Age-related degeneration leads to gliosis but not regeneration in the zebrafish retina

AUTHORS

Raquel R. Martins[1,2], Mazen Zamzam[3], Mariya Moosajee[4,5,6,7], Ryan Thummel[3], Catarina M. Henriques[1,2§], Ryan B. MacDonald[4§]

Affiliations:

1. Bateson Centre, University of Sheffield, Sheffield, S10 2TN, UK.
2. Department of Oncology and Metabolism, University of Sheffield, Sheffield, S10 2TN UK.
3. Department of Ophthalmology, Visual and Anatomical Sciences, Wayne State University School of Medicine, Detroit, MI 48201, USA.
4. Institute of Ophthalmology, University College London, London, EC1V 9EL, UK.
5. Moorfields Eye Hospital NHS Foundation Trust, London, EC1V 2PD, UK
6. Great Ormond Street Hospital for Children NHS Foundation Trust, London, WC1N 3JH, UK
7. The Francis Crick Institute, London, NW1 1AT, London

§Co-corresponding authors: c.m.henriques@sheffield.ac.uk and ryan.macdonald@ucl.ac.uk

ABSTRACT

Ageing is a significant risk factor for degeneration of the retina. Harnessing the regenerative potential of Müller glia cells (MG) in the retina offers great promise for the treatment of blinding conditions, such as age-related macular degeneration. Yet, the impact of ageing on their regenerative capacity has not yet been considered. Here we show that MG retain their ability regenerate after acute damage in the aged zebrafish retina. Despite this, we observe chronic age-related neurodegeneration in the retina, which is insufficient to stimulate MG proliferation and regeneration. Instead of regeneration, ageing leads to a gliotic response and loss of vision, recapitulating hallmarks of human retinal degeneration with age. Therefore we identify key differences in the MG regenerative response to acute versus chronic damage, a key consideration for stimulating endogenous regenerative mechanisms to treat human retinal disease.

KEY WORDS: retina, Müller glia, ageing, proliferation, degeneration, gliosis, zebrafish, telomerase, regeneration

INTRODUCTION

The physiology and structure of the healthy human eye is well known to degrade with age. Hallmarks of ageing in the retina include tissue thinning, neuronal loss and reduced visual function, especially in the macula [1-6]. Ageing is a significant risk factor for degeneration of the retina, such as age-related macular degeneration (ARMD) or primary open angle glaucoma[5-7]. As the population is ageing worldwide, the identification and potential treatment of the underlying cellular and molecular dysfunctions in the aged retina will be critical for global health going forward [8]. Retinal degeneration is often a process that plays out over months or years, whereby neurons gradually die leading to dysfunction [5,6]. Regenerative-based therapies, such as stimulating endogenous MG to regenerate, offer great promise for the treatment of various types of blindness that are caused by the loss of retinal neurons. Thus, determining the effects of ageing on the regenerative potential of the retina is an important consideration for the efficacy of such treatments going forward.

The principal cells tasked with maintaining the retina throughout life are the Müller glia (MG). MG provide retinal neurons with a myriad of support functions, including trophic support, neurotransmitter recycling and energy metabolism [9]. While these functions are critical for healthy retinal function, MG also have a prominent role during disease and after neuronal insult. In the mammalian retina, neuronal damage results in a MG gliotic response, involving the up-regulation of stress proteins, proliferation and morphology changes. This response is thought to be neuroprotective initially, but may ultimately culminate in dysfunction and death via loss of metabolic support or tissue integrity [10]. In the zebrafish retina after acute damage MG undergo a brief reactive gliosis-like phase that transitions into a regenerative response: a de-differentiation and proliferation of the MG progenitor cell to specifically generate new neurons and restore vision [11-13]. Intensive efforts have been made to uncover the molecular mechanisms regulating these endogenous regenerative responses in the retinas of lower vertebrates [14-19]. While the mammalian retina lacks significant regenerative capacity [20,21], re-introduction of key molecular signals into MG has shown great promise to stimulate the genesis of new neurons in mice after damage [22-24]. These molecular mechanisms are largely studied in the zebrafish using acute injury models, such as those generated by intense light or toxins [16,25]. Multiple acute injuries do not appear to reduce the overall regenerative response of MG; however, there does appear to be chronic inflammation after successive rounds of injury [26].

In contrast to acute injury, damage to the retina caused by degenerative disease or in ageing manifest themselves over much longer timescales and result in the gradual

accumulation of DNA damage and cell death[6]. As such, chronic degeneration may significantly differ from injury models, especially in its capacity to induce a regenerative response from glia. The zebrafish is an emerging ageing model, as it displays key human-like hallmarks of ageing in most tissues, including the retina [27,28]. These include an age-related decrease in cell density and increased cell death, suggesting chronic degeneration of the retina with increasing age [27]. Depending on the tissue, hallmarks of ageing also include telomere shortening, DNA damage, decreased proliferation and apoptosis or senescence [29-31]. Accordingly, the telomerase mutant (*tert*$^{-/-}$) zebrafish model is now an established accelerated model of ageing [29-31], where regeneration is known to be impaired in tissues such as the heart [32]. The *tert*$^{-/-}$ model therefore offers the possibility of studying key aspects of ageing in a shorter amount of time, and it allows the identification of telomerase-dependent mechanisms of ageing, potentially involved in retinal regeneration [29-31].

Here, we used the well-established zebrafish retina as a model study the impact of ageing on retinal regenerative capacity, with a particular focus on proliferation and maintenance of neuronal structure and function [19,33-37]. We show that MG retain their regenerative potential into old age in response to acute damage, and are therefore capable of proliferating to regenerate neurons dying throughout life. However, despite this potential, naturally aged and telomerase deficient retinas display known hallmarks of retinal ageing, including tissue thinning, accumulation of DNA damage and neuronal death. We show, by PCNA staining and EDU labelling, that MG in fact do not proliferate in response to this chronic neuronal degeneration. Instead, and in contrast to acute injury, ageing leads to a gliotic response and loss of vision, recapitulating hallmarks of human retinal degeneration with age. We therefore identify key differences in the MG regenerative response to acute versus chronic damage, a key consideration in potential therapies aiming to stimulate endogenous regenerative mechanisms to treat human retinal disease.

