



## Plasticity of cell proliferation in the retina of *Austrolebias charrua* fish under light and darkness conditions

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### ABSTRACT

*Austrolebias* annual fishes exhibit cell proliferation and neurogenesis throughout life. They withstand extreme environmental changes as their habitat dries out, pressuring nervous system to adapt. Their visual system is challenged to adjust as the water becomes turbid. Therefore, this study focused on how change in photic environment can lead to an increased cell proliferation in the retina. We administered 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU) at different temporal windows to detect cell proliferation in natural light and permanent darkness. Stem/progenitor cells were recognized as IdU+/CldU + nuclei co-labeled with Sox2, Pax6 or BLBP found in the ciliary marginal zone (CMZ). The expression pattern of BLBP + glial cells and ultrastructural analysis indicates that CMZ has different cell progenitors. In darkness, the number of dividing cells significantly increased, compared to light conditions. Surprisingly, CMZ IdU+/CldU + cell number was similar under light and darkness, suggesting a stable pool of stem/progenitor cells possibly responsible for retinal growth. Therefore, darkness stimulated cell progenitors outside the CMZ, where Müller glia play a crucial role to generate rod precursors and other cell types that might integrate rod-dependent circuits to allow darkness adaptation. Thus, the *Austrolebias* fish retina shows great plasticity, with cell proliferation rates significantly higher than that of brain visual areas.

### 1. Introduction

In contrast with mammals, teleost fishes grow throughout life, increasing massively in body size. The growth in size of the nervous system accompanies body growth and this process must therefore involve continuous generation of new cells, including neurons. The growth rates may vary between different regions of the nervous system depending on the biological characteristics of the individuals. In the case of the eye, and in particular of the retina, it has been seen that it is

capable of growing at a rate greater than that of the body (Johns and Easter, 1977; Kubota et al., 2002). These results imply a requirement for the rearrangement of the cytoarchitecture of the eye in order to maintain a balance between eye and body growth, as well as of the different tissues forming the retina that must somehow coordinate to expand at the same rate (Wan et al., 2016; Tsingos et al., 2019).

The retina is the light-sensitive part of the eye that consists of the neural retina that contains light-detecting cells (rods and cones), and the retinal pigmented epithelium, which supports these cells. Many studies conducted in teleosts showed that the retina grows throughout life and

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**Abbreviations**

BLBP	Brain lipid binding protein
BrdU	5-bromo-2'- deoxyuridine
CE	ciliary epithelium
CMZ	ciliary marginal zone
CldU	5-chloro-2'-deoxyuridine
DAPI	4',6-diamidino-2-phenylindole
GCL	ganglion cell layer
HuC	Hu-antigen C protein
IdU	5-iodo-2'-deoxyuridine
INL	inner nuclear layer
OLM	outer limiting membrane
ONL	outer nuclear layer
Pax6	Paired box protein Pax-6
PB	phosphate buffer
PBT	PB plus Triton X-100
PFA	paraformaldehyde
Sox2	SRY (Sex Determining Region Y)-Box 2 Transcription Factor
TEM	transmission electron microscopy
XAP-2	HBV X-associated protein 2

that it is capable of regenerating all cell types after damage (Raymond and Hitchcock, 1997; Easter and Hitchcock, 2000; Otteson and Hitchcock, 2003; Stenkamp, 2007; Sun et al., 2018). Unlike in homeothermic vertebrates, the retina of fishes and amphibians grows continuously by adding rings of new cells at the periphery in a region described as a circumference of germinal tissue, called the ciliary marginal zone (CMZ) (Straznicky and Gaze, 1971; Johns and Easter, 1977; Kubota et al., 2002; Miles and Tropepe, 2021). The CMZ is located between the neural retina and the ciliary epithelium (CE). Unlike in other vertebrates, in fishes the CMZ is present in the mature retina and consists of a germinal zone of stem/progenitor cells (Perron and Harris, 2000; Reh and Fischer, 2001; Raymond et al., 2006; Fischer et al., 2014). The presence and diversity of stem/progenitor cells and their different rates of proliferation in the CMZ show characteristics that are common to the neurogenic niches described in the adult nervous system of vertebrates (Álvarez-Buylla et al., 2001; Ming and Song, 2005). However, the identity and features of the different kinds of progenitors in the CMZ are not yet well understood. One of the most intriguing discoveries in vertebrate neurogenic “niches” is that adult neural stem cells exhibit some properties of glial cells and that neurons in certain regions of the developing embryonic mammalian and in adult fishes, reptiles and avian brains derive from radial glia (Noctor et al., 2002; Doetsch, 2003; Zupanc and Clint, 2003; Mori et al., 2005; González-Granero et al., 2011). The Müller glia (MG) present in the embryonic and adult retina of fishes and other vertebrates are considered to be radial glia with stem/progenitor cells characteristics (Fischer and Reh, 2003; Raymond et al., 2006; Gallina et al., 2014; Gorsuch and Hyde, 2014; Lenkowski and Raymond, 2014). Müller glial cells in juvenile fishes are distributed in many regions of the retina including the CMZ (Bernardos et al., 2007). In zebrafish it was demonstrated that Müller glia cells re-enter the cell cycle, and that they can generate multipotent neuronal progenitors by asymmetrical division that regenerate the missing retinal cell types after damage (Bernardos et al., 2007; Fimbel et al., 2007; Kassen et al., 2009; Hamon et al., 2016; Nagashima et al., 2013, 2020; Powell et al., 2016; Sifuentes et al., 2016). Furthermore, it has been shown that some of these new neurons possess normal morphology and connectivity (McGinn et al., 2018). In addition, analysis of 5-bromo-2'- deoxyuridine (BrdU) uptake and label retention show that the cells in the extreme peripheral edge of the CMZ -that are presumably radial glia-divide slowly whereas the cells in the central part of the CMZ divide faster (Ohnuma et al., 1999; Perron and Harris, 2000;

Xue and Harris, 2012).

Outside the CMZ, in the outer nuclear layer (ONL), rod progenitor cells were described in many species of fishes (Morris et al., 2008). In normal conditions, these progenitors usually only produce rods, but in the presence of damage it was thought that rod progenitors were capable of generating all types of retinal neurons. The hypothesis that the rod progenitors are the primary source of regenerating retinal neurons has been challenged by work indicating that Müller glial cells respond to retinal damage by proliferating and producing the inner nuclear layer (INL) stem cells (Wu et al., 2001, Wan and Goldman, 2016; Sifuentes et al., 2016, Langhe and Pearson, 2020). It was also shown that dark-adaptation increases rod precursor cell proliferation in the ONL zebrafish retina (Lahne et al., 2019).

Fish with unique life history traits have provided opportunities for understanding plasticity of the visual system as a naturally adaptive mechanism (Stenkamp, 2007). Therefore, for the first time, we will describe the *Austrolebias* CMZ and its different cell progenitors by morphological characteristics, marker expression and dynamics of cell proliferation. We also show how cell proliferation increases in darkness conditions in all retinal layers where we think that MG play a fundamental role. Our question remains open: which cells are the progenitors in the retina at different layers, Müller glia? Or are there other progenitor cells? We don't know, but MG is the first candidate.

Annual fishes are a unique example of extreme environmental stress tolerance among vertebrates. They inhabit temporary savannah and pampa pools of Africa and South America that become dry when fishes are still in active reproduction (Wourms, 1972; Podrabsky et al., 2010; Berois et al., 2015). The species are maintained by embryonated eggs buried in the mud. Our model, *Austrolebias charrua*, is an annual fish species with a lifespan of 8 months that presents active and fast neurogenesis in several adult brain regions (Fernández et al., 2011). These fish live in temporary puddles of water that progressively lose luminosity as they dry when animals are still actively mating. Thus, we hypothesized that the demands for retinal cell proliferation increases as puddle luminosity decreases and the reproductive activity imposes the finding of couples to mate and preserve the species. In accordance, it has been shown that a greater number of rods will be necessary to improve vision under low luminosity (Locket, 1970; Wagner et al., 1976; Chiu et al., 1995; Ali et al., 1990).

In previous studies we have mapped and quantified cell proliferation in various brain regions along ventricular areas of *Austrolebias* (Fernández et al., 2011), estimated the proportion of different populations of proliferating cells and also the putative stem cells using two proliferation markers applied at different temporal windows (Rosillo et al., 2016; Torres-Pérez et al., 2017). Now, using the same methodological approaches, we have analyzed if the retina presents rapid neurogenesis to compensate decreased luminosity. We have also explored if there are related structural changes including the presence of stem/progenitor cells in other retinal regions besides the CMZ.

## 2. Material and methods

### 2.1. Animal collection, care and processing

*Austrolebias charrua* fish (Cyprinodontiformes-Rivulidae) are freshwater teleosts that inhabit temporary ponds. When the ponds dry, all adult fish die, but desiccation-resistant embryonated eggs remain buried into the mud in developmental arrests or “diapauses” during 4 or 5 months. Once the ponds flood in the next rainy season, early in April, most embryos hatch. Fish employed in this study were collected during September to November in transient ponds close to the major rivers in Rocha, Uruguay (fishing permission by DINARA, abbreviation in Spanish of the name of the National Direction for Aquatic Resources). Therefore, fish used in experiments were males between 5 and 6 months of age and had an average length of 5 cm. Males were chosen for this study since *Austrolebias* fishes present phenotypic and behavioral sexual

dimorphisms (Berois et al., 2015). Moreover, differences in the nervous system between males and females could exist as reported in many other species (Dulac and Kimchi, 2007). Once the specimens were collected, they were kept in 30 L glass aquariums with aerated, de-chlorinated tap water (pH 7–7.5;  $19 \pm 1$  °C) and exposed to natural light (14 h light/10 h darkness). Water was partially changed every 5 days. Fish were fed daily with live *Tubifex* sp. Feeding and housing was maintained in standard conditions since the capture until the processing. Before processing, fish were deeply anesthetized by adding a 1:1000 v/v solution 10% Eugenol (Sigma-Aldrich Corporation, St. Louis, MO, USA) to the aquarium water until opercular movement ceased. After that, each fish was intracardially perfused with saline solution to wash the vascular system and then with specific fixative solutions according to the method employed (Rosillo et al., 2016). All procedures were approved by the local Committee for Animal Care and Research (CHEA, UdelaR and CEUA, IIBCE), which follows NIH guidelines for maintenance and use of laboratory animals.

## 2.2. Transmission electron microscopy and boraxic methylene blue staining

Transmission electron microscopy (TEM) was used to examine the ultrastructural features of the cell types in the neurogenic niches located in the ciliary marginal zone in the retina. Six fish (light) were perfused with 2.5% glutaraldehyde plus 2% paraformaldehyde (PFA) (Sigma-Aldrich Corporation) in 0.1 M phosphate buffer (PB; pH 7.2). Eyes were removed and kept in the same fixative for 2 h at 4 °C. Then, tissue was transferred to PB overnight. The eyes of fish or 200 µm vibratome sections were post fixed in 2% osmium tetroxide for 1.5 h, rinsed with PB, dehydrated through ascending ethanol series (50, 75, 95 and 100%) and then with anhydrous acetone before final embedding with epoxy resin (Durcupan, Sigma-Aldrich). Semi-thin sections (1 µm) were obtained using a PowerTome XL ultramicrotome (RMC, Az) and stained with 1% boraxic methylene blue before being observed in a Nikon Eclipse E 200 microscope. Images were taken using a Nikon COOLPIX 8400 digital camera. Ultrathin (60 nm) sections were cut with a glass knife, mounted on Formvar-coated slot grids (2 × 1 mm) and contrasted with 2% uranyl acetate and lead citrate in accordance with Reynolds' method (Reynolds, 1963). Individual cell types were characterized by examination using both a Jeol 100CX- II and a Jeol JEM1010 TEM equipped with 4000 a.m. DVC and HAMAMATSU C-4742-95 digital cameras, respectively.

