Successful optic nerve regeneration in the senescent zebrafish despite age-related decline of cell intrinsic and extrinsic response processes

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Abstract

Dysfunction of the central nervous system (CNS) in neurodegenerative diseases or after brain lesions seriously affects life quality of a growing number of elderly, since adult mammals lack the capacity to replace or repair damaged neurons. Despite intensive research efforts, full functional recovery after CNS injuries remains challenging, especially in an aging environment. As such, there is a rising need for an aging model in which the impact of aging on successful regeneration can be studied. Here we introduce the senescent zebrafish retinotectal system as a valuable model to elucidate the cellular and molecular processes underlying age-related decline in axonal regeneration capacities. We found both intrinsic and extrinsic response processes to be altered in aged fish. Indeed, expression levels of growth-associated genes are reduced in naive and crushed retinas, indicative of neuronal aging, and the injury-associated increase in innate immune cell density appears delayed, suggesting retinal inflammaging. However, despite a clear deceleration in regeneration onset and early axon outgrowth, these cellular senescent processes do not withstand successful reinnervation of the optic tectum and full visual recovery.

Keywords

Aging, Zebrafish, Axonal regeneration, Central nervous system, Cellular senescence, Inflammation

List of abbreviations

CNS	Central nervous system
dpi	days post injury
Gap-43	Growth-associated protein 43
IHC	Immunohistochemistry
ON	Optic nerve
ONC	Optic nerve crush
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PNS	Peripheral nervous system
RGC	Retinal ganglion cell

1. Introduction

Considering the increasing number of elderly in the world's population today, developing effective treatments for neurodegenerative diseases is one of the biggest challenges in modern medical research. Currently, their irreversibility and lack of effective treatments impinge a high social and economical burden, and urge the development of therapeutic strategies to ameliorate and/or resolve neuropathology. This could in part be achieved by triggering endogenous stem cells to form new neurons, i.e. neuronal regeneration, or protect damaged cells/axons from undergoing cell death, i.e. neuroprotection. In both cases, new and/or injured neurons have to regrow a functional axon and restore synaptic contacts, thereby establishing axonal regeneration. Yet, regenerative capacities are very limited in the adult mammalian CNS, and they are affected by age. Indeed, several studies in rodents have shown that the age at the time of neuronal damage determines neuronal survival rate (Wang et al., 2007) as well as axonal regrowth potential (Geoffroy et al., 2016; Verdu et al., 2000). As such, most research into mammalian CNS regeneration using young animal models might eventually fail successful translation into therapeutic approaches applicable in an aging environment. Clearly, well-characterized aging models, in which regeneration can be studied in a senescent environment, are highly needed.

Zebrafish (*Danio rerio*) are one of the most versatile animal research models. Their robust regenerative capacity continues to drive comparative research, and a remarkably high similarity in the signaling pathways underlying CNS regeneration in mammals and zebrafish has already been unraveled (Becker and Becker, 2014; Elsaeidi et al., 2014; Fleisch et al., 2011; Wang and Jin, 2011). Now, they are also increasingly valued in gerontology, as it is recently established that zebrafish - like most vertebrates - are subject to processes of gradual aging (Gerhard, 2003; Gerhard and Cheng, 2002; Keller and Murtha, 2004; Kishi, 2011; Kishi et al., 2003; Van houcke

et al., 2015). Indeed, also in these small teleosts, reparative capacities diminish with aging. Yet, while the neurogenic potential in the brain (Edelmann et al., 2013) and axonal regeneration capacities of the peripheral nervous system (PNS) (Graciarena et al., 2014) are shown to decline with age, the underlying molecules/pathways contributing to this phenomena remain elusive.

The visual system forms a powerful model to study neuronal survival and axonal regrowth in the CNS. It is relatively accessible, and both structurally and functionally well conserved among vertebrates. As in mammals, the zebrafish retina is a layered structure and projects visual signals towards the brain via the optic nerve (ON), containing the axons of the retinal ganglion cells (RGCs). However, in contrast to mammals, the adult zebrafish visual system shows a high reparative capacity. When the zebrafish ON is injured, a substantial part of the RGCs is able to survive and regenerate (McCurley and Callard, 2010b; Zhou and Wang, 2002; Zou et al., 2013). After a short injury response, the RGCs start preparing for axonal outgrowth by upregulation of growth-associated genes from 1 till 4 days post injury (dpi) (Lemmens et al., 2016; McCurley and Callard, 2010b). Subsequently, the RGC axons regrow towards the optic tectum and reinnervation is completed between 5 and 14 dpi (Bhumika et al., 2015; Kaneda et al., 2008; Lemmens et al., 2016). Finally, full recovery of visual function is achieved by 20-25 dpi, although synaptic refinement of the retinotectal topography is known to continue until 30 to 90 days after injury (Becker and Becker, 2008; Kaneda et al., 2008; McCurley and Callard, 2010b; Zou et al., 2013).

In this study, we examine the impact of aging on the axonal regeneration potential of the zebrafish retinotectal system, and show that despite a significant delay in the initiation of axonal regeneration and early outgrowth, aged zebrafish are capable of successful ON regeneration. Indeed, regardless of age-related alterations in both intrinsic and extrinsic cellular response processes, zebrafish can reestablish visual function after optic nerve crush (ONC), even upon

aging.

2. Material and methods

2.1. Fish

Zebrafish (*Danio rerio*) on an AB background, either wild-type or the *Tg(Gap-43:eGFP)* and *Tg(coro1a*:*eGFP*) lines, were used and maintained under standard conditions (Westerfield, 2000). The ages chosen for ON regeneration analyses represent young and aged adults of 5, 12, 18, 24 and 36 months old of either sex. Both fish density and food supply were under strict regulation over the entire life span of the fish, resulting in uniform fish sizes within each age group. Fish size was measured as the standard length, i.e. the distance from the snout to the base of the tail fin, and ranged from 29,67 ± 1,12 mm in 5-month-old fish to 36,20 ± 0,73 mm in 36-month-old individuals. All animal experiments were approved by the KU Leuven Animal Ethics Committee and executed in strict accordance with the European Communities Council Directive of 20 October 2010 (2010/63/EU).

2.2. Optic nerve crush (ONC)

ON injury was performed as previously described (Bhumika et al., 2015; Lemmens et al., 2016; Zou et al., 2013). Briefly, zebrafish were anesthetized in 0.02% buffered tricaine (MS-222, Sigma Aldrich). Sterile forceps (Dumont No. 5, FST) were used to remove the connective tissue surrounding the left eye, allowing to lift the eyeball out of its orbit and expose the ON. By carefully placing the sterile forceps around the (left) ON while avoiding damage to the ophthalmic artery, a crush of 10 s at 500 µm distance of the ON head was performed, which was kept fixed at all ages and fish sizes. Of note, bilateral ON lesions were used to evaluate optokinetic responses (Elsaeidi et al., 2014; Zou et al., 2013).

