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Developmental expression patterns of acetylcholinesterase and choline acetyltransferase in zebrafish retina

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SUMMARY

During recent years a key role as morphogen has been postulated for the neurotransmitter acetylcholine in the developing Central Nervous System. Acetylcholine released from growing axons regulates growth, differentiation and plasticity. The acetylcholine distribution is frequently defined by acetylcholinesterase and choline acetyltransferase expression patterns. The cholinergic/cholinoceptive system in the adult zebrafish retina has been described. Nevertheless, there are no data regarding the developing retina. The acetylcholinesterase and choline acetyltransferase distribution patterns during zebrafish retinal development are very similar. In both cases the first positive elements appear in the plexiform layers and in later stages reactive amacrine cells have been observed in the ganglion cell layer and inner nuclear layer. In the adult retina a cholinergic and cholinoceptive neuropile band is observed in the inner plexiform layer. Displaced amacrine cells and amacrine cells positive to both markers have been observed. Transient expressions of choline acetyltransferase in the optic nerve and outer plexiform layer and of acetylcholinesterase in amacrine cells and displaced amacrine cells are observed during retinal development coinciding with the arrangement of the pioneering retinal projections into the optic tectum. The mature distribution pattern of the cholinergic/cholinoceptive system in the adult retina is conserved along the phylogenetic scale, thus it seems to be a primary feature acquired relatively early during the evolution of vertebrates.

Key words: Amacrine cell – Cholinesterase – Cholinoceptive – Cholinergic – Optic nerve – Transient expression

INTRODUCTION

During embryonic development the first morphological evidence of eye formation is a bilateral growth of the prosencephalon at the end of neurulation (Geneser, 1993; Chow and Lang, 2001). During successive stages, the optic primordium proliferates and constitutes the optic cup. Then the optic cup divides into two layers, the external stratum produces pigments constituting the pigmentary epithelium (Robinson et al., 1995), whereas the inner stratum forms the neural retina. At the end of the morphogenesis of the retina, retinal cells migrate to their final position and differentiate into specific retinal cell types. The mature laminar pattern of the neural retina is composed of three nuclear and three plexiform lay-

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ers, which, from the outermost to the innermost surface, are: the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and optic nerve fibre layer (ONFL).

The zebrafish visual system has been characterised from anatomical (Burrill and Easter, 1994; Schmitt and Dowling, 1994, 1999) genetic (Trowe et al., 1996; Karlstrom et al., 1997), and physiological (Easter and Nicola, 1996, 1997) points of view. In zebrafish, the optic vesicles develop at 11.5 hours post-fertilisation (hpf) (Malicki, 1999; Li et al., 2000), and cellular differentiation is first evident at 36 hpf with the histogenesis of ganglion cells (Burrill and Easter, 1995; Schmitt and Dowling, 1996). Other retinal cell types appear in a specific time sequence and can be identified between 72 and 96 hpf (Raymondl et al., 1995; Schmitt and Dowling, 1996). In the retina, the cell differentiation wave originates in central positions and progresses towards the periphery (Grun, 1975; Holt et al., 1988). This process is regulated by direct cell-to-cell contact, hormonal influences and gradients of morphogens (Meinhardt, 1983a, b; Lawrence and Struhl, 1996). In addition to their main synaptic function, classical neurotransmitters, such as acetylcholine (ACh), could act as morphogens during Central Nervous System (CNS) development (Lauder, 1993). Thus, the neurotransmitter ACh plays an active role in the refinement of the visual cortical circuitry during maturation (Kuczewski et al., 2005). Accumulated evidence suggests that ACh released from growing axons regulates growth, differentiation and plasticity of the developing CNS (Lipton et al., 1988; Lauder, 1999).

ACh is synthesised in the cytoplasm of cholinergic neurons by the enzyme choline acetyltransferase (ChAT) and is degraded at the synaptic cleft by the enzyme acetylcholinesterase (AChE). It has been reported that AChE may also be involved in other functions during embryogenesis (Ross et al., 1992; Sternfeld et al., 1998). During the development of zebrafish brain it has been reported that the first AChE-positive neurones appear before synaptogenesis occurs (Ross et al., 1992) and it has been proposed that this enzyme could be involved in neuritogenesis (Sternfeld et al., 1998). Different reports have shown that AChE has, in addition to a catalytic domain, a neuritogenic domain (Sternfeld et al., 1998). In mutant zebrafish models with a deletion of the neuritogenic domain, AChE continues degrading ACh but cannot stimulate the neurite growth, demonstrating the independence between the catalytic function and the neuritogenic activity of the enzyme (Sternfeld et al., 1998). Nonetheless, other studies in mutant zebrafish report that the deletion of both catalytic and neuritogenic domains does not affect axonal growth, but reduces the ACh receptors in the postsynaptic element, suggesting a key role for AChE in the stabilisation of synapses (Downes and Granato, 2004).