## 2.3. Temporal discrimination of cell proliferation: 5-Chloro-2'-deoxyuridine (CldU) and 5-Iodo-2'-deoxyuridine (IdU) application

The administration of different halogenated thymidine analogs that can be distinguished by specific primary antibodies together with careful timing between administrations allowed the discrimination of proliferating cell subpopulations in the brains of amniotes (Vega and Peterson, 2005; Llorens-Martín et al., 2010), anamniotes (Grandel et al., 2006; Alunni et al., 2010) and invertebrates (Sullivan et al., 2007). Therefore, to study dynamics of cell proliferation in the CMZ of the *Austrolebias charrua* retina, eighteen adult fish (9 for light and 9 darkness) were injected via intraperitoneal with two halogenated thymidine analogs (IdU, CldU, Sigma-Aldrich). Two fish were used as controls and were injected with 50 µl of saline solution (the same volume was injected with proliferation markers). The dark condition was obtained by putting the fish in aquariums with black walls. Under these conditions, only some light enters creating an ambient with much lower luminosity (~480 lux in light and ~40 lux in darkness, measured at 12 p. m.). The darkness group was conditioned in the dark aquarium during 4 days before the application of the first proliferation marker (IdU). Administration of IdU (57.5 mg/kg body weight) was at day 0 and that of CldU (42.5 mg/kg body weight) was 29 days later. At day 30 (1 and 29 days after CldU and IdU injection, respectively), fish were anesthetized

and fixed with 10% PFA (Fig. 1A). In all experiments, IdU was dissolved in 0.7% NaCl and 0.04 N NaOH and CldU in 0.7% NaCl, respectively. Six fish were injected with a single dose of BrdU (100 mg/kg, Sigma-Aldrich) and after 30 days were fixed. This marking would then be combined with an antibody that recognized the brain lipid binding proteins (BLBP) for detecting radial glial cells. In all cases, injections and animal processing were done at 12 p.m., to avoid interferences of the circadian cycle on the cell proliferation rate as reported in zebrafish (Akle et al., 2017).

## 2.4. Immunohistochemistry

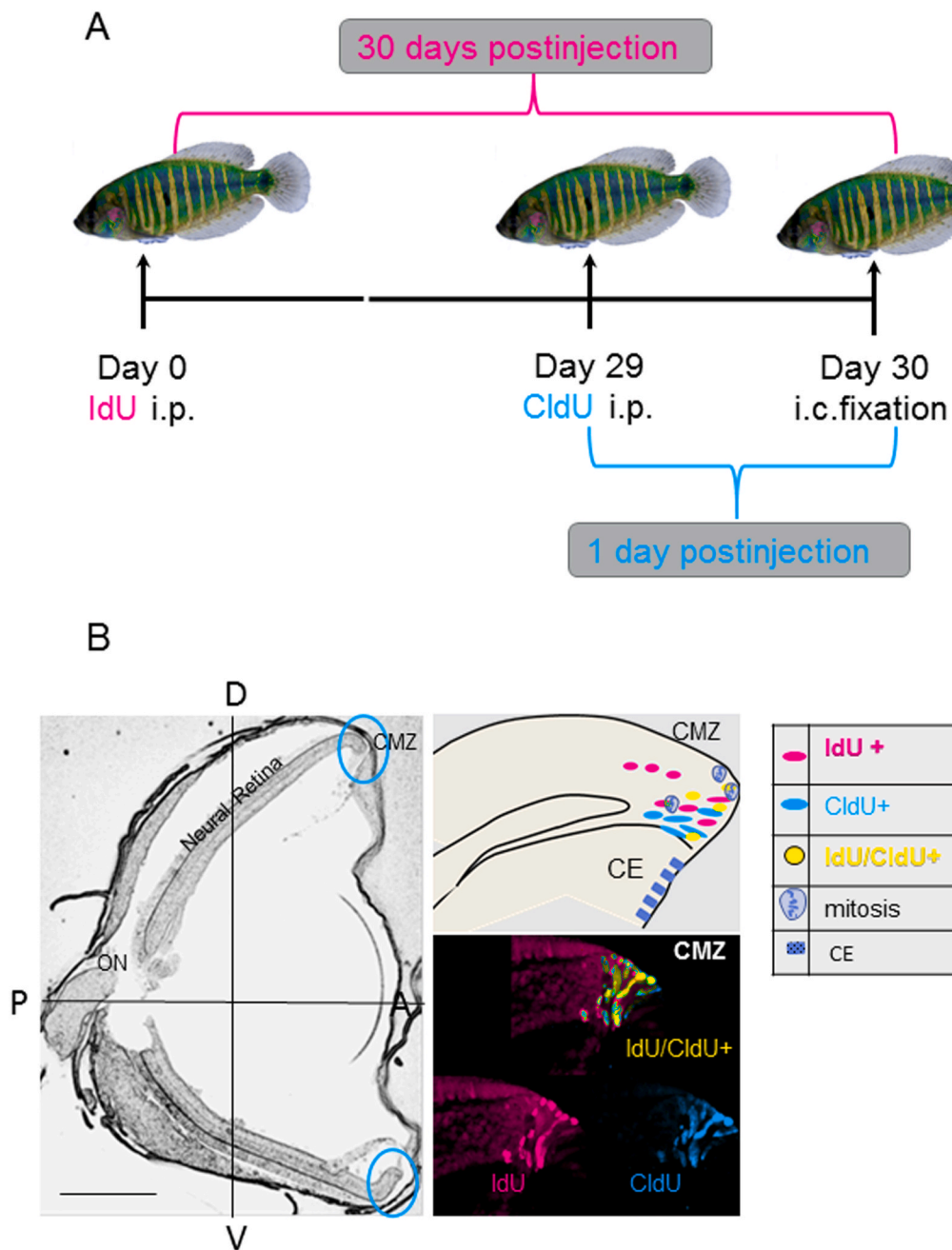
After fixation, dissection and post-fixation, eyes were transferred to PB and maintained at 4 °C until processing. Fixed eyes were serially sectioned in parasagittal sections of 60 µm thickness in a Vibratome S1000 (Leica, Buffalo Grove, IL, USA). Finally, sections were transferred to multi-well plates for further free-floating immunohistochemistry processing.

Proliferation markers incorporated during the S-phase of the cell cycle were analyzed in tissue sections pretreated with 2 N HCl in PB containing 0.3% Triton X-100 (PBT) for 45 min at room temperature to break double-stranded DNA into single strands, as previously described in Fernández et al. (2011). After 3 washes of 10 min with PB, proliferating cells were recognized immunohistochemically using antibodies to detect BrdU, (DSHB Cat# G3G4; RRID: AB\_2618097; mouse, monoclonal) IdU (BD Biosciences Cat# 347580, RRID: AB\_10015219, mouse, monoclonal) or CldU (Accurate Chemical and Scientific Corporation Cat # OBT0030, RRID: AB\_2313756, Rat, monoclonal) (see Table 1). In addition, double immunolabeling against BrdU and the neuronal protein marker HuC, (Abcam Cat# ab78467, RRID: AB\_1566163; rabbit; polyclonal) or β III tubulin (Abcam Cat# ab41489, RRID: AB\_727049, chicken; polyclonal) was done to identify the proliferating cells that were differentiated into a neuronal phenotype, or double immunolabeling against BrdU and BLBP to detect radial glial cells. Incubation was made for 48 h at 4 °C. After rinsing with PB (3 × 10 min), recognition of each label was performed with secondary antibodies conjugated to the fluorescent markers Alexa 488 and Alexa 633 (Invitrogen, CA, USA), both diluted 1:1000 in PBT.

To visualize CldU and IdU, tissue sections were incubated 48 h at 4 °C with 1:500 dilutions of both rat anti-CldU/BrdU (Accurate, New York, USA) and mouse anti-IdU/BrdU (Becton Dickenson, New Jersey, USA). Sections were rinsed in PB (3 × 10 min) and incubated in donkey anti-rat biotinylated secondary antibody at 1:500 (Jackson ImmunoResearch, West Grove, PA, USA) in PBT for 1 h. After that, sections were quickly washed 3x in PB and incubated in a mixture of streptavidin conjugated to Cy3 at 1:500 and donkey anti-mouse Alexa 488 at 1:500 in PBT for 90 min. Combinations of CldU and IdU together with anti-Pax6 (Abcam, Cat# ab135147, RRID: AB\_2891134, rabbit, polyclonal) and Sox2 (Abcam Cat# ab97959, RRID: AB\_2341193; rabbit, polyclonal) antibodies (both transcription factors of neurodevelopmental differentiation usually expressed in stem cells), or anti-BLBP were employed to identify progenitor and radial glial cells. All sections were mounted with glycerol with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and imaged using a confocal FV300 Olympus microscope with Fluoview 5.0 software (Olympus Fluoview 300 Confocal Microscope, RRID: SCR\_020339). Sequential imaging and multi-plane view analysis were done using 20x (0.50 N.A.), 40x (0.75 N.A.) and 60x (1.42 N.A.) lenses.

### 2.4.1. Primary antibodies used and controls

A summary of the antibodies employed appears in Table 1. All antibodies were previously validated by other researchers and ourselves or indicated by the manufacturer as able to be used in zebrafish, as is the case of the anti-BrdU antibody that was employed in many fishes including zebrafish (Grandel et al., 2006) and *Austrolebias* (Rosillo-Martín et al., 2010; Fernández et al., 2011). IdU and CldU antibodies were used in *Gymnotus omarorum* by Olivera-Pasilio et al. (2014). In all cases, to



**Fig. 1.** Experimental design and proliferating cells analyzed. A: Schematic representation of the experimental design showing the time of intraperitoneal (i.p.) administration of IdU and CldU at day 1 and 29, respectively. Fish were processed by intracardiac (i.c.) fixation at 30 days post-injection of IdU and at 1 day post-injection of CldU. B: The microphotograph on the left shows a sagittal unstained section of the eye with the CMZ encircled in cyan. In the upper right panel, there is a schematic representation of the proliferative cellular types located in the CMZ with abundant mitotic figures and the CE. The bottom right panel shows a confocal image with cell nuclei labeled with both cell proliferating markers IdU (magenta) and CldU (cyan), and the merged image showing double labeled (IdU+/CldU+) cells in yellow. Abbreviations: CMZ-ciliary marginal zone; CE-ciliary epithelium; ON- optic nerve; D-dorsal; V- ventral; P- posterior; A-anterior. Scale bar in B: 300  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

determine optimal conditions for antibody incubation, including its dilution, intestine sections from rats injected with BrdU, CldU, IdU or vehicle were used as positive or negative controls, respectively.

Among the antibodies used to recognize cell phenotypes in *Austrolebias*, the anti-BLBP antibody was employed by Wen et al. (2010) in *Trachinotus blochii* fish; the anti-HuC antibody that is able to be used in zebrafish as the manufacturer (Abcam) indicates was utilized by Arslan-Ergul et al. (2016), and the anti-Pax6 and anti-Sox2 antibodies that were used to recognize stem/progenitor cells were previously employed by Sakurai and Osumi (2008). For the recognition of rod photoreceptors,

we used an antibody from the Hybridoma Bank, the anti-Rod XAP-2 (DSHB Cat# XAP-2 (Clone 5B9), RRID: AB\_528087, mouse; monoclonal). In each case, we determined optimal concentration and incubation conditions by exposing *Austrolebias* and zebrafish brain sections at the same time in equal conditions.

