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Authors: FJ Arenzana, A Santos-Ledo, A Porteros, J Aijón, A Velasco, JM Lara, R Arévalo

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Research Highlights

We analyse the distribution of zn-1, CR and GFAP during zebrafish lifespan

Postmitotic cells of the retina are immunopositive to Zn-1 and calretinin

Optic nerve transiently expresses GFAP when it arrives at the optic tectum

CR is expressed in different cellular types and neuropile in the visual system

Title page

Characterisation of neuronal and glial populations of the visual system during zebrafish lifespan.

*#Arenzana FJ, *Santos-Ledo A, Porteros A, Aijón J, Velasco A, Lara JM and Arévalo R

* These authors contributed equally to this work

Dpto. de Biología Celular y Patología. Universidad de Salamanca. Instituto de Neurociencias de Castilla y León. Salamanca, E 37007 (Spain)

Grupo de Neurobiología del Desarrollo-GNDe, Hospital Nacional de Parapléjicos, Finca La Peraleda s/n Toledo, E-45071, Spain.

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Corresponding author:

Dr. Rosario Arévalo
Departamento de Biología Celular y Patología.
Universidad de Salamanca.
Instituto de Neurociencias de Castilla y León.
C/ Pintor Fernando Gallego 1
E-37007 Salamanca. Spain
Phone: + (34) (923) 294500 ext. 5322
Fax: + (34) (923) 294549
E-mail: mraa@usal.es

Abstract

During visual system morphogenesis, several cell populations arise at different time points correlating with the expression of specific molecular markers We have analysed the distribution pattern of three molecular markers (zn-1, calretinin and glial fibrillary acidic protein) which are involved in the development of zebrafish retina and optic tectum. Zn-1 is a neural antigen expressed in the developing zebrafish central nervous system. Calretinin is the first calcium-binding protein expressed in the central nervous system of vertebrates and it is widely distributed in different neuronal populations of vertebrate retina, being a valuable marker for its early and late development. Glial fibrillary acidic protein (GFAP), which is an astroglial marker, is a useful tool for characterising the glial environment in which the optic axons develop.

We describe the expression profile changes in these three markers throughout the zebrafish lifespan with special attention to ganglion cells and their projections. Zn-1 is expressed in the first postmitotic ganglion cells of the retina. Calretinin is observed in the ganglion and amacrine cells of the retina in neurons of different tectal bands and in axons of retinofugal projections. GFAP is localised in the endfeet of Müller cells and in radial processes of the optic tectum after hatching. A transient expression of GFAP in the optic nerve, coinciding with the arrival of the first calretinin-immunoreactive optic axons, is observed. As axonal growth occurs in these regions of the zebrafish visual pathway (retina and optic tectum) throughout the lifespan, a relationship between GFAP expression and the correct arrangement of the first optic axons may exist.

In conclusion we provide valuable neuroanatomical data about the best characterised sensorial pathway to be used in further studies such as teratology and toxicology.

Key words: Calretinin, Development, GFAP, zn-1

1. Introduction

The zebrafish has become one of the standard vertebrate models for Developmental Biology and human disease studies (reviewed in: Lieschke and Currie, 2007) including neurological pathologies such as fronto-temporal dementia (Paquet *et al*., 2009), Alzheimer'sdisease (Newman *et al*., 2009; Paquet *et al*., 2009), Huntington's disease (Henshall *et al*., 2009), Parkinson's disease (Sheng *et al*., 2010) and visual disorders (Goldsmith and Harris, 2003; Fadool and Dowling, 2008; Sager *et al*., 2010). Neuroanatomical studies have described different central nervous system (CNS) populations (catecholaminergic: Holzschuh *et al*., 2001; Arenzana *et al*., 2006; cholinergic: Clemente *et al*., 2004; Arenzana *et al*., 2005; dopaminergic: Filippi *et al*., 2007; 2010; Kastenhuber *et al*., 2010; GABAergic: Candal *et al*., 2008; Mueller and Guo, 2009; histaminergic: Kaslin and Panula, 2001; nitrergic: Holmqvist *et al*., 2004; orexinergic/hypocretinergic: Prober *et al*.., 2006; Faraco *et al*., 2006; noradrenergic: Filippi *et al*., 2007; 2010; Kastenhuber *et al*., 2010; serotoninergic: McLean and Fetcho, 2004) which have allowed the analysis of the structural alterations present in the mutants obtained after genetic screening (Amsterdam and Hopkins, 2006; Sprague *et al*., 2008) as well as the analysis of their putative alterations after different pharmacological treatments and/or small molecular screens (i.e. drugs of abuse, xenobiotics, pesticides, nanoparticles...). Most of the studies focus on either development or adulthood, but very few describe the distribution pattern of proteins throughout lifespan. These kinds of analysis are necessaries for a better knowledge of the dynamic expression of the proteins, as some of them change their location during development. The analysis throughout lifespan would provide valuable information that can be combined with behavioural and teratogenic analysis. Moreover, the zebrafish visual system has became a model for behaviour, teratogenia and gene expression studies (Parng et al., 2007; review in Renninger et al., 2011).