2.3. Tracing and quantification of tectal (re)innervation

Reinnervation of the optic tectum was assessed using biocytin as an anterograde tracer (Fig. S1), all as previously described (Bhumika et al., 2015; Lemmens et al., 2016). In short, biocytin, taken up by the regenerated axons, was visualized within 50 µm coronal vibratome sections by means of the Vectastain ABC kit (Vectastain laboratories), using diaminobenzidine as a chromogen. Imaging was carried out at 10x using a microscope Zeiss Imager Z1. Tectal (re)innervation was quantified via an in house developed Image J script, in which the biocytin-labeled area was measured using a preset threshold. Next, axonal density was defined as the ratio of the biocytin^{+}-area to the area of reinnervation, being the stratum fibrosum et griseum superficiale and the stratum opticum of the optic tectum. Per fish, tectal (re)innervation was evaluated on at minimum four sections containing the central optic tectum (Wulliman et al., 1996) and averaged. Importantly, axonal density in naive young adults was considered maximal and set as a 100% reference value. (Re)innervation values of all other conditions were expressed relative to this reference value.

2.4. Optokinetic response test

To evaluate visual recovery after ON injury, adult zebrafish were subjected to an optokinetic response test at various time points after bilateral ONC. Fish were anesthetized briefly in 0.02% buffered tricaine to enable positioning in a custom-made glass chamber. Next, a continuous water flow was provided, reawakening the fish and allowing them to breathe while immobilized. The flow-through chamber was then placed in an OptoMotry device (CerebralMechanics) for visual stimulation (Mueller and Neuhauss, 2010). Visual acuity was assessed by determining the maximal spatial frequency of the fish, while velocity and contrast were kept fixed at 10 deg/s and 100%, respectively. After an adaptation period of 40 seconds, each trial was initiated with a spatial frequency of 0.02 (c/d), which increased stepwise following a staircase model.

2.5. Visualization and analysis of axonal elongation

Early outgrowth and elongation of RGC axons were assessed by visualization of regrowing RGC axons in horizontal visual system sections of *Tg(Gap-43:eGFP)* fish (Diekmann et al., 2015; Udvadia et al., 2001). Thereto, fish were euthanized in 0.1% buffered tricaine and transcardially perfused with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). The complete visual system as a whole was dissected, fixed overnight and processed for horizontal cryosectioning (10 µm sections). Imaging of sections was carried out using an Olympus FV1000 confocal microscope at 20x. The distance grown by the axons, being the length between the crush site and the axonal growth tips, was determined on a minimum of four sections containing the complete ON tract, and averaged per fish.

2.6. Retinal explant assay

To assess the outgrowth potential of young and aged RGCs *in vitro*, retinal explants were made at 3 days post ONC, according to previously published methods (Veldman et al., 2007). The explants were allowed to grow for 24 hours in a humidified, ambient air incubator at 28°C, and then fixed in 4% PFA. Neurites were visualized via IHC, using mouse anti-α-tubulin (1:300, Sigma Aldrich, T9026) and Alexa-488 conjugated secondary antibody (1:200, Invitrogen) for detection. The explant body was stained using DAPI. Explants were then imaged with an Olympus FV1000 confocal microscope at 4x. Neurite outgrowth was analyzed with a previously published custom script (Gaublomme et al., 2014), which makes use of a size selection tool to extract neurites attached to the explant body. As such, the total neurite area was measured and categorized into segments by drawing concentric circles with increasing diameter from the explant body: 0 to 100 µm, 100 to 200 µm, 200-300 µm and more than 300 µm from the explant edge, which enables to evaluate neurite elongation. To avoid possible neurite outgrowth differences between central or peripheral retina, orientation of the retina was taken into account when making the explants.

2.7. Tracing and quantification of the number of regenerated RGCs

To evaluate the number of regenerating RGCs, we used again biocytin, this time enabling retrograde transport (Fig. S1). Hereto, fish were sedated (0.02% buffered tricaine) and the left ON was then exposed and cut right behind the crush site. A gelfoam with the dissolved tracer was applied on the proximal nerve end and the eye was placed back into its socket. Fish were revived for three hours, allowing the tracer to be retrogradely transported and label the RGC cell bodies in the retina. After three hours, the fish were euthanized in 0.1% buffered tricaine, the left eye was dissected and fixed overnight in PFA, and then processed for serial sagittal cryosectioning (10 µm sections). Biocytin was visualized using Alexa-488-coupled streptavidin (1:200, Invitrogen) and fluorescent imaging of the retina was performed using an Olympus FV1000 confocal microscope at 20x. Biocytin positive ($^{+}$) cells were counted on six central retinal cross-sections at 80, 160 and 240 µm distance at either side of the ON head, and averaged per fish.

2.8. Analysis of RGC cell death

Cell death was visualized on 10 µm cryosections of the left eye, processed as described above. After application of rabbit anti-activated-caspase-3 (1:70, Biovision, 3015-100) and detection via the TSA™ FT/Cy3 System (PerkinElmer), imaging of the stained sections was performed using an Olympus FV1000 confocal microscope at 20x.

2.9. RNA isolation and quantitative real time PCR

Quantitative real-time PCR was performed to assess the expression of *gap43* and *tuba1a* in retinal tissue of young and aged fish. Whole retinas were dissected and homogenized in Tri reagent (Sigma-Aldrich). Total RNA was extracted by NucleoSpin RNA isolation kit (Machery-Nagel, Germany) and RNA was reverse transcribed to cDNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen, Belgium). The qPCR reactions were

performed using SYBR Green master mix (Applied Biosystems) and a StepOne Plus Real Time PCR system (Applied Biosystems). All reactions were run in triplicate and at least 3 independent samples (consisting of 3 - 4 pooled retinas each) were analyzed per experimental condition. Using GeNorm (qBase software, (Vandesompele et al., 2002)), hypoxanthine phosphoribosyl-transferase 1 (hprt1) and succinate dehydrogenase complex subunit A flavoprotein (sdha) were selected as reference genes. Analysis of gene expression levels was performed using qBase software, which is based on the ΔΔCt quantification method (Hellemans et al., 2007). Primer specificity (Table 1) was confirmed via dissociation curve analysis at the end of each qPCR reaction.

Table 1: Primers used for qPCR analysis

Gene	Primer sequence (5' -> 3')	Reference
gap43	F - CAGCCGACGTGCCTGAA	(McCurley and Callard, 2010a)
	R - TCCTCAGCAGCGTCTGGTTT	
tuba1	F - GGAGCTCATTGACCTTGTTTTAGATA	(McCurley and Callard, 2010a)
	R - GCTGTGGAAGACCAGGAAACC	
hprt1	F - TGGACCGAACTGAACGTCTG	(Bhumika et al., 2015)
	R - TGGGAATGGAGCGATCACTG	
Sdha	F - ACGCACCCAATGCCAAAGAC	
	R - TCTTTATCCGGCCCAACACC	

2.10. Visualization and analysis of the innate immune response

To visualize the inflammatory responses upon axonal injury, *Tg(coro1a:eGFP)* reporter fish were used, in which microglia/macrophages appear green (Zou et al., 2013). Fish were euthanized at defined time points post injury in 0.1% buffered tricaine and their eyes were extracted. Eyes were then fixed for one hour in 4% PFA. The retinas were dissected and post-fixed in 4% PFA, again for one hour. Next, the retinal whole mounts were stained with mouse anti-GFP (1:250, Millipore), using an Alexa-488 conjugated secondary antibody (1:200, Invitrogen). Imaging was performed with an Olympus FV1000 confocal microscope at 20x.

