

Differential expression of calretinin in the developing and regenerating zebrafish visual system

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Summary. Calretinin is a calcium-binding protein which participates in a variety of functions including calcium buffering and neuronal protection. It also serves as a developmental marker of retinal ganglion cells (RGCs). In order to study the role of calretinin in the development and regeneration of RGCs, we have studied its pattern of expression in the retina at different developmental stages, as well as during optic nerve regeneration by means of immunohistochemistry. During development, calretinin is found for the first time in RGCs when they connect with the optic tectum. Optic nerves from adult zebrafish were crushed and after different survival times, calretinin expression in the retina, optic nerve tract and optic tectum was studied. From the day of crushing to 10 days later, calretinin expression was found to be downregulated within RGCs and their axons, as was also observed during the early developmental stages of RGCs, when they are not committed to a definite cell phenotype. Moreover, 13 days after lesion, when the regenerating axons arrived at the optic tectum, a recovery of calretinin immunoreactivity within the RGCs was observed. These results indicate that calretinin may play an important role during optic nerve regeneration. Thus, the down-regulation of Calretinin during the growth of the RGC axons towards the target during development as well as during their regeneration after injury, indicates that an increase the availability of cytosolic calcium is integral to axon outgrowth thus recapitulating the pattern observed during development.

Key words: Optic nerve, Renegeneration, Zebrafish, Calretinin, RGC

Introduction

Fish retinal ganglion cells (RGCs) are able to regenerate their axons after nerve damage and to re-establish their appropriate pattern of synaptic

connections in the optic tectum, a process which underlies the recovery of visual function (Sperry, 1948). This capacity contrasts with that of the adult mammalian central nervous system (CNS) in which axon regeneration rarely occurs (Li and Black, 1996).

Several calcium-binding proteins (CaBPs), such as calretinin (CR) and calbindin, are well conserved across species and are expressed in several types of neurons. This characteristic has led to their use as specific neuronal markers (Westerfield, 1993; Celio and Pauls, 1996). Previous studies have shown that calcium binding proteins may play an important role in neuroprotection after experimental manipulation, such as ischemia or neurotoxin application (Meiri and Grafstein, 1984; Nitsch et al., 1989; D'Orlando et al., 2002). The high calcium-binding capacity of calretinin makes it a suitable protein for calcium buffering. In this sense it has been reported that calretinin plays a critical role in the development and maturation of human retinal neurons (Nag and Wadhwa, 1999). We have previously shown that calbindin is expressed during the development, axogenesis and differentiation of trout RGCs; moreover, it is absent for a period of time after commitment to the RGC cell phenotype (Vecino et al., 1993; Vecino, 1998). A temporospatial gradient of calbindin expression was found in the adult retina; in peripheral areas of the retina, where more immature RGCs are located, less calbindin immunoreactivity was found, while in the central retina, which contains more mature cells, robust calbindin immunoreactivity was observed in RGCs. Therefore, the peripheral-center spatial gradient of calbindin-immunoreactivity in the adult retina seems to recapitulate that which occurs during different stages of development of RGCs (Vecino, 1998). The presence of a peripheral area where new retinal ganglion cells are continuously added to the retina during adult life has also been detected in zebrafish (Marcus et al., 1999).

The zebrafish has become an important model for developmental neuroscience for several reasons. The similarities of the zebrafish visual system to that of other vertebrates make this animal a valuable model for visual neuroscience. Concerning visual development, the eye begins with the optic primordia which appear at about 12 hours post-fertilization (hpf) (Schmitt and Dowling,

1994). By 24 hpf, the eyecups are well developed and by about 30 hpf, ganglion cells are found in a small area of the ventronasal retina (Schmitt and Dowling, 1994; Burril and Easter, 1995; Hu and Easter, 1999; Schmitt and Dowling, 1999). At 50 hpf, the retinal layers become apparent across parts of the retina (Schmitt and Dowling, 1999). Although the first ganglion cell axons reach the optic tectum by 48 hpf, the mature structure of the retina is observed later at 60 hpf (Easter and Nicola, 1996).

In the light of the ability of fish RGCs to regenerate their axons (Benhardt et al., 1996; Becker and Becker, 2000; Liu et al., 2002), as well as the role of calcium-binding proteins in development and neuronal protection, we aimed to elucidate if calretinin is implicated in the regeneration of zebrafish RGC axons and to evaluate the possible role of this molecule in developing and regenerative processes.