Zebrafish visual system is one of the best characterised sensorial pathways in vertebrates. Different experimental approaches, such as anatomical (Burrill and Easter, 1994; Schmitt and Dowling, 1994; Liu *et al*., 1999), genetic (Karlstrom *et al*., 1996; Trowe *et al*., 1996; Cerveny *et al*., 2010) and physiological (Easter and Nicola, 1996; Emran *et al*., 2010), have been used. After the evagination of the optic vesicles, some of the most distal cells originate the neural retinal layer and the cells that connect the optic vesicle to the forebrain form the optic stalk and differentiate as glial cells (review in Wilson and Houart, 2004). Retinal axons leave the retina at 36 hpf, although optic growth cones do not reach the optic tectum (OT) until 46 hpf (Stuermer, 1988). The optic axons project topographically onto the OT where they form several bands of terminals. The mesencephalic OT is a multi-layered encephalic region constituted by six layers according to Vanegas *et al.* (1984) and Meek and Nieuwenhuys (1998). All these layers are originated during development from two regions called periventricular grey zone (PVGZ) and superficial white zone (SWZ) (Sharma, 1975). This nomenclature has been used in several works (Miguel-Hidalgo *et al*., 1991; Arévalo *et al*., 1995; Diaz *et al*., 2002; Arenzana *et al*., 2006; Clemente *et al*, 2008).

Zn-1, calretinin (CR) and glial fibrillary acidic protein (GFAP) are molecular markers that allow the characterisation of specific cell populations in the developing visual pathway of zebrafish. Zn-1 is a neural antigen from zebrafish embryos whose distribution pattern has been used for describing the segmental organisation of zebrafish hindbrain (Hanneman *et al*., 1988; Trevarrow *et al*., 1990). CR is the first detectable calcium-binding protein during fish CNS development (Porteros *et al*., 1997, 1998; Candal *et al*., 2008) and its expression is located, among other types, in the ganglion cells of the retina (Weruaga *et al*., 2000). In fish, GFAP has been observed in different glial cellular types like ependimocytes or radial glia in both adult

animals (Cardone and Roots, 1990; Ito *et al*., 2010) and embryos (Marcus and Easter, 1995; Bernardos and Raymond, 2006).

Although these three molecular markers have been previously used to understand the development of the zebrafish visual system, the cell populations that express them, their involvement in retinal and optic tectum differentiation and their role in the axonal guidance of the optic nerve remain unclear. In the present study, we have analysed the distribution pattern of zn-1, CR and GFAP in the zebrafish visual pathway throughout the lifespan, providing useful neuroanatomical data for the identification of mutants with visual system disorders after genetic screening (Li *et al*., 2010), for studies in teratology and toxicology, and for pharmacogenetic screening in zebrafish models of human visual disorders.

