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Decline in constitutive proliferative activity in the zebrafish retina with ageing

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Abstract

It is largely assumed that the fish retina shows continuous and active proliferative and neurogenic activity throughout life. This is based on studies in teleost models. However, work in lampreys and cartilaginous fishes has shown that proliferative and mitotic activity is almost absent in adult individuals of these ancient fish groups. Interestingly, when deepening in the teleost literature one finds that claims of a highly active and continuous proliferation in the adult retina are based on studies in which proliferation was not quantified in a comparative way at different life stages or was mainly studied in juveniles/young adults. Here, we performed a systematic and comparative study of the constitutive proliferative activity of the retina from early developing (2 days post-fertilization) to aged (up to 3-4 years post-fertilization) zebrafish. Cell proliferation was analysed by using immunofluorescence against pH3 (marker of mitotic cells) and PCNA (marker of proliferating cells). We observed a decline in cell proliferation in the whole retina with ageing, even despite the occurrence of a wave of secondary proliferation during sexual maturation. Interestingly, during this wave of secondary proliferation the distribution of proliferating and mitotic cells changes from the

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inner to the outer nuclear layer in the central retina. Importantly, in aged zebrafish there is a virtual disappearance of mitotic activity. Our results showing a decline in proliferative activity of the zebrafish retina with ageing are of crucial importance since it is largely assumed that the fish retina grows continuously throughout life from progenitor cells located in the periphery.

Introduction

Neurogenesis is the process by which neural progenitor cells give rise to mature neurons and glial cells. Early in development, the central nervous system (CNS) is formed from a highly active neurogenic neuroepithelium. As development progresses, proliferative and neurogenic activities are gradually lost in most CNS regions, and in postnatal life, neurogenic activity is restricted to specific regions called neurogenic niches (Doetsch, 2003; Álvarez-Buylla and Lim, 2004). Moreover, the presence of postnatal neurogenic activity in the CNS was also progressively lost during vertebrate evolution (reviewed in Ferretti, 2011; Zupanc and Sîrbulescu, 2011; Than-Trong and Bally-Cuif, 2015; Alunni and Bally-Cuif, 2016; Zupanc, 2021). Accordingly, different vertebrate species show different postnatal/adult proliferative and neurogenic rates and different numbers of neurogenic niches in the CNS, which are more abundant in teleost fishes (reviewed in Ferretti, 2011; Zupanc and Sîrbulescu, 2011; Than-Trong and Bally-Cuif, 2015; Alunni and Bally-Cuif, 2016; Zupanc, 2021). Some postnatal neurogenic niches are found in the retina of vertebrates. These include the ciliary marginal zone (CMZ), which is a circumferential ring of cells located in the peripheral retina (Harris and Perron, 1998; Raymond et al., 2006; Fisher et al., 2013; Marcucci et al., 2016, Bélanger et al., 2017); the Müller glial cells of the inner nuclear layer (INL) of the central retina (Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007; Nagashima et al., 2013); the retinal pigment epithelium (Okada, 1980; Engelhardt et al., 2005; Ma et al., 2009); the pigment epithelium of the ciliary body (Tropepe et al., 2000); a pseudostratified region at the junction between the retina and the ciliary body (Eymann et al., 2019); and the ciliary epithelium (Fischer and Reh, 2001, 2003; Das et al., 2005, 2006). The proliferative and neurogenic capacity of each of these retinal neurogenic niches varies in different vertebrate species (reviewed in Reh and Fischer, 2001; Amato et al., 2004; Moshiri et al., 2004).

Based on studies in teleost species (see references in Table 1), it is largely assumed that the retina of fish, in contrast to mammals, has continuous proliferative activity throughout life and that this is responsible for continuous eye growth, even during adulthood. This idea emerges in relevant articles

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on this topic during the last decades: “Fish retinas differ fundamentally from those of other vertebrates because they continue to grow throughout the life of the animal, both by adding new neurons and by stretching existing retinal tissue.” (Fernald, 1991); "In fish and amphibia, retinal stem cells located in the periphery of the retina, the ciliary marginal zone (CMZ), produce new neurons in the retina throughout life.” (Perron and Harris, 2000); "The retina of many fish and amphibians grows throughout life, roughly matching the overall growth of the animal. The new retinal cells are continually added at the anterior margin of the retina, in a circumferential zone of cells, known as...” (Kubota et al., 2002); "In the retina of teleost fish, new ganglion cells are generated from a circumferential peripheral growth zone at the edge of the eye throughout life.” (Paulsen et al., 2010); "The retinas of lower vertebrates grow throughout life from retinal stem cells (RSCs) and retinal progenitor cells (RPCs) at the rim of the retina.” (Wan et al., 2016); “In the retina of teleost fish, cell addition continues throughout life involving proliferation and axonal growth.” (García-Pradas et al., 2018) to name a few. However, in recent studies from our group in the sea lamprey, *Petromyzon marinus*, and the catshark, *Scyliorhinus canicula*, we reported the loss of proliferative and mitotic activity in the retina of adult individuals of these ancient vertebrate groups (Villar-Cheda et al., 2008; Hernández-Núñez et al., 2021). This suggested that a continuous proliferative and active mitotic activity throughout life in the retina could be a derived characteristic of modern teleost fishes and not the ancestral character common to all fish groups (Hernández-Núñez et al., 2021).