The number and distribution of inflammatory cells was evaluated using a recently published Image J script, combined with spatial statistical techniques (Davis et al., 2017).

2.11. Experimental design and statistical analysis

In all cases, raw data were first tested for normality and homoscedasticity. Comparisons were performed using t-tests, ANCOVA and one- or two-way ANOVA followed by the Tukey post hoc test. All values are represented as mean ± SEM and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Tectal reinnervation after axonal injury is delayed upon aging

To study the impact of aging on CNS regeneration, reinnervation of the optic tectum, quantified via anterograde biocytin tracing of the regenerating RGC axons (Fig. S1), was evaluated after ONC injury in fish of 5, 12, 18, 24 and 36 months old (Fig. S2). First, it is worth mentioning that zebrafish growth already coincides with small changes in naive axonal density values (Fig. 1A). Seven days after axonal injury to the ON, reinnervation of the tectum progressively reduces with aging, and is significantly declined in 24 (64,3 ± 5,3 %) and 36 (58,1 ± 6,3 %) month-old fish, when compared to young adults (86,7 ± 5,9 % at 5 months of age). By 14 dpi, however, tectal reinnervation is restored at all ages, suggesting the reinnervation process to be only delayed (Fig. 1A). Importantly, we anticipated a possible influence of fish size on this read-out, as it is well established that teleosts display an indeterminate growth through lifespan, whereby also the size of the visual system increases with aging (Johns and Easter, 1977; Mack et al., 2004). Regrowth of the injured axons within the ON may therefore take longer in larger, aged fish, where they have to traverse a greater distance. Nonetheless, analysis of covariance

clearly shows the increase in reinnervation latency to be age-specific and independent of fish size (p = 0,577, ANCOVA).

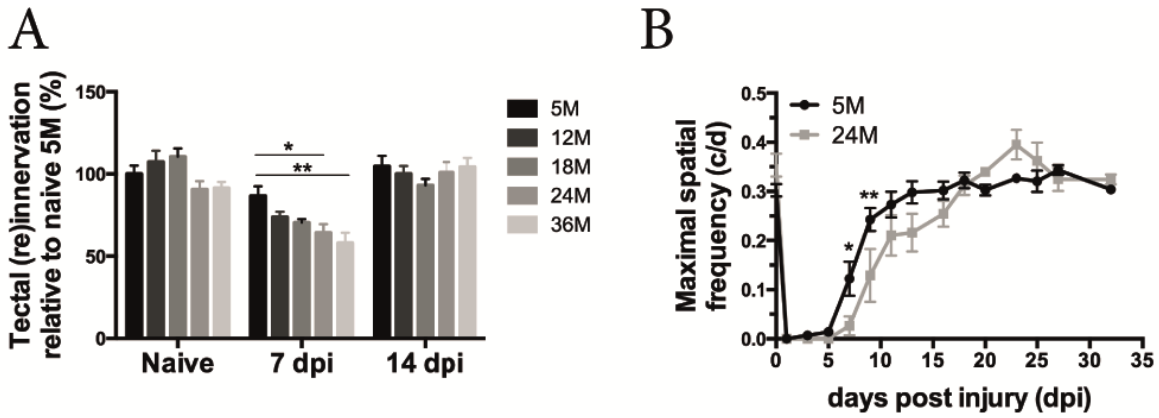

Fig. 1 Optic nerve regeneration is delayed in aged zebrafish

(A) Reinnervation of the optic tectum, quantified as the axonal density within the stratum fibrosum et griseum superficiale and the stratum opticum after anterograde biocytin tracing of the regenerating retinal ganglion cell axons, is delayed upon axon injury in aged fish. At 7 days post injury (dpi) to the optic nerve, tectal reinnervation is significantly reduced in 24- and 36-month-old fish, compared to young adults of 5 months old. By 14 dpi, all fish have restored axonal density to naive age-matched values, indicating that tectal reinnervation by the RGC axons is merely decelerated in aged fish. Values are means ± SEM, n≥5. (B) Visual recovery was evaluated by determining the maximal spatial frequency eliciting an optokinetic response. While young adult fish already show return of this primary reflex from 3 dpi on, aged individuals only start regaining sight at 7 dpi. Full restoration to naive visual acuity is achieved by 16 dpi in young adults, yet only by 20 dpi in aged fish. Values are represented as mean ± SEM, n=6.

3.2. Functional recovery after axonal injury is delayed in aged fish

In order to confirm age-related reinnervation latency, an optokinetic response test was used to evaluate functional recovery, which generally correlates well with tectal reinnervation by RGC axons (Kaneda et al., 2008). Visual acuity was measured by determining the maximal spatial frequency eliciting an eye response of the fish, and assessed at regular time points after bilateral

ONC injury in 5- (young adults) and 24-month-old (aged) fish (Fig. 1B). Notably, sight is preserved with aging (maximal spatial frequency of 0,302 ± 0,013 c/d in young versus 0,355 ± 0,024 c/d in aged fish). Immediately after damage to the ON, eyesight is completely lost, but it slowly recovers over time. In young adults, primary vision already starts to regain at 3 dpi (0,007 ± 0,007 c/d) and visual acuity is restored to naive values by 16 dpi (0,302 ± 0,018 c/d). However, aged fish only start retrieving vision from 7 dpi on (0,027 ± 0,019 c/d) and achieve complete visual recovery beyond 20 days post ONC (0,340 ± 0,009 c/d). Although aged zebrafish are thus able to functionally recover from axonal injury, these results endorse an age-dependent delay in the ON regeneration process.

3.3. Axonal outgrowth is decelerated in aged fish

As tectal reinnervation and visual recovery were found to be delayed upon aging, we further investigated axonal outgrowth and elongation. As growth-associated protein 43 (Gap-43) is known to be expressed in outgrowing axons, regrowth of the injured axons was visualized within horizontal visual system sections of *Tg(Gap-43:eGFP)* fish (Fig. 2A) (Holohan, 2015). The distance regrown by the axons was assessed at different time points after ONC, which enabled to estimate growth rate over time (Fig. 2B). Our results show that at 2 dpi most axons are still situated at the level of the crush site. One day later, a substantial amount of axons has crossed the site of crush, yet the number of regenerating axons is clearly reduced in aged fish, when compared to young adults. Moreover, the distance regrown by these axons comprises only 60 % (444,15 ± 104,54 µm) of that in young adults (740,72 ± 41,83 µm) at 3 dpi, indicating that aging significantly impacts axonal outgrowth initiation. Curiously, aged fish then seem to demonstrate an increased axonal growth speed, as represented by the elevated slope of the graph (Fig. 2B). Yet, by 5 dpi the RGC axons are still, although slightly, behind of those in young adults. As demonstrated above, aged fish are thus able to successfully regenerate upon ON injury, but early RGC outgrowth is clearly decelerated with aging. Next, the number of

regenerating RGCs was quantified using a retrograde biocytin tracing paradigm (Fig. S1 and 2C). The number of retrogradely labeled RGCs is similar in naive fish of 5- and 24-month-old. However, and as expected, aged fish consistently display a lower number of regenerating RGCs after crush, which is already visible from 2 dpi on and lasts over time. Nevertheless, at 7 dpi, the number of biocytin^{+} cells is equal to that in age-matched naive fish, indicating all RGCs are eventually able to regenerate. Taken together, our data reveal an important impact of aging on axonal outgrowth initiation, reflected by an age-related, transient reduction in the number of regenerating RGCs and their early (out)growth capacity.