2. Material and methods

2.1 Subjects

AB strain zebrafish embryos were obtained by natural mating from our laboratory colony and maintained according to standard procedures (Westerfield, 1995). Ages of embryos are given as hours postfertilization (hpf) or days postfertilization (dpf). Embryos (from fertilization to hatching) of 24, 36, 48 and 60 hpf, larvae (from hatching to yolk re-absorption) of 3, 4 and 5 dpf, juveniles of 10, 15, 21, 30 and 60 dpf, and adults of 90 dpf and 1 year old were analysed. The specimens were anaesthetised with 0.03% tricaine methanesulfonate (MS 222, Sigma-Aldrich Inc., St. Louis, MO). All procedures were in accordance with the European Communities Directives (86/ 609/ EEC; 2003/65/EC) and the current Spanish legislation for the use and care of animals in research (RD 1201/2005; BOE 252/34367-91, 2005), and conformed to NIH guidelines.

2.2 Western blot analysis

In order to verify the specificity of the GFAP antibody in zebrafish CNS, we carried out a Western blot analysis of extracts of retina and brain. Both retinas and brains of zebrafish and mouse were dissected and immediately lysed with 50-100 µl of 25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate supplemented with protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 20 µg/ml aprotinin, 20 µg/ml leupeptin; Sigma-Aldrich, Inc.). After 20 min on ice the solubilised proteins were obtained by centrifugation, boiled in Laemmli sample buffer [2% sodium dodecyl sulphate (SDS), 10% glycerol, 140 mM β-mercaptoethanol, 60 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue]. The proteins were measured with a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) (Bradford method). Proteins (20-50 µg) were separated through 7.5-12% SDS-polyacrylamide gels under reducing conditions. Pre-stained protein molecular mass standards (Bio-Rad Laboratories) were also run in the same gel. After electrophoresis, the proteins were transferred to nitrocellulose filters (Boehringer Mannheim, Indianapolis, IN), blocked with 5% (w/v) powdered defatted milk in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 90 min at room temperature and incubated overnight at 4ºC with primary mouse monoclonal antibody anti-GFAP (1:4000). The biotinylated secondary antibody horse anti-mouse immunoglobulin G (Vectastain, Vector Laboratories, Inc.) was immunodetected using streptavidin-horseradish peroxidase conjugate (Vector Laboratories Inc.) diluted 1:1000 in TBST and signals were revealed using an enhanced chemiluminiscence detection (ECL) system (Amersham Bioscience, Aylesbury, UK). Negative controls were performed by omitting the primary antibody or by substituting it with non-immune rabbit IgG.

2.3 Immunohistochemistry

Embryos, larvae, and heads of juveniles and adults were fixed by immersion overnight at 4 ºC with paraformaldehyde 4%. Transverse and parasagittal sections (25 µm thick for adults and juveniles; 12 µm for larvae and embryos) were obtained on a cryostat (Leica, Nussloch, Germany) and thaw-mounted on gelatin-coated slides. The immunohistochemical technique was performed as previously described (Clemente *et al*., 2004, Arenzana *et al*., 2005; Arenzana *et al*., 2006). Mouse monoclonal antibody (1:300) against zn-1 (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit polyclonal antibody (CR 7696; Swant, Bellinzona, Switzerland) (1:10000) against CR, and mouse monoclonal antibody (1:400) against GFAP (Incstar, Stillwater, MN) were employed. The secondary antibodies for anti-zn-1 and anti-GFAP were biotinylated horse anti-mouse immunoglobulin G (Vectastain, Vector Laboratories, Inc., Burlingame, CA) or Cy5-goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories Inc., West Grove, PA), while for CR immunodetection the secondary antibodies employed were biotinylated goat anti-rabbit immunoglobulin G (Vectastain, Vector Laboratories Inc.) or Cy2-goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories Inc.). Nuclei were counterstained with propidium iodide (1:4000).

2.4 Image Analysis

Sections were analysed with a Leica DMLS (Leica Microsystems, Bensheim, Germany) equipped with brightfield condensers. Brightfield digital images were obtained with an Olympus OP-70 digital camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus Provis AX70 photomicroscope. The capture software was connected to a trichromatic sequential filter (Cambridge Research & Instrumentation Inc., Boston, MA). Fluorescence

images were obtained from a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems) coupled to a Leica DM IRE2 (Leica Microsystems) inverted microscope with argon- and helium-neon lasers. The original images were processed digitally with Adobe® Photoshop® 7.0 (Adobe Systems, San Jose, CA) software. The sharpness, contrast and brightness were adjusted to reflect the appearance seen through the microscope.