### 2.5. Quantitation of proliferating cell number under light and darkness

The medial sections of the eye were selected for quantification, discarding the sections containing the poles where the configuration of the

**Table 1**  
Antibodies, their antigens and manufacturer information.

Antibody	Immunogen	Manufacturer, catalog number, species, type	Dilution
Anti-HuC antibody	Synthetic peptide near the N terminus of human/rat/mouse HuC	Abcam Cat# ab78467, RRID: <a href="#">AB_1566163</a> , rabbit, polyclonal	1:100
Anti-beta III Tubulin antibody	Three different synthetic peptides conjugated to KLH. These synthetic peptides corresponded to different regions of the beta III Tubulin gene product, but are shared between human and rat.	Abcam Cat# ab41489, RRID: <a href="#">AB_727049</a> , chicken, polyclonal	1:1000
BrdU (Bromodeoxyuridine) antibody	5-bromo-2'-deoxyuridine-BSA	DSHB Cat# G3G4, RRID: <a href="#">AB_2618097</a> , mouse, monoclonal	1:80
BrdU antibody, Clone BU1/75 (ICR1) (Used to detect CldU)	The details about of the immunogen are not available in the Datasheet of the company.	Accurate Chemical and Scientific Corporation Cat #OBT0030, RRID: <a href="#">AB_2313756</a> , rat monoclonal	1:500
BrdU antibody, Clone B44. (Used to detect IdU)	Derived from hybridization of Sp2/0-Ag14 mouse myeloma cells with spleen cells from BALB/c mice immunized with iodouridine conjugated ovalbumin	BD Biosciences Cat# 347580, RRID: <a href="#">AB_10015219</a> , mouse, monoclonal	1:500
Anti-BLBP antibody	Synthetic peptide corresponding to Human BLBP (C terminal) conjugated to Keyhole Limpet Haemocyanin (KLH).	Abcam Cat# ab110099, RRID: <a href="#">AB_10866432</a> , goat, polyclonal	1:500
Anti-PAX6 antibody	Synthetic peptide corresponding to a region within the internal amino acid sequence of Human PAX6	Abcam, Cat# ab135147, RRID: <a href="#">AB_2891134</a> , rabbit, polyclonal	1:250
Anti-SOX2 antibody	Synthetic peptide. This information is proprietary to Abcam and/or its suppliers	Abcam Cat# ab97959, RRID: <a href="#">AB_2341193</a> , rabbit, polyclonal	1:500
Mouse Anti-Xenopus XAP-2 Photoreceptor, rods only Monoclonal Antibody, Unconjugated	Immunosuppressed against Xenopus embryos, immunized with Xenopus tadpole optic nerves and retinae	DSHB Cat# XAP-2 (Clone 5B9), RRID: <a href="#">AB_528087</a> , mouse, monoclonal	1:20

ciliary marginal zone is missing. The number of the sections counted in each eye was eighteen. CldU positive and IdU positive nuclei present in retinal sections were counted in six pairs of eyes. To do this, image stacks of 30  $\mu\text{m}$  with steps of 1  $\mu\text{m}$  were obtained to quantitate cells labeled either with one or both proliferation markers. The total numbers, as well as the percentage of CldU (cyan), IdU (magenta) and IdU/CldU (yellow) cells in each region per fish were counted for light and dark conditions. Recognition of positive cells was done using the ImageJ free software (ImageJ, RRID: [SCR\\_003070](#)). Descriptive statistics, comparisons among groups and plotting were done by using GraphPad Prism 6.0 (GraphPad

Prism, RRID: [SCR\\_002798](#)). Non-parametric Kruskal Wallis test followed by Dunn's comparison was employed to compare the data among different groups. Data are presented as the mean  $\pm$  SEM. Significant statistical difference was determined at  $p < 0.05$ .

### 3. Results

#### 3.1. Cytoarchitecture of the CMZ by TEM

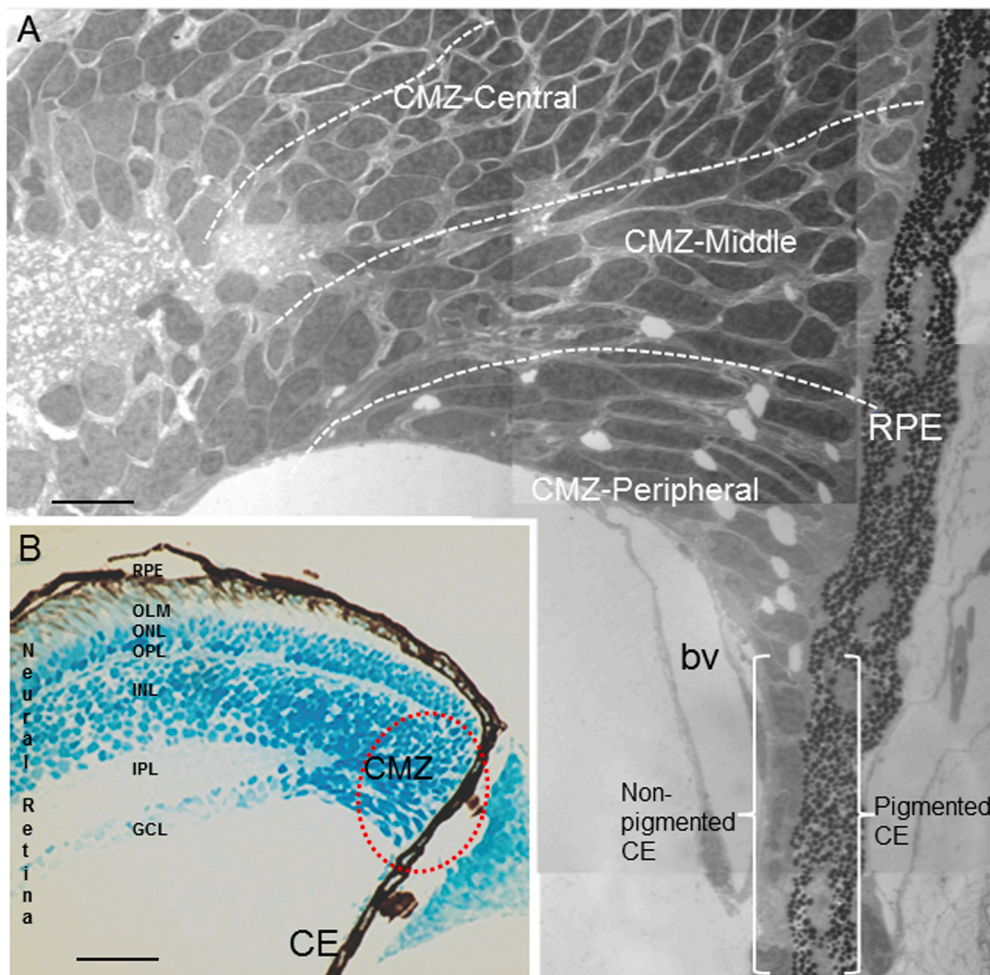
The vertebrate eye consists of the neural retina and the retinal pigmented epithelium. In the adult fish and amphibians, retinal tissues continue growing from a ring-shaped stem cell niche located in the retinal periphery, the CMZ. The semi-thin sections allow us a panoramic view of the whole neural retina with the CMZ located in an edge limiting with the pigmented epithelium that covers the retina and continues covering the single cell row of the non-pigmented CE (Fig. 2B). The electron microscopy analysis of the CMZ of *A. charrua* fish showed a regionalization consistent with the compartmentalization described for zebrafish CMZ after using various molecular expression techniques (Raymond et al., 2006) (Fig. 2A). Our data showed the three CMZ regions (Central, Middle and Peripheral) bearing cell types with different morphologies (Fig. 2B). A fourth region formed by the row of non-pigmented CE cells presents cell proliferation and expression of neural stem cell markers.

The CMZ-Peripheral has the most elongated cells, with 7–18  $\mu\text{m}$  of length in their major axis, scanty cytoplasm and nuclei with condensed chromatin denoting significant proliferative activity (Fig. 3A). These cells differentiate from the other progenitor cells because they have little cytoplasm and are similar to neighboring cells except for the space between them, which may evidence that they are moving toward the retinal layers (Fig. 3E). The CMZ-Middle cells have major axis between 6 and 12  $\mu\text{m}$  and are larger than those located in CMZ-Central. The most relevant feature of the CMZ-Middle is that all cells appear to be at some stage of mitosis as suggested by the appearance of chromatin and mitotic figures in many cells (Fig. 3B). The CMZ-Central that borders the rest of the neural retina shows the smallest progenitor cells of 5–8  $\mu\text{m}$  with different sizes and shapes. They have irregular shapes and nuclei with dense chromatin, few cytoplasm and some space between them (Fig. 3C).

The ciliary epithelium continues outside the neural retina and is made up of two layers of cells: the pigmented and the non-pigmented. The pigmented CE contains round pigments and is located externally to the non-pigmented ciliary epithelium. The non-pigmented ciliary epithelium shows particular characteristics. The cells have rectangular shape and possess multiple cytoplasm invaginations on the free edge. On the outside of the free edge, a basal membrane can be seen (Fig. 3D). The cytoplasm containing invaginations is attached to a basal membrane while the borders attached to the pigmentary epithelium are free of invaginations. (Fig. 3F).

#### 3.2. Immunohistochemistry of Sox2, Pax6 and cell proliferation markers

Some eye sections from animals injected with IdU and CldU were incubated with antibodies against Sox2 or Pax6. Sox2 is a transcription factor that is essential for maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells (Okuda et al., 2006). This factor was expressed in cell proliferating regions co-localized with CldU/IdU. Many of the double labeled cells (yellow) co-localized with Sox2 in the CMZ-Middle. Sox2 was also expressed in the first segment next to the CMZ-Central at the top of the inner nuclear layer, as well as in the CE where it co-localized with IdU label (Fig. 4A, B, E). Sox2 did not co-localize with BLBP + cells in the CMZ and its expression was not observed in the CMZ-Peripheral, where BLBP was slightly expressed (Fig. 4F). Furthermore, Pax6, a transcription factor with key functions in the development of the eye (Macdonald and Wilson, 1997), also had an important expression in the three regions of CMZ, co-localizing with



**Fig. 2.** Transmission electron microscopy images of the CMZ. A: The image shows a photocomposition that allowed the reconstruction of the CMZ. Dashed white lines define the histological landmarks of the three areas identified in the CMZ (Peripheral, Middle, Central) and a fourth zone consisting of the non-pigmented CE attached to the pigmented CE, that are continuous with the retina. B: Part of the retina, including the CMZ, stained with borax methylene blue. The region analyzed in the electron microphotography is framed in red dotted lines. Abbreviations: CMZ-ciliary marginal zone; CE-ciliary epithelium; RPE- Retinal pigmented epithelium; OLM-outer limiting membrane; ONL-outer nuclear layer; OPL-outer plexiform layer; INL-inner nuclear layer; IPL-inner plexiform layer; GCL-ganglion cellular layer; bv: blood vessel. Scale bar in A: 15  $\mu$ m; in B: 100  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