The distribution patterns of ChAT and AChE in the developing retina have been described in different vertebrate groups, such as amphibians (Cheon and Saito, 1999; López et al., 2002), and mammals (Dann, 1989; Criswell and Brandon, 1993; Hutchins et al., 1995; Camargo De Moura Campos and Hokoc, 1999; Kim et al., 2000) whereas in reptiles only the distribution of cholinergic elements has been studied (Nguyen and Grzywacz, 2000). The distribution of the cholinergic/cholinoceptive system has been described in the adult zebrafish retina (Clemente et al., 2004), but only preliminary data on the development of the cholinergic elements are available (Arenzana et al., 2005). In this study we provide a detailed analysis of the distribution patterns of both cholinergic and cholinoceptive elements in the developing and adult zebrafish retina. Moreover, we compare our data with available data in other vertebrates to shed light on the evolution of the cholinergic/cholinoceptive systems from ontogenic and phylogenetic points of view.

Materials and Methods

Zebrafish embryos were obtained by natural mating from our laboratory colony of AB strain zebrafish (wild-type, originally acquired from the Zebrafish International Resource Center, Eugene OR) and maintained according to standard procedures (Westerfield, 1995). All procedures were in accordance with the European Communities Directives (86/609/EEC and 2003/65/CE), the current Spanish legislation (RD 12001/2005; BOE 252/34367-91, 2005) and conformed to NIH guidelines for the use and care of animals in research.

Embryos (12, 24, 36, 48, 60, 66 hpf), larvae (3, 4 and 5 days postfertilisation [dpf]), juveniles (10, 15, 21, 30, 60 dpf) and adults (90 dpf and 1 year) were analysed. 10 animals per stage were used. The specimens were deeply anaesthetised with 0.2 g/l MS-222 (Sigma, St. Louis, MO, EEUU). Then they were fixed in 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline, pH 7.4 (PBS) for 20 minutes for embryos and larvae and 4 hours for juveniles and adults. The eyes were dissected out from 2 adults for a posterior labelling with carbocyanine dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, D282, Eugene, OR, EEUU). After several washes in PBS, the tissue was embedded in agar and cryoprotected in 30% (w/v) sucrose in PBS. Coronal, horizontal and parasagittal 12 µm (embryos and larvae) and 20 µm (juveniles and adults) thick sections were obtained with a cryostat (Leica, Nussloch, Germany) and thaw-mounted on gelatine-coated slides.

ChAT immunohistochemistry

To deactivate endogenous peroxidase, the sections were treated with 0.3% H_2O_2 in PBS for 20 minutes and then pre-incubated with 5% normal donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA, EEUU) and 0.2% Triton X-100 (Probus, Badalona, Spain) diluted in PBS for 1 hour at 4ºC. The tissue was incubated in primary goat polyclonal antibody against ChAT (Chemicon International, Temecula, CA, EEUU), 1:250 in PBS containing 5% normal donkey serum (Santa Cruz Biotechnology) and 0.2% Triton X-100 for 3 days at 4°C. The sections were washed in PBS and incubated with biotinylated donkey anti-goat IgG (Santa Cruz Biotechnology), 1:250 in PBS containing 5% normal donkey serum and 0.2% Triton X-100 for 1 hour at room temperature followed by Vectastain® Elite® ABC reagent (Vector Labs., Burlingame, CA, EEUU) diluted in PBS containing 0.2% Triton X-100 for 1.5 hours at room temperature. The reaction product was visualized by using 0.025% 3,3-diaminobenzidine (DAB) (Sigma) and 0.0033% H_2O_2 in 0.2 M Tris-HCl, pH 7.6 and monitored. Finally, sections were dehydrated in an increasing ethanol series, cleared with xylene, and coverslipped using Entellan® (Merck, Darmstadt, Germany). Control tissue was incubated with normal donkey serum (1:100) instead of the primary antibody, the secondary antibody, or the ABC reagent. Non-specific staining in the ONL was observed in the control without the primary antibody.

The primary antibody used in this study has been previously used in zebrafish (Clemente et al., 2004; Mueller et al., 2004; Arenzana et al., 2005; Clemente et al., 2005). The specificity of the antibody has been tested previously by Western blot analysis of brain protein extracts from rat, dogfish, sturgeon and trout (Anadón et al., 2000).