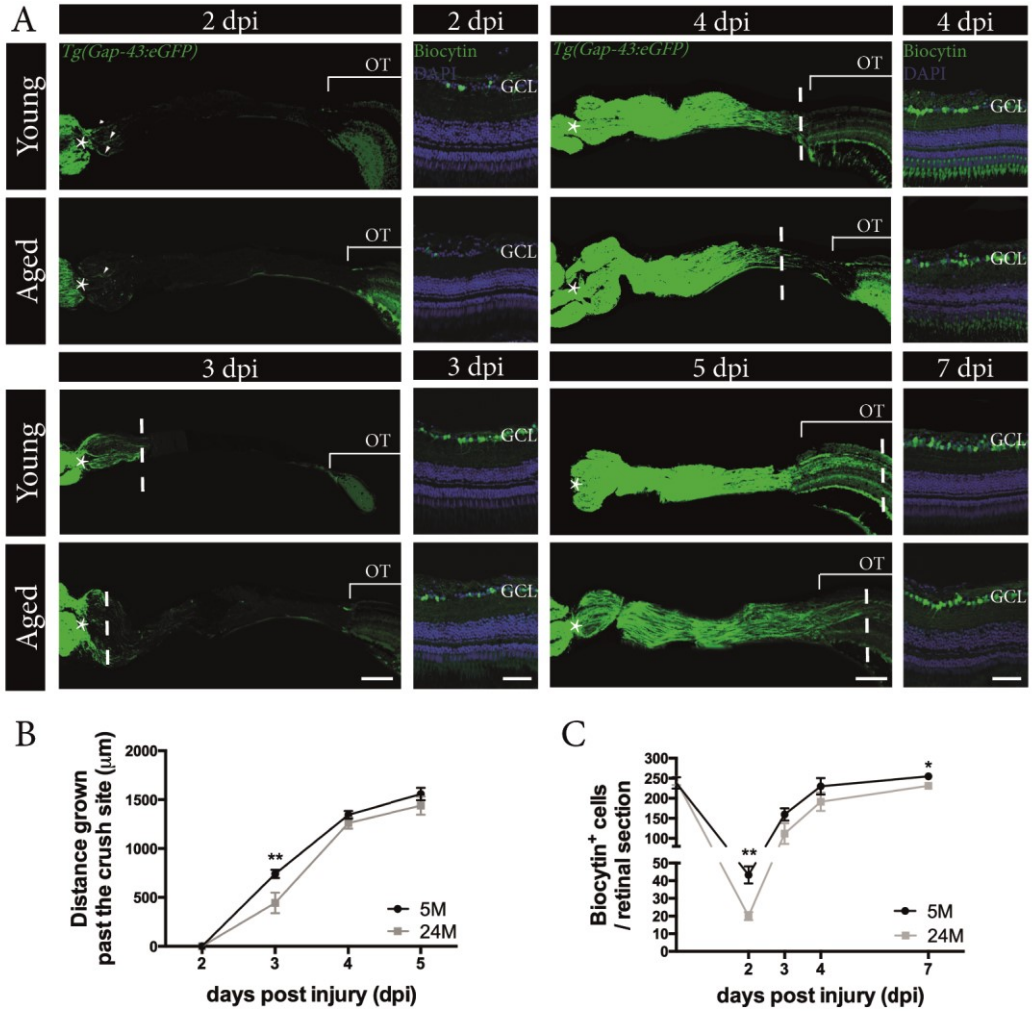

Fig. 2 Initiation and early retinal ganglion cell (RGC) axonal outgrowth are decelerated in aged fish

(A) Regrowing RGC axons were visualized in horizontal visual system sections of young (5 months) and aged (24 months) *Tg(Gap-43:eGFP)* fish. Insets represent a more detailed view of the axonal growth front at that time. At 2 days post injury (dpi), regrowing axons are still situated at the site of crush (indicated by *) in both young adult and aged fish. One day later, a significant amount of RGCs has already regrown an axon past the crush site in young adults, while outgrowth is still negligible in aged fish. Consequently, axons reach the optic tectum (OT) by 4 dpi in young fish and only by 5 dpi in aged fish, although they are only slightly behind in distance at that time. Scale bar = 200 µm. (B) Morphometric analysis of regrowing RGC axons in young (5M) and aged (24M) *Tg(Gap-43:eGFP)* fish uncovers an age-related delay in outgrowth initiation at 3 dpi, and reveals that the average distance regrown by the RGC axons is consistently smaller in aged compared to young adults ($p = 0{,}004$). (C) Quantification of the number of regenerating RGCs, as visualized on retinal sections after retrograde biocytin tracing, shows that the number of biocytin positive ($^{+}$) RGCs per retinal section is smaller in aged fish, when compared to young adults, starting from 2 dpi on ($p = 0{,}031$). Values are means ± SEM, $n \geq 5$.

3.4. The age-related decline in axonal outgrowth potential is recapitulated in an *ex vivo* retinal explant model

To further support the observed age-related decline in axonal outgrowth potential, growth onset and progression were assessed in an *ex vivo* setting, wherein retinal explants from young and aged injured fish were cultivated for 24 hours, and outgrowing neurites were subsequently stained for α-tubulin. Our results demonstrate that the total area covered by neurites is drastically reduced in aged retinal explants, as compared to explants of young adult fish (Fig. 3A and B), thereby affirming a reduction in early outgrowth capacity with aging. In addition, also axon elongation appears disturbed, as explants from aged fish never grow neurites above 300 µm of length. Overall, these data thus endorse the age-related decline in axonal outgrowth. Since external factors are absent in this *ex vivo* assay, these results indicate that an important

part of the defects in axonal regeneration onset and progression are either intrinsic, and/or the result of alterations in the immediate environment of the cells.