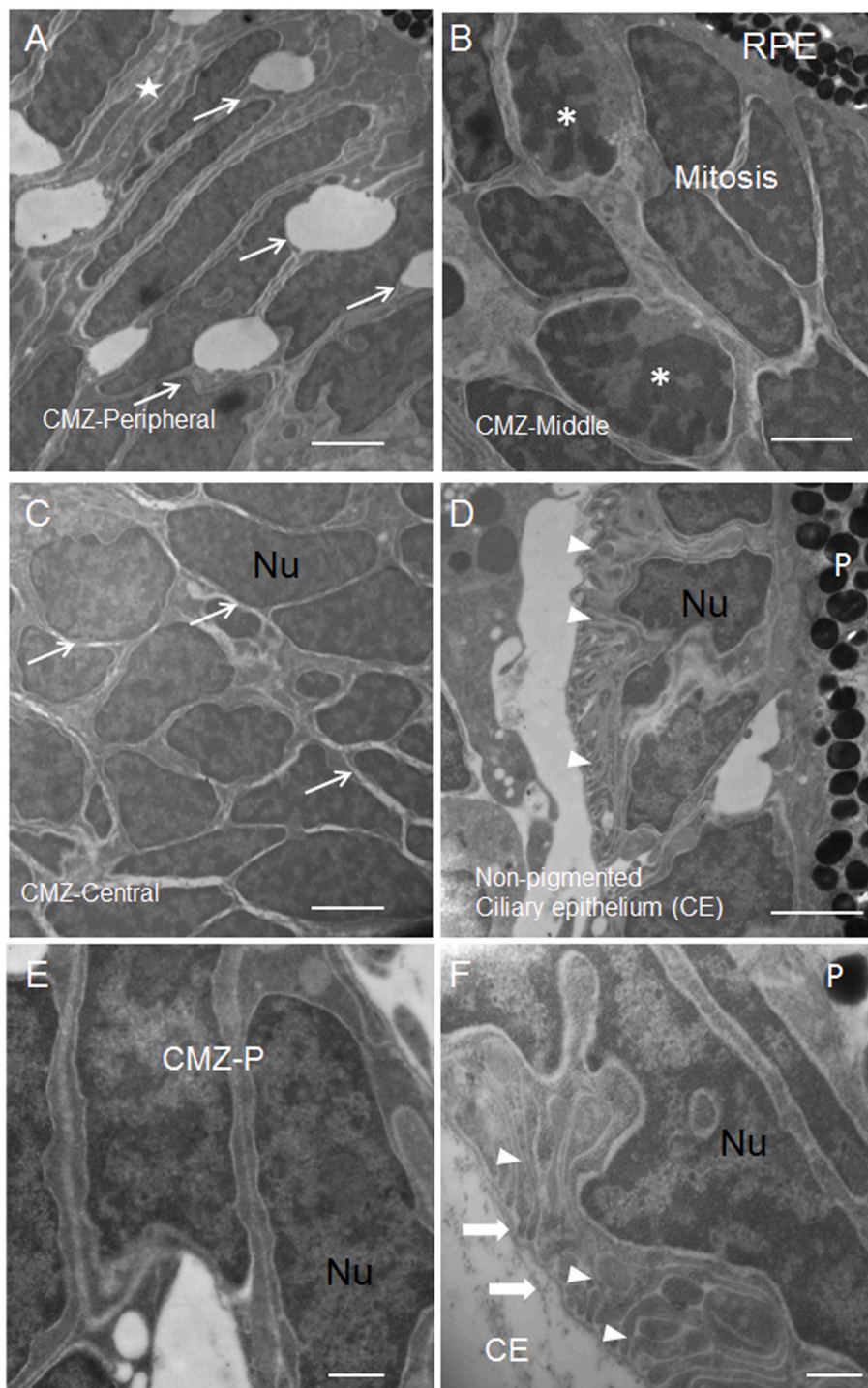
proliferative round yellow (IdU/CldU) nuclei, as shown in the orthogonal planes from confocal images (Fig. 5). However, unlike Sox2 expression, Pax6 was found diffusely in many other regions of the retina. In summary, the findings showing that Sox2 and Pax6 co-localize in the IdU/CldU nuclei and are strongly expressed in the CMZ indicate that this region is an active neurogenic niche.

### 3.3. Retinal glial cells and neurogenesis

We performed some experiments injecting 6 adult fish with a single dose of BrdU and allowing 30 days of survival under normal light conditions. In samples from these experiments, anti-BrdU recognition was combined with antibodies against radial glial markers, such as BLBP, or neuronal markers, such as HuC and  $\beta$  III tubulin, to detect neurogenesis. BrdU detection under normal light conditions showed a clear cell proliferation in the outer nuclear layer of the retina and in the peripheral, middle and central regions of the CMZ (Fig. 6A). Surprisingly, BLBP expression was strongly observed throughout the retina but with a very clear predominance in the region close to the CMZ up to  $\sim$ 200–400  $\mu$ m, decreasing towards to the center of the retina. There is also strong expression at the level of the optic nerve outlet. BLBP + cells were found in the CMZ-Central, little BLBP expression was seen in the CMZ-Peripheral, but was absent in CMZ-Middle. BLBP + cells were observed in the CMZ-Central co-localizing with BrdU (Fig. 6). The biggest BLBP + cell bodies were found in the INL, consistent with the description of the morphology of Müller radial glia. The BLBP + radial glial somas were located at different levels of the INL (level 2 in Fig. 6B and C) and had different morphology. The sizes of cell bodies were

variable between 8 and 20  $\mu$ m in their major axis, with their processes reaching all layers of the retina. The apical processes showed small terminal triangular feet aligned beyond the ONL (level 1 in Fig. 6B and C) as a closure integrated to the outer limiting membrane (OLM) (Fig. 6, small arrows). At the ONL level, small cells with BrdU + round nuclei were wrapped by thin BLBP + processes. These BrdU + cells could be progenitors generated by asymmetric division from Müller glia in the INL (Morris et al., 2008). Robust BLBP + terminal cell processes were found at the bottom of the ganglion cell layer (GCL) and seemed to envelope flattened nuclei cells (level 3 in Fig. 6B and C) with elongated nuclei (Fig. 6C, inset). Few of these cells were BrdU+ (Fig. 6A, C, white arrow). Cells with slight BLBP expression were also found in the ciliary epithelium (Fig. 6). Triple label of CldU/IdU/BLBP performed in fishes in permanent darkness showed that BLBP + cells are a proliferating population that co-localizes with some CldU/IdU yellow cells or only with CldU or IdU + nuclei (data not shown). Therefore, MG respond to darkness by increasing cell proliferation.

Neurogenesis in the retina was demonstrated by immunolabeling against the ribosomal neuronal marker HuC. This marker was strongly expressed in the GCL and the INL, whereas there was no expression in the ONL. Double label HuC/BrdU was found in the CMZ-Central and -Middle. HuC was expressed in neuroblasts and in mature neurons. The  $\beta$ III tubulin antibody recognizes the axonal microtubules and is strongly present in differentiating neurons. Double label signals of  $\beta$ III tubulin together with BrdU were found in the central and middle region of the CMZ, the ONL, the INL and the GCL (Fig. 7).

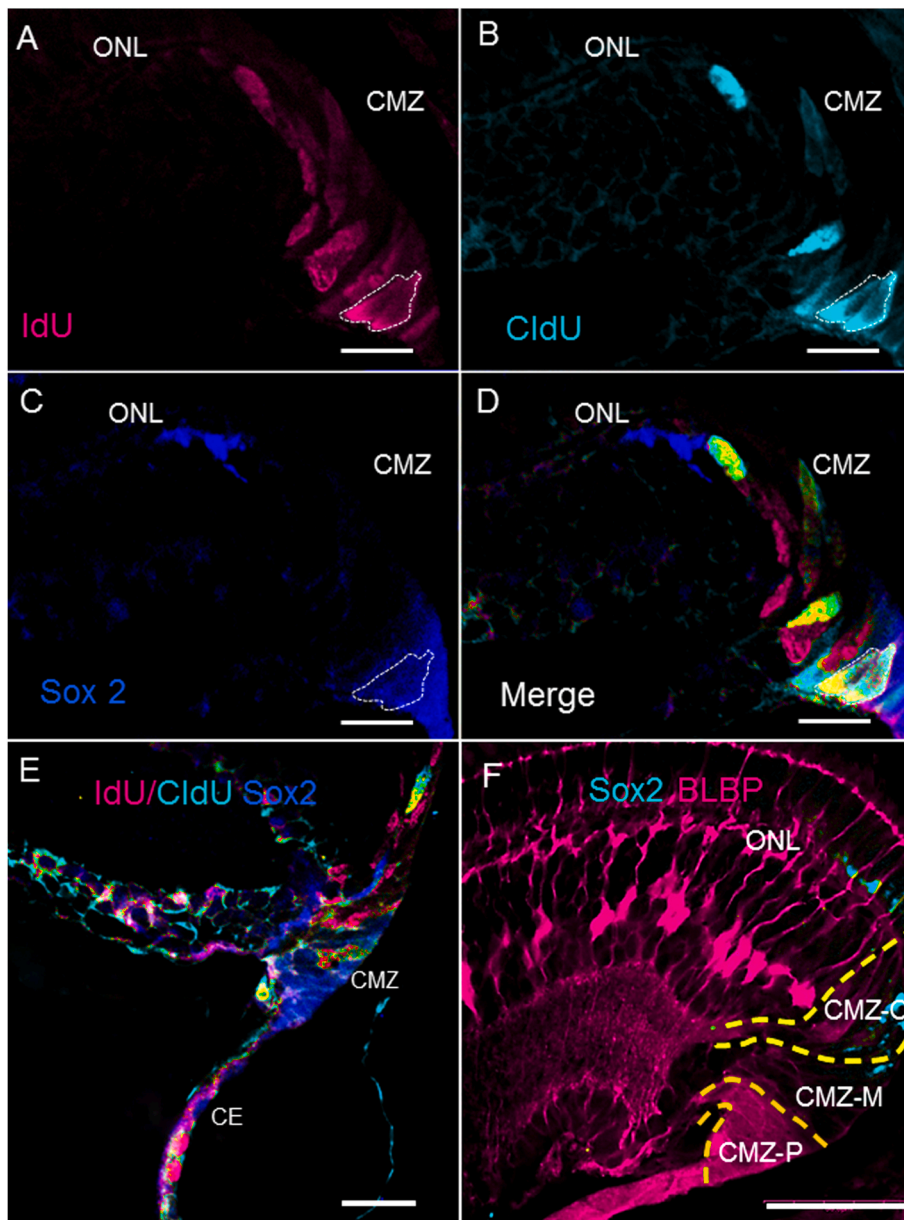


**Fig. 3.** Transmission electron microscopy images of cell types in the CMZ. A: The CMZ-Peripheral contains elongated cells with scanty cytoplasm, elongated nuclei and large intercellular spaces (white arrows). The star indicates the intricate cytoplasm interdigitations. B: Many cells in the CMZ-Middle present a proliferative phenotype with very condensed chromatin. Abundant cells in this region are in some phase of mitosis with visible condensed chromosomes (asterisks). C: Cells from the CMZ-Central possess polygonal shapes and show thin spaces between them (white arrows). D: The cells from the non-pigmented CE show a peculiar cytoplasm with plenty of interdigitations (white arrowheads) and irregular nuclei. E: A higher magnification of the elongated cells in the CMZ-Peripheral. F: A higher magnification of CE cells showing deep foldings in the cytoplasm membrane (arrowheads), which are in contact with the basal lamina (white arrows). Abbreviations: CMZ-ciliary marginal zone; CE-ciliary epithelium; RPE-Retinal pigmented epithelium; P- pigments; Nu-cellular nuclei. Scale bars in A–D: 2  $\mu$ m; in E, F: 1  $\mu$ m.