AChE histochemistry

A modified Karnovsky-Roots method (Karnovsky and Roots, 1964; Darvesh et al., 1998) was used to demonstrate the presence of AChE activity. After washing in 0.1 M maleate buffer pH 6.0, sections were incubated at room temperature for 30 minutes in a reaction medium containing 65 mM maleate buffer pH 6.0 (Probus), 0.5 mM sodium citrate (Probus), 0.47 mM cupric sulphate (Probus), 0.05 mM potassium ferricyanide (Probus), 0.37 mM acetylthiocholine iodide (Sigma) and 0.06 mM ethopropazine (Sigma) as an inhibitor of non-specific esterases.

AChE activity was visualized enhancing the intensity of the staining with 0.0125% DAB and 0.0033% H_2O_2 in 0.2 M Tris-HCl buffer, pH 7.6. The reaction was monitored under the microscope and stopped by washing the sections in the same Tris-HCl buffer. Sections were dehydrated in an ethanol series, cleared with xylene and cover-slipped with Entellan® (Merck). As a control, the substrate acetylthiocholine iodide was omitted and no residual activity was observed.

DiI Labelling

After the eye dissection, a small DiI crystal (~ 10 µm in diameter) was placed on the prechiasmatic optic nerve with a glass pipette. This tracer labels neuronal processes and somata in both anterograde and retrograde directions by lateral diffusion in the cell membrane (Honig and Hume, 1989). The fixed eyes with the DiI crystal were embedded in agar, cryoprotected for 3 days at 25ºC and cut in 12 µm thick sections which were processed for AChE histochemistry as described above.

Analysis

Sections were examined under an epifluorescence microscope (Leica Aristoplan; Leica Microsystems, Bensheim, Germany) equipped

with appropriate filter blocks for viewing DiI fluorescence and with brightfield condensers. The background was controlled and the photomultiplier voltage (800 V) selected for maximum sensitivity in the linear range. Digital images were obtained with an Olympus OP-70 digital camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus Provis AX70 photomicroscope. After conversion into black and white, the sharpness, contrast, and brightness were adjusted to reflect the appearance seen through the microscope. The original images were processed digitally with Adobe® Photoshop® 8.0 (Adobe Systems, San Jose, CA, EEUU) software.

Results

During the development of the zebrafish retina ChAT immunoreactivity and AChE activity were observed in neuronal elements and no glial cells were positive. A 12 hour delay was observed in the onset of expression between ChAT and AChE. The first positive elements observed for ChAT and AChE were fibres in the plexiform layers during embryonic development. During the retinal morphogenesis ChAT transient expression was observed in the optic nerve (ON) and in the OPL. At juvenile stages we observed cells positive for ChAT and AChE in the INL and in the GCL. In the adult retina scattered, round neurons were positive to AChE and ChAT in the vitrealmost part of the INL and in the scleralmost part of the GCL. Two neuropile bands reactive to both markers appeared in the IPL.

ChAT immunocytochemistry

The first ChAT immunoreactive (ChAT-ir) elements were observed in the plexiform layers at 48 hpf (Fig. 1A). The centralmost part of the IPL and the OPL showed weakly stained fibres forming a neuropile band. In the following stage analysed (60 hpf) the staining in the plexiform layers of the retina increased, although the immunoreactivity in the IPL was more intense than in the OPL (Fig. 1B). In addition, optic axons of ganglion cells were ChAT immunostained, whereas their somata were always negative (Fig. 1B). At 66 hpf the density of stained fibres in the optic nerve (ON) was greater than in previous stages.

At 72 hpf ChAT staining was absent in the ON. We observed positive fibres in both the IPL and OPL throughout the retina. The ChAT-ir neuropile in the IPL was increased with respect to previous stages, whereas the staining in the OPL gradually decreased during larval and juvenile development. By the end of larval development (5 dpf) ChAT immunoreactivity was observed in cells located in the centralmost part of the INL. These cells presented a round morphology and a large size, typical of amacrine cells.

At 10 dpf ChAT-ir cells were distributed throughout the whole extension of the INL (Fig. 1C). In addition, several round cells could be observed in the centralmost part of the GCL close to the boundary with the IPL. Due to their morphology, size and location, we have identified them as displaced amacrine cells. A new ChAT positive neuropile band was observed at this stage in the IPL. Thus, in juvenile stages, two intensely stained cholinergic bands were observed in this layer, one close to the INL and another in the boundary with the GCL. The staining observed in the ONL (Fig. 1C) was non-specific, as controls without the primary antibody demonstrated.

At 15 and 30 dpf the density of ChAT-ir cells in the INL was greater than in previous stages, whereas labelled displaced amacrine cells in the GCL were scarce. Both subpopulations showed immunopositive projections directed towards the IPL, establishing the two cholinergic neuropile bands that appeared in this layer. The projections of the displaced amacrine cells constituted the band located in the vitrealmost part, whereas the amacrine cells established the band situated in the scleralmost part of the IPL.