RESULTS

Müller glia retain the ability to regenerate after acute damage until old age

Studies on retinal regeneration typically focus on the young adult retina, and do not take old age into account for the regenerative response. Several acute damage paradigms induce retinal neuron death and elicit a regenerative response in zebrafish [26,38-40]. Chronic damage in the adult retina, specifically between 9-18 months of age, results in MG continuously re-entering the cell cycle to proliferate and regenerate lost neurons, although they do show signs of chronic activation at later timepoints [26]. However, it remains unclear if MG retain

their ability to proliferate and regenerate the retina throughout life. To test this, we used the light-damage model where aged zebrafish, at three stages of their lifecycle, were treated with light to elicit photoreceptor damage. To detect increased proliferation of MG in response to damage, a 3-day pulse of BrdU was performed, followed by a 28-days chase period (**Fig 1A**). After a 3-day pulse, BrdU should label both MG and their daughter cells, the newly-formed progenitors (**Fig 1B**). However, since the BrdU staining dilutes in the rapidly-dividing progenitor cells and MG only divide once, after a 28-days chase, the majority of the BrdU staining is retained in MG (**Fig 1B**). Our results show that light-lesion in aged retinas leads to a loss of photoreceptors, which is accompanied by a strong increase in microglia in the ONL, typical of photoreceptor degeneration in this damage model (**Fig 1C, C'**). Moreover, there are no differences in the incorporation of BrdU with ageing on any layer, at 72 hours post-light damage (hpL), when the initial regenerative response is mounted, or at 28 dpL, when the number of MG that initially re-entered the cycle can be identified **(Fig 1D, D')**. Both the unaltered immediate timing of MG response and the overall capacity to regenerate each neuronal layer are maintained with increased age. Together these results suggest that the regenerative response remains intact throughout zebrafish lifespan.

The aged zebrafish retina displays neurodegeneration independently of telomerase

Similar to humans, the zebrafish retina consists of three nuclear layers separated by two synaptic plexiform layers. The nuclear layers consist in the outer nuclear layer (ONL), containing photoreceptors; the inner nuclear layer (INL) containing bipolar cells (BCs), amacrine cells (ACs), horizontal cells (HCs) and MG; and the ganglion cell layer (GCL) mainly containing retinal ganglion cells (RGCs) (see diagram in **Figure 2A**). The macula is a specialised region of the human retina thought to be the region central for vision [41]. It remains unclear if the zebrafish has a true macula, however the central vision in fishes does focus on a particular zone within the retina[42]. A recent report suggests molecular and cellular specialised zones critical for visual acuity [43], so it is possible that a specialised "macula-like" zone is present in the central region of the zebrafish retina. This central region can be considered the "oldest" part of the retina, as it is generated at early developmental stages, and where there is usually very little proliferation to generate new neurons [28]. In contrast to the peripheral region of the retina, where the proliferative ciliary marginal zone (CMZ) resides[17], which is a population of progenitors found in the peripheral retina that is thought to contribute to retinal growth throughout life.

Given that the zebrafish retina maintains its regenerative potential until old ages, then we hypothesised that MG would regenerate dying neurons lost from the central retina until old ages, thereby counter-acting any age-related neurodegeneration. If this were the case, then removing telomerase, known to be important for proliferation [30,31] and regeneration in zebrafish [32], should reduce MG's ability to proliferate, thereby accelerating retinal degeneration. However, despite the MG's regenerative potential, the naturally aged wild type (WT) central retina displayed hallmarks of age-related retinal degeneration. Cells in the aged aged zebrafish retina accumulate DNA damage, as evidenced by the presence of strong γH2AX nuclear foci, a molecular marker of the DNA Damage Response (**Fig 2B, B'**). This was accompanied by a significant increase in cell death, as assessed by the early cell death labeling terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (**Fig 2C, C'**). Increased DNA damage and cell death with ageing occur in a telomerase-independent manner, as no differences are identified between genotypes at any time-point analysed. Retinal thinning is a likely consequence of increased cell death and is a known hallmark of human retinal ageing [44]. Accordingly, we show that zebrafish central retina progressively thins with ageing, consistent with previous studies [27,28]. Moreover, as for increased DNA damage and cell death, we show that this occurs largely independently of telomerase (**Supplementary Fig 1A**).

We next sought to determine whether the cell death, and resulting retinal thinning, is specific to any particular neuronal type. We used immunohistochemistry with several molecular markers specific for different neuronal populations throughout the lifespan of zebrafish, in the presence and absence of telomerase (*tert*$^{-/-}$*).* As expected, there was an overall reduction in neuron numbers with ageing in the zebrafish retina (**Fig 2D**). Importantly, this neuronal loss is not due to a decreased number of RGCs in the GCL (**Fig 2F**), but due to a decreased number of HuC/D-expressing ACs (**Fig 2G**) and PKC-expressing BCs in the INL layer (**Fig 2H**). Additionally, in the aged zebrafish retina, BCs display disorganised axon terminals in the IPL. The RGCs, ACs and BCs are neurons that come together to make connections in the major synaptic neuropil, called the inner plexiform layer (IPL). Thus, degeneration of these neuronal populations is expected to affect connectivity in the IPL. Accordingly, we confirmed alterations in the synaptic IPL layer, namely a reduction and disorganisation of pre-synaptic terminals with ageing (**Supplementary Fig 1B**). None of these neurodegenerative phenotypes were affected by depletion of telomerase, as there were no differences between WT and *tert*$^{-/-}$ at any of the tested ages.

The loss of photoreceptor integrity is one of the key features of human retina ageing and disease [45] and we show that rod photoreceptor outer segments in the zebrafish retina

undergo dramatic structural changes with ageing (**Supplementary Fig 1C**), confirming previous results [27]. These structural changes are accompanied by disruption of the tight junction protein zonula occludens (ZO-1) (**Supplementary Fig 1D**), a marker for the outer limiting membrane thought to be involved in photoreceptor degeneration [28]. These defects are also not accelerated in the *tert*$^{-/-}$ at any age tested, suggesting this is likely a telomerase-independent phenomenon. Together, these data suggest that, despite maintaining regenerative potential into old age, MG does not completely maintain the central retina in the context of age-related damage.