3. Results

In this study, we analysed the neurochemical evolution of the zebrafish retina and OT from the embryonic period to adult configuration using the immunohistochemical detection of zn-1, CR and GFAP. In order to analyse the relationship between the growing CR-ir optic axons and GFAP transient expression in optic nerve at 3 dpf, we carried out a double immunolabelling study using confocal microscopy.

As a previous step we tested the specificity of GFAP antibody. Western blotting results for GFAP showed a single band of 50 kDa in all biological samples, demonstrating the specificity of the GFAP antibody in zebrafish CNS (Fig. 1). The specificity of zn-1 (Developmental Studies Hybridoma Bank) and CR (Castro *et al*., 2006) had been previously reported.

3.1 Zn-1

Zn-1 immunoreactivity was observed for the first time in the visual pathway at 36 hpf in the retina and the OT. The retina displayed zn-1 immunoreactive (zn-1-ir) pyriform cells situated in the central part of the presumptive ganglion cell layer of the retina (GCL) (Fig. 2a), while the OT showed zn-1-ir cells located in the periventricular grey zone (PVGZ) (Fig. 2b). The density of zn-1-ir elements in the zebrafish visual pathway increased at 48 hpf, especially

in the retina where staining extended from the central part to the periphery (Fig. 2c). Stained neuropile in the outer plexiform layer of the retina (OPL) and in the inner plexiform layer of the retina (IPL) were observed. Fusiform zn-1-ir cells were situated in the inner nuclear layer of the retina (INL) showing stained prolongations at the cellular poles. Cell bodies and axons of ganglion cells that constitute the optic nerve were immunolabelled for zn-1 (Fig. 2c).

There were no evident changes in the distribution pattern of zn1 until 4dpf when the retina showed a great reduction of zn-1 immunoreactivity and zn-1-ir cells were only observed in the outer most scleral part of the retina. Cells in the scleral part of the INL were segregated into two groups. One was characterised by an elongated morphology and an arrangement parallel to the OPL (Fig. 2d). The other was constituted by zn-1-ir cells with a thick stained process directed towards the OPL, or reaching the nuclear layer of the retina (ONL) (Fig. 2e). Because of their morphology and localisation we identified these cells as horizontal cells. At this stage of development, there was no immunoreactivity for zn-1 in the axons of the ganglion cells that constitute the optic nerve. At 4 dpf, zn-1-ir elements were observed in both the dorsal-most part of the superficial white zone (SWZ) and in the PVGZ of the OT. The ventral-most part of the SWZ did not present immunolabelled elements for zn-1 (Fig. 2f). From 4 dpf onwards there was no immunoreactivity for zn-1 either in the visual pathway or in other regions of the zebrafish CNS.

3.2 Calretinin

At 48 hpf, CR immunoreactive (CR-ir) elements in the anlage of the retinal INL were observed. The stained prolongations of these cells arborised into the presumptive IPL originating a scarce immunolabelled neuropile. These cells were identified as amacrine cells. Some CR-ir ganglion cells were observed in the central part of the GCL. However, the axons

of the ganglion cells that constitute the optic nerve did not show immunoreactivity for CR at 48 hpf (Fig. 3a). Regarding the OT, CR-ir cells were observed only in the lateral portions. These cells were situated in both the SWZ and the PVGZ, displaying a rounded morphology and immunolabelled processes (Fig. 3a).

The CR distribution pattern in the retina at the 60 hpf stage was very similar to that observed at the previous stage. The OT showed CR-ir cells and neuropile in both the lateral and medial portions of the two germinal zones of the OT: SWZ and PVGZ (Fig. 3b).

After hatching, the density of CR-ir elements in the retina and OT increased. The CR distribution pattern in the retina extended from the central part to its whole extension. In addition, immunoreactivity for CR was detected in the optic nerve fibre layer of the retina (ONFL) (Fig. 3c). Immunolabelled axons were observed in the optic nerve, optic chiasm and optic tracts, and remained immunostained until adult life.