At 60 dpf, the relative density of ChAT positive cells in the INL and GCL increased with respect to previous stages (Fig. 1D). ChAT-ir amacrine cells in the INL were arranged in a row at the boundary with the IPL, whereas ChAT-ir displaced amacrine cells were more scattered throughout the whole extension of the GCL.

At the adult stage, two different cholinergic amacrine cell populations could be observed: amacrine cells located in the vitrealmost part of the INL and displaced amacrine cells in the GCL. Two ChAT-ir neuropile bands were observed in the IPL, one close to the INL and the other at the boundary between the IPL and the GCL. During development the immunopositive neuropile band in the OPL decreased and could not be observed from 90 dpf onwards.

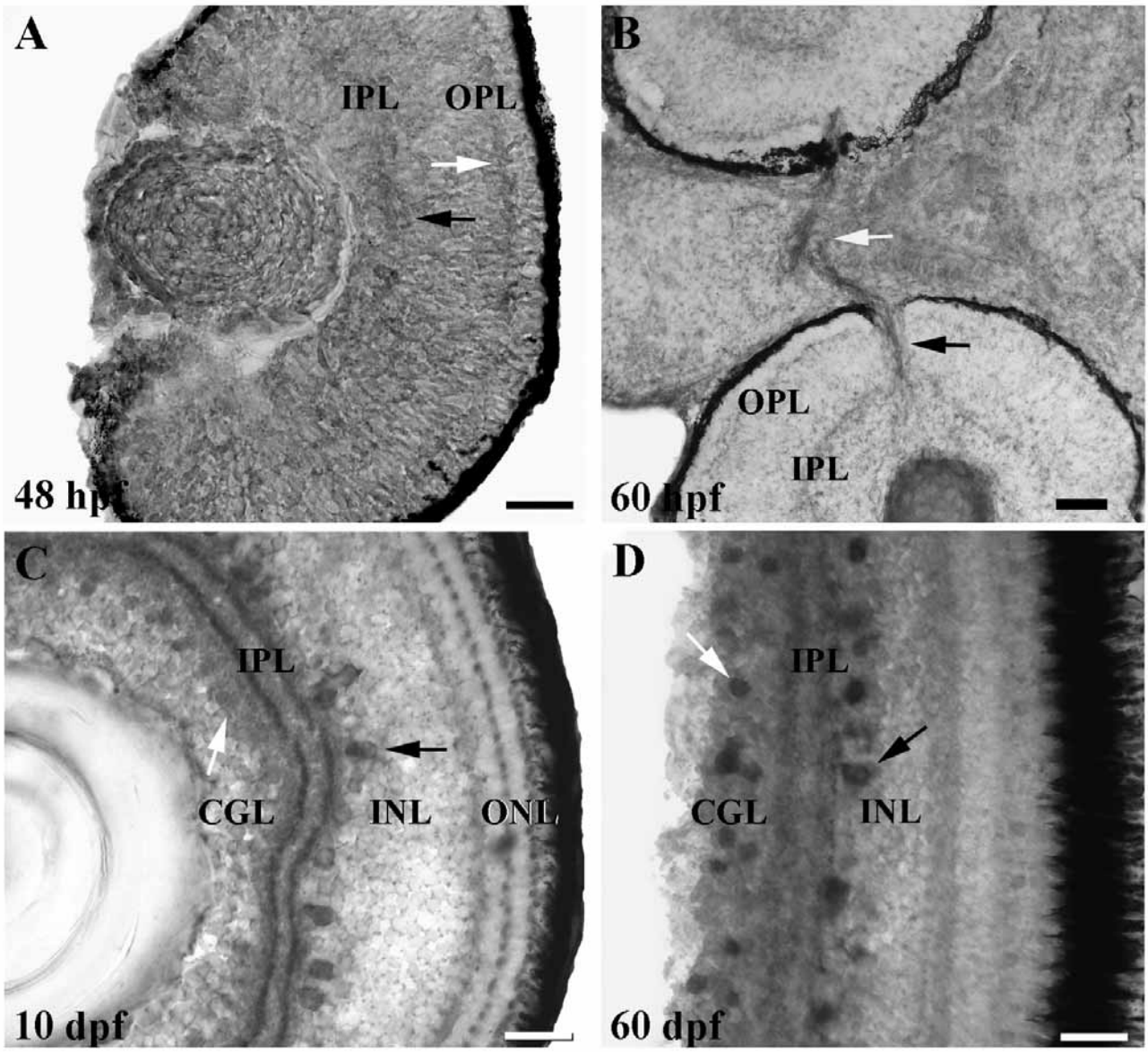

Figure 1. Distribution pattern of ChAT immunoreactivity during zebrafish retinal development. **A:** Horizontal section from a 48 hpf retina showing a cholinergic neuropile band in the inner plexiform layer (IPL) (black arrow) and in the outer plexiform layer (OPL) (white arrow). **B**: Horizontal section (rostral to the left, caudal to the right) of a 60 hpf embryo showing ChAT immunoreactive (ChAT-ir) optic nerve (black arrow) and the optic chiasm (OC) (white arrow). A cholinergic neuropile band in the inner plexiform layer (IPL) and in the outer plexiform layer (OPL) can be observed. **C**: Retina of a 10 dpf juvenile zebrafish. Positive amacrine cells (black arrow) appear in the inner nuclear layer (INL) and displaced amacrine cells (white arrow) are clearly distinguishable in the ganglion cell layer (GCL). **D**: Distribution of ChAT-ir in the juvenile retina. Cholinergic amacrine cells are observed in the ganglion cell layer (GCL) (white arrow) and in the inner nuclear layer (INL) (black arrow). Two bands of ChAT-ir neuropile formed by the amacrine cells are discerned in the inner plexiform layer (IPL). Scale bar: **A, C, D** = 20 μm; **B** = 30 μm.

AChE histochemistry

The onset of AChE activity in the retina occurred at the end of the embryonic period (60 hpf) (Fig. 2A, B), consisting of a reactive neuropile band located in the centralmost part of the IPL. At 72 hpf an overexpression of AChE in the inner nuclear layers was observed. AChE-positive cells (Fig. 2C) appeared in both the INL and GCL, close to the boundary with the IPL.

48 hours later (5dpf), the expression level in the INL and GCL was lesser. Two AChE-positive cell populations were located in the INL and GCL, both close to the boundary with the IPL (Fig. 2D). Both populations were similar, with round morphology and large size, typical characteristics of amacrine cells.

At juvenile stages (from 10 dpf to 60 dpf) the AChE-positive cells in both INL and GCL were located throughout the whole extension of these layers (Fig. 2E) and they presented stained projections. These projections constituted two AChE-positive neuropile bands located in the vitrealmost and scleralmost part of the IPL (Fig. 2E).