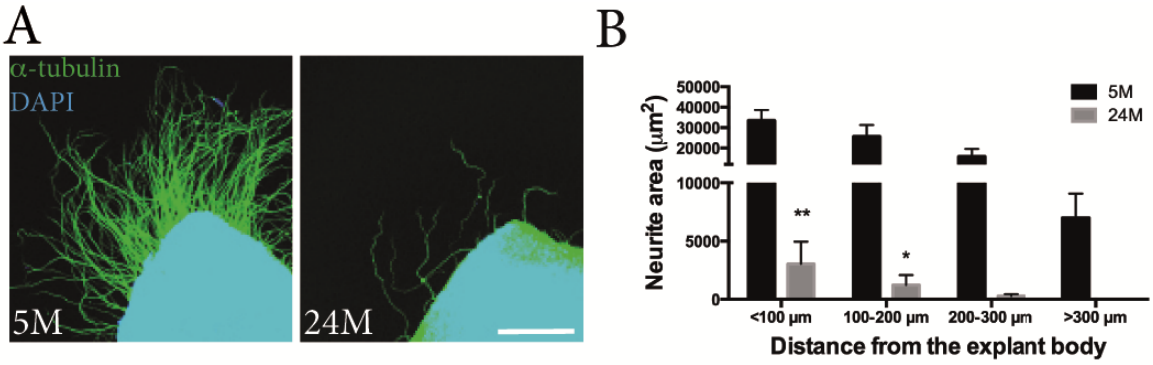

Fig. 3 The *ex vivo* axonal growth potential is declined in aged zebrafish

(A) Immunostainings for α-tubulin on retinal explants of young (5M) and aged (24M) fish expose a clear decline in neurite outgrowth with aging. Scale bar = 200 μm. (B) Quantification of the immunodetected neurite outgrowth area, at 100 um increments from the explant body, shows that total neurite outgrowth is reduced within explants of aged fish, and that also axonal extension is impaired. Explants of aged fish have consistently less neurites at all distances analyzed and do not possess neurites longer than 300 μm ($p<0,0001$). Values are means ± SEM, $n\geq4$.

3.5. All RGCs are able to survive axonal injury, even upon aging

Although our results show that even in aged fish all RGCs are eventually able to regenerate after ON injury, the occurrence of cell death early in the process may contribute to a delay in RGC outgrowth initiation. As such, apoptosis was evaluated at 1 dpi, using immunohistochemistry (IHC) for activated caspase-3 on retinal sections. As previously described in young adult fish, we do not detect RGC death after ONC (Zou et al., 2013), as no apoptotic cells are detected in the ganglion cell layer of both young and aged fish (Fig. S3). These results prove that, even upon aging, all RGCs are able to survive early after ONC injury.

3.6. Aging results in a diminished intrinsic growth potential

Although aged RGCs are not more vulnerable to cell death after injury to the ON, a diminution in their intrinsic growth potential could possibly underlie the decline in early RGC outgrowth. Therefore, intrinsic neuronal growth capacity was evaluated by determining retinal mRNA expression levels of growth-associated genes *gap-43* and *tuba1a.* Quantitative real-time PCR on retinal tissue of young and aged fish shows that the baseline expression of both genes is significantly reduced in aged retinas (*gap-43*: 1,00 ± 0,15 in young adults *vs.* 0,16 ± 0,02 in aged fish) (*tuba1a*: 1,00 ± 0,21 in young adults *vs.* 0,29 ± 0,13 in aged fish) (Fig. 4). Furthermore, mRNA levels remain significantly lower upon ONC in aged fish, even though axonal damage results in a clear upregulation of expression, as in young adults. These findings thus reveal the zebrafish retina to be subject to neuronal aging, resulting in a significant decline in intrinsic growth potential.

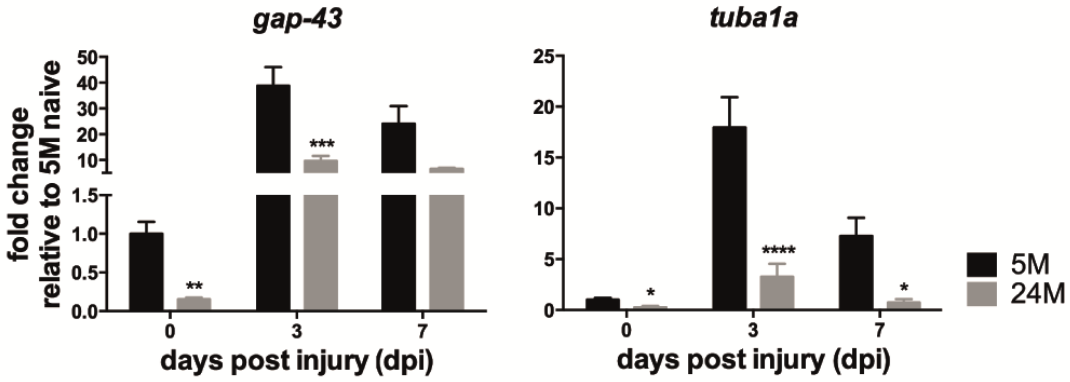

Fig. 4 Aged retinal ganglion cells (RGCs) show a reduced outgrowth capacity

RT-PCR of the growth-associated genes *gap-43* and *tuba1a* reveals a significant decline in naive expression levels with aging (*gap-43:* p=0,010, *tuba1a:* p=0,024), indicative of neuronal aging. Also injury response levels are significantly reduced after optic nerve crush in aged fish (24M), when compared to young adults (5M) (*gap-43:* p=0,001, *tuba1a:* p<0,0001), suggestive of a decreased outgrowth capacity. Values are means ± SEM, n≥3.

3.7. Aging leads to a deceleration of the extrinsic inflammatory response

Next, the potential effect of an aged cellular environment on the diminished regenerative potential in old fish was investigated. In light of the importance of inflammation for neural repair (Benowitz and Popovich, 2011; Bollaerts et al., 2017; Kyritsis et al., 2014), the innate immune response upon ONC was studied in young and aged *Tg(coro1a*:*eGFP*) fish - a reporter line expressing green microglia and macrophages (Fig. 5A). As already previously reported (Zou et al., 2013), our morphometric analyses of retinal microglia/macrophages show that ON injury in young adults is followed by an increase in the number of, innate immune cells, which peaks at 7 dpi, before returning to naive values at 28 days after the ONC (Fig. 5B). With aging, we reveal inflammaging to be apparent in the zebrafish retina, as the number of microglia is higher compared to young adults (792 ± 42 in young adults *vs*. 910 ± 32 in aged fish). Moreover, in contrast to the swift innate immune response in young adults, aged individuals only reach a maximal increase in microglial/macrophage number by 21 dpi (Fig. 5B). In addition, analysis of innate immune cell localization within the entire retina illustrates an injury-associated redistribution of the cell population towards the center of the retina (Fig. 5C). Importantly, in young adults most microglia/macrophages are situated at the center of the retina at 7 dpi (Fig. 5D). However, in aged fish the innate immune cells only redistribute by 14 dpi. Thus, consistent with the observed age-dependent delay in axonal regeneration, the inflammation response upon ON injury appears decelerated with aging.