MG do not proliferate in response to chronic retinal neurodegeneration with ageing

While the progressive age-related retinal neurodegeneration would suggest a lack of regenerative response by MG, it remains unclear whether these degenerations are being counteracted, at least to some degree, by proliferation in the retina. To test this, we used an EDU pulse-chase strategy to identify any cell divisions in the retina, which would be indicative of potential regeneration. To characterise the steady state regenerative capacity of the central and peripheral CMZ until old age, we carried out a 3-day pulse of EdU followed by 0- and 30-days chase, in young and old WT and *tert*$^{-/-}$ zebrafish (**Fig 3A, B**). We observe few EdU-positive cells and, instead of a compensatory proliferation response, we detect even less EdU-positive cells with ageing, suggesting overall reduced proliferative capacity in the central retina (**Fig 3C**). These levels are further reduced after a 30-days chase (**Fig 3D**), suggesting that there are very few cells proliferating in the aged central retina. In the peripheral retina, where the proliferative CMZ resides, we see double of the EdU-retaining cells than in the central retina at 0-days chase, (**Fig 3C)**. Nevertheless, as in the central retina, proliferation in the peripheral retina (**Fig 3 C and D**) decreases with ageing, suggesting that there is no compensatory proliferation in response to the increased cell death with ageing. Removing telomerase has no further effect on the already low and decreasing levels of proliferation with ageing in the retina. Reduced proliferation with ageing is therefore unlikely to be the driving mechanism for retinal degeneration.

As proliferation of MG is the primary source of neurons after injury in fish [14,38], we sought to determine whether there was any compensatory proliferation occurring specifically within this cell population. We co-labelled cells with the MG specific marker glutamine synthetase (GS), and counted the GS-positive; EdU-positive cells. We detected very few GS-positive; EdU-positive cells in the central retina in both WT and *tert*$^{-/-}$, at any of the time-points characterised throughout their lifespan (**Fig 3E, E')**, suggesting MG are also not proliferating

in the central retina at old ages. Thus, contrary to acute damage, our data show that age-related chronic cell death does not trigger proliferation of MG cells. Finally, rods can originate from rod-specific progenitors [46,47], which are found in the ONL and are derived from MG that slowly divide in the WT retina [17,38,48,49]. To test whether there was any compensatory proliferation with ageing occurring from rod-specific progenitors found scattered throughout the ONL of the retina [36,46], we quantified levels of EdU positive cells in the ONL. Although we observe EDU+ cells in the central ONL of 5 months WT (likely to be rod precursors dividing), there are few detected in >30 months old aged WT. Once again *tert*$^{-/-}$ mutants had no further effect (**Fig 3F**). Together, our data show that there is no compensatory proliferation in response to age-related degeneration in the zebrafish retina, by any of the known sources of regeneration and neurogenesis: CMZ, rod precursor cells or MG.

The zebrafish retina shows signs of gliosis with ageing

In the mammalian retina gliosis is a hallmark of retinal damage [50], but also commonly observed in the aged retina [51]. Microglia are the innate immune cells found in the retina (reviewed in [52]. Microglia are also key players in maintaining tissue homeostasis throughout life and part of the gliosis process. They are activated in many neurodegenerative diseases, with increased number at sites of damage, including in the photoreceptor layer in many forms of retinal degeneration (reviewed in [51]. As we do not observe regeneration in the aged zebrafish retina in response to neuronal death, we asked whether there are alterations in the number of microglia (4C4-positive cells) found in the tissue. In contrast to what is observed in acute damage paradigms (Fig 1B; ref) we observed very few microglia in the central retina with no increase with ageing (**Fig 4A, A'**).

MG respond to damage or injury in most, if not all, neurodegenerative diseases [10]. The characteristic mammalian response is gliosis, whereby MG change shape and up-regulate structural proteins like GFAP to promote a neuroprotective effect [9,10]. In the zebrafish retina, the initial regenerative response to acute damage is similar to mammalian gliosis [11,26], and gliosis can be aggravated if MG proliferation is blocked [12]. Here, we identified MG using the well-described GS immunolabelling and characterised MG reactivity using the gliosis marker GFAP [53,54]. Interestingly, we do not observe an overall loss of MG with ageing (**Fig 4 B, B'**), in contrast to the observed neuronal death observed at the same stages (**Fig 2**). Despite this, there is a significant change in the morphology of the MG cells in the WT aged retina. Aberrations in the aged MG cells include disruptions in the radial morphology along the synaptic IPL and basal lamina (**Fig 4C, C'**), which are known hallmarks of gliosis in retina

degeneration [51]. Qualitative assessment further shows that while all young fish display long and aligned MG basal processes, 100% of the old fish show morphological disorganisation, a hallmark of gliosis. Thus, similarly to humans, chronic neurodegeneration with ageing elicits a gliotic response in the zebrafish retina rather than the regeneration typically observed after acute injury.

Zebrafish vision declines with ageing, independently of telomerase

Loss of visual acuity and contrast sensitivity with advancing age in humans has been well documented [5,6]. To determine whether the molecular and structural changes we observe have a pathological consequence on the retinal function, we tested whether zebrafish have impaired vision with ageing. Visual testing in the zebrafish has been used to screen for mutants with defects in retinal development and function [55-58], but had yet to be tested in the context of ageing. The optokinetic response (OKR) is a well-established assay to measure innate visual responses and provides readout of visual acuity [55,59] (**Fig 5A**). Our results show a decreased number of eye rotations per minute in aged fish (**Fig 5B, B' and videos 1 and 2**), suggesting that zebrafish vision declined with ageing. As for most of the hallmark phenotypes of ageing described so far, (**Supplementary Table 1**), telomerase is not a limiting factor for visual acuity, since the *tert*$^{-/-}$ zebrafish do not display an accelerated reduced visual acuity at 5 months or 12 months of age, close to the end point of *tert*$^{-/-}$ life (**Fig 5B, B' and videos 3 and 4**).

DISCUSSION

Using a combination of cell labelling strategies throughout adulthood into old age, we show that the zebrafish retina retains its potential to regenerate in response to acute damage into old age. Therefore, the lack of compensatory proliferation in response to chronic, age-associated cell death in the ageing zebrafish retina is not due to a loss of capacity for MG to proliferate *per se*, but likely due to the absence or insufficient levels of the required stimuli for MG engagement. Indeed, contrasting to acute damage, we show that age-related retinal damage is insufficient to trigger compensatory proliferation by any of the known sources of regeneration and neurogenesis, namely the CMZ, rod precursor cells and MG. Moreover, we show that MG's inability to heal the aged retina is not due to telomerase-dependent proliferative limits. Overall, instead of regeneration, ageing leads to a gliotic response and loss of vision, reminiscent of human retinal ageing.