### 3.4. CldU/IdU double labeling in CMZ under normal light and darkness conditions

We used CldU and IdU labeling to obtain information about the origin, dynamics and migration of proliferating cells. Short (1 day, CldU) and long (30 days, IdU) experiments (Fig. 1) showed the temporal discrimination of the proliferating cell populations in the retina. Three populations of proliferative cells were identified: 1) CldU+ (cyan) fast cycling cells that were located in the three regions of the CMZ but less in the CMZ-Peripheral. No CldU + nuclei were found in the non-pigmented ciliary epithelium; 2) IdU+ (magenta) nuclei were found in all regions of the CMZ with less presence in the CMZ-Middle and frequently located in

the non-pigmented ciliary epithelium. IdU + cells were also found outside the CMZ, such as in the ONL and the GCL. As IdU + cells were recognized 30 days after IdU application, it is possible that these cells were found in the ONL and GCL due to their migration from their origin in the CMZ or to in situ generation; 3) CldU+/IdU+ (yellow) slow proliferating cells that do not migrate but reenter the cell cycle after 29 days as indicated by the uptake of the second proliferation marker (Fig. 8A). The analysis of confocal planes of retinal serial sections demonstrated the precise co-localization of CldU/IdU markers, yellow stained cells, that were mostly confined into the CMZ and very few were found in other regions of the neural retina. Some of the yellow cells co-localized with the stem cell markers Sox2 and Pax6 showed before



**Fig. 4.** IdU/CldU/Sox2 triple labeling. A–C: Immunohistochemistry against IdU (magenta), CldU (cyan) and Sox2 (blue) in the CMZ. Dotted white lines show a couple of cells with triple labeling. D: Merged images of A–C showing the co-localization of IdU, CldU and Sox2 (white) confirming the presence of stem/progenitor cells in the CMZ-P region. E: Confocal image of triple immunolabeling against IdU (magenta), CldU (cyan) and Sox2 (blue), showing Sox2 expression in the CMZ-C and part of the CMZ-P co-localizing with both cell proliferation markers. In the ciliary epithelium, Sox2 co-localizes with IdU + nuclei. F: Photomicrography showing BLBP (magenta) and Sox2 (cyan) double labeling without co-localization. Note that Sox2 signal is in the CMZ-C and in the adjacent retina in the ONL, whereas BLBP is strongly expressed in CE but negative in CMZ-M. Dashed yellow lines indicate the different CMZ regions. Abbreviations: CMZ-ciliary marginal zone; CMZ-P- CMZ-Peripheral; CMZ-M- CMZ-Middle; CMZ-C- CMZ-Central; CE-ciliary epithelium; ONL-outer nuclear layer. Scale bars: in A–D: 10  $\mu$ m; in E: 100  $\mu$ m; in F: 30  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 5).

Quantitation of the three cell populations identified showed a permanent cell proliferation activity in the CMZ under light conditions. The IdU + cell population (magenta) represents more than twice that of CldU+ (cyan), which is reasonable given that they could have divided more than once during the 30 days after IdU application. There is a significant proportion of CldU+/IdU + cells (yellow) that are around 36% of the total population of proliferative cells (Fig. 8C, plain columns).

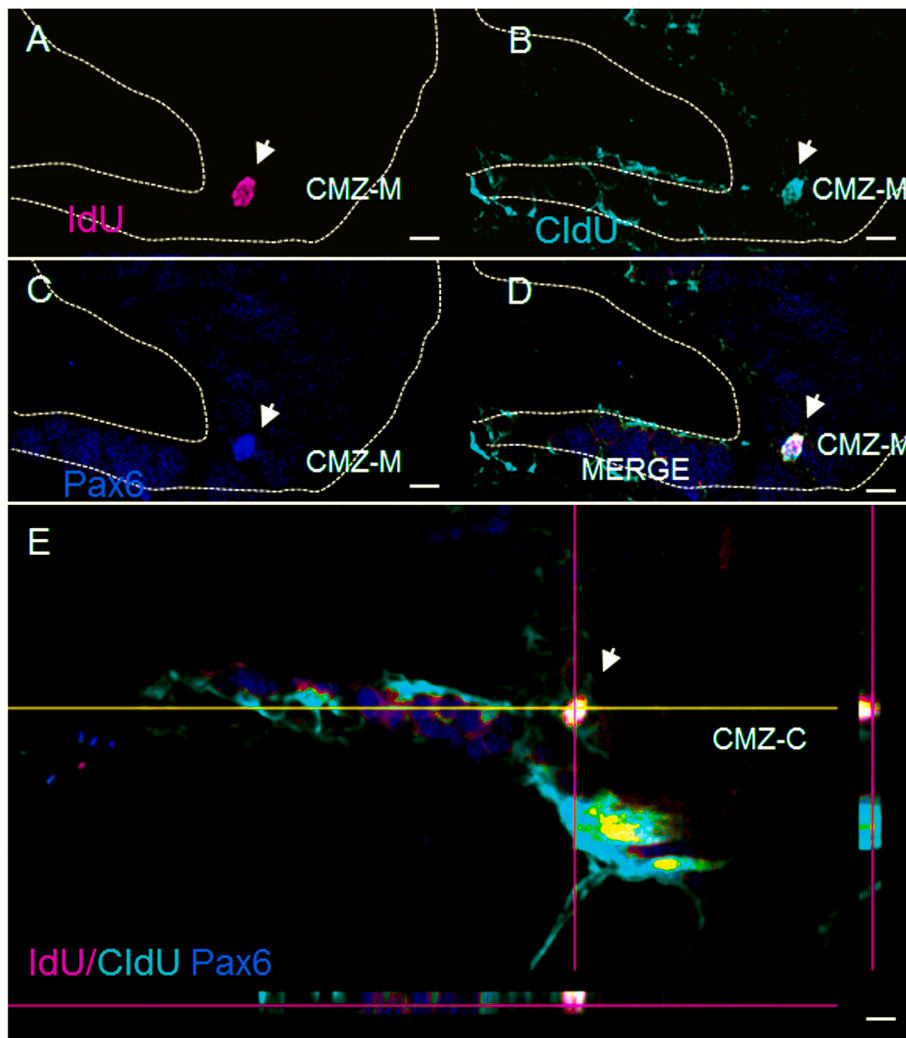
The experiments performed in darkness conditions showed a statistically significant increase in cell proliferation. Unlike what happens under normal light conditions, in darkness, cell proliferation was found in other retinal layers (Fig. 8A). Cell proliferation was found in the bipolar cell layer and GCL, but predominantly in the outer nuclear layer corresponding to the photoreceptor nuclei (Fig. 8B). Quantitation of the total number of IdU+ (magenta) and CldU+ (cyan) cells showed statistically significant increases in darkness over light conditions (Fig. 8C). However, the number of double-marked cells (IdU+/CldU+, yellow nuclei) did not show significant differences between light and dark

experimental conditions (Fig. 8C). Therefore, we propose that there is a constant population of proliferating double labeled cells (yellow) that include the stem/progenitor cells in the CMZ.

Analysis of rod photoreceptors using the XAP-2 antibody, and comparing the retina of fish exposed to light versus darkness, showed more rod rows in darkness, coincident with an increased cell proliferation in the ONL (Fig. 9A, C).

Analysis of cell proliferation in different retinal cell layers in light and dark conditions show significant increased number of BrdU + nuclei in the ONL as well as in other retinal layers (Fig. 10A and B). Semi-thin sections also revealed differences in the retinal cytoarchitecture with the ONL being  $\sim$ 30% thicker in darkness with respect to light. Under light conditions, the ONL shows a row of irregular cells with nuclei intensely stained with boraxic methylene blue that are intercalated among the nuclei of rods and cones. In darkness, the amount of these intensely stained cells tripled (Fig. 10C and D). Transmission electron microscopy in the region of rods and cones clearly show a higher density of rods in retinas from dark conditions, whereas in light, cones are the predominant cell type (Fig. 10E and F).





**Fig. 5.** IdU/CldU/Pax6 triple labeling. A–C: Immunohistochemistry against IdU (magenta), CldU (cyan) and Pax6 (blue) in the CMZ. D: Merged images of A–C showing the co-localization of IdU, CldU and Pax 6. White arrows point to a nucleus that was labeled with the three markers. White dotted lines frame the CMZ region and surroundings. E: Orthogonal planes of a 30 µm stack of the CMZ at the level of the triple labeled nucleus confirming co-localization of the three markers. This demonstrated the presence of a stem/progenitor cell in the CMZ-C. Abbreviations: CMZ-ciliary marginal zone; CMZ-C- CMZ-Central. Scale bars: 5 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

#### 4. Discussion

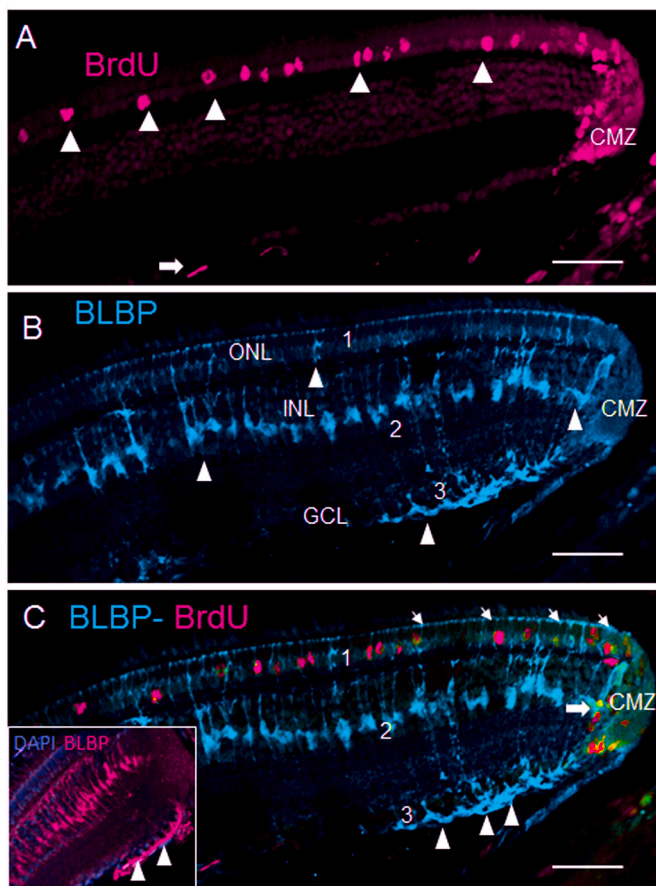
In this study we have addressed two main aspects of retinal plasticity: the regionalization of CMZ progenitor cells by morphological and molecular characteristics, and retinal cell proliferation dynamics under light and permanent darkness to simulate the light context of drying temporary puddles. Comparison of cell proliferation in the same animals under light conditions showed that the retina has greater proliferative activity than brain visual areas. In addition, CMZ has a stable population of stem cell candidates that is three times that of those previously detected in brain regions involved in visual processing (data previously published in [Torres-Pérez et al., 2017](#)). In darkness, proliferating cells in the layers of the differentiated retina (ONL, INL, and GCL) were abundant, while in the CMZ no significant changes were observed.

##### 4.1. Identification of retinal cell progenitors

The analysis by electron microscopy allowed morphological identification of four types of retinal progenitor cells: three in the CMZ and a fourth in the CE, all mostly differentiable by their distinctive morphology and expression of markers as indicated by immunolabelling ([Fig. 12](#)). The regionalization of the *Austrolebias* CMZ found in this work seems consistent with the data reported by [Raymond's group \(2006\)](#), who demonstrated, by gene expression analysis of the CMZ zebrafish, a regional compartmentalization in three sectors with different progenitor cells that express different proneural and stem cell genes. Molecular

studies reveal that cells from peripheral to central CMZ have different expression profiles of transcription factors, signaling molecules and cell cycle genes ([Agathocleous and Harris, 2009](#); [Cervený et al., 2012](#); [Johns and Easter, 1977](#); [Raymond et al., 2006](#)). The clonal analysis and imaging studies in adult zebrafish retina show that stem/progenitor cells reside at the extreme peripheral edge of the CMZ, and that, as the retina develops, they divide increasingly asymmetrically in the radial orientation in the same way that occurs in the embryo ([Wan et al., 2016](#)).