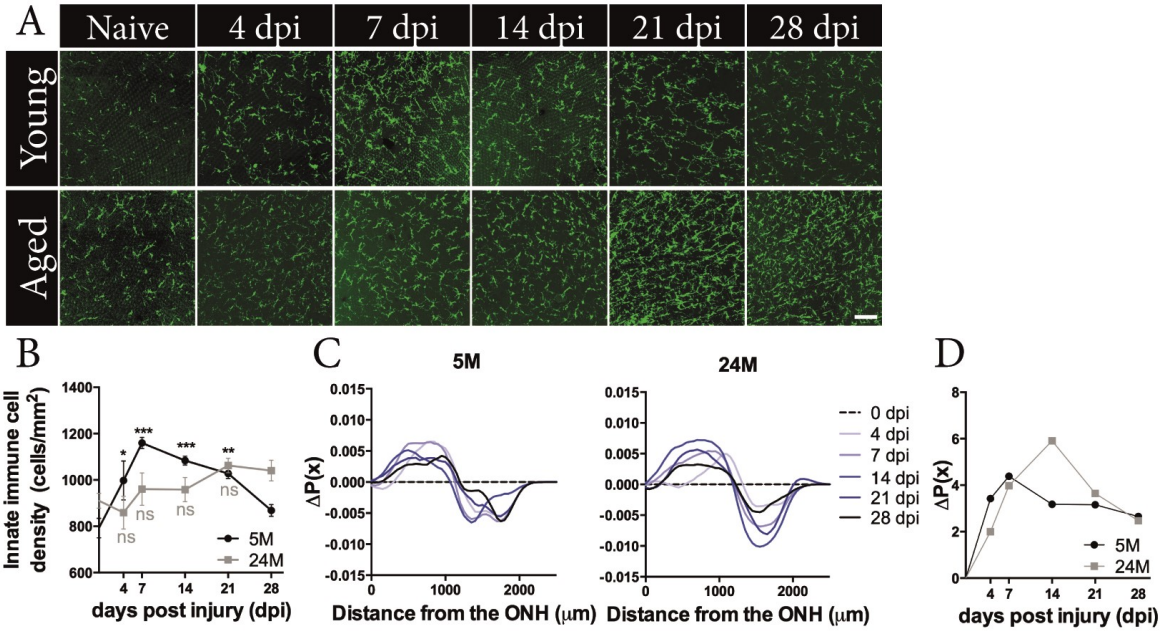

Fig. 5 The innate immune response is decelerated upon optic nerve injury in aged fish (A) Pseudocolour maps of innate immune cell density and spatial organization in retinal whole mounts of *Tg(Coro1a:eGFP)* fish illustrate an age-related retardation in the inflammatory response upon axonal injury. Scale bar = 500 μm. (B) Inflammaging is apparent in the senescent zebrafish retina, as innate immune cell density is significantly higher in naive aged fish (24M), compared to young adults (5M) (p = 0,039). Upon optic nerve damage, microglia/macrophage density only peaks by 21 days post injury (dpi) in 24M fish, in contrast to the fast rise at 4 and 7 dpi in young fish. Values are means ± SEM, n=6. (C) Detailed analyses of immune cell probability distributions in relation to the distance from the optic nerve head (ONH), reveal a redistribution of the immune cell population from the periphery towards the center of the retina upon axonal injury in both young and aged fish, visible as the upward and downward shift of all the optic nerve crush (ONC) curves (in blue) below and above 1000 μm distance from the ONH, respectively. (D) Starting from the probability distribution graphs (C), the area under each curve of each treatment group was calculated. This shows that immune cell redistribution in response to ONC is maximal at 7 dpi in young fish, and at 14 dpi in aged fish. For both ages, cells have not yet regained normal distribution by 28 days post ONC.

4. Discussion

In the mammalian peripheral nervous system (PNS), where regeneration is more robust, ample evidence is available indicating that axons exhibit an age-associated decline in reparative capacities (Verdu et al., 2000). Only recently however, Geoffroy *et al*. (2016) revealed that also in the regeneration-induced CNS, growth-promoting effects are greatly diminished with aging. Indeed, in both the corticospinal and rubrospinal tract, two systems with different regenerative abilities (Blesch and Tuszynski, 2009), substantially less axons were found to regenerate beyond the lesion site of aged *Pten* deleted mice (Geoffroy et al., 2016). Considering the aging context of many neurodegenerative diseases, exploring the impact of aging on regenerative capacities will thus be enormously important for mechanistic, preclinical, and future clinical studies to promote CNS repair after injury or disease. However, elucidating the etiology of age-related regenerative decline might be less challenging in a vertebrate animal model with high regenerative abilities.

The current study addresses the axonal regeneration capacity of the aged zebrafish visual system, which proves to retain a remarkable regenerative capacity. Although we did not yet investigate synaptic refinement and the restoration of retinotopy - which only occur 1 to 3 months after ONC in young adult zebrafish (Becker and Becker, 2008; Kaneda et al., 2008; McCurley and Callard, 2010b; Zou et al., 2013), we show that aged fish are capable of complete reinnervation of the optic tectum and successful visual recovery after ON injury. Nevertheless, we find these processes to be slowed upon aging, which is in line with findings in the goldfish spinal cord, where age likewise delays functional recovery (Bernstein, 1964). Further analysis revealed the observed age-related retardation in the ON regeneration process to be most likely the result of a delay in the initiation of the regenerative response, as was also described in the PNS of aged zebrafish (Graciarena et al., 2014). Indeed, we show aged zebrafish initially

display fewer regenerating axons with less extensive outgrowth after injury, findings which are in agreement with those reported in the aged, regeneration-induced mammalian CNS (Geoffroy et al., 2016). Yet, in contrast to goldfish spinal cord regeneration (Bernstein, 1964), we here report that almost all RGCs are eventually able to regenerate, even upon aging. Importantly, these age-dependent effects on early RGC outgrowth could be repeated in an *ex vivo* retinal explant model, thereby excluding the impact of several external factors, such as macrophage infiltration, presence of myelin-associated proteins and navigation cues, on the diminished RGC regeneration capacities in aged fish. As such, in-depth research into the mechanisms that could underlie the observed age-related delay in zebrafish ON regeneration was focused on intrinsic processes such as RGC viability and intrinsic growth capacity on the one hand, and extrinsic factors within their close environment, i.e. the injury response of glial cells, on the other.