Telomerase-dependent and -independent hallmarks of zebrafish retinal ageing

Previous work suggested that telomerase and telomere length are important for human retinal health. In particular, the retinal pigment epithelium (RPE) has been reported to have shorter telomeres than the neural retina, and it accumulates senescence over-time [60]. Accordingly, reactivation of telomerase has been described to ameliorate symptoms of AMD [61,62] and telomerase activators are currently in clinical trials (e.g. NCT02530255). However, retina homeostasis requires more than RPE maintenance. It requires a steady-state level of proliferation of different cell types involved in multiple aspects of retina function. As in other proliferative tissues [29-31], it would make sense that telomerase levels would influence zebrafish retina homeostasis. We therefore hypothesised that the zebrafish retina would degenerate in a telomerase-dependent manner, leading to vision loss. However, our data suggest that most age-related changes in the zebrafish retina, including vision loss, are largely telomerase independent, since depletion of telomerase (*tert*$^{-/-}$) does not accelerate or exacerbate any of these phenotypes. Telomere dysfunction is known to affect mostly highly proliferative tissues, reviewed in [63] and what our data show is that, in the region most affected by ageing phenotypes, the central retina, there is very little proliferation to start with. This suggests that replicative exhaustion of telomeres is not a limiting factor in the age-associated degeneration of the central retina. Instead, it is likely that chronic exposure to damaging agents such as oxidising UV radiation is the key driver of degeneration in the retina, as was proposed in the "Wear and Tear Theory" (reviewed in [6]). However, we cannot exclude that telomere-associated damage may still be a contributing factor to the observed increased levels of DNA damage and cell death in the central retina. Telomeres are known to be damaged by oxidative stress and can act as sinks of DNA damage, irrespectively of length and levels of telomerase [64,65]. In fact, this is a likely contributor to RPE damage with ageing [66,67], potentially explaining why depleting telomerase has no effect on the proliferation of the ONL, where photoreceptors reside, nor does it accelerate vision loss.

Chronic vs. acute damage in MG responses to natural ageing

Our results show that zebrafish develop vision loss with ageing and that this is underpinned by retinal neurodegeneration and gliosis. This could seem counter-intuitive, given that it is well established that the zebrafish retina is capable of regenerating after acute damage. Therefore, the lack of regeneration after age-related cell death observed in this study suggest that there are critical differences between chronic ageing and repair after an acute injury. Further supporting this, we show that this lack of regeneration in the context of ageing is not due to an intrinsic inability of MG to proliferate *per se*, as we show they are still capable of doing so in response to an acute injury in old animals. Several tissues in the zebrafish retain the ability to regenerate throughout the lifespan of the zebrafish, including

the fin and the heart [68,69]. Likewise, the optic nerve crush paradigm has shown that there is successful recovery and function in the zebrafish retinotectal system [70], suggesting that regeneration is still possible at advanced age in the retina. The regenerative capacity of vertebrate tissues tends to decrease after repeated injury and as animals advance in age. This is likely due to reduced progenitor cell proliferation potential and subsequent differentiation [71-74]. In the context of the central nervous system, this is aggravated by gliosis, a reactive change in glial cells in response to damage. In the mammalian retina, MG cells undergo gliosis in response to damage and in many retina degenerative diseases [10]. However, in the zebrafish retina MG cells undergo the initial reactive gliotic response after acute damage but quickly shift to the regenerative pathway. Here we show that while we observe morphology alterations in MG cells in response to age-related neurodegeneration**,** MG in the aged retina retain their ability to regenerate in response to acute damage of photoreceptors. It is unclear if alternative damage paradigms will also lead to similar regenerative response and potential in the aged zebrafish retina. It also remains unclear what is the consequence of the gliotic response observed on retinal neurons in the aged retina, as gliosis is classically thought of as “Janus-faced”, with both pro- and anti-neuroprotective functions [10]. Importantly, the kinetics of these MG morphology and molecular changes relative to neurodegeneration may provide further clues to the precise cellular breakdown in the retina causing widespread neuronal death and dysfunction. That is the MG cells react first, thereby abandoning their neuronal support functions and precipitating neuronal damage, or do they purely respond to neuronal degeneration. Despite the gliosis detected in the MG with ageing, their numbers are maintained, despite neuron loss, suggesting that MG may be protected from age related death caused by the accumulation of DNA damage or free radicals. It remains unclear if there is a molecular mechanism inferring this protection or if MG gliosis is in itself protective to cell death.

Our proposed model: A molecular “tipping point” required to stimulate regeneration in ageing

Regeneration studies so far have relied on several damage paradigms, including phototoxic [26] and ouabain induced lesions [75], which induce rapid cell death post-insult. While acute damage models are suitable to explore the cellular and molecular mechanisms underpinning the regenerative potential of the retina, they do not allow testing whether this regenerative response is also occurring with natural ageing in the retina. “Natural ageing” and associated stress-induced neuronal death play out over months or even years and we now show that they do not elicit the same regenerative response. This may be because the slow degeneration is not producing a strong enough signal in a short amount of time to induce

MG to undergo the cellular and molecular process of regeneration (**Fig 6; proposed model**). It has been shown that the level of cell death can induce a differential response of MG cells. Whilst a large amount of rod death causes a regenerative response, small amounts do not [25,76]. Furthermore, there may be distinct differences in signals released after apoptosis or necrosis in ageing vs damage models[77,78]. In support of this concept, recent work suggests that there may be key signalling differences underpinning the difference between a "regenerative" or a "reparative" response to injury [79]. Thus, we propose that a molecular signal, or expression changes of such signal, will regulate the "tipping point" required to elicit a MG regenerative response in ageing. Research in the context of acute damage paradigms in the zebrafish retina has uncovered many of the molecular mechanisms regulating this regenerative response (reviewed in [19, 37]). For instance, proliferation appears to be a key mechanism for the initiation of the regenerative response as blocking proliferation after damage in the zebrafish retina results in MG gliosis, and not regeneration, similar to humans [18]. Moreover, age-associated degeneration of retinal neurons and their synapses, as we observed to occur in this study, may result in a loss of neurotransmitter release, such as GABA, which has been shown to facilitate the initiation of MG proliferation [80]. Recently, it has been suggested that electrical stimulation may promote MG proliferation and expression of progenitor markers [81]. Thus, dysregulation of neurotransmitters upon neurodegeneration could inhibit the key molecular pathways regulating regeneration. Alternatively, the initial inflammatory response has also been shown to be determinant for the repair process. Upon light-induced retinal damage, overexpression and subsequent release of TNF by apoptotic photoreceptors seems to induce MG proliferation [11,82]. After acute damage, there is also an increased number of microglia in the retina [75,83] and they have been shown to be essential for retinal regeneration [84,85]. In contrast, microglia numbers do not increase with ageing and therefore may compromise the regenerative response. Nonetheless, it is important to consider that the available immunohistochemistry techniques to identify microglia number has a few limitations. Namely, there may be a spike in microglia number in the aged retina that is rapidly resolved and missed at our timepoints or microglia may undergo apoptosis similar to retinal neurons. Thus, we cannot exclude the possibility that microglial cells are involved in the observed retinal degenerations or play a critical role in the lack of a regenerative response observed in the aged retina.