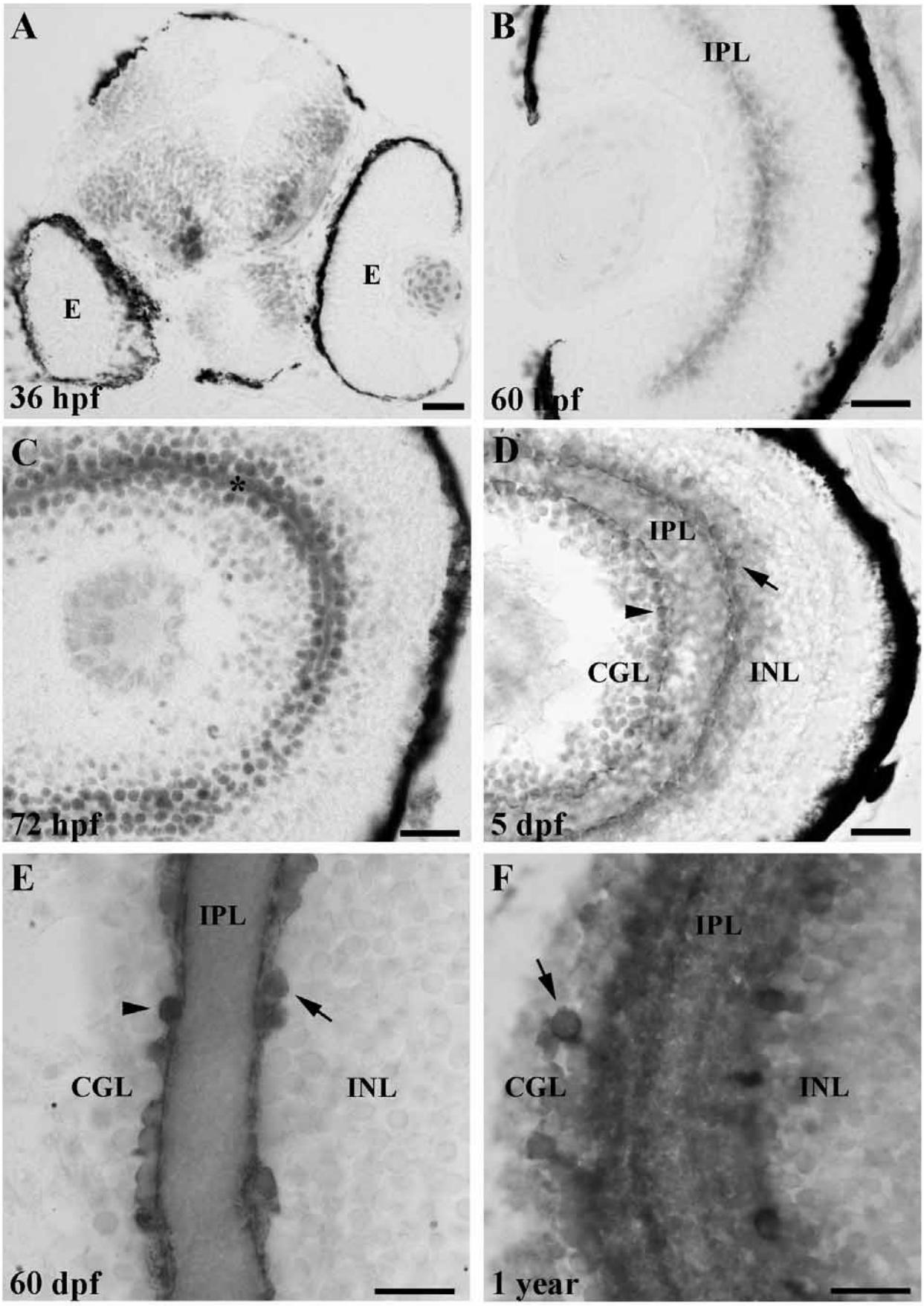

Figure 2. AChE distribution pattern in the developing zebrafish retina. **A**: Coronal section of a 36 hpf embryo. No staining is observed in the eye (E). **B:** An AChE-positive neuropile band located in the inner plexiform layer (IPL) at 60 hpf. **C**: At 72 hpf AChE-positive cells appeared in both the INL and GCL, close to the boundary with the IPL (asterisk). In the retina of a 5 dpf larva (**D**) and a 60 dpf juvenile (**E**) positive displaced amacrine cells (black arrowheads) and amacrine cells (black arrows) are observed. Two reactive neuropile bands in the inner plexiform layer (IPL) can be discerned. **F**: Vitrealmost part of the adult retina. Displaced amacrine cells (arrow) are located in the ganglion cell layer (GCL) and emit a stained process towards the inner plexiform layer (IPL). Scale bar: **A**= 30 μm; **B, C, D** = 20 μm; **E**, **F**: 10 μm

In the adult zebrafish retina we observed two AChE reactive cell populations: amacrine cells in the INL and other cells located in the GCL (Fig. 2F) presenting a stained prolongation directed towards the IPL. Both populations were situated close to the boundary with the IPL and shared morphological features. The relative density of the cells located in the GCL was lower in adult stages than in juvenile stages. After DiI retrograde labelling of ganglion cells combined with AChE histochemistry (Fig. 3A, B), colocalization of tracer labelling and AChE staining in the same cell was not observed. Therefore, we identified AChE-reactive cells in the GCL as displaced amacrine cells.

Discussion