Based on our recent work in sharks (Hernández-Núñez et al., 2021), we decided to revisit the teleost literature on this topic (see Table 1). We observed that claims of a continuous proliferative and neurogenic activity in the retina of teleost fishes are mainly supported by work on juveniles and young adults, by work using animals in which the age is not clearly indicated or by studies that did not systematically quantified the proliferative and active mitotic activity at different developmental and postnatal stages (see Table 1). So, based on existing literature, we do not actually know whether the teleost retina shows continuous and high proliferative activity throughout life or whether there is a decline in cell proliferation during ageing.

Here, we decided to perform a systematic and comparative study of retinal proliferative and mitotic activity from early developing (2 days post-fertilization) to aged (up to 3-4 years post-fertilization) zebrafish, *Danio rerio*. Zebrafish is by far the most widely used teleost model and, in our study, we have covered all major life stages (from early development to sexual maturity and ageing). Our results show that there is a progressive loss of proliferative activity in the retina throughout life in

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zebrafish, even despite the occurrence of a wave of secondary proliferation that occurs during sexual maturation. Importantly, mitotic activity is virtually absent in the retina of old animals.

Material and Methods

Animals

Two days post-fertilization (2 dpf, n = 16), 4 dpf (n = 17), 7 dpf (n = 11), 1.5 months post-fertilization (mpf, n = 5), 2.5 mpf (n = 10), 3 mpf (n = 8), 8.5 mpf (n = 5), 18-20 mpf (n = 9) and 3-4 years post-fertilization (ypf, n= 5) zebrafish (*Danio rerio*) specimens were used in this study. Zebrafish were kept in aquaria under standard conditions of temperature (28 °C), light cycle (14 h of light and 10 h of darkness) and pH (7.0) until use for experimental procedures. All experimental procedures were performed according to the regulations and laws established by the European Union (2010/63/UE) and by the Spanish Royal Decree 1386/2018 for the care and handling of animals in research and were approved by the Bioethics Committee of the University of Santiago de Compostela.

Tissue preparation for histology

Animals were deeply anesthetized with 0.0016% tricaine methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, MO), euthanized and fixed by immersion in 4% paraformaldehyde in 0.1M phosphate-buffered saline pH 7.4 (PBS) for 2 h (from 2 dpf to 7 dpf) and 1 day (from 1.5 mpf to 3-4 ypf) at 4 °C. After fixation, the lens was removed from the eye in specimens from 1.5 mpf onwards. Eyes were dissected out from the rest of the body in specimens from 8.5 mpf onwards. After rinsing in PBS, the animals or eyes were cryoprotected with 30 % sucrose in PBS, embedded in Neg-50TM (Thermo Scientific, Kalamazoo, MI) and frozen with liquid nitrogen-cooled isopentane. Transverse sections (18 µm thick) were obtained on a cryostat and mounted on Superfrost Plus slides (Menzel-Glasser, Madison, WI).

Haematoxylin-eosin staining

Some sections from 2 dpf and 1.5 mpf specimens were stained with haematoxylin-eosin following standard protocols. Briefly, cryostat sections were dried at room temperature (RT), rinsed in 0.05 M Tris-buffered (pH 7.4) saline (TBS) for 10 min and stained with haematoxylin solution for 10 min.

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Sections were subsequently rinsed in tap water until removal of the excess of haematoxylin, in distilled water for 10 min and then stained with eosin for 2 min. Finally, the sections were dehydrated and mounted in DPX mounting medium (Scharlau, Sentmenat, Spain).

Immunofluorescence

Sections were first pre-treated with 0.01 M citrate buffer pH 6.0 for 30 min at 90 °C for heat-induced epitope retrieval, allowed to cool for 20 min at RT and rinsed in TBS for 5 min. Then, sections were incubated overnight at RT with a combination of two primary antibodies: a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1:500; Sigma-Aldrich; catalogue number P8825; RRID: AB_477413) and a rabbit polyclonal anti-phospho-histone 3 (pH3) (1:300; Millipore; Billerica, MA; catalogue number 06-570; RRID: AB_310177). Sections were rinsed 3 times in TBS for 10 min each and incubated for 1 h at RT with a combination of fluorescent dye-labelled secondary antibodies: a Cy3-conjugated goat anti-rabbit (1:200; Sigma-Aldrich; catalogue number A10520) and a FITC-conjugated goat anti-mouse (1:200; Sigma-Aldrich; catalogue number F2761). All antibody dilutions were made in TBS containing 15 % normal goat serum (Millipore), 0.2 % Triton X-100 (Sigma-Aldrich) and 2 % BSA (Sigma-Aldrich). Sections were then rinsed 3 times in TBS for 10 min each and in distilled water for 30 min, allowed to dry for 30 min at 37 °C and mounted in MOWIOL® 4-88 (Calbiochem, Darmstadt, Germany).