Conclusions

Our work demonstrates that, in the context of age-induced neuronal degeneration, the MG in the zebrafish retina react by undergoing a response more akin to gliosis, rather than

regeneration. This resembles what happens in the aged human retina. Importantly, we identify lack of proliferation and increasing gliosis as a key difference in MG response after acute damage compared to the damage done through chronic aging.

MATERIALS AND METHODS

Zebrafish husbandry

Zebrafish were maintained at 27-28ºC, in a 14:10 hour (h) light-dark cycle and fed twice a day. The OKR was performed in the UCL Institute of Ophthalmology and the phototoxic lesions and regeneration experiments were performed at Wayne State University School of Medicine (USA). All other experiments were performed in the University of Sheffield. All animal work was approved by local animal review boards, including the Local Ethical Review Committee at the University of Sheffield (performed according to the protocols of Project Licence 70/8681) and the Institutional Animal Care and Use Committee at Wayne State University School of Medicine (performed according to the protocols of IACUC-19-02-0970).

Zebrafish strains, ages and sex

Three strains of adult zebrafish (*Danio rerio*) were used for these studies: wild-type (WT; AB strain), *tert*$^{-/-}$ (*tert*$^{AB/hu3430}$) and *albino* (*slc45a2*$^{b4/b4}$). t*ert*$^{-/-}$ zebrafish is a premature model of ageing and therefore, age and die earlier than the naturally aged zebrafish. While *tert*$^{-/-}$ fish have a lifespan of 12-20 months, WT fish typically die between 36-42 months of age [29,30]. In order to study age-related phenotypes in the zebrafish retina, here we used young (5 months) WT and *tert*$^{-/-}$ fish, alongside with middle aged (12 months) WT fish, and old WT and *tert*$^{-/-}$ fish. 'Old' was defined as the age at which the majority of the fish present age-associated phenotypes, such as cachexia, loss of body mass and curvature of the spine. These phenotypes develop close to the time of death and are observed at >30 months of age in WT and at >12 months in *tert*$^{-/-}$ [29,30]. In addition, we used adult *albino* zebrafish for retinal regeneration studies (described in detail below). Importantly, none of the animals included in this study displayed visible morphological alterations in the eyes (e.g. cataracts). Whenever possible, males where chosen to perform the experiments.

OKR assay

Fish were anaesthetised in 4% tricaine methanesulfonate (MS-222; Sigma-Aldrich) and placed in a small bed-like structure made of sponge, located inside a small petri dish containing fish water. During the experiment, fish were maintained still by the strategic use

of needles that sustained the sponges close to the fish so that the fish could not move. The petri dish was then placed inside a rotation chamber with black and white striped walls (8mm-thick stripes). After fish recovered from anaesthesia, the trial began, and the walls of the chamber started rotating at 12rpm (for 1min to the left side followed by 1min to the right side). Eye movements were recorded using a digital camera throughout the experiment. After the experiment, the number of eye rotations per minute was measured by video observation and manually counting. The counting was performed blindly by two independent researchers. In the end, the results were normalised for the WT young from the same day / batch, in order to control for different days of experiments.

Intense light-damage paradigm with BrdU incorporation

A photolytic damage model in adult *albino* zebrafish was utilised to destroy rod and cone photoreceptors and elicit a regenerative response [25]. Briefly, adult *albino* zebrafish were dark-adapted for 10 days prior to a 30 min exposure to ~100,000 lux from a broadband light source. Next, fish were exposed to ~10,000 lux of light from four, 250 W halogen lamps for 24 hrs. Following 24 hrs of light treatment, fish were transferred to a 1L solution containing 0.66 g of NaCl, 0.1 g Neutral Regulator (SeaChem Laboratories, Inc. Stone Mountain, GA, USA), and 1.5 g BrdU (5mM; B5002; Sigma-Aldrich) for 48 hrs. This timeframe for BrdU incubation was based on previous studies in order to label MG cell-cycle re-entry [26]. Following a 48 hour incubation in BrdU, the fish were split into two groups: one group was euthanised by an overdose of 2-Phenoxyenthanol and eyes were processed for immunohistochemistry as described below; the second group returned to normal husbandry conditions for an additional 25 days (or 28 days after light onset) prior to euthanasia and tissue collection. During this time, BrdU incorporation dilutes in actively dividing cells, allowing for clear visualization of the number of MG in the INL that only divide a single time. It also serves as an indirect measure of regenerative capacity (i.e. an equal loss of BrdU-positive cells at 28 dpL between experimental groups would indicate similar numbers of progenitor cell divisions earlier in the regenerative process).

Tissue preparation: paraffin-embedded sections and cryosections

Adult fish were culled by overdose of MS-222, followed by confirmation of death. Whole fish or dissected eyeballs were then processed for paraffin-embedded sections or for cryosections, as follows:

Paraffin-embedded sections. Whole fish were fixed in in 4% paraformaldehyde (PFA) buffered at pH 7.0, at 4ºC for 48-72h, decalcified in 0.5M EDTA at pH 8.0 for 48-72h, and embedded in paraffin by the following series of washes: formalin I (Merck & Co, Kenilworth, NJ, USA) for 10min, formalin II for 50min, ethanol 50% for 1h, ethanol 70% for 1h, ethanol 95% for 1h 30min, ethanol 100% for 2h, ethanol 100% for 2h 30min, 50:50 of ethanol 100% : xilol for 1h 30min, xylene I for 3h, xylene II for 3h, paraffin I for 3h and paraffin II for 4h 30min. Paraffin-embedded whole fish were then sliced in sagittal 4µm-thick or coronal 16µm-thick sections, using a Leica TP 1020 cryostat.