Materials and methods

Embryos and larvae were obtained by natural mating from a laboratory colony. Embryos at 42, 60 and 76 hours post-fecundation (hpf) and larvae at 5 and 23 days post fecundation (dpf) were directly fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 followed by overnight cryoprotection in 30% sucrose (w/v) in PB.

In the present study, we employed twenty-seven mature zebrafish (*Danio rerio*) obtained from a local breeder. The animals were kept under standard conditions throughout the course of the experiments (Westerfield, 1993). Following anesthesia of zebrafish with 0.03% tricaine methanesulphonate (MS-222, Sigma), the left optic nerve was crushed at the level of the eyeball with an ophthalmic forceps until a clear gap in the nerve was observed, while the right nerve was left as a control without injury. Non-operated animals were used as controls. After a survival period of 3, 6, 10, 13, 19, 23, 30 and 60 days, three animals from each time group were perfused transcardially with 5 ml of fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Once perfused, the brains and eyes were removed from the skulls and postfixed in the same fixative for 4 h followed by overnight cryoprotection in 30% sucrose (w/v) in PB.

Larvae and embryos, as well as eyes, optic nerves and optic tectum of adult animals, were cut into 14 μ m thick sections with a cryotome and collected on slides coated with chrome-alum gelatin. Sections were immersed in phosphate-buffer saline (PBS) containing 0.25% Triton X-100. Afterwards, the sections were incubated overnight at 4 °C in a moist chamber with anti-calretinin polyclonal antiserum in PB (1:3000) with 1% BSA. The sections were washed in PBS and then incubated for 1 h at room temperature with anti-rabbit immunoglobulin G (Vector, 1:200) followed by a 1-h incubation in avidin-biotin-peroxidase complex. Tissue bound peroxidase was visualized with 0.05% 3,3'-diaminobenzidine and 0.05% H₂O₂ in Tris-HCl buffer

(pH 7.4). Finally, sections were dehydrated in an increasing ethanol series, cleared with citrosol and coverslipped using DPX. Immunohistochemical controls consisted of omission of the primary antibody, omission of the secondary antibody and use of the corresponding non-immune serum.

Results

Development

Four stages of zebrafish development were selected to show CR expression in the retina (Fig. 1A-D) and a recapitulation of these developmental events could be found in the adult peripheral retina (Fig. 1E).

At the development stage of 42 hpf, no CR immunoreactivity was found in any layer of the retina. This stage is analogous to that which occurs in the most peripheral retina of the adult zebrafish (Fig. 1E).

At 60 hpf CR-immunoreactive cells were only found in the central retina, possibly corresponding to amacrine cells. An incipient inner plexiform layer (IPL), mainly in the central retina showed CR-immunoreactivity. A different gradient from the ventral to dorsal retina was observed. Thus, in the ventral retina we found fewer CR-immunoreactive cells and fibers than in the centro-dorsal retina (Fig. 1A). No CR-immunoreactive fibers were found in the optic nerve, optic tract or optic tectum (Fig. 2A).

At 76 hpf, the number of CR immunoreactive cells increased. A large number of ganglion cells appeared labeled at this stage in the ganglion cell layer (GCL) as well as amacrine cells in the inner nuclear layer (INL). Many labeled fibers were found in the IPL. However, very few fibers were observed in the outer plexiform layer (OPL); these were mainly located in the periphery of the retina (Fig. 1B). Calretinin immunoreactive fibers were observed in the optic nerve, optic tract and entering the optic tectum (Fig. 2B).

At 5 dpf, the pattern of CR-immunoreactivity observed in the GCL, IPL and INL was similar to that observed earlier. Moreover, the different layers of the retina were better defined. Thus the outer nuclear layer was apparently more organized at this stage (Fig. 1C). A higher number of CR-immunoreactive fibers was observed in the optic tectum in comparison to that observed at previous stages. Moreover, the optic nerve presented very intense CR-immunoreactivity (Fig. 2C).

At 23 dpf, CR-immunoreactivity was distributed as in previous stages. It was mainly detected in RGCs and in some cells in the INL. Amacrine- and bipolar-like cells were also labeled, as occurs in the adult retina (Fig. 1D). However, in the three-month-old adult retina, it was possible to distinguish a gradient of expression of calretinin. Thus it appears that the different developmental stages are represented in the retina, with the younger retina being represented at the most peripheral retina and the more mature, adult retina being represented in the central retina (Fig. 1E).