Specificity of antibodies

Anti-PCNA antibodies (including the one used in this study) have been traditionally used to label proliferating cells in the retina of different fish (including zebrafish): *Oryzias latipes* (Negishi et al., 1990); *Haplochromis burtoni* (Mack and Fernald, 1995, 1997); *Onchorynchus mykiss* (Julian et al., 1998); *Tinca tinca* (Velasco et al., 2001; Cid et al., 2002; Jimeno et al., 2003); *Carassius auratus* (Negishi et al., 1990; Cid et al., 2002); *Salmo trutta fario* (Candal et al., 2005); *Danio rerio* (Cid et al., 2002; Amini et al., 2019*); Scyliorhinus canicula* (Ferreiro-Galve et al., 2010a, b; 2012; Bejarano-Escobar et al., 2012; Sánchez-Farías and Candal, 2015; 2016; Hernández-Núñez et al., 2021); and *Petromyzon marinus* (Villar-Cheda et al., 2008). Anti-pH3 antibodies (including the one used in this study) have been also commonly used to label mitotic cells in the retina of fish (including zebrafish): *Carassius auratus* (Otteson et al., 2001); *Scyliorhinus canicula* (Ferreiro-Galve et al., 2010a; Bejarano-Escobar et al., 2012; Hernández-Núñez et al., 2021); and *Danio rerio* (Jensen et al., 2001; Godinho et al., 2007; Weber et al., 2014).

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Image acquisition

Brightfield images of haematoxylin-eosin stained sections were taken with an Olympus BX51 microscope equipped with an Olympus DP71 camera. Images of fluorescent labelled sections were taken with a Leica TCS-SP2 confocal microscope with a combination of blue and green excitation lasers. Confocal optical sections were taken at steps of 1 µm along the z-axis. Collapsed images were obtained with the LITE software (Leica, Wetzlar, Germany). For figure preparation, contrast and brightness of the images were minimally adjusted using Adobe Photoshop CS4 (Adobe, San Jose, CA).

Cell quantifications and statistical analyses

We quantified the number of mitotic cells (pH3+) in the whole retina (peripheral and central retina) and proliferative cells (PCNA+) in the central retina. The number of PCNA+ cells was not quantified in the peripheral retina because at some stages the high number of positive cells impeded to clearly differentiate between cells individually. The number of retinas per experimental group was as follows: 2 dpf (n = 32), 4 dpf (n = 33), 7 dpf (n =21), 1.5 mpf (n = 10), 2.5 mpf (n = 20), 3 mpf (n = 16), 8.5 mpf (n = 10), 18-20 mpf (n = 17) and 3-4 ypf (n = 10).

The number of pH3+ and PCNA+ cells was manually counted under a fluorescence microscope in one out of each two consecutive retinal sections (18 µm). The limit between the peripheral and the central retina was established based on the expression pattern of PCNA, which is mainly found in the most peripheral region of the retina. In 2 dpf specimens, we did not separate pH3+ cell quantifications of the peripheral and central retinas because at this developmental stage PCNA was highly expressed throughout the entire retina, which impeded to establish a clear limit between both regions. Then, we calculated the mean number of cells per section for each retina and used that value for statistical analyses. We also quantified the differential distribution of pH3+ and PCNA+ cells in the different layers of the central retina from 4 dpf onwards: the ganglion cell layer (GCL), the INL and the outer nuclear layer (ONL).

Statistical analyses were performed with Prism 8 (GraphPad software, La Jolla, CA). Normality of the data was determined with the D'Agostino and Pearson test. An ordinary one-way ANOVA followed by a Turkey's multiple comparison test was used to determine statistically significant differences in normally distributed data. A Kruskal-Wallis test followed by a Dunn's multiple

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comparison test was used to determine statistically significant differences in non-normally distributed data.

Results

The zebrafish retina exhibits the typical morphology and structure of the vertebrate retina, with a peripheral retina located at the retinal margin containing different types of progenitor cells and a highly organized central retina (Fig. 1A), which can be observed from 2.5 dpf (Malicki et al., 1996), formed by two plexiform layers: the outer plexiform layer (OPL) and the inner plexiform layer (IPL); and three cell layers: the ONL, where the nuclei of photoreceptors are located; the INL, where horizontal, bipolar, amacrine and Müller glia cells are located; and the GCL, which contains the ganglion cells.

In this study, we analysed zebrafish of different developmental/life stages: 2 dpf, 4 dpf, 7 dpf, 1.5 mpf, 2.5 mpf, 3 mpf, 8.5 mpf and 18-20 mpf and 3-4 ypf. 2 to 7 dpf coincides with a period of early zebrafish development. From 1.5 mpf to 3 mpf, zebrafish are in the process of sexual maturation, and at 8.5 mpf they are sexually mature and in peak fertility. After this, sexual efficiency decreases, and senescence processes begin.

In 2 dpf animals, the retina did not show the typical layered organization of a mature vertebrate retina and it is mainly formed by neuroepithelial cells (Fig. 1B). As it can be observed in haematoxylin-eosin stained sections, the cell nucleus occupies almost the entire cell body in most retinal cells, which is characteristic of proliferating tissues (Fig. 1B). From 4 dpf, we observed the typical layered organization of the central retinal and that the peripheral retina was progressively reduced and restricted to the most marginal region with ageing (Figs. 1B-D).

Changes in mitotic and proliferative activity with age

To compare the number of proliferating and mitotic cells in the zebrafish retina at different developmental/life stages we used double immunofluorescence to detect the expression of PCNA, which is present in proliferating cells during every phase of the cell cycle except in the M-phase, and pH3, a marker of mitotic cells. The number of PCNA+ or pH3+ cells is given as the mean number of

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cells per section for each retina to allow for a comparison between specimens of different age and size.

pH3+ cells were mainly observed in the peripheral retina and in this region most of them were located in the apical surface, i.e. near the ventricle (Figs. 2A-C). However, some ectopic mitoses were also observed in the different layers of the central retina at the different developmental/life stages (Figs. 2A, C). Mitotic cells were almost absent in the whole retina of aged specimens (Fig. 2D). Most of the PCNA+ cells were also located in the peripheral retina (Figs. 2A-D). However, as with pH3+ cells, PCNA+ cells were also present in the different cell layers of the central retina (Fig 2C). The number of proliferating (PCNA+) cells was highly reduced in the whole retina of aged specimens (Fig. 2D).