Cryopreservation and cryosections. Dissected eyeballs were fixed in 4% PFA at 4ºC, overnight (ON). Then, they were washed in cold 1x PBS and immersed in 30% sucrose in phosphate-buffered saline (PBS), ON at 4ºC, for cryopreservation. Single cryopreserved eyeballs were then embedded in mounting media – optimal cutting temperature compound (OCT, VWR International), snap-frozen at −80°C, and stored at -20ºC until cryosectioning. Cryosections were sliced at a 13µm thickness using a Leica Jung Frigocut cryostat or a Leica CM1860 cryostat.

Immunohistochemistry (IHC)

Before immunofluorescence staining, cryosections were hydrated in PBS at room temperature (RT) for 10min, and paraffin-embedded sections were deparaffinised and hydrated as follows: histoclear (Scientific Laboratory Supplies, Wilford, Nottingham, UK) 2x for 5min, followed by ethanol 100% 2x for 5min, ethanol 90% for 5min, ethanol 70% for 5min, and distilled water 2x for 5min. After antigen retrieval in 0.01M citrate buffer at pH 6.0 for 10min, the sections were permeabilised in PBS 0.5% Triton X-100 for 10min and blocked in 3% bovine serum albumin (BSA), 5% Goat Serum (or Donkey Serum), 0.3% Tween-20 in PBS, for 1h. The slides were then incubated with the primary antibody at 4℃ ON. After washes in PBS 0.1% Tween-20 (3x 10min) to remove excess to primary antibody, the sections were incubated with secondary antibody at RT for 1h. Finally, the slides were incubated in 1µg/ml of 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) at RT for 10min, washed in PBS 1x, and mounted with vectashield (Vector Laboratories, Burlingame, CA, USA). The primary and secondary antibodies used in this study are described in Table 1 and Table 2, respectively.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining

In paraffin-embedded sections, TUNEL was performed using the *In Situ Cell Death Detection Kit*, *Fluorescein* (Merck & Co), following the manufacturer's instructions. Briefly, after deparaffinisation, hydration, antigen retrieval and permeabilization, as described above, the slides were incubated in enzyme and label solution (1:10) at 37ºC for 1h. The slides were then washed in 1x PBS (2x 10min) before blocking and incubation with primary antibody.

5-Ethynyl-2'-deoxyuridine (EDU) labelling

EdU labelling was detected using the Click-iT® EdU Imaging Kit (Thermo Fisher Scientific), following manufacturer's instructions. Briefly, fish were injected with 5µl of 10mM EdU diluted in dimethyl sulfoxide (DSMO), by intraperitoneal (IP) injection, for 3 consecutive days (3-day pulse). In order to differentiate proliferating cells and low-proliferative EdU-retaining cells, the fish were separated into two groups: 0-day chase and 30-days chase groups. The fish from the first group were culled 2h30min after the last injection of Edu, whereas the fish from the second group were culled 30 days after the last injection of EdU. After culling, whole fish were processed for paraffin-embedded sections as described above. In order to detect EdU labelling in paraffin-embedded sections, the slides were deparaffinised, hydrated, underwent antigen retrieved, were permeabilised and washed in 1x PBS. The slides were incubated in freshly made EdU-labelling solution (per 1ml of solution: 860 1x Click-iT®EdU reaction buffer, 40 $CuSO_4$, 2.5 Alexa Fluor® 647 azide working solution and 100 10x EdU reaction buffer additive) at RT for 30min. Finally, the slides were washed in 1x PBS before blocking and incubation with primary antibody (ON, at 4ºC). The incubation in secondary antibody was performed as previously described.

Imaging and quantifications

Paraffin-embedded sections were imaged by epifluorescence microscopy, using a DeltaVision microscope with a 40x oil objective. Cryosections were imaged by laser scanning confocal imaging, using a Leica SP5 microscope or a Nikon A1 Confocal microscope, with a 40x oil objective. In both cases, multiple 0.2-0.6µm thick z-stacks were acquired in order to capture the whole section. For each staining, a total of 4 images were taken per retina, 2 from central and 2 from peripheral retinal. The central retina was defined to be the centre point between opposing CMZs (A minimum of ~1000µm from the periphery), while images of the peripheral retina contain the limits of the retina, including the CMZ. The peripheral retina was defined as the tissue directly adjacent to the CMZ.

In order to quantify the staining, after a z-projection was generated, three boxes of 100x100µm were drawn in each field of view. The total number of positive cells was then manually counted for each labelling. Rhodopsin, ZO1, ribeye and GFAP staining were an exception to this, for which a qualitative assessment was performed instead. To do so, structural and morphological defects were identified as follows. For the rhodopsin staining, as young WT retinas usually display long and aligned outer segments, all short and/or misaligned outer segments were considered defective. For the ZO1 staining, the average number of breaks in the ZO1-labelled membrane per animal was quantified. The average of breaks in the WT young animals was used as a reference, and any fish presenting an average number of breaks bellow this average was considered to present defects in the membrane. For the ribeye staining, young retinas usually present two distinguished layers of pre-synaptic ribbons. Thus, staining where the two layers of pre-synaptic ribbons are not distinguished, was considered defective. GFAP staining usually reveals long and aligned MG processes in young WT retinas, and therefore short and/or misaligned MG processes are considered gliotic. Through this qualitative assessment it was calculated the percentage of fish per group presenting structural and morphological defects. Finally, raw images were used for quantification purposes. The images were then processed with Adobe Illustrator 21.0.2 for display purposes.

Statistical analysis

Statistics were performed using the GraphPad Prism v7.00. Normality was assessed by Shapiro-Wilk test. For normally distributed data unpaired t-test was used to compare 2 data points and one-way ANOVA followed by Bonferroni post-hoc test was used to compare more than 2 data points. For non-normally distributed data, Mann-Whitney test and Kruskal-Wallis test followed by Dunn's post-hoc test were used instead. Two-way ANOVA was used in order to compare more than 2 data points in 2 groups different groups (genotypes). Chi-square was performed to analyse structure and morphological changes in the retina based on qualitative assessment, having into account the number of animals per group displaying defects versus not displaying defects. A critical value for significance of $p<0.05$ was used throughout the analysis.