In the OT, the thickness of the SWZ was greater than previous stages and two regions were discerned. The superficial most region had fusiform CR-ir cells and fibres. The deepest region, in contact with the PVGZ, showed only CR-ir fibres and the density of CR-ir cells in the PVGZ increased with respect to previous stages. At 3dpf CR-immunoreactivity was also observed in the retino-tectal projections that arrived at the rostral most part of the OT (Fig. 3c).

At 4 dpf, in addition to the ganglion cells, immunoreactivity for CR in another cell type in the GCL, with a pear-shaped morphology and single stained process directed towards the IPL was observed. Due to morphology and localisation, these cells were identified as displaced amacrine cells (Fig. 3d).

At 30 dpf the distribution of CR immunoreactivity in the retina was similar to that observed at previous stages and in adult animals. Axons of the retino-pretectal-hypothalamic and retino-hypothalamic pathways showed immunoreactivity for CR (Fig. 3e). From 3dpf to 30

dpf a refinement of the CR distribution was observed in the OT until all the layers were developed. The OT displayed all the strata that will constitute the adult OT at this stage. From the pial surface inwards the first tectal stratum was the marginal stratum (MS), which showed CR-ir cells for the first time at this stage. The CR-ir cells had rounded morphology with one immunolabelled prolongation. The optic stratum (OS) showed CR-ir fibres that correspond with the axons of the ganglion cells. The superficial fibrous and grey stratum (SFGS) displayed both CR-ir fibres and CR-ir rounded and fusiform cells. The central grey stratum (CGS) and the central white stratum (CWS) showed a lower density of CR-ir cells compared with the deepest stratum, the periventricular stratum (PVS). In the PVS, CR-ir cells showed a single immunolabelled prolongation directed towards the more superficial tectal strata, arriving in some cases at the SFGS (Fig. 3f). Using previous morphological criteria by Golgi impregnation (Meek and Schellart, 1978), we identified these cells as type XIV neurons. There were no significant changes in the CR distribution pattern between 30 dpf and 60 dpf.

In the retina of adult animals (90 dpf and 1 year), the CR-ir elements were ganglion cells and displaced amacrine cells in the GCL, amacrine cells in the INL and immunolabelled neuropile in the IPL (Fig. 3g). The retinofugal (retino-tectal, retino-pretecto-hypothalamic and retino-hypothalamic) projections remained CR immunoreactive, as observed in previous stages. Finally, the main change in the CR distribution pattern in the adult OT was the decrease in the density of CR-ir cells in the MS, which displayed a single stained process directed towards the OS (Fig. 3h).

3.3 Glial fibrillary acidic protein

At 36 hpf, GFAP-ir cell bodies and processes delimiting the lateral regions of the mesencephalic OT were observed (Fig. 4a). The intensity of the staining was stronger in the

ventral region (presumptive PVGZ) than in the dorsal region (presumptive SWZ). The boundary between the caudal portion of the OT and the rhombencephalon also showed immunoreactivity for GFAP.

At the end of embryonic life (60 hpf), zebrafish optic nerve showed immunoreactivity for GFAP in both cell bodies and processes. The ventrolateral optic tract also displayed immunoreactivity for GFAP (Fig. 4b). The rostral part of the OT was delimited by GFAP-ir processes.

After hatching, GFAP-ir elements were observed in the retina close to the vitreal region. GFAP-ir was also present in both cell bodies and processes of the optic nerve (Fig. 4c).

At the 5 dpf stage, the ONFL of the retina showed an increase in the density of GFAP-ir processes with respect to previous stages (Fig. 4d). The immunoreactivity for GFAP was identified as the endfeet of Müller cells. This staining was also observed in adult animals. No immunolabelled processes were observed in the optic nerve.

At 30 dpf, GFAP-ir processes in all tectal bands were observed (Fig. 4e). The density of GFAP-ir processes was higher in the rostral most portion of the OT than in the caudal most region. There were no significant differences in the GFAP distribution pattern between 60 hpf and 30 dpf. In adult animals GFAP-immunolabelled processes were observed in both the limit of the OS and in the SFGS of the OT, close to the radial stained prolongations of the subpial limitant (Fig. 4f).