In the whole retina, the number of mitotic (pH3+) cells significantly and progressively decreased during early development from 2 to 7 dpf (Fig. 2E; Supplementary file 1). Interestingly, we observed a significant increase in mitotic activity in the whole retina from 7 dpf to 1.5 mpf (which did not reach levels of 2 dpf animals; Fig. 2E; Supplementary file 1). From 1.5 mpf onwards, we observed a significant and progressive decline in the number of mitotic cells with ageing (Fig. 2E; Supplementary file 1). Importantly, mitotic cells were almost absent in the retina of aged specimens (from 8.5 mpf to 3-4 ypf, Fig. 2E; Supplementary file 1).

Very similar trends in temporal expression patterns were observed when looking separately at the number of pH3+ cells per section in the peripheral (Fig. 2F; Supplementary file 1) and central retinas (Fig. 2G; Supplementary file 1) or at the number of PCNA+ (proliferating) cells per section in the central retina (Fig. 2H; Supplementary file 1).

Our results indicate that mitotic and proliferative activity decreases with age in both the peripheral and central regions of the zebrafish retina, despite the occurrence of a secondary wave of proliferation during sexual maturation (i.e. 1.5 to 3 mpf). Importantly, mitotic activity is virtually absent in aged specimens.

Changes in the location of mitotic and proliferating cells of the central retina with age

We quantified separately the number of mitotic (pH3+) and proliferating (PCNA+) cells in the cell layers of the central retina (GCL, INL and ONL) at different developmental and life stages (Fig. 3; Supplementary file 2). Interestingly, in early developing 4 dpf specimens the number of mitotic and

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proliferating cells was significantly higher in the INL than in the GCL or ONL (Fig. 3; Supplementary file 2), whereas from 7 dpf to 18-20 mpf (both included) the number of mitotic and proliferating cells was significantly higher in the ONL than in GCL or INL (Fig. 3; Supplementary file 2). As can be observed in Figure 3, this difference is highly significant during sexual maturation (1.5 to 3 mpf). In the oldest animals (3 to 4 ypf) the very few mitotic or proliferating cells located in the central retina did not show differential distribution in the cell layers (Fig. 3; Supplementary file 2). These results suggest that the progenitor cells that remain in the mature central retina after early development could mainly contribute to the production of mature cell types of the ONL.

Discussion

As indicated in the introduction, it is largely assumed that the retina of fishes shows continuous and active proliferation and neurogenesis throughout life. We have shown that this does not apply to lampreys or cartilaginous fishes, in which proliferative or mitotic activity is virtually absent in adult animals (Villar-Cheda et al., 2008; Hernández-Núñez et al., 2021). This assumption is based on previous work in teleost models in which the presence of proliferating cells was only studied in juveniles or young adults, in animals in which the age was not clearly defined or knwon by the authors of the study or without performing quantitative comparisons between different life stages or ages (see Table 1). Here, we obtained quantitative data comparing cell proliferation in the zebrafish retina at different ages and covering all major life stages. Our results show that there is a clear decline in proliferative activity with age in the zebrafish retina, despite the occurrence of a secondary wave of proliferation during sexual maturation (i.e. from 1.5 to 3 mpf). In fact, in sexually mature and old animals there is a drastic reduction in the number of proliferating cells and mitotic cells are virtually absent in old animals.

Our results agree with previous studies reporting the presence of proliferating cells in the retina of young adult zebrafish or in the juvenile/adult retina of other teleost species (see references in Table 1). But this is the first study quantitatively showing a decline in proliferative and mitotic activity with age in the teleost retina. Some of the previous studies provided qualitative descriptions that also suggested a loss of proliferating cells with age (see Table 1). For example, Johns and Fernald (1981) reported that, when studying African cichlid and goldfish juveniles and adults, dividing cells in the ONL were "easier to demonstrate" in younger fish. In zebrafish, Marcus et al. (1999) also indicated that BrdU labelled cells were "greater" in the embryos. Our quantitative results in zebrafish reveal a

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similar pattern to that reported in our work in lampreys and sharks showing a loss of proliferating and mitotic cells in the adult retina (Villar-Cheda et al., 2008; Hernández-Núñez et al., 2021); however, it seems that retinal proliferative and mitotic activity is maintained at a higher rate in adult zebrafish than in adult lampreys (no PCNA+ cells; Villar-Cheda et al., 2008) or sharks (very few PCNA+ cells and pH3+ cells were almost absent; Hernández-Núñez et al., 2021). Our quantitative data comparing retinal cell proliferation at different life stages/ages in zebrafish provides crucial knowledge for future studies on the constitutive retinal neurogenesis of mature zebrafish and for research with zebrafish as a model of retinal degeneration and ageing.