For the study of the vertebrate cholinergic/cholinoceptive system different antibodies against ACh (Geffard et al., 1985), AChE (Brimijoin et al., 1986), ChAT (Sugimoto et al., 1984; Clemente et al., 2004), cholinergic receptors (Wenthold et al., 1990) and vesicular ACh transporter (Ichikawa et al., 1997) were employed. Previous reports (Clemente et al., 2004; Arenzana et al., 2005) are in general agreement with the present results. The AChE and ChAT expression patterns during the early embryogenesis were similar; however, a delay of the cholinoceptive phenotype acquisition has been reported. Amacrine and displaced amacrine cells and two neuropile bands in the IPL were positive to both markers. Cholinergic transient expressions in the ON and OPL have been observed.

Distribution of ChAT

In the retina of most vertebrates studied until now, the first ChAT-ir elements were cells (Von Bartheld et al., 1991; Cheon and Saito, 1999). In contrast, in zebrafish retina stained fibres were located in the plexiform layers. These fibres appeared 3 days before any stained amacrine somata could be detected. In the early embryogenesis, the concentration of ChAT in the somata could be too low to be detected by immunocytochemistry related with a high rate of transport to the synaptic terminal in these stages.

Two different phases in the development of the cholinergic system have been reported (Filogamo and Marchisio, 1971). The 'earlier' system appears temporarily in the differentiating neuroblasts, whereas the 'later' one develops with the formation of synapses in cholinergic pathways (Filogamo and Marchisio, 1971). In zebrafish retina, synaptogenesis starts at 72 hpf (Schmitt and Dowling, 1999). These data suggest that ACh characterised in the plexiform layer at 48 hpf may be involved in the maturation and/or differentiation of the different cell types in the nuclear layers of the retina.