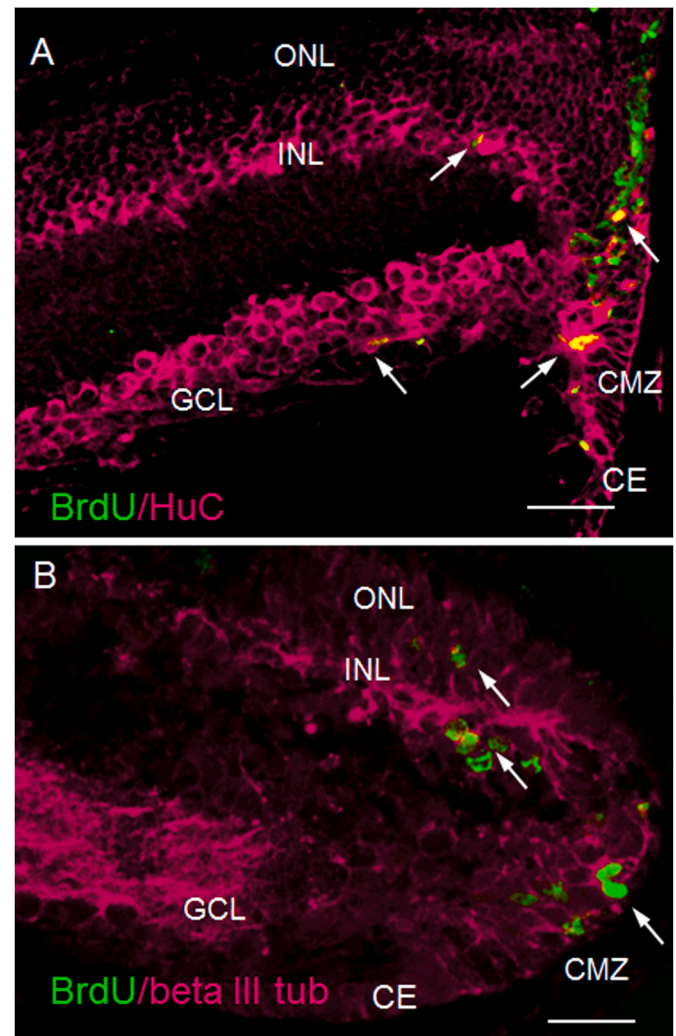
Another site recognized as a neurogenic niche is the ciliary non pigmented epithelium ([Otterson and Hitchcock, 2003](#); [Raymond et al., 2006](#); [Bernardos et al., 2007](#)). The cells in the *Austrolebias* CE have a very peculiar morphology. They proliferate and express Sox2, Pax6 as well as BLBP, suggesting they can be stem cells as proposed by [Raymond et al. \(2006\)](#). The cells in the CMZ-Peripheral are elongated, proliferate, and express Sox2, Pax6 and a slight BLBP signal ([Figs. 4, 5 and 12](#)). These cells are the putative stem cells that divide slowly and maintain the stem cell progeny ([Miles and Tropepe, 2021](#)). Between the elongated cells there are spaces that could be interpreted as resulting from the movements produced by cell proliferation and migration or as spaces through which signals flow to maintain the normal activity of the neurogenic niche. These spaces only appeared in the CMZ-Peripheral and were consistently found in all fish analyzed. Similar spaces between cells in movement were previously described in different neurogenic niches through diverse species ([Álvarez-Buylla and García-Verdugo, 2002](#)). Elongated cells from the CMZ-Peripheral remind of the neuroepithelial-like cells described in stem cell compartments of the



**Fig. 6.** Immunohistochemistry of BrdU and BLBP in Müller glia cells under light condition. **A:** Confocal image of retina at 30 days after BrdU injection. Many BrdU + nuclei (magenta, white arrowheads) are present in the CMZ and ONL. A flat nucleus positive to BrdU is found at the level of the GCL (white arrow). **B:** BLBP expression (cyan) was observed in the whole retina except in the CMZ-Central and CMZ-Peripheral. In level 1 there are BLBP processes that surround photoreceptor cell bodies (white arrowhead) and end contributing to the OLM layer. In level 2 there are the typical large cell bodies of Müller glia in the INL (white arrowheads) and in level 3 there are flattened cells surrounded by strong BLBP + processes (arrowhead). **C:** Merge from A and B images. Yellow color indicates co-localization of both markers some at the ONL level (1) but mainly in the CMZ-Peripheral. Small white arrows show the BLBP end processes contributing to the OLM layer. Inset shows double BLBP/DAPI (magenta/cyan) labeling to evidence the presence of cell bodies adjacent to the GCL (white arrowheads). Note that strong BLBP + processes surround flat nuclei. Abbreviations: CMZ-ciliary marginal zone; OLM-outer limiting membrane; ONL-outer nuclear layer; INL-inner nuclear layer; GCL-ganglion cell layer. Scale bars in A–C: 50  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

adult zebrafish brain that were postulated by Lindsey's group as "primed to modulate their cellular behavior upon change of the environment or sensory stimuli compared with radial glial cells" (Lindsey and Tropepe, 2014; Lindsey et al., 2019).

In *Austrolebias*, elongated cells with morphology similar to neuroepithelial-like cells, are present in many neurogenic niches of the brain related to growth of structures, like the dorsal and ventral extremes of the Tectum Opticum or transitional places between two brain structures such as that of the Telencephalon-olfactory bulb (Rosillo et al., 2016; Torres-Pérez et al., 2017). These cells seemed to be more proliferative compared to radial glia (Raymond and Rivlin, 1987; Otteson et al., 2001; Fernández et al., 2002; Lindsey et al., 2019). The CMZ-Middle is the highest proliferative region, with long-round cells in which CldU/IdU co-localize with Sox2 or Pax6. In the CMZ-Central there

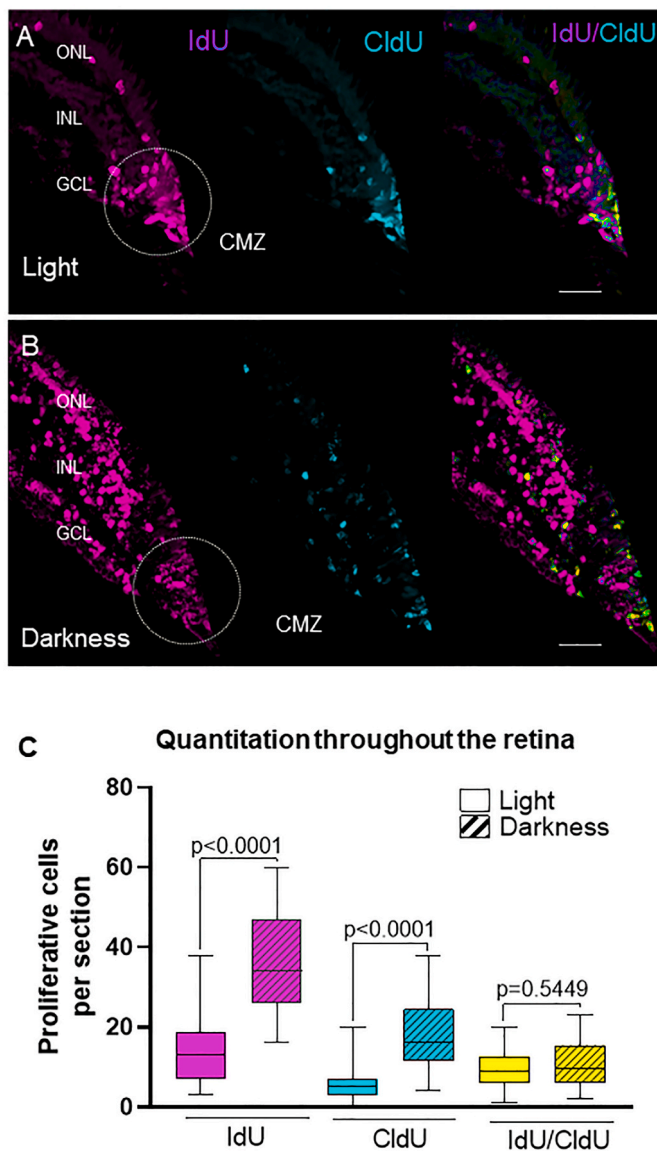


**Fig. 7.** Neurogenesis in the retina. **A:** Confocal image showing the double immunostaining of BrdU (green) and the neuronal marker HuC (magenta) at 30 days after BrdU injection. Double-labeling evidences newly born neurons in the INL, CMZ-Central and in the GCL (white arrows). **B:** Co-immunolabeling against BrdU (green) and the early neuronal marker beta III tub (magenta). The image shows the co-localization of both signals in the CMZ, ONL and INL as pointed by white arrows. Abbreviations: beta III tub-beta III tubulin; CMZ-ciliary marginal zone; ONL-outer nuclear layer; INL-inner nuclear layer; GCL-ganglion cell layer. Scale bars in A: 30  $\mu$ m; in B: 20  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

is strong expression of BLBP that co-localizes with BrdU (Fig. 6). CMZ-Central contains the cells that are the most similar to the rest of the retina, strongly suggesting that the newly generated cells in CMZ-C provide the cells that will integrate the different cell layers.

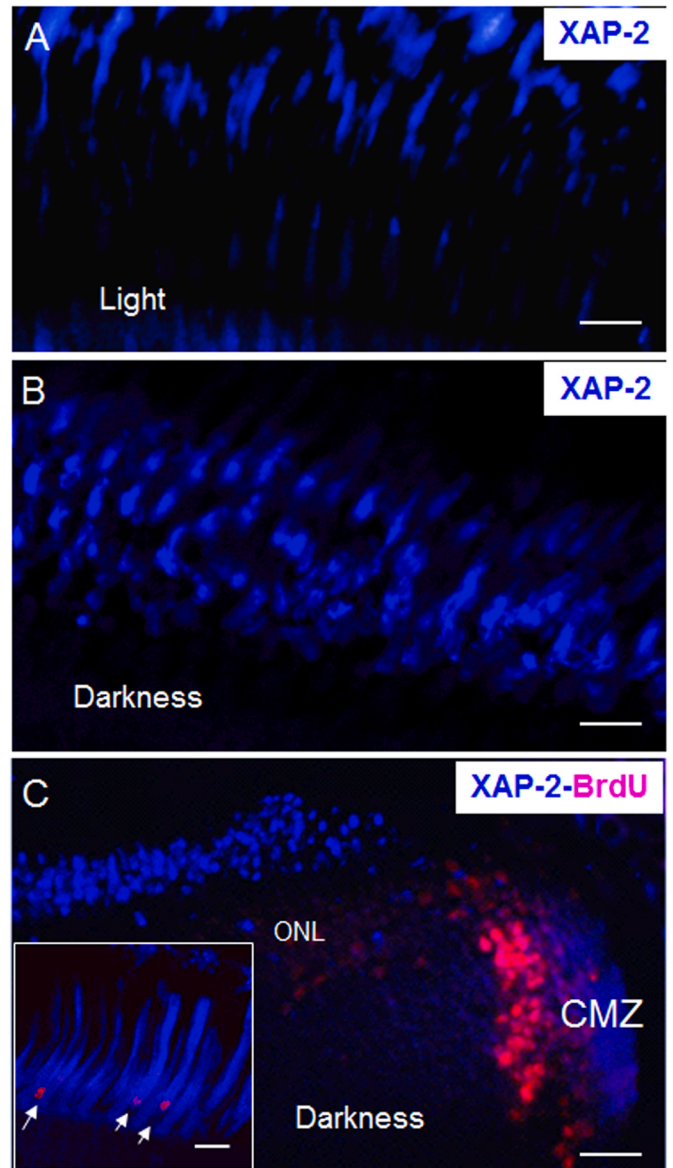
#### 4.2. Müller glia of the adult *Austrolebias* retina