582 **Table 1. Primary antibodies used for immunostaining.**

Antibody, species and type	Dilution factor	Catalogue number; Company, City, Country
yH2AX rabbit polyclonal	1:500	GTX127342; GeneTex, Irvine, CA, USA
p53 rabbit polyclonal	1:200	AS-55342s; Labscoop, Little Rock, AR, USA
PCNA mouse monoclonal	1:500	NB500-106; Novus Biologicals, Littleton, CO, USA
PCNA rabbit polyclonal	1:500	GTX124496; GeneTex, Irvine, CA, USA
7.4.C4 (4C4) mouse monoclonal	1:100	A gift from A. McGown
HuCD mouse monoclonal	1:100	A21271; Thermo Fisher Scientific, Waltham, MA, USA
PKCβ1 rabbit polyclonal	1:100	Santa Cruz, Dallas, TX, USA
Ribeye rabbit polyclonal	1:10,000	A gift from Teresa Nicholson
GFAP rabbit polyclonal	1:200	Z0334, Agilent DAKO, Santa Clara, CA, USA
GFAP mouse monoclonal	1:100	zrf1, ZIRC
Glutamine Synthase (GS) mouse monoclonal	1:150	mab302, Merck, Kenilworth, NJ, USA
Rhodopsin rabbit	1:5,000	A gift from David Hyde
BrdU rat	1:200	OBT0030A, Accurate Chemical & Scientific, Westbury, NY, USA

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Table 2. Secondary antibodies used for immunostaining.

Antibody, species and type	Dilution factor	Catalogue number; Company, City, Country
Goat anti-rabbit IgG Alexa Fluor® 488	1:500	A11008;Invitrogen, Carlsbad, CA, USA
Goat anti-rat IgG Alexa Fluor® 488	1:500	A11006; Thermo Fisher Scientific, Waltham, MA, USA
Goat anti-rabbit IgG Alexa Fluor® 568	1:500	10032302; Thermo Fisher Scientific, Waltham, MA, USA
Donkey anti-rabbit IgG Alexa Fluor® 647	1:500	A31573; Thermo Fisher Scientific, Waltham, MA, USA
Goat anti-mouse IgG Alexa Fluor® 488	1:500	A11001; Thermo Fisher Scientific, Waltham, MA, USA
Goat anti-mouse IgG Alexa Fluor® 568	1:500	10348072; Thermo Fisher Scientific, Waltham, MA, USA
Goat anti-mouse IgG Alexa Fluor® 647	1:500	A21235; Thermo Fisher Scientific, Waltham, MA, USA

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COMPETING INTERESTS

The authors declare no competing interests.

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808 **Figures & legends**

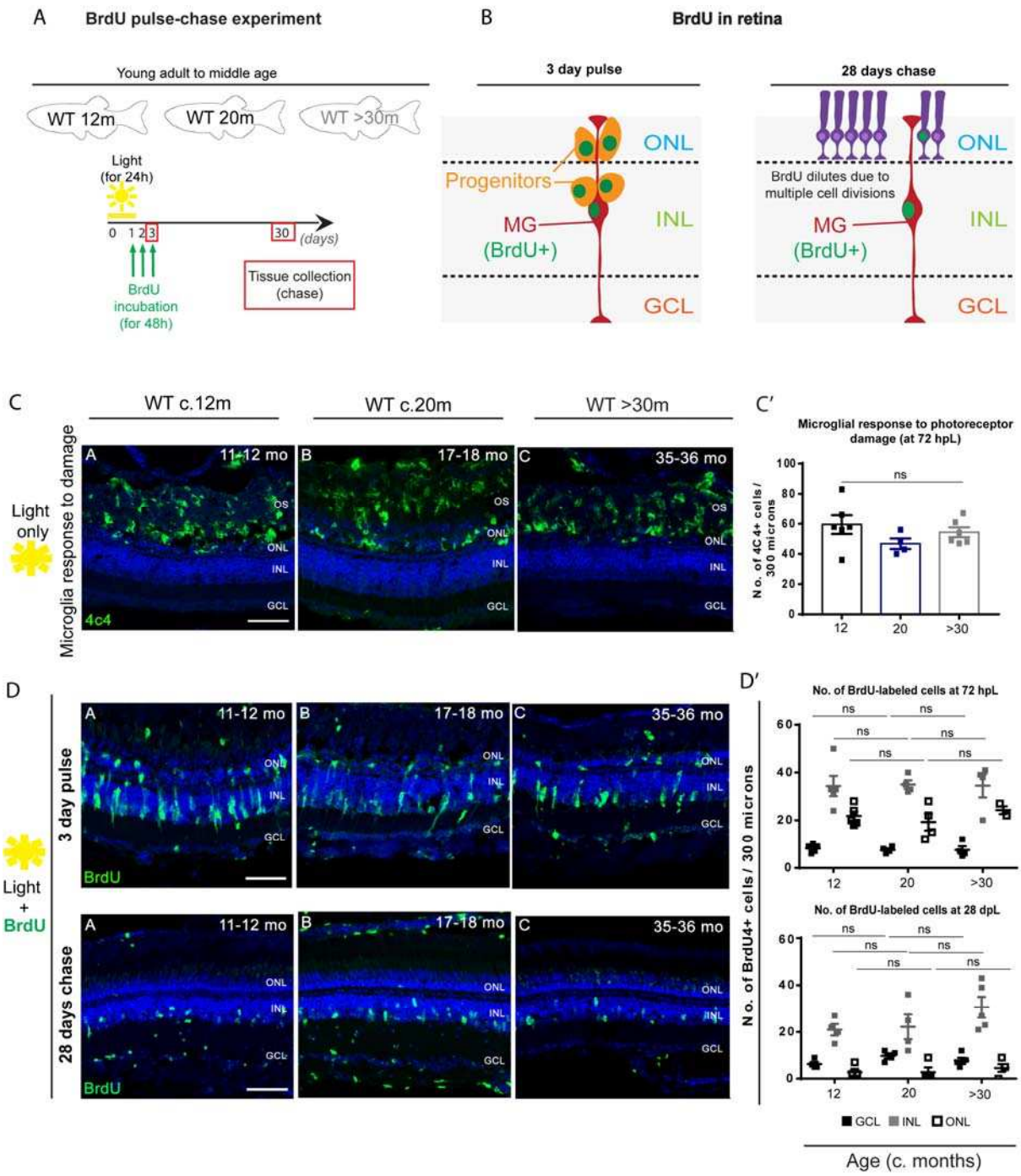

Figure 1. Zebrafish MG regenerative capacity upon acute damage is maintained until old ages. (A) Schematic image of the experimental design. (B) Schematic image of the expected results. Fish were light treated for 24 hours and BrdU incorporation occurred between 24-72 hrs (green), which allowed for BrdU to be washed out the proliferating progenitors, leaving only MG which re-entered