The cellular and extracellular changes that accompany aging render neurons vulnerable (Mattson and Magnus, 2006). Accordingly, two-year-old mice show a faster time course of RGC loss following ON injury than mice of only 2 months (Wang et al., 2007). Fish however, normally show no or very limited RGC death upon axonal injury (Zhou and Wang, 2002; Zou et al., 2013), as also confirmed by our data in young adults. Furthermore, our results indicate that also aged zebrafish RGCs are able to survive ON injury, as apoptosis is absent from the GCL of both young and aged fish at 1 day post ONC. While our data thus suggest that aging does not lead to an increased susceptibility of zebrafish RGCs early upon axonal injury, we do show their intrinsic growth potential to be clearly impacted by age. In mammals, both GAP-43 and α-tubulin expression levels are often found to be reduced in the aged CNS, associated with an overall decline in synaptic plasticity and memory (Casoli et al., 2004; Casoli et al., 1996; Riascos et al., 2014; Vanguilder and Freeman, 2011; Zhang et al., 2015). Here, we uncover a similar aging phenotype in the zebrafish, in which the expression levels of both growth-

associated genes diminish with age, and prove the zebrafish retina to be subject to neuronal aging. More importantly, also the injury-associated expression levels of both genes are significantly curtailed in aged fish and may seriously influence regeneration capacity. In line with these results, one study already reported a gradual age-dependent loss of a sustained *Gap-43* response upon seizure-induced synaptic plasticity in the rat hippocampus (Schmoll et al., 2005). More aging research in certainly needed here, but generally the induction of growth-associated genes is well known to be of major importance for successful regeneration. Indeed, overexpression of this protein is known to enhance mouse spinal cord regeneration (Bomze et al., 2001), while its downregulation hampers the generation of reactive sprouts upon climbing fiber axotomy (Allegra Mascaro et al., 2013). Taken together, we thus believe that neuronal aging of the zebrafish retina results in the insufficient upregulation of growth-associated genes upon injury, and that this may lead to the observed delay in outgrowth initiation, and an overall retardation of the axonal regeneration process.

Apart from these intrinsic neuronal aging symptoms, also external factors can significantly affect neuronal functioning and repair (Bollaerts et al., 2017; Van houcke et al., 2015). Both macro- and microglia undergo morphological and functional changes with age, known as cellular senescence. Furthermore, aged microglia alter their secretion pattern in favor of pro-inflammatory cytokines, leading to a chronic low-grade inflammation status, a phenomenon called ‘inflammaging’. The aged mammalian CNS also displays a clear increase in microglial number (Damani et al., 2011; Streit et al., 2014). This, however, does not implicate increased neuroprotection, but is assumed to compensate for the diminished activity of individual aged microglia. Notably, also acute responses to injury seem age-dependent, as senescent microglia show a reduced mobilization to the injury site while their resolution is retarded (Damani et al., 2011; Wasserman et al., 2008). Increasing evidence for a detrimental effect of inflammaging on the regenerative outcome is emerging, but has only been scarcely described in fish. In a

model of ON remyelination (Munzel et al., 2014), decreased recruitment of microglia/macrophages to the site of injury was suggested to underlie regeneration failure in 15- to 18-month-old fish, as the early rise in myeloid cell number seen in young adults appeared blunted upon aging. Likewise, our data demonstrate the manifestation of inflammaging in the aged zebrafish retina, characterized not only by an increase in naive microglial number with aging, but also a clear absence of an early inflammatory response upon axonal injury. Indeed, aged fish show a decelerated increase in innate immune cell density, an their inflammatory response upon ON injury is still not resolved by 28 dpi, in contrast to young adults. In addition, our data illustrate an injury-associated shift in innate immune cell location from the periphery towards the center of the retina, possibly representing the migration of these myeloid cells towards the site of injury, which occurs significantly later in aged fish. It will be most interesting to elucidate if the resolution of this redistribution is likewise delayed with aging, as is described after focal laser injury in the retina of aged mice (Damani et al., 2011). Altogether, the immune response upon ONC injury appears deregulated in aged zebrafish. However, at this point, we can only speculate about the underlying mechanisms that link a timed immune response to successful ON regeneration in young adults, and about the implications of aberrant inflammation on the regenerative process. As microglia/macrophages remain present in the retina for several days in young adults - at the time that RGC axonal regeneration is fully ongoing -, and since they have been proposed to beneficially affect neuroregeneration in another CNS injury model in zebrafish (Kyritsis et al., 2012), we hypothesize that they exert a supportive role in ON regeneration. In young adult zebrafish, the inflammatory response upon ONC has indeed been suggested to positively contribute to regeneration, as intravitreal application of zymosan, - a yeast cell wall glucan known to efficiently stimulate inflammation in the retina, - enhances ON regeneration (Bollaerts et al., 2017; Zou et al., 2013). Future studies addressing microglial/macrophage activation and polarization state - a largely unexplored area

in zebrafish regeneration research - are undoubtedly needed and will be of utmost importance to elucidate the exact function of inflammation/inflammaging in successful CNS regeneration.

5. Conclusions

Our findings show that aged fish are able to functionally recovery from axonal injury in the CNS. However, despite the conservation of robust regenerative capacities, both intrinsic as well as extrinsic factors impact the normally flawless ON regeneration process and could underlie the observed age-related decline in regeneration onset and early outgrowth of axons. Further in-depth research into the molecules underlying neuronal aging and immune senescence will provide novel insights, and may contribute to the identification of innovative therapeutic targets to stimulate regeneration in an aging environment.

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7. Disclosure statement

The authors declare no conflicts of interests.

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Suppl. Figures

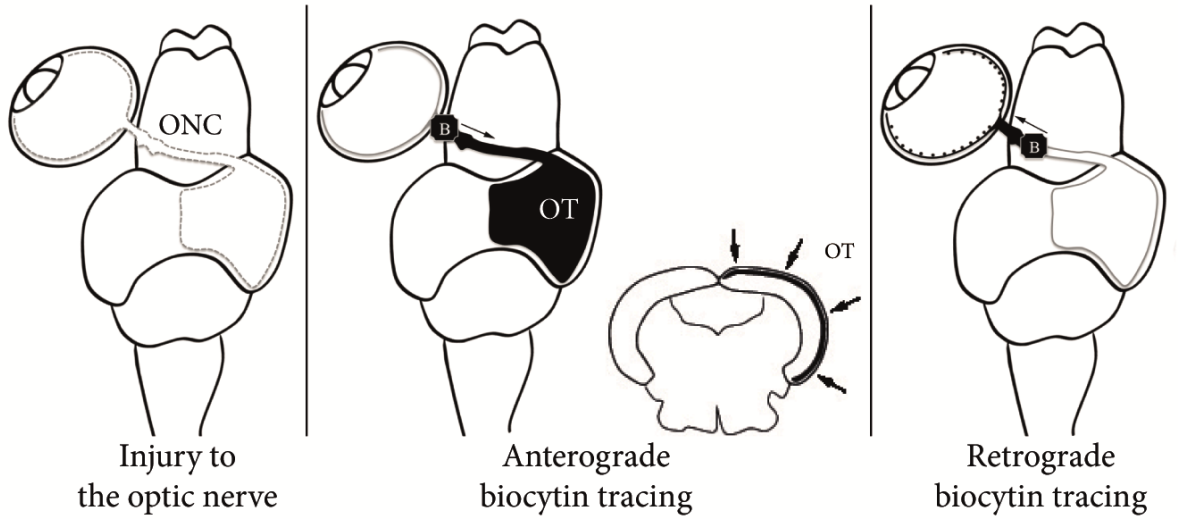

Fig. S1 Anterograde and retrograde tracing in the zebrafish retinotectal system

In this study, biocytin (B) tracings where used to study regeneration of the retinal ganglion cell (RGC) axons after optic nerve crush (ONC) injury. As biocytin is a passive tracer, its application to the optic nerve allows both anterograde and retrograde labeling. In a first set-up, the optic nerve was cut between the optic nerve head and the crush site, and a foam with biocytin was applied between both nerve ends, which enabled anterograde labeling of regrowing axons in the contralateral optic tectum (OT). Subsequent staining of the traced axons on coronal brain sections then permitted quantification of tectal reinnervation. In a second approach, the soma of regenerating RGCs in the retina were visualized via retrograde tracing, this time inserting the foam directly past the site of crush. As such, the number of regenerating RGCs, - able to regrow an axon past the crush site, - was counted on retinal sections.