Calretinin in zebrafish visual system

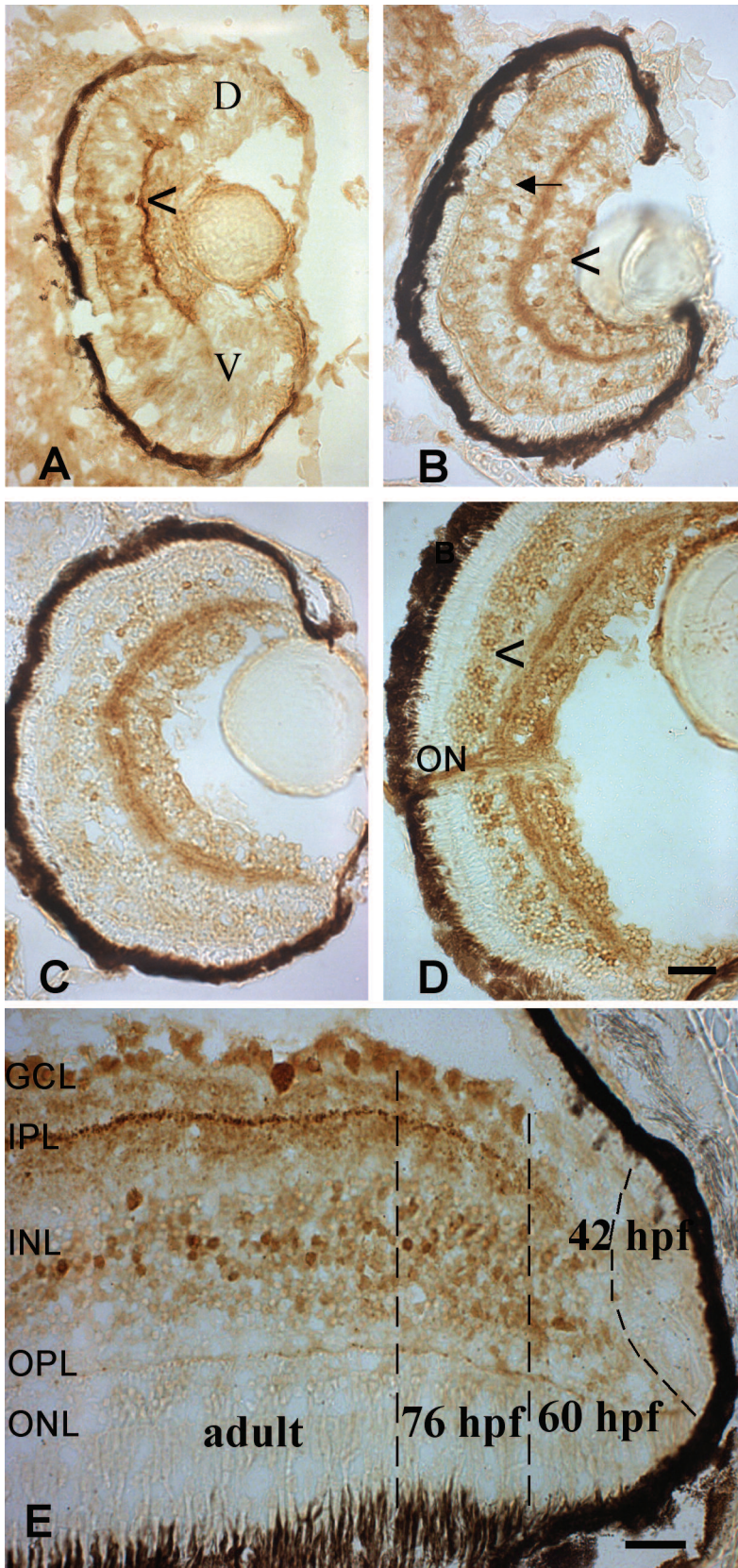


Fig. 1. Transverse sections of the zebrafish eye immunostained for calretinin at four developmental stages: 60 hpf (**A**), 76 hpf (**B**), 5 dpf (**C**) and 23 dpf (**D**). A section of retina (peripheral area) from a 3-month-old zebrafish is illustrated in (**E**). **A.** The only calretinin immunoreactive cells are located in the inner nuclear layer (INL) at the central retina. Note also the immunopositive fibers in the inner plexiform layer (arrowhead). **B.** At 76 hpf, the ganglion cell layer (GCL) presents a large number of cells (arrowhead) and also some cells in the inner nuclear layer, as observed during earlier stages (arrow). **C.** At 5 dpf, a similar pattern of expression is observed. However, the different retinal layers are more clearly defined. **D.** At 23 dpf, immunoreactive cells are detected in the ganglion cell layer and inner nuclear layer (arrowhead) where more immunoreactive cells are observed than during previous developmental stages. **E.** Transverse section of the retina from a 3-month-old zebrafish. A centro-peripheral gradient of calretinin immunoreactivity is observed. Thus the most peripheral areas show a pattern of calretinin-expression which is typical of the embryonic developmental stage which is found at 42 hpf. Towards more central areas of the retina, more mature stages of development (60 and 76 hpf respectively) appear to be represented. dpf: days post-fertilization; hpf: hours post-fertilization. Scale bars: 100 μ m.

Regeneration

A series of sections from the retina (Fig. 3A-D), optic nerve tract (Fig. 3E-G) and optic tectum (Fig. 3H-K) were selected to illustrate the results of regeneration of the zebrafish visual system.

In control animals, CR-immunoreactivity was mainly detected within the retina in RGCs and in the nerve fiber layer (NFL). In addition, some amacrine and bipolar cells within the central retina were also immunoreactive to calretinin (Figs. 1E, 3A). Retinal ganglion cell axons which make up the optic nerve, chiasm and tract presented intense CR-immunoreactivity throughout their extension (Fig. 3E). Moreover, within the *stratum opticum* of the optic tectum, we observed numerous CR-immunoreactive fibers (Fig. 3H). This stratum receives most of its afferents from retinal axons.