The annual fishes of the *Austrolebias* genus increase their body size many times in a short period of time. The nervous system growth is accompanied by cell proliferation, where radial glial cells play a crucial role, as we previously detected (Torres-Pérez et al., 2017). Now, we found that in the retina of adult *Austrolebias* fish there is an essential presence of BLBP + radial glial cells not only in the central and peripheral CMZ but also widely distributed in most of the retina. However, in the zebrafish, after two months of life the BLBP + Müller glia is reduced and observed only in the CMZ (Johns and Fernald, 1981; Raymond et al., 2006). The comparative analysis among vertebrates shows



**Fig. 8.** IdU/CldU double labeling experiments under light and darkness. A: Confocal images of a 30  $\mu\text{m}$  stack showing IdU+ (magenta) and CldU+ (cyan) nuclei as well as the merged (yellow) image in normal light conditions. Note that cell proliferation is predominantly found in the CMZ (dashed white circle), but there are some proliferating nuclei in the ONL. B: Confocal images of a 30  $\mu\text{m}$  stack showing IdU+ (magenta), CldU+ (cyan) and IdU+/CldU+ (yellow) nuclei under darkness, evidencing that cell proliferation is widespread in all cell layers of the retina. C: The chart shows the averaged total number of IdU+, CldU+ and IdU+/CldU+ nuclei under light and darkness conditions throughout the retina. Under light conditions, the quantitation shows an important population of progenitor yellow cells that remains inside the CMZ and reenters the cell cycle in a month. In darkness, there were statistically significant increases in IdU and CldU cells in all the retinal cellular layers but double.labeled cells remained unchanged in both conditions. The p values were determined with Kruskal Wallis test (Statistic = 50.87) and Dunn's multiple comparisons among different experimental groups. Significance level was determined at  $p < 0.05$ . Abbreviations: ONL-outer nuclear layer, INL-inner nuclear layer, GCL-ganglion cell layer; CMZ-ciliary marginal zone. Scale bar in A-B: 50  $\mu\text{m}$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

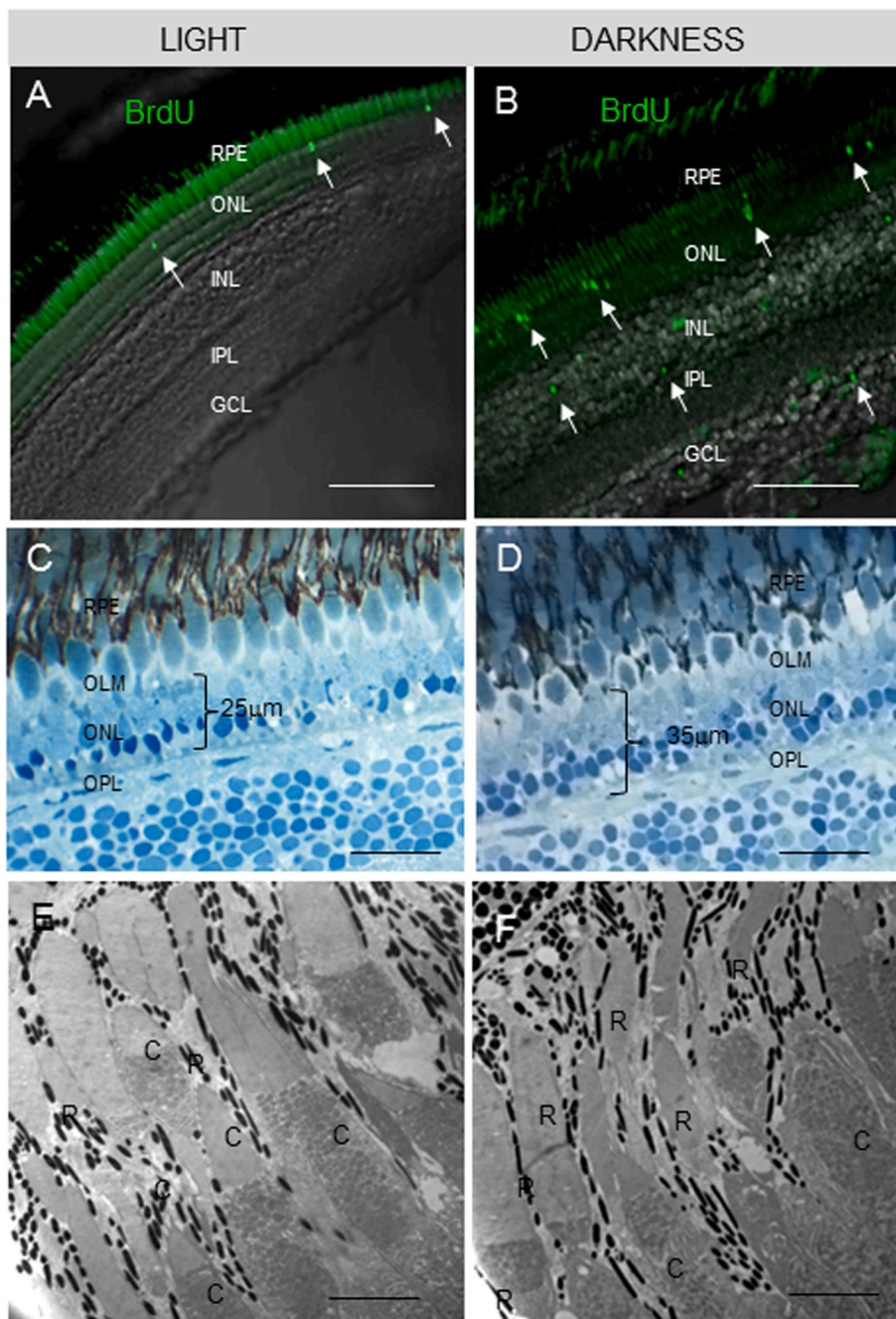
that the CMZ has been gradually diminished during vertebrate evolution (Kubota et al., 2002). Probably *Austrolebias* fishes are challenged by environmental conditions and this implies an accelerated growth to complete their short life cycle in a very changing habitat. Thus, we



**Fig. 9.** Darkness induced increased density of rod photoreceptors. A: Representative confocal image showing the immunoreactivity against the rod photoreceptor cell type specific marker XAP-2 (blue) under light conditions. B: Confocal image of the same marker under darkness evidencing stronger signal and increased density of rod photoreceptors. C: Co-immunostaining of XAP-2 (blue) and BrdU (magenta) under dark conditions evidencing increased cell proliferation in the CMZ and in other retinal regions. Inset: XAP2+ rods (blue) that show BrdU + nuclei (magenta) indicating the generation of new rods (arrows). Abbreviations: CMZ-ciliary marginal zone; ONL-outer nuclear layer. Scale bars: in A, B: 50  $\mu\text{m}$ , C: 100  $\mu\text{m}$ , inset: 10  $\mu\text{m}$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

propose that glial cells act as neuronal progenitors that might be in a latent proliferative state, contributing to growth and responding to environmental demands as has been previously reported in zebrafish (Fausett and Goldman, 2006). It could also be possible that there is a population of latent pluripotent stem cells (double label "yellow nuclei", not identified yet, some with BLBP + cells), as has been proposed for the adult zebrafish retina by Cameron (2000).

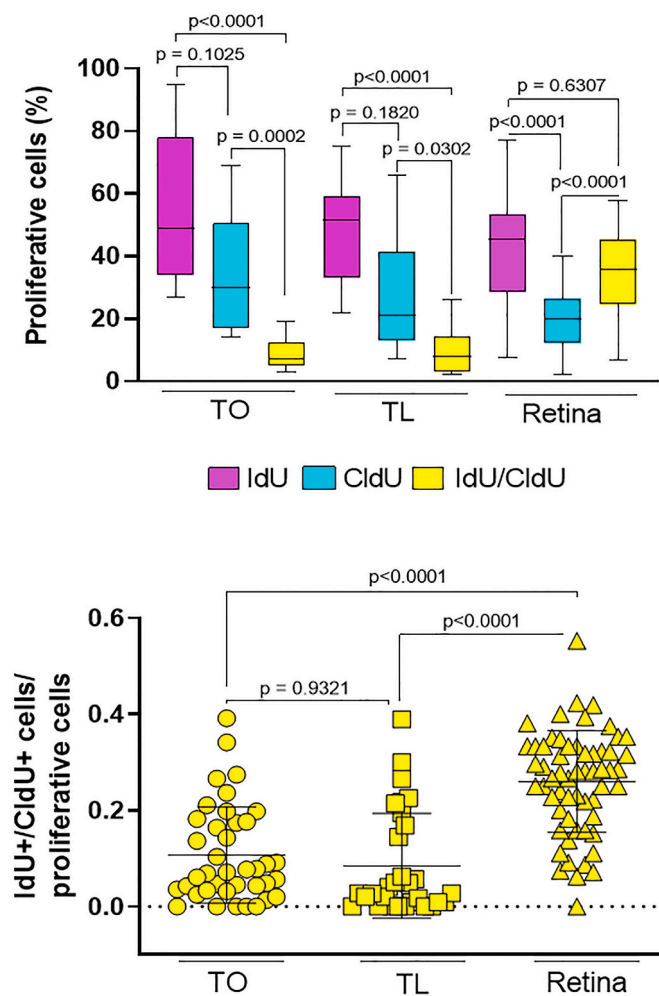
We found BLBP + cell bodies in the central and peripheral CMZ and in the INL at different levels. BLBP expression was found around cell somas in ONL, which can be cell processes that terminate over the ONL, forming the outer limiting membrane and contributing to the strong



**Fig. 10.** Differences in cell proliferation and in the cytoarchitecture of the retina in light and darkness. A, B: Representative confocal images showing a single focal plane of BrdU immunofluorescence (green) together with differential interference contrast illumination in equivalent sections of the retina in light (A) and darkness (B) experiments. Under light conditions few nuclei positive to BrdU are found only in the ONL. In darkness, there are more abundant BrdU + nuclei in the ONL and also in the INL and the GCL (white arrows). C,D: Semi-thin sections stained with borax methylene blue to evidence the retinal cytoarchitecture under light (C) and darkness (D) conditions. Note that in darkness, the retinal ONL layer is around 30% thicker than in light. In darkness, the ONL also has two or three rows of cells with more intensely stained nuclei than in light conditions. E,F: Electron microphotograph of the retina at the level of cones (C) and rods (R) showing a different distribution of both cell types under light (E) and darkness (F) with a prevalence of cones in light and of rods in darkness. RPE pigments are seen as dark grains surrounding the outer segment of rods and cones. Abbreviations: RPE- Retinal pigmented epithelium; ONL-outer nuclear layer; OPL-outer plexiform layer; OLM-outer limiting membrane; INL-inner nuclear layer; IPL-inner plexiform layer; GCL: ganglion cell layer. Scale bars in A, B: 100  $\mu$ m; in C,D: 30  $\mu$ m, in E, F: 5  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

linear density of OLM adherent junctions. Small BLBP + cells found in the ONL might be cell progenitors (rod precursors) originated from Müller glia in the INL that migrated to the ONL as described previously in zebrafish (Fausett and Goldman, 2006). Müller glial cells have been shown to be also able to undergo a reprogramming process to reacquire a stem/progenitor state, allowing them to proliferate and generate new neurons for repair following retinal damages, a process that is more common in fishes than in mammals (Otterson and Hitchcock, 2003; Fausett and Goldman, 2006; Bibliowicz and Gross, 2009; Gorsuch et al., 2017). Comparison of the Müller glia of zebrafish with the regenerative capacity of mouse Müller glia suffering retinal degeneration revealed several new categories of biologic responses that could promote endogenous retinal regeneration, including Wnt, NF- $\kappa$ B signaling, PGE2 synthesis, expression of central clock genes and signaling metabolic pathways associated with stem cells, which are shared in both biological

models (Hamon et al., 2016; Sifuentes et al., 2016). Unlike that of mammals, the zebrafish retina responds to neuronal damage by proliferation of Müller glia, which can replace all neuron types, including photoreceptors. Müller glia are located so that they can monitor the entire retina and contribute to retinal structure and function (Wan and Goldman, 2016; Iribarne, 2019). In adult *Austrolebias*, BLBP + Müller cells are much more abundant than in adult zebrafish. In this context it is possible that *Austrolebias* Müller glia might be the stem/cell progenitors that can activate and respond to demands associated to lower luminosity, which facilitates and increases the proliferation in INL, ONL and GCL. The immunohistochemical detection of CldU/IdU + positive cells in INL together the BLBP+ was frequently found in darkness condition (data not shown). Recent data from adult zebrafish dark-adapted retinas have shown increased rod precursor cell proliferation while the number of proliferating Müller glia did not significantly change (Lahne et al.,