3.4 Double immunostaining

At 3 dpf**,** the optic nerve of zebrafish larvae displayed immunoreactivity for both CR and GFAP. The staining for CR was observed only in axons (Fig. 5a), while immunoreactivity for

GFAP was located in glial processes (Fig. 5b). Co-localisation of the immunostaining for CR and GFAP was not observed (Fig. 5c).

4. Discussion

In this study we have analysed the distribution pattern of three molecular markers (zn-1, CR, GFAP) during the morphogenesis of the zebrafish visual system. The immunodetection of these proteins allows the study of the evolution of the cell populations and projections into their adult configuration and the environment in which they develop.

In both zebrafish retina and OT, zn1 is expressed very early during embryonic development (36 hpf), suggesting a putative role in morphofunctional differentiation of the visual system. In the retina, CR and GFAP onsets are observed at the same stage of development (48 hpf), while the OT displays an earlier expression of GFAP (36 pf) than CR (48 hpf). Moreover, a transient expression of GFAP in cell bodies and processes in the optic nerve is observed around hatching.

4.1 Zn-1 in the fish visual pathway

The studies using zn-1 are restricted to the rhombencephalon and myelencephalon of zebrafish during embryonic development (Hanneman *et al*., 1988; Trevarrow *et al*., 1990) and the auditive pathway of juvenile and adult zebrafish (Bang *et al*., 2001). Regarding zn-1 functional implications, it has been suggested that zn-1 may be involved in the latter stages of differentiation of the statoacoustic and auditive system in the axolotl *Ambystoma mexicanum* (Kornblum *et al*., 1990). The zn-1 immunoreactivity in zebrafish retina (present results) is

observed in the primordia of the GCL at the 36 hpf stage, when the first ganglion cell axons exit the optic vesicle (Stuermer, 1988).

4.2 Calretinin in the fish visual pathway

During vertebrate development, CR is expressed in different cellular types and neuropile and the onset of the immunoreactivity for CR coincides with the differentiation of the retinal histological layers (Doldan *et al*., 1999; present results).

The distribution pattern of the immunoreactivity for CR in the retina has been previously analysed in three groups of teleostean fish with different habitats: cyprinidae (tench *Tinca tinca*: Weruaga *et al*., 2000; zebrafish: García-Crespo and Vecino, 2004; Castro *et al*., 2006), scophthalmidae (turbot *Psseta maxima*: Doldan *et al*., 1999), salmonidae (rainbow trout *Oncorhynchus mykiss*: Weruaga *et al*., 2000) and petromyzontidae (lamprey: Villar-Cheda *et al*., 2006). The CR expression pattern is similar among the teleostean species studied since it localises in neurons of the GCL and INL, where ganglion and amacrine cells are immunolabelled for CR. However, there are some differences between species in the retinal cellular types immunolabelled for CR. Previous works detected bipolar cells in different teleosts (rainbow trout, Weruaga *et al*., 2000; turbot, Doldan *et al*., 1999; lamprey, Villar-Cheda *et al*., 2006) and also in the INL of zebrafish (Yazulla and Studholme, 2001). We have not seen descending and ascending projections in the positive cells in the INL, thus we identify them as amacrine cell. Double immunohistochemistry with specific antibodies of amacrine and bipolar cells should be performed in order to answer this question. We have not detected horizontal cells, which have been described in the turbot (Doldan *et al*., 1999) and in the lamprey (Villar-Cheda *et al*., 2006).

During the development of the vertebrate retina, it has been suggested that a minimal degree of cell and tissue differentiation is required for the CR expression (Ellis *et al*., 1991; Doldan *et al*., 1999). The appearance of CR in the retina occurs concurrently with the establishment of the early laminar organisation when the proliferation capability of neuroblast is reduced (Mack and Fernald, 1997) and retinal cells acquire their neurochemical identity (Negishi and Wagner, 1995). At this stage in ontogeny, CR could participate in the formation of the firing pattern of neurons (Miller and Baimbridge, 1983), as well as triggering some enzymes in the signal transduction machinery of cells (Yamaguchi *et al*., 1991).