The spatiotemporal pattern of the onset of ChAT in the vertebrate retina differs among species. In zebrafish retina ChAT immunore-

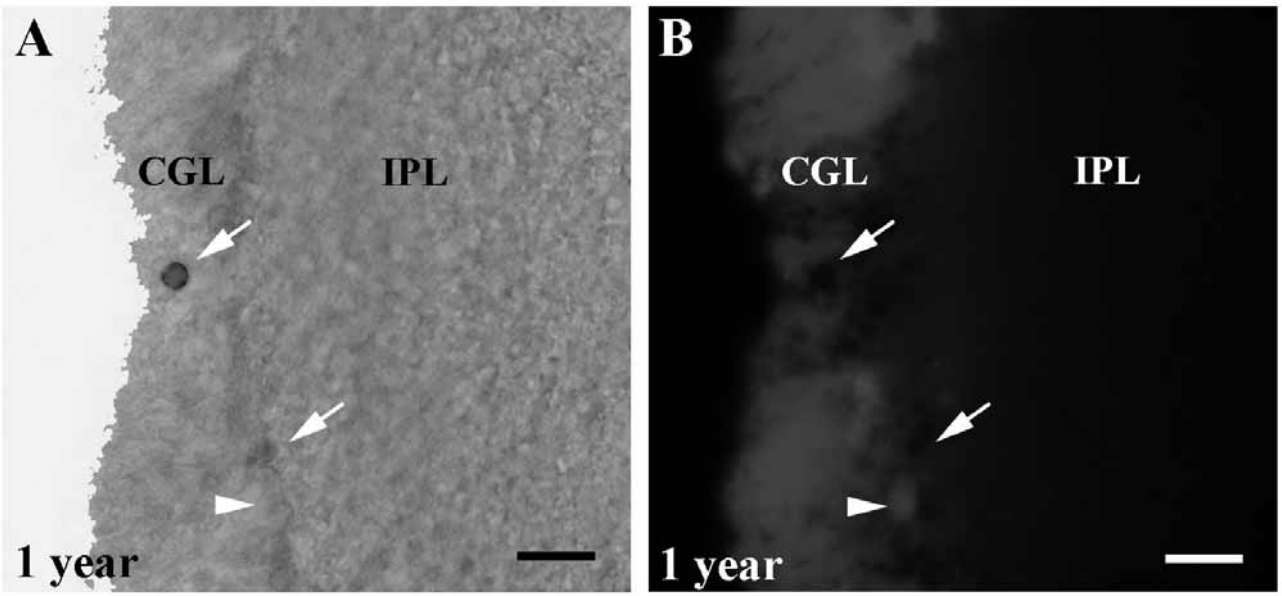

Figure 3. Double staining for DiI label and AChE histochemistry in the adult retina. Optical (**A**) and fluorescence (**B**) microscopy images of the vitrealmost part of the retina. Displaced amacrine cells (arrow) are AChE-positive (**A**) and DiI-negative (**B**), whereas ganglion cells (arrowhead) are AChE-negative and DiI-positive. Scale bar: **A**, **B**= 20 μm.

activity appeared five days earlier in amacrine cells than in displaced amacrine cells. Nevertheless, in other vertebrates the first cholinergic cells observed during development are displaced amacrine cells, as in lamprey (Pombal et al., 2003), rat (Mitrofanis et al., 1988), opossum (Camargo De Moura Campos and Hokoc, 1999), and turtle (Nguyen and Grzywacz, 2000). In frog (López et al., 2002) and in the chicken retina (Millar et al., 1985), both amacrine cell populations have a coincident onset of ChAT expression.

Between 60 and 72 hpf, we observed a transient cholinergic expression in the ON. ACh receptors on the surface of the projections of retinal ganglion cells and in the OT have been described in frog (Sargent et al., 1989). This could be in relation to the arrangement of the pioneering retinal projections. A transient cholinergic expression in the OPL has also been observed. A cholinergic neuropile band in this layer appeared at 48 hpf, and progressively decreased during juvenile development. In rat, a transient expression of ChAT and vesicular acetylcholine transporter in horizontal cells during postnatal development has been reported (Kim et al., 2000). Moreover, it has also been reported that ACh receptors were transiently expressed in the OPL of the developing ferret retina (Hutchins, 1994). These data suggest that ACh released from horizontal cells may play a role in neuronal maturation in the outer layers of the retina.

The distribution pattern of cholinergic neurons in the retina is conserved along the phylogenetic scale (Brandon, 1991; Kim et al., 2000; Nguyen and Grzywacz, 2000). In juvenile stages two cholinergic amacrine cell populations located in the INL (5 dpf) and the GCL (10 dpf) could be observed. Previous reports have shown that the amacrine and displaced amacrine cells in adult zebrafish present a cholinergic phenotype (Clemente et al., 2004; Arenzana et al., 2005). Studies in goldfish have demonstrated that the ganglion cells of teleosts are not cholinergic (Tumosa and Stell, 1986).The dogfish (Brandon, 1991), the opossum (Camargo De Moura Campos and Hokoc, 1999), the turtle (Guiloff and Kolb, 1992) and the chicken (Millar et al., 1987) have two cholinergic subpopulations in the INL and one positive population in the GCL, whereas in goldfish two cholinergic subpopulations in the INL and two in the GCL have been reported (Tumosa et al., 1984). These data show that, although the cholinergic distribution pattern is conserved along the phylogenetic scale, there are some differences in the number, size and shape of the ChAT-ir cells.