In our comparative study we also observed the presence of a secondary wave of cell proliferation in the zebrafish retina during sexual maturation (i.e. from 1.5 to 3 mpf) that had not been detected in previous studies. This increase in cell proliferation did not reach levels of the early developing (2 dpf) period but it was significantly higher than in 7 dpf specimens. This secondary wave of proliferation could be related to an earlier peak of cell death that occurs in the retina (especially in the ONL) of 7 dpf zebrafish (Biehlmaier et al., 2001) to replace lost cells. In relation to behaviour, this second wave of proliferation does not appear to be necessary for visual abilities related to feeding, since the zebrafish retina becomes functional at 3 to 4 dpf, shortly before larvae begin active feeding (Schmitt and Dowling, 1999). We are tempted to speculate that this secondary wave of proliferation could be related to retinal adaptations that might be needed for sexual behaviours, especially since the integration of multi-sensory information between olfaction and vision has been implicated in mating-like behaviours in zebrafish (Li et al., 2017). However, current data has only implicated dopaminergic interplexiform and retinal ganglion cells in this olfacto-visual centrifugal pathway (Li et al., 2017), which would not explain why proliferation and neurogenesis are needed in the ONL (see below). Future studies should decipher whether this secondary wave of proliferation is only needed to replace lost retinal cells or if it is related to retinal adaptations needed for sexual (or adult) behaviours in zebrafish.

By looking at the distribution of proliferating/mitotic cells in the cell layers of the central retina at different ages we observed that in early developing 4 dpf specimens the number of mitotic and proliferating cells was higher in the INL, whereas in older animals they were more abundant in the ONL. This indicates that progenitor cells in the mature central retina are probably focused on the generation of new photoreceptors. Previous studies showed that the juvenile/adult retina of goldfish produces new rods (Johns and Fernald, 1981; Johns, 1982; Otteson et al., 2001), which fits well with

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our data in zebrafish. The zebrafish retina presents 5 main types of photoreceptors (4 cones and 1 rod) and all of them are generated during early development (Crespo and Knust, 2018). Perhaps specific photoreceptors are needed for mating/courtship/adult behaviours and could be generated in extra numbers during sexual maturation. In zebrafish, males show a slightly more intense yellow coloration compared to females and female zebrafish discriminate between sexes using visual cues in which this yellow coloration is critical, particularly during courtship and spawning (Hutter et al., 2012). In addition, it has been suggested that there might be sex differences in the ultraviolet (UV) spectrum in zebrafish (Hutter et al., 2012). Future work should attempt to study the phenotype of these dividing progenitor cells and their destiny to mature cell types, especially during the previously undetected secondary wave of proliferation at the time of sexual maturation.

Conclusions

Our results reveal a decline in constitutive proliferative activity in the teleost retina with ageing. Importantly, mitotic activity is virtually absent in aged animals. Claims of continuous and high proliferative activity in the fish (teleost) retina throughout life should be tuned down in the future and, actually, these claims do not even apply to all fish (lampreys: Villar-Cheda et al., 2008; sharks: Hernández-Núñez et al., 2021). Interestingly, our systematic study also detected the presence of a secondary wave of cell proliferation during sexual maturation in the zebrafish retina. A possible relationship to the generation of specific photoreceptors needed for sexual/adult behaviours is suggested. However, future work should try to decipher the origin and destiny of these progenitor cells and their relationship to zebrafish behaviour.

Table

Table 1. Studies demonstrating the presence of proliferating cells in the juvenile/adult retina of different teleost species.

Only studies that included juvenile/adult specimens in their analyses were added to the table. Studies looking at cell proliferation during regenerative processes were not included. Note that most of the studies only looked at a specific stage/age/size and that these were usually juveniles or young adults. Sometimes the authors only referred to the animals as “adults” without any indication or age or size.

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In a few cases in which several life stages were compared no systematic quantifications or statistical analyses were performed. Moreover, only one study in goldfish juveniles used a marker of mitotic cells (pH3; Otteson et al., 2001).

Figure captions

Figure 1. A. Schematic drawing of the mature zebrafish retina showing the retinal pigment epithelium (RPE) and the neural retina with two differentiated regions: the peripheral retina, which contains different types of progenitor cells (RPCs), and the central retina with a layered structure, which contains the outer (OPL) and inner (IPL) plexiform layers and three cell layers [outer nuclear layer (ONL) with cone (C) and rod (R) photoreceptors; inner nuclear layer (INL) with bipolar (B), amacrine (A), horizontal (H) and Müller glia (MG) cells; and a ganglion cell layer (GCL) with ganglion cells (G)]. **B-D**. Hemaetoxylin-eosin-stained transverse sections of the retina of 2 dpf (B) and 1.5 mpf (C, D) zebrafish showing the maturation of retinal organization. The dotted lines in C indicate the limit between the peripheral and central retinas. Figure D shows a detail of the central retina squared in figure C. Abbreviations: CE: ciliary epithelium; CMZ: ciliary marginal zone. Scale bars: B: 50 µm; C: 200 µm; D: 100 µm.