CR expression in adult OT has been analyzed in tench (Arévalo *et al*., 1995) and zebrafish (Castro *et al*., 2006). Staining is very similar between these species but divergences are described in the MS, with CR-ir cells in zebrafish (Castro *et al*., 2006; present results), but not in the tench (Arévalo *et al*., 1995). The existence of CR-ir cells in the MS during juvenile development (present results) could be a consequence of the continuous neurogenesis and migration processes that this encephalic region presents in fish (review in Zupanc, 2008; Ito *et al*., 2010). The Cajal-Retzius cells in the marginal zone (prospective layer I) of the mammalian cortex show CR-immunoreactivity during cortinogenesis (Weisenhorn *et al*., 1994). It has been suggested that these cells engaged in migration may need CR during this migratory period. The CR-ir neurons in the MS of the fish OT could be considered a homologous cellular type of the CR-ir Cajal-Retzius cells.

4.3 Glial fibrillary acidic protein in the fish visual pathway

The GFAP distribution pattern described in our studies is similar to that described in other teleostean species (goldfish *Carassius auratus*: Nona *et al*., 1989; carp *Cyprinus carpio*: Kalmán, 1998; tench: Jimeno *et al*., 1999: zebrafish: Bernardos and Raymond, 2006; Yazulla

and Studholme, 2001). GFAP-ir cells in the retina have been considered as astrocytic cells although some authors have observed some oligodendroglial characteristics since these cells can be identified using specific markers of both astrocytes and oligodendrocytes (Kumpulainen *et al*., 1983; Norenberg and Martínez-Hernández, 1979).

The optic nerve of zebrafish shows a transient expression of GFAP in glial cells around hatching. In the teleostean optic nerve, only one type of astrocyte is considered, which is denominated reticular (Maggs and Scholes, 1990), morphologically characterised by its regular distribution along the nerve and the organisation of their prolongations that participate in the nerve bundling. The GFAP, as a member of the cytoskeletal protein family, is thought to be an important modulator of astrocyte motility and shape by providing structural stability to astrocytic processes (Eng *et al*., 2000).

The transient expression of GFAP in the optic nerve coincides with the arrival of the majority of the zn-1-ir and CR-ir optic axons. Thus, glial cells could support early axon outgrowth as it occurs in the zebrafish hindbrain (Marcus and Easter, 1995) and forebrain comissures (Barresi *et al*., 2005) and could be involved in the axonal guidance as it happens in the chicken optic nerve (Gerhardt *et al*., 2000).

The retinorecipient tectal strata and the retina are the two main regions that support axon guidance in the zebrafish visual pathway and they show GFAP immunoreactiviy during ontogeny. Radial glia could be involved in the migration of neuroblasts from the PVS to the rest of the tectal strata (Schmatolla and Erdmann, 1973; Ito *et al*., 2010), as well as in other regions of the fish CNS (Bauchot *et al*., 1979; Tomizawa *et al*., 2000).

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Figure legends

Fig. 1: Western blots of zebrafish and mouse retina and brain extracts with the anti-GFAP monoclonal antibody displaying a single band around 50 kDa in all lanes.

Fig. 2: (a) Parasagittal section of the rostralmost part of an embryo at 36 hpf showing zn-1-ir elements in both the central part of the presumptive GCL (arrowhead) and the PVGZ (arrow). (b) Parasagittal section of an embryo at 36 hpf showing zn-1-ir elements in the PVGZ (arrows) of the OT. (c) Immunoreactivity for zn-1 in the ganglion cell axons constituting the optic nerve (arrow) at 48 hpf. (d, e) Retina of larvae at 4 dpf showing two groups of zn-1-ir cells. One of them is constituted by elongated adjacent zn-1-ir cells, in a parallel orientation with respect to the retinal lamination (arrow in d). The other group is composed by cells in a perpendicular orientation with a thick stained process directed towards the OPL or the ONL (arrowheads in e). (f) OT of larva at 4 dpf showing zn-1 immunolabeling in the dorsal part of the SWZ (arrow) and in the PVGZ (arrowheads in inset). Scale bar: c, d, e = 50 µm; a, b, f, inset in f = 100 µm. D: diencephalon; dpf: days post-fertilization; GCL: ganglion cell layer; H: hypothalamus; hpf: hours post-fertilization; M: mesencephalon; ONL: outer nuclear layer; OPL: outer plexiform layer; OT: optic tectum; PVGZ: periventricular grey zone; T: telencephalon.