Distribution of AChE

During retinal development two cholinoceptive neuropile bands were observed in the scleralmost and vitrealmost part of the IPL. In the retina of newt (Cheon and Saito, 1999), ferret (Hutchins et al., 1995), cat and rat (Criswell and Brandon, 1993) the first AChE-positive elements were also fibres in the same layer, coinciding with our results.

A great peak in AChE expression was observed at 72 hpf in round undifferentiated cells in the INL and GCL. At this stage (72 hpf) we observed the highest expression level of AChE throughout the development, moreover, this overexpression coincided with the cholinergic transient expression in the ON. In ferret retina (Hutchins et al., 1995) an AChE-positive transient expression in the nerve fibre layer has been observed, due to the reactivity in the growing tips of ganglion cell neurites.

At 5 dpf, the two reactive cell subpopulations located in the INL and GCL shared a morphology and projection pattern typical of amacrine cells, sending their projections towards the IPL. DiI tracing experiments demonstrated that AChE-positive cells in the GCL were not ganglion cells but displaced amacrine cells. Our results are in agreement with the data observed in ferret (Hutchins et al., 1995), newt (Cheon and Saito, 1999), chicken (Spira et al., 1987), cat and rat (Criswell and Brandon, 1993) retinas.

In the zebrafish retina AChE reactivity described at 5 dpf is conserved until the adult stage and coincides with the results reported for the adult retina of newt (Cheon and Saito, 1999), ferret (Hutchins, 1994), cat and rat (Criswell and Brandon, 1993).

Functional implications

The existence of a delay between the onset of AChE and ChAT activities raises the matter of Ach degradation during the 12 hour gap. Butyrylcholinesterase (BChE) is the cholinesterase responsible for the degradation of ACh during retinal morphogenesis and synaptogenesis in chicken (Layer, 1991). Whereas BChE and ChAT expression patterns seem to be analogous, BChE and AChE might be exclusive, because BChE has been described as the "histological precursor" of AChE (Layer,

1991). The temporary expression of AChE and BChE is related to the morphogenic functions of both cholinesterases. Whereas BChE is involved in proliferation of neuroblasts, AChE is resposible for differentiation and lamination of the retinal cells (Willbold and Layer, 1994; Robitzki et al., 1997). Moreover, BChE inhibition increases AChE expression and stimulates the retinal cell differentiation (Willbold and Layer, 1994).

During chicken retinal development (Layer, 1991; Reiss et al., 1997) BChE was present in all layers, disappearing when AChE activity occurred. The BChE distribution throughout the retina is in agreement with the morphogenic role of ACh during early development and would act as a morphogen over greater distances (Wassélius et al., 1998). The cholinergic phenotype acquisition in zebrafish retina is similar to the BChE developmental expression pattern in chicken retina (Reiss et al., 1997). In both cases the first positive elements appeared in the plexiform layers, before any positive cells in the nuclear layers were observed. Moreover, BChE-positive transient expression in the ganglion, horizontal and photoreceptors cells has been described, coinciding with ChAT transient expression in the ON and OPL.

Between 66 and 72 hpf, zebrafish embryos hatch and the adaptation to free life involves morphological and functional changes in all the sensorial systems, including the visual system. At this stage (72 hpf) the retinal projection neurons, the ganglion cells, send their axons towards the optic tectum. The visual projections develop in a consistent topographic pattern, constituting retinotopic maps in the OT. The arrangement of the pioneering retinal projections is a very complicated and precise process, in which diverse morphogens are involved. It has been reported that ACh plays an active role in the refinement of the visual cortical circuitry (Kuczewski et al., 2005) and in the growing axons (Lipton et al., 1988; Lauder, 1999). At 72 hpf, we observed transient expression of ChAT in the ON. These data suggest that released ACh could be involved in the refinement of the retinal projections during development.

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Abbreviations

ACh: acetylcholine
AChE: acetylcholinesterase
BChE: butyrylcholinesterase
ChAT: choline acetyltransferase
ChAT-ir: choline acetyltransferase immunoreactive
CNS: central nervous system
dpf: days postfertilisation
E: eye
GCL: ganglion cell layer
hpf: hours post-fertilization
INL: inner nuclear layer
IPL: inner plexiform layer
OC: optic chiasma
ON: optic nerve
ONL: outer nuclear layer
OPL: outer plexiform layer
OT: optic tectum

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