Three to six days after optic nerve damage, both the number and intensity of labeling of CR-immunoreactive RGCs decreased (Fig. 3B). However, no evident changes in CR-immunoreactivity were observed in other retinal cells present in the INL. Calretinin immunoreactivity was not observed in the optic nerve, chiasm or optic tract, i.e. the area located between the optic chiasm and the optic tectum (Fig. 3F). In addition, we did not observe immunoreactivity in the stratum opticum of the optic tectum (Fig. 3I) at this stage.

Ten days after optic nerve damage, CR-labeling within the retina was slightly more evident than at 3 days post damage (Fig. 3C), but not all RGCs were intensely labeled. The optic nerve presented some calretinin immunoreactivity. A few calretinin-positive fibers were also found within the optic tectum (Fig. 3J).

From 13 to 60 days after optic nerve damage, calretinin immunoreactivity in the retina, optic nerve

tract and optic tectum was observed to be similar to that observed in control animals (Fig. 3D,G,K).

The temporal progression of optic nerve regeneration and calretinin re-expression is summarized in Fig. 4.

Discussion

In a previous study we have shown that the time course of expression of calbindin in the trout retina parallels, in general terms, that which occurs during the peripheral to central progression of differentiation (Vecino et al., 1993; Vecino, 1998). However, calbindin (and also calretinin; present study) immunoreactivity was observed to be expressed earlier in amacrine cells than other cells. In this study, calretinin immunoreactivity in the developing zebrafish retina showed a similar pattern. Thus, we can say that at least for ganglion cells, calretinin was not present for several hours after commitment to a definitive cell phenotype. The periphery-central spatial gradient of calretinin immunoreactivity reflects the maturation of the retina. Thus the central area of the retina is more mature, in contrast to more peripheral regions (Fig. 1E). Within the most peripheral areas of the mature retina, the pattern of calretinin immunoreactivity is similar to that observed during initial stages of development (42 hpf) and as we look more centrally to the retina, the pattern of calretinin expression resembles that observed during later developmental stages (60 hpf and 76 hpf).

The re-expression of calretinin during regeneration of RGCs after optic nerve section may be related to the expression of calretinin during development. Thus, the timing of axonal regeneration observed in the present study was similar to that reported using tracing