Figure 2. A-D. Transverse sections of the retina of 2 dpf (A), 7 dpf (B), 1.5 mpf (C) and 3-4 ypf (D) zebrafish specimens showing the presence of PCNA (arrowheads) or pH3 (arrows) positive cells. In 2 dpf to 1.5 mpf zebrafish (A-C) PCNA or pH3 expressing cells were mainly located in the peripheral retina (CMZ) but also in the central retina, while in 3-4 ypf specimens mitotic and proliferating cells almost disappeared (D). Scale bars: A, B: 50 µm; C: 100 µm; D: 200 µm. **E.** Graph showing significant changes in the number of pH3+ cells/section in the whole retina at different ages (Kruskal-Wallis test, $p < 0.0001$). **F.** Graph showing significant changes in the number of pH3+ cells/section in the peripheral retina at different ages (Kruskal-Wallis test, $p < 0.0001$). **G.** Graph showing significant changes in the number of pH3+ cells/section in the central retina at different ages (Kruskal-Wallis test, $p < 0.0001$). **H.** Graph showing significant changes in the number of PCNA+ cells/section in the central retina at different ages (Kruskal-Wallis test, $p < 0.0001$). Mean ± S.E.M. data and data on statistical multiple comparisons related to these graphs can be found on Supplementary file 1. Asterisks indicate different levels of statistical significance: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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Figure 3. Graphs showing the differential distribution of mitotic (pH3+) and proliferating (PCNA+) cells in cell layers of the central retina at different developmental and life stages. 4 dpf specimens: pH3 (Kruskal-Wallis test, $p = 0.0018$), PCNA (Kruskal-Wallis test, $p = 0.0023$). 7 dpf specimens: pH3 (Kruskal-Wallis test, $p = 0.0296$), PCNA (Kruskal-Wallis test, $p = 0.0036$). 1.5 mpf specimens: pH3 (Kruskal-Wallis test, $p < 0.0001$), PCNA (one-way ANOVA, $p < 0.0001$). 2.5 mpf specimens: pH3 (Kruskal-Wallis test, $p < 0.0001$), PCNA (Kruskal-Wallis test, $p < 0.0001$). 3 mpf specimens: pH3 (Kruskal-Wallis test, $p < 0.0001$), PCNA (one-way ANOVA, $p < 0.0001$). 8.5 mpf specimens: pH3 (one-way ANOVA, $p = 0.0001$), PCNA (one-way ANOVA, $p < 0.0001$). 18-20 mpf specimens: pH3 (Kruskal-Wallis test, $p = 0.0007$), PCNA (Kruskal-Wallis test, $p < 0.0001$). 3-4 ypf specimens: pH3 (Kruskal-Wallis test, $p = 0.8977$), PCNA (Kruskal-Wallis test, $p = 0.6217$). Mean ± S.E.M. data and data on statistical multiple comparisons related to these graphs can be found on Supplementary file 2. Asterisks indicate different levels of statistical significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Supplementary File 1. Mean ± S.E.M. data and data on statistical multiple comparisons related to graphs shown in Figure 2.

Supplementary File 2. Mean ± S.E.M. data and data on statistical multiple comparisons related to graphs shown in Figure 3.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: IH-N, FA, EC and AB-I. Acquisition of data: IH-N, AQ-R, LS. Analysis and interpretation of data: IH-N, FA, EC and AB-I.

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Drafting of the manuscript: IH-N, FA, EC and AB-I. Critical revision of the manuscript: IH-N, AQ-R, LS, FA, EC and AB-I. Obtained funding: IH-N, EC.

