the patterning events that gave rise to these two different structures during ontogeny. With a more refined analysis, in the future it will be possible to discover which molecules are qualitatively or quantitatively responsible for such a big
difference in the final organization of the dorsal pallial areas in these two taxa.

Based on the observation that \textit{pcp4a} expression seems to be enriched in sensory relay nuclei, including telencephalic areas that are hypothesized on the basis of hodological approaches to receive sensory inputs (Nieuwenhuys, 1998), we investigated whether the expression of \textit{pcp4a} alone or in combination with other markers could provide information on the molecular differences between dorsal pallial areas of the late larval zebrafish telencephalon.

A graded expression of different genes (EphA7, Id2, tbr1, and others: Rubenstein et al., 1999; \textit{Ermx}, \textit{Pax6}, \textit{Cad6}, and \textit{Cad8}; Bishop et al., 2000) characterizes different regions of the mammalian isocortex during development. It has been suggested that the combinatorial expression of these genes marks functionally distinct areas of the mammalian isocortex, thus allowing one to distinguish, primarily, motor versus sensory cortices, but also visual, auditory, and somatosensory cortical areas. A complication in applying this approach to other vertebrates is that most of the molecular markers used in mouse are also expressed outside the isocortex or dorsal pallium, in other areas of the dorsal telencephalon, namely, in the medial, lateral, and ventral pallium (Puelles et al., 2000). As the localization of these dorsal telencephalic divisions in teleosts is still a matter of debate, and the distribution of the above transcripts in the zebrafish larval telencephalon is largely unknown, the use of these markers is, at the moment, problematic. Therefore we decided to compare the localization of \textit{pcp4a} transcripts in different areas of the developing zebrafish dorsal pallium with that of two other dorsal telencephalic genes that we have previously characterized in details (\textit{tbr1}, Mione et al., 2001; reelin, Costagli et al., 2002). These genes have important attributes related to the present study: the zebrafish olfactory system, including the area dorsalis pars posterior (Dp), which is known to receive olfactory projections (Nieuwenhuys, 1998), is totally devoid of \textit{reelin} expression (Costagli et al., 2002) but is enriched for \textit{tbr1} transcript.
**pep4 in Zebrafish Neurons**