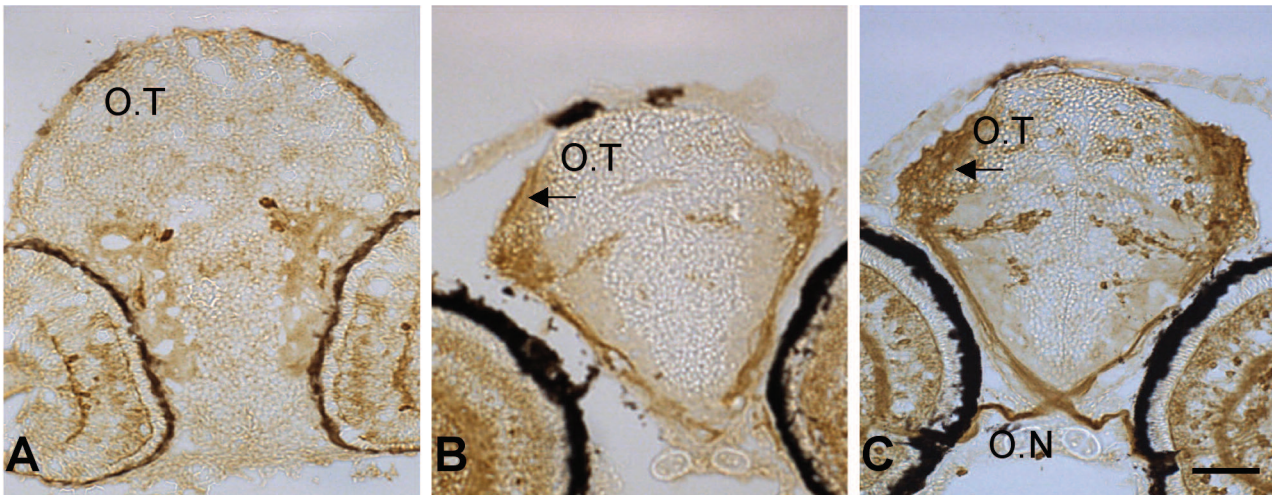


Fig. 2. Transverse sections of the optic tectum (O.T), optic nerve (O.N) and retinas of zebrafish at the optic chiasm level: 60 hpf (A), 76 hpf (B) and 5 dpf (C). A. No calretinin immunoreactive fibers are found at this stage. B. Only incipient immunopositive fibers are found entering the OT (arrow). C. The number of calretinin immunoreactive fibers located in the OT (arrow) is superior in comparison to previous stages. The O.N. shows strong immunolabeling. Scale bars: 50 μ m.

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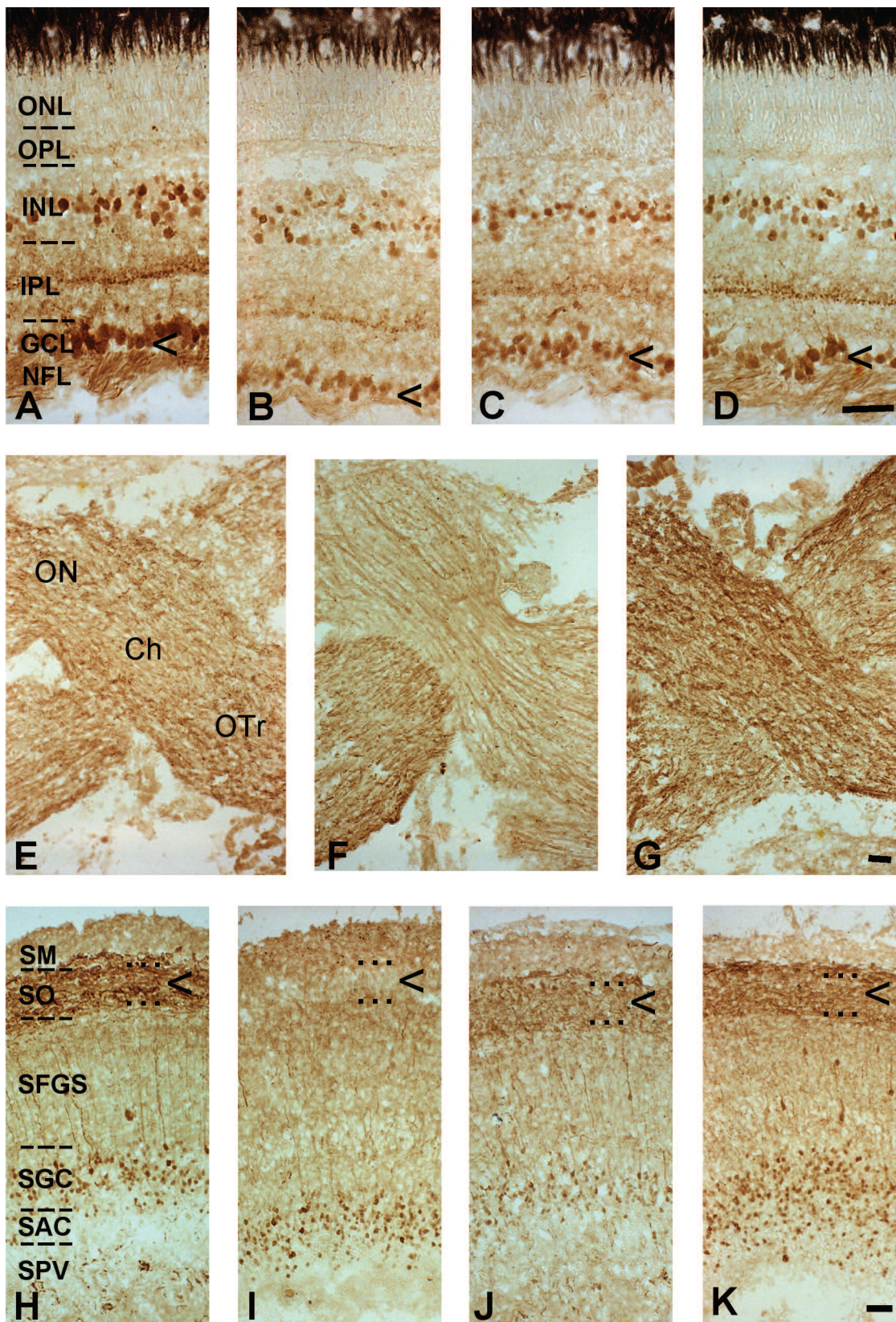