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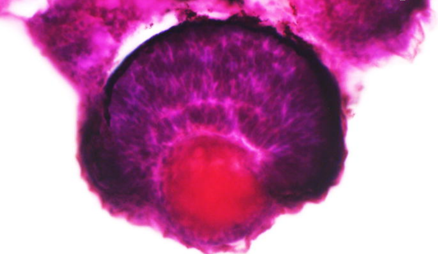
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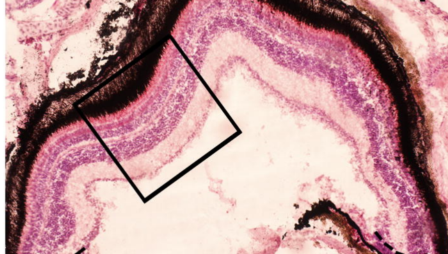
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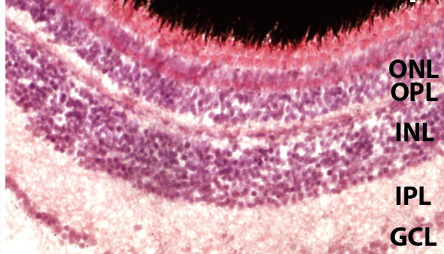
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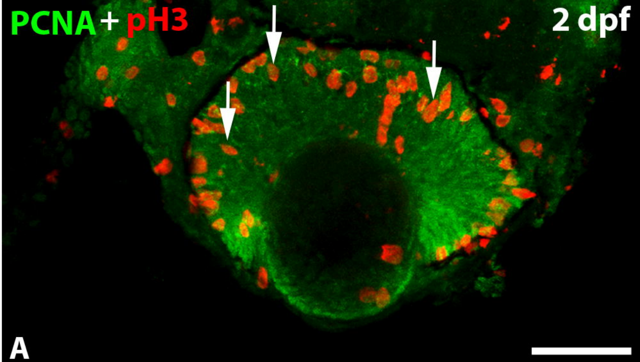
PCNA + pH3
2 dpf
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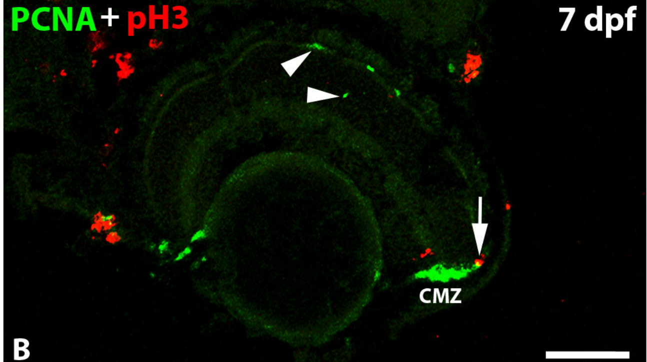
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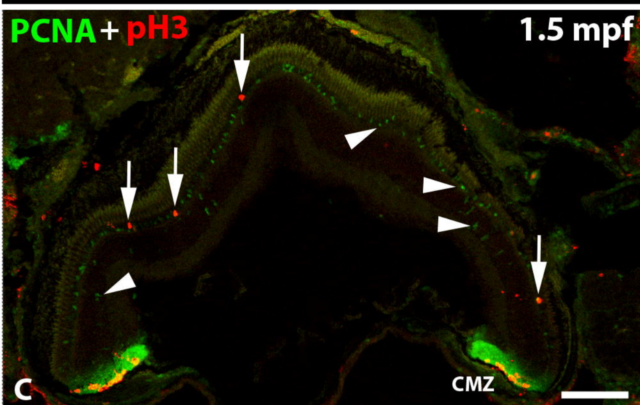
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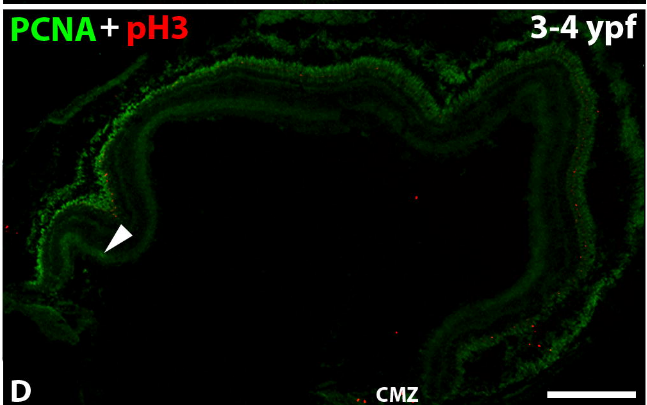
PCNA + pH3
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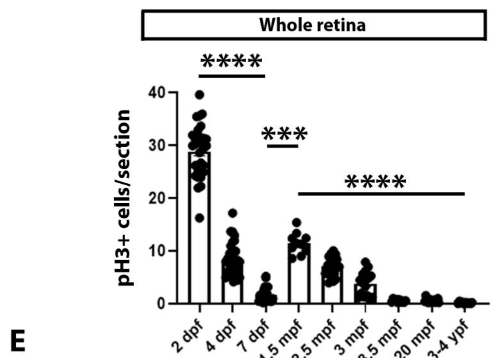
Whole retina
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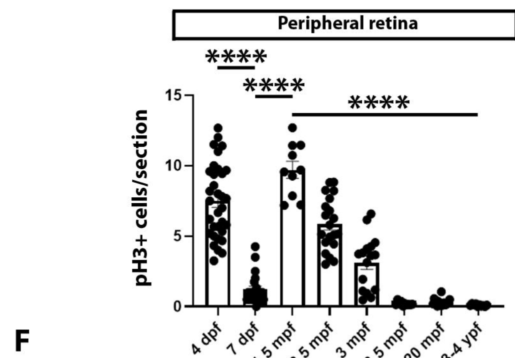
Peripheral retina
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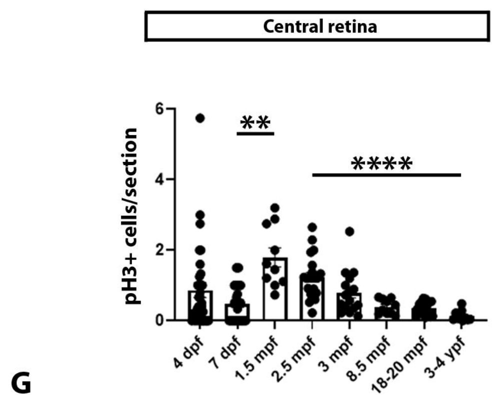
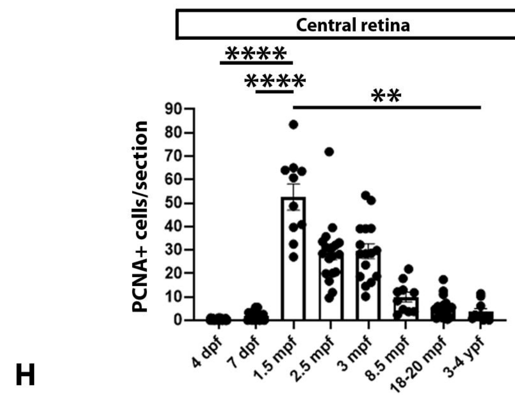
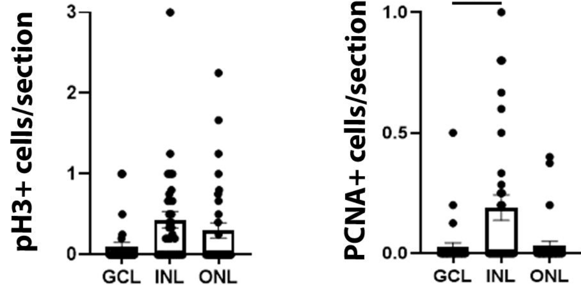
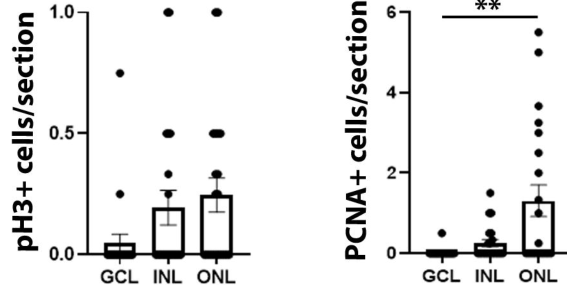
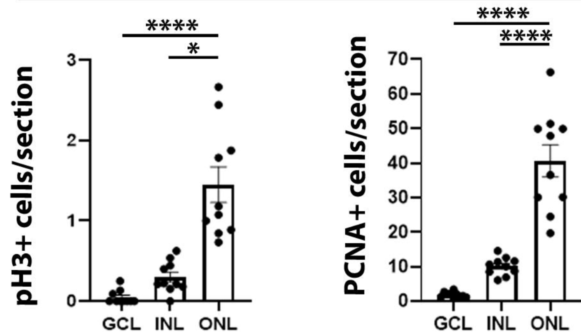
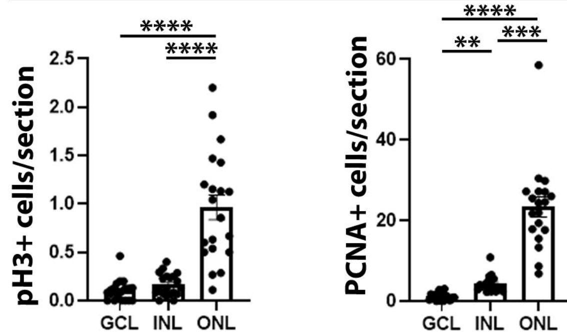