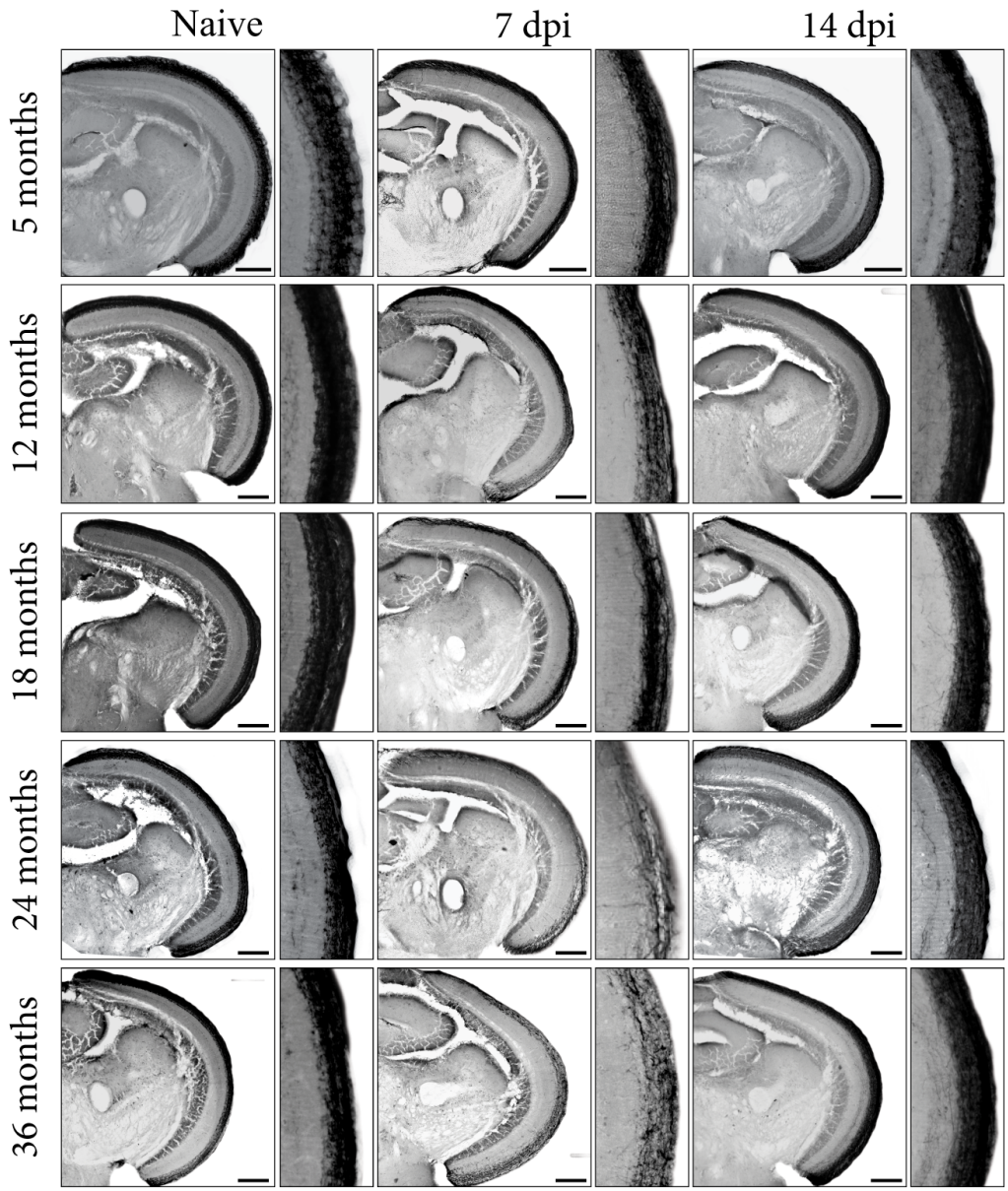

Fig. S2 Tectal reinnervation is delayed after optic nerve crush in aged zebrafish.

Anterograde biocytin tracing of regenerating retinal ganglion cell (RGC) axons (Fig. 1) reveals an age-related delay in the reinnervation of the contralateral optic tectum. At 7 days post injury (dpi) to the optic nerve, the optic tectum is less reinnervated in aged fish. Comparison of 5-, 12-, 18-, 24-, and 36-months old fish reveals that axonal density in the reinnervated tectum gradually decreases with aging. By 14 dpi however, reinnervation of the optic tectum is completely restored to the naive situation at all ages, indicating that the regeneration process is merely decelerated in aged fish. Scale bar = 200 μm.

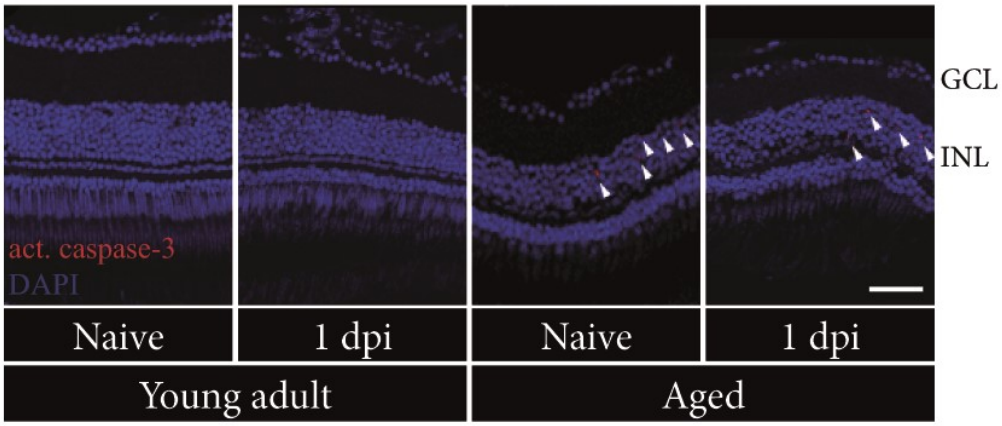

Fig. S3 Aged retinal ganglion cells (RGCs) survive optic nerve injury

Immunostainings for activated caspase-3 on retinal sections of young (5M) and aged (24M) zebrafish indicate that all RGCs are able to survive optic nerve crush, even upon aging. Despite some activated caspase-3^{+} cells in the inner nuclear layer (INL) of aged fish, no apoptotic cells are detected within the ganglion cell layer (GCL) at 1 day post injury (dpi). Scale bar = 50 μm.