Fig. 3. Calretinin immunohistochemical-stained sections showing different parts of the zebrafish visual system. The retina (**A-D**), longitudinal sections of the optic nerve (ON), chiasm (CH) and tract (OTr) (**E-G**) and the optic tectum (**H-K**) are illustrated in control conditions (**A, E, H**) and during the regeneration of the optic nerve following crushing at 3 (**B, F, I**), 10 (**C, J**) and 13 (**D, G, K**) days post-damage. In **A**, the different layers of the retina are delimited as follows: nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL). In **H**, the different layers of the optic tectum are delimited as follows: stratum periventriculare (SPV), stratum album centrale (SAC), stratum griseum centrale (SGC), stratum fibrosum et griseum superficiale (SFGS), stratum opticum (SO) and stratum marginale (SM). Within the stratum opticum of the optic tectum, many immunoreactive fibers are found in control sections (**H**) and in sections from regenerated animals (**K**). None are observed at 3 days post-lesion (**I**) but a few are found 10 days post-lesion (**J**) (arrowheads). Note the intense labeling of ganglion cells (arrowheads) in **A** and **D** which contrasts with the weak labeling of the ganglion cells in **B** and **C**. The two optic nerves and tracts of control animals (**E**) and those of animals

13 days post-damage (**G**) are labeled. In contrast, the right-hand optic nerve 3 days post-damage (**F**) is not labeled, while labeling is apparent in the undamaged left-hand nerve (**F**). Scale bars: 100 μ m.

experiments in zebrafish (Benhardt et al., 1996; Becker and Becker, 2000; Liu et al., 2002). On the basis of the re-appearance of calretinin immunoreactivity in areas distal to optic nerve crushing, it appears that RGC axon regeneration starts 10 days after optic nerve damage; other studies have reported that regenerating axons start to arrive at the optic tectum 10 days after optic nerve damage. On the other hand, Benhardt et al. (1996) found the first regenerating axons at 7 days after optic nerve damage, while Becker and Becker, (2000) found the entry of regenerating axons into the optic tectum at about 10 days after optic nerve damage. These minor differences could be due to the distance of optic nerve damage to the eye, which in this very small animal, is difficult to estimate precisely, even when the damage is as close to the eye as possible without damaging the ophthalmic arteria.

Down-regulation of calretinin in RGCs was obvious between 3 and 10 days after optic nerve damage, and calretinin immunoreactivity remained at low levels 10 days after optic nerve damage. During this period, RGCs have to be able to extend their axons from the damaged area to the optic tectum, as occurs during development. The return to a normal pattern of expression of calretinin, 13 days after optic nerve damage, may

indicate that RGCs are functional again. A parallelism may be drawn between the regeneration of RGC axons, and thus functional recovery, and the developmental moment in which RGC axons in the expression of a calcium-binding protein has been found. The present results suggest that calretinin is downregulated during functional recovery of the visual system but not during refinement of neural connections, which occurs during later stages of regeneration.