Fig. 3: (a) Transversal section of an embryo at 48 hpf showing CR-ir cells in the presumptive GCL (arrowhead) and in the presumptive INL. CR-ir cells are present in the PVGZ of the OT (arrow). (b) Parasaggital section of the zebrafish encephalon at 60 hpf showing CR-ir cells in the T, in the PVGZ and SWZ of the OT and in the ventral part of the H.

(c) CR-ir elements are present in the SWZ (arrow) and in the PVGZ of the OT. Immunolabelled axons (arrowhead) can be observed in the optic nerve, optic chiasm and optic tracts. (d) Section of a retina at 4 dpf stage, another cell type with a pear-shaped morphology and single stained process directed towards the IPL is observed (arrow). (e) Parasaggital section at 30 dpf that shows the immunostaining in the optic nerve (arrow) and in the retino-pretectal-hypothalamic pathway (arrowhead). (f) CR distribution pattern in all the tectal bands showing CR-ir neuropile in all tectal bands and stained cells in the MS (arrow) and the SFGS (arrowhead). (g) In the retina of adult animals, CR-ir elements are ganglion cells and displaced amacrine cells in the GCL, amacrine cells in the INL and immunolabelled neuropile in the IPL. The optic nerve is positive for CR (arrowhead). (h) At 1 year, positive cells for CR are observed in the MS (arrow) although in a reduced number. Scale bar: a, b, c, f, g= 100 µm; e = 200 µm. CGS: central grey stratum; CWS: central white stratum; dpf: days post-fertilization; GCL: ganglion cell layer; hpf: hours post-fertilization; H: hindbrain; INL: inner nuclear layer; IPL: inner plexiform layer; M: mesencephalon; MS: marginal stratum; ONL: outer nuclear layer; OPL: outer plexiform layer; OS: optic **s**tratum; OT: optic tectum; PE: pigmentary epithelium; PVGZ: periventricular grey zone; PVS: periventricular stratum; SFGS: superficial fibrous and grey stratum; SWZ: superficial white zone; T: telencephalon.

Fig. 4: (a) Parasaggital section of the zebrafish CNS at 36 hpf that shows the boundaries of mesencephalic OT by GFAP-ir processes (arrow). (b) Transversal section at diencephalic level at 60 hpf showing GFAP staining in both cell bodies and processes of optic tract in the dorsomedial portion of this encephalic region (arrows). (c) GFAP-ir cell bodies and processes in the optic nerve (arrow) at 3 dpf (magnification in enlarged area). (d) At 5 dpf, GFAP-ir processes (arrow) of the retina are observed in the ONFL. (e) GFAP-ir distribution pattern in

the different strata of the OT at 30 dpf. All the tectal strata show GFAP stained processes with the exception of the MS. (f) The staining for GFAP in zebrafish OT at 1 year is only observed in the SFGS and in the OS. Scale bar: inset in c: 10 µm; c, d: 50 µm; a, b, e, f = 100 µm. CGS: central grey stratum; CWS: central white stratum; dpf: days post-fertilization; hpf: hours post-fertilization; M: mesencephalon; MS: marginal stratum; ONFL: optic nerve fibre layer; OS: optic stratum; OT: optic tectum; PVS: periventricular stratum; R: rombencephalon; SFGS: superficial fibrous and grey stratum.

Fig. 5: Horizontal sections of zebrafish brain at 3 dpf showing the optic nerve and double immunohistochemical detection of calretinin (CR) and glial fibrillary acidic protein (GFAP). (a) CR immunoreactivity in zebrafish optic nerve is observed only in neuropile (arrow). (b) GFAP-ir processes in the optic nerve (asterisks). (c) The CR-ir optic axons (arrow) and GFAP-ir glial processes (asterisks) are close but co-localization is not observed. The nuclei are counterstained with propidium iodide (PI). Scale bar: a, b, c = 40 µm. INL: inner nuclear layer; ONL: outer nuclear layer.

Figure 1