**Fig. 11.** Quantitative analysis and comparison of cell proliferation in TO, TL and retina. A: Different proportions of the proliferative cell populations in the TO, TL and retina, showing that TO and TL have the same distribution among IdU+ (magenta), CldU+ (cyan) and double labeled cells (yellow). However, in the retina, the population of double labeled cells (yellow) is larger, denoting that a significantly higher percentage of cells re-enter the cell cycle and uptake both markers. B: Analysis of the double marked cell population (yellow) in TO, TL and retina. Note that mean values in retina almost reached 40%, more than three times that of the brain regions analyzed (around 10%). It is important to consider that the analysis in all regions was performed on the same animals. Significant differences and p values were determined by Kruskal Wallis test (Statistic = 149.5) with Dunn's comparisons among groups. Abbreviations: TO-Tectum Opticum; TL- Torus Longitudinalis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2019). Our results indicate that there is an increase in cell proliferation in the ONL in dark conditions. It is likely that rod precursors are the proliferating population that increases the thickness of the ONL observed in the semi-thin sections. However, we also believe that in the INL there is an increase in the proliferation of BLBP + glia that gives rise to new rod precursors.

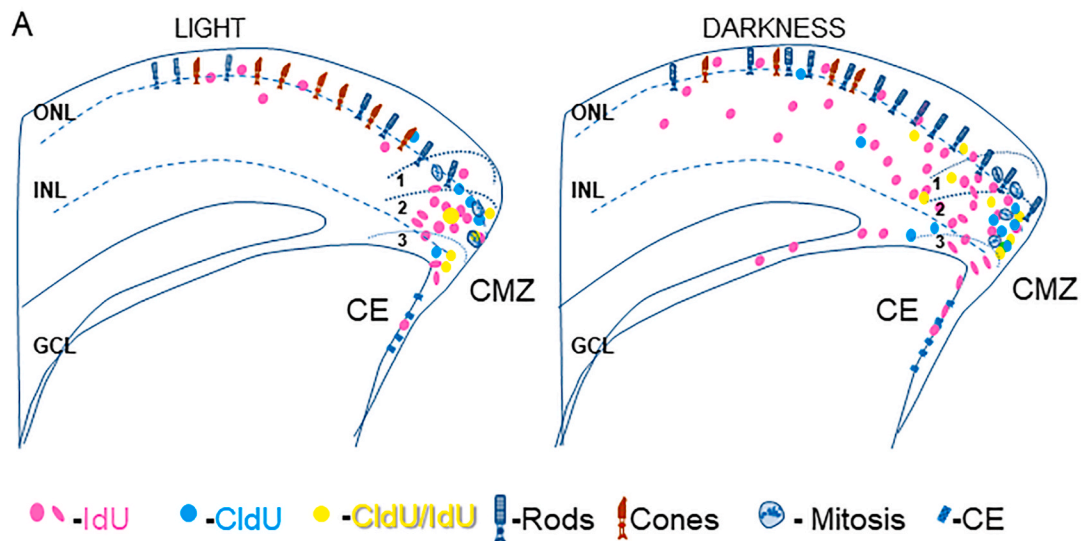
#### 4.3. Retinal cell proliferation dynamics under light and darkness

Under normal light conditions we found that *Austrolebias* CMZ was almost the exclusive proliferation zone, with spare cell proliferation in the INL, ONL and GCL. In normal light conditions scanty cell proliferation was reported in the INL of the mature retina in rainbow trout *Onchoryncus mykiss* (Julian et al., 1998). In *Austrolebias*, proliferating

cells were found in all CMZ regions with a predominance of double IdU+/CldU+ cells in the CMZ- Middle. Under permanent darkness, we found a greater number of proliferative cells throughout all retinal cell layers with significant increases in total number of IdU+ and CldU+ cells (Fig. 8). However, the proportion of the yellow proliferative cells (putative stem/progenitor cells) in the CMZ was stable and had the same absolute number that under light conditions, implying that the pool of candidates to be the stem/progenitor cells in CMZ seemed constant. Remarkably, in darkness, we also found cell proliferation in all layers of the retina with special emphasis on the ONL and INL, sustaining the idea that the greater generation of rods found (Figs. 9 and 10) may compensate higher demands when luminosity decreases. This is in accordance with many studies conducted in different species demonstrating that a greater number of rods is necessary to improve vision under low luminosity (Locket, 1970; Wagner et al., 1976; Chiu et al., 1995; Ali et al., 1990; Nag and Bhattacharjee, 2002; Lahne et al., 2019). As an example, the *Haplochromis burtoni* fish showed a daily rhythm in the retinal cell proliferation, being higher at night and in dark conditions (Chiu et al., 1995). Our results from darkness experiments suggest that the demand of rods increases to compensate for the low level of ambient light and consequently the cell proliferation in all the layers of the retina increases to complete the neural circuits that allow for the proper functioning of the rods. Therefore, we can suggest that retinal plasticity in *Austrolebias* is latent and could mainly rely on other neurogenic niches different from the CMZ, such as the Müller glia in the INL or progenitor cells from the ciliary non pigmented epithelium. Behavioral studies carried out by our group in other species of *Austrolebias* (*A. reicherti*) showed that females kept for 4 months in the dark improved visual acuity at the time of selecting the male for mating (Berrostequieta Rodríguez, 2020). On the other hand, the analysis of the adult retina by electron microscopy of *A. charrua* shows a distribution of the melanosomes of the pigmented epithelium around the photoreceptors presenting characteristics of both dark- and light-adapted fishes. This provides an additional element that helps to understand the adaptive plasticity at different levels that *Austrolebias* species exhibit under the pressure of their changing environment (Berrostequieta et al., 2018). In the mammalian ciliary epithelium, cells can be induced to acquire restricted progenitor cell-like characteristics in response to exogenous signals (Fischer et al., 2014; Sifuentes et al., 2016). Several studies show that the mature retina may have a conserved propensity for homeostatic growth and plasticity in response to adaptation and response to damage or dysfunction, thanks to the regulatory activity of the CMZ (Miles and Tropepe, 2021).

#### 4.4. Comparison of proliferation rates between brain visual regions and CMZ of *Austrolebias charrua* fish

We have evaluated cell proliferation and different populations of proliferating cells under normal light conditions in the CMZ and brain regions related to visual information processing (Torus Longitudinalis and Tectum Opticum) in the same experimental animals. Results obtained showed a brain population of putative stem/progenitor cells (IdU+/CldU+ cells) of about 10% of all detected proliferating cells. This proportion was maintained in different regions of the brain (Torres-Pérez et al., 2017). Analysis of the same proliferative cell populations in the retina showed that stem/progenitor cell candidates were more than 35% of the total proliferating cells in the CMZ (Fig. 11). Therefore, cell proliferation rate in the ciliary marginal zone is more than three times higher than that found in the brain neurogenic niches responsible for the processing of visual information. We suggest that increased CMZ cell proliferation rates may account for higher retinal growth rates when compared to the rest of the body including the brain. Further studies are directed to analyze the response of brain visual areas to decreased luminosity. These results will collaborate to the understanding of retinal plastic and eventually reparative capabilities. In Fig. 12 the most relevant data of this work is summarized.



**B Summary**

	Cell morphology	BrdU	CldU	IdU	CldU/IdU	BLBP	Sox2	Pax6
1 CMZ-Middle	Long, 6-12 μm	+++	+++	++	+++	--	++	++
2 CMZ-Central	Round, 5-8 μm	++	++	+	+	+++	+	-
3 CMZ-Peripheral	Long, 7-18 μm	++	++	+	+	+	+	++
Non-pigmented CE	Square, 6 μm	+	--	++	+	+	+	+
ONL	Long, 25-30μm	++	+	++	+	-	++	+
INL	Round, 6-8 μm	+	+	+	+	+++	-	-
GCL	Round, 10-12 μm	+	-	+	-	++	-	-

**Fig. 12.** Summary of the main results obtained. A: Schemes show the differences found in the retina when comparing dark with light conditions. Note the increased cell proliferation in all layers of the retina, as well as more rods in the ONL. Colors represent different labeled nuclei: IdU (magenta), CldU (cyan) and double labeled cells IdU/CldU (yellow). Abundant mitotic figures were found in the CMZ-Middle. Square cells are found in the non-pigmented CE. B: Table summarizing the main cell features in each retinal zone, and a semiquantitative evaluation (labeled by + and - indicating level of expression) of stem/progenitor cells and cell proliferation under light condition. In darkness, increased cell proliferation was the main change observed. Abbreviations: CMZ-ciliary marginal zone; CE-ciliary epithelium; INL-inner nuclear layer; ONL-outer nuclear layer; GCL: ganglion cell layer. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**5. Conclusion**

We can conclude that the retina of adult *Austrolebias charrua* has several potential places where cell proliferation could occur. The CMZ is a neurogenic niche par excellence that has a higher proliferation rate than the brain; it retains a stable stem cell population, responsible for retina growth. The great plasticity of the retina is evident when the demand for more photoreceptors is urgent. In this case, the radial glia outside the CMZ may act as active progenitors capable of generating new rod precursors and cells reinforcing rod-circuits in other retinal layers. These processes may occur in dark puddles when reproduction is crucial to preserve the species before the puddles are completely dry.

This may explain why, in adult retina of *Austrolebias* fish, the radial glia is highly present: they are capable of reactivating to generate all types of cells when the environmental pressure requires so. This speaks of a peculiar plasticity in the retina at the service of a species pressed by time as annual *Austrolebias charrua* fish are. Finally, our studies may collaborate to the better understanding of retinal cell proliferation plastic response once challenged by environmental light variations.

**Data accessibility statement**

Data are available upon substantiated request to the corresponding authors.

**CRedit authorship contribution statement**

**Inés Berrostequieta:** Conceptualization, Investigation, Methodology, Data curation, Validation, Writing – review & editing, preparation, Visualization, Software. **Juan Carlos Rosillo:** Conceptualization, Investigation, Methodology, Data curation, Validation, Writing – review & editing, preparation, Visualization, Software. **María Laura Herrera:** Investigation, Methodology, Data curation, Validation, Writing – review & editing, preparation, Visualization, Software. **Silvia Olivera-Bravo:** Conceptualization, Formal analysis, Validation, Writing – original draft, preparation, Writing – review & editing, Visualization, Software. **Gabriela Casanova:** Investigation, Methodology, Validation, Writing – review & editing, Visualization, Software. **Vicente Herranz-Pérez:** Writing – original draft, Writing – review & editing. **José Manuel García-Verdugo:** Writing – original draft, Writing – review & editing. **Anabel Sonia Fernández:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crneur.2022.100042>.

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