Although the specific role of calcium-binding proteins in regenerating RGCs has not yet been precisely established, some studies have shown that these proteins contribute to the survival of neurons and the mobility of growing neurites, as well as to the proliferation of glial cells (Donato, 1991; Al-Mohana et al., 1992). Other studies have shown that an increase in the levels of cytoplasmic Ca^{2+} in regenerating axons may be responsible for the enhanced rate of recovery of visual function in goldfish (Meiri and Grafstein, 1984). Although there are a variety of mechanisms for controlling the levels of cytosolic calcium, the downregulation of calretinin between 1 and 10 days after optic nerve damage may promote an increase in the levels of free cytosolic calcium, which may be necessary for the survival and recovery of RGCs. Cytosolic Ca^{2+} has thus been indirectly observed to be elevated during axonal regeneration in goldfish and in zebrafish (present study); nevertheless, in mammalian neuronal systems, elevated Ca^{2+} can be neurotoxic. Clearly, comparative studies are necessary in order to understand the role played by calcium-binding proteins during regeneration.

The interruption of visual input due to axonal damage leads to a lack of nervous excitation, which is likely to result in a decrease in the expression of excitatory amino acids. Interestingly, it has been reported that calcium binding protein expression is upregulated in the presence of excitatory amino acids (Mattson et al., 1991; Batini et al., 1997). Thus, regeneration may involve the re-establishing of electrical activity, when RGCs finally re-connect with the optic tectum, re-expression of excitatory amino acids, and subsequently, restoration of calretinin expression.

In conclusion, our results indicate that the pattern of expression of calretinin which occurs during the early stages of retinal ganglion cell regeneration resembles that which is found during the early stages of RGC development, and thus supports the idea that calretinin plays an important role during axon regeneration in the visual system. Moreover, it seems reasonable to suggest that calretinin can be employed as an excellent marker for studying the regeneration of the visual pathway as well as the time-course of RGC maturation.

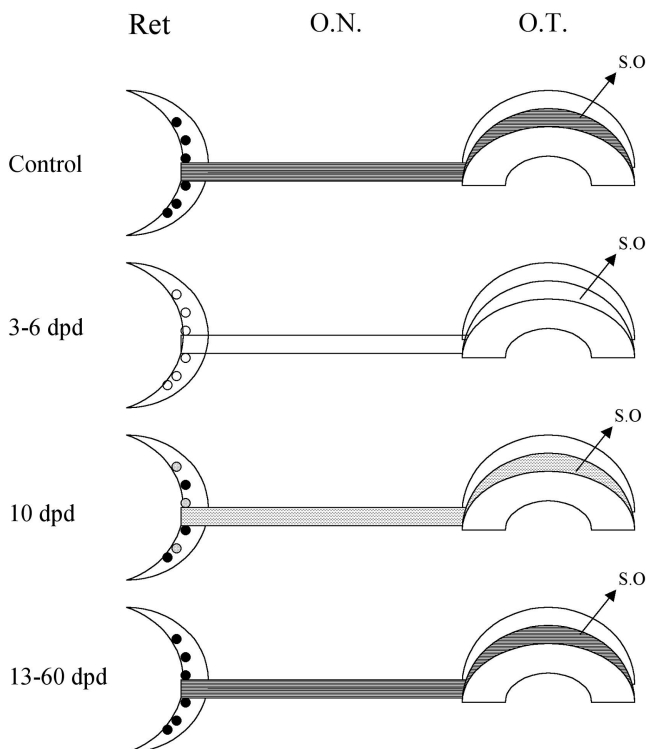


Fig. 4. Schematic drawing representing the temporal progression, in days post-damage (dpd), of optic nerve regeneration and the re-expression of calretinin in the retina (Ret), optic nerve (ON), optic tectum (OT) and stratum opticum (SO). Note that in the control situation, recovery is complete by day 13 post-damage.

Acknowledgements. Supported by grants from the European Community (Grant Pro Age Ret, QLK6-CT-2001-00385), Univ. Basque Country (E-14887/2002;15350/2003), Spanish Ministry of Science and Technology (BFI 2003-07177) to E.V. The authors would like to express their thanks to the agency ACTS (Academic Consulting and Translating Services) for having corrected the English of our manuscript.