the cell cycle to be labelled. (C) Representative images of the central retina labelled with 4C4 (microglia, in green), after light onset, in young (12 months), middle aged 20 months) and old (>30 months) *albino* zebrafish. The majority of the microglia response to damage occurs within the outer segments (OS) and nuclear layer (ONL) of the retina, where a debris field is present due to photoreceptor degeneration. (C') Quantification of the number of microglial cells which responded to photoreceptor damage in the different aged groups (young represented in black, middle aged in blue and old in grey). (D) Representative images of the central retina 72 hours after light onset, immunolabeled with BrdU (proliferation, in green). (D'). Quantification of the number of cells proliferating in the ganglion cell layer (GCL, represented in black squares), inner nuclear layer (INL, represented in grey squares), and outer nuclear layer (ONL, represented in white squares) in each age group. (D) Representative images of the central retina 28 days after light onset. (D'). Quantification of the number of BrdU+ cells observed in the GCL, INL, and ONL of each aged group. Error bars represent the standard error of the mean (SEM). N=4-5. Stats: One-way ANOVA followed by Turkey post-hoc tests.

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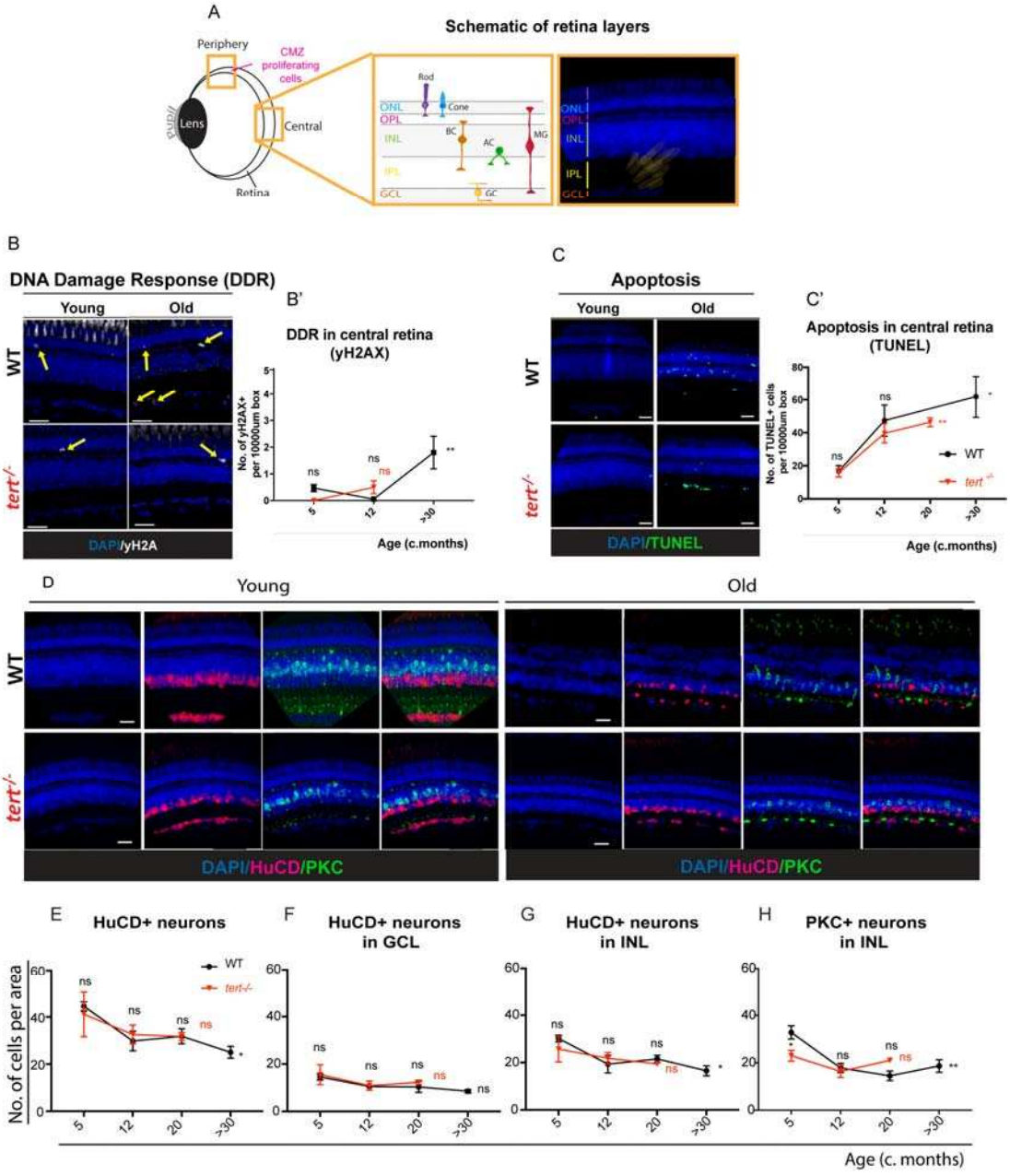

Figure 2. The ageing zebrafish retina displays neurodegeneration, independently of telomerase. (A) Schematic figure of the zebrafish retina with respective layers and cell types. (B-C) The central retina immunolabeled with (B) γH2AX (DNA damage, in white) and (C) TUNEL (apoptotic cells, in green) in both WT and *tert*$^{-/-}$, at young (5 months) and old ages (>30 months in WT and 12 or 20 in *tert*$^{-/-}$). Scale bars: 20μm. (B) Yellow arrows highlight γH2AX^{+} cells. (B'-C') Quantifications of the number of (B') γH2AX^{+} cells and (C')

TUNEL^{+} cells. (D) The central retina immunolabeled with HuC/D and PKC (amacrine in magenta and bipolar cells in green, respectively), in both WT and *tert*$^{-/-}$, at young (5 months) and old ages (>30 months in WT and 12 months in *tert*$^{-/-}$). Scale bars: 20µm. (E-H) Quantifications of the number of HuCD-positive neurons (E