Central retina
pH3+ cells/section
**
****
4 dpf
7 dpf
1.5 mpf
2.5 mpf
3 mpf
8.5 mpf
18-20 mpf
3-4 ypf
G

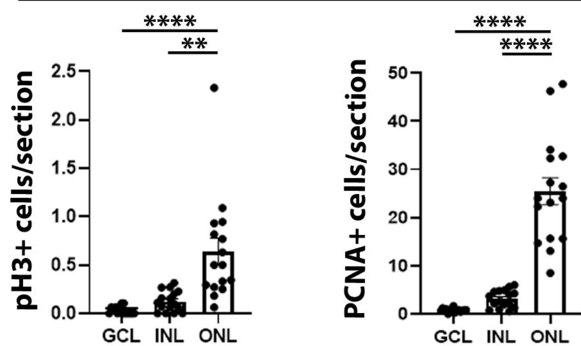

Central retina
PCNA+ cells/section
****
****
**
4 dpf
7 dpf
1.5 mpf
2.5 mpf
3 mpf
8.5 mpf
18-20 mpf
3-4 ypf
H

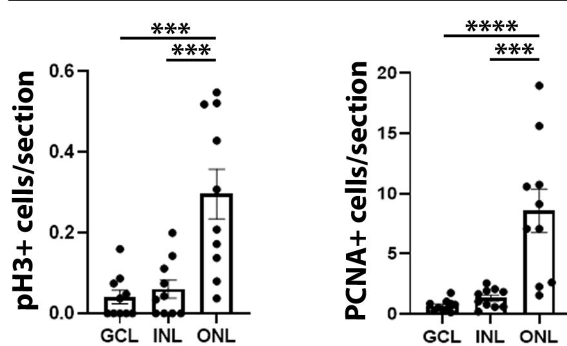

4 dpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
**

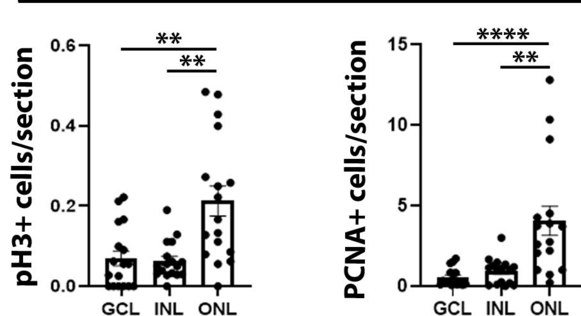

7 dpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
*
**

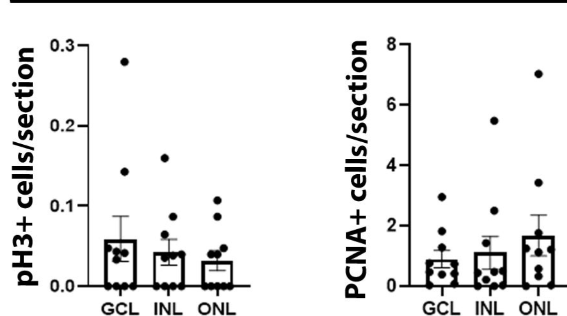

1.5 mpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
****
*


2.5 mpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
****
**
***


3 mpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
****
**


8.5 mpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
***
****


18-20 mpf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL
**
****


3-4 ypf
pH3+ cells/section
PCNA+ cells/section
GCL
INL
ONL