*Calretinin in zebrafish visual system***References**

- Al-Mohana F.A., Cave J. and Cowan W.M. (1992). A narrow review of intracellular calcium concentration is optimal for neurite outgrowth in rat sensory neurones. *Dev. Brain. Res.* 70, 287-290.
- Batini C., Guegan M., Palestini M., Thomase M. and Vigot R. (1997). Upregulation of calbindin D-28K immunoreactivity by excitatory amino acids. *Arch. Ital. Biol.* 135, 161-166.
- Becker C.G. and Becker T. (2000). Gradients of ephrin-A2 and Ephrin-A5b mRNA during retinotopic regeneration of the optic projection in adult zebrafish. *J. Comp. Neurol.* 427, 469-483.
- Benhardt R.R., Tongiorgi E., Anzini P. and Schachner M. (1996). Increased expression of specific recognition molecules by retinal ganglion cells and by optic pathway glia accompanies the successful regeneration of retinal axons in adult zebrafish. *J. Comp. Neurol.* 376, 253-264.
- Burril J.D. and Easter S.S. Jr. (1995). The first retinal axons and their microenvironment in zebrafish: Cryptic pioneers and the pretact. *J. Neurosci.* 15, 2935-2947.
- Celio M.R. and Pauls T. (1996). Guidebook to calcium-binding proteins. Oxford University Press.
- Donato R. (1991). Perspectives in S-100 protein biology. *Cell Calcium* 12, 731-726.
- D'Orlando C., Celio M.R. and Schwaller B. (2002). Calretinin and calbindin D-28k, but not parvalbumin protect against glutamate-induced delayed excitotoxicity in transfected N18-RE 105 neuroblastoma-retina hybrids cells. *Brain Res.* 945, 181-190.
- Easter S.S. Jr. and Nicola G.N. (1996). The development of vision in the zebrafish (*Danio rerio*). *Dev. Biol.* 180, 646-663.
- Hu M. and Easter S.S. Jr. (1999). Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. *Dev. Biol.* 207, 309-321.
- Li Y. and Black M.M. (1996). Microtubule assembly and turnover in growing axons. *J. Neurosci.* 16, 531-544.
- Liu Q., Londraville R.L., Azodi E., Babb S.G., Chiaoini-Williamson C., Marrs J.A. and Raymond P.A. (2002). Up-regulation of Cadherin-2 and cadherin-4 in regenerating visual structures of adult zebrafish. *Exp. Neurol.* 177, 396-406.
- Marcus R.C., Delaney C.L. and Easter S.S. Jr. (1999). Neurogenesis in the visual system of embryonic and adult zebrafish (*Danio rerio*). *Vis. Neurosci.* 16, 417-424.
- Mattson M.P., Rychlik B., Chu C. and Christakos S. (1991). Evidence for calcium-reducing and excito-protective roles for the calcium-binding protein calbindin-D28K in cultured hippocampal neurones. *Neuron* 6, 41-51.
- Meiri H. and Grafstein B. (1984). Local application of calcium-modulating agents to a crushed goldfish optic nerve modifies visual recovery. *Exp. Neurol.* 83, 403-413.
- Nag T.C. and Wadhwa S. (1999). Developmental expression of calretinin immunoreactivity in the human retina and a comparison with two other EF-hand calcium-binding proteins. *Neuroscience* 91, 41-50.
- Nitsch C., Scott A., Sommacal A. and Kalt G. (1989). GABAergic hippocampal neurones resistant to ischemia-induced neuronal death contain the Ca²⁺-binding protein parvalbumin. *Neurosci. Lett.* 105, 263-268.
- Schmitt E.A. and Dowling J.E. (1994). Early eye morphogenesis in the zebrafish retinal development. *Vis. Neurosci.* 16, 881-888.
- Schmitt E.A. and Dowling J.E. (1999). Early retinal development in the zebrafish, *Danio rerio*: light and electron microscopic analyses. *J. Comp. Neurol.* 404, 515-536.
- Sperry R.W. (1948). Patterning of central synapses in regeneration of the optic nerve in teleost. *Physiol. Zool.* 21, 351-361.
- Vecino E. (1998). Spatiotemporal development of the fish retina: Distribution of calbindin D-28K. *Sem. Cell Dev. Biol.* 9, 271-277.
- Vecino E., García-Briñón J., Velasco A., Caminos E. and Lara J. (1993). Calbindin D-28K distribution in the retina of the developing trout (*Salmo fario* L.). *Neurosci. Lett.* 152, 91-95.
- Westerfield M. (1993). The zebrafish book. University of Oregon Press.

Accepted May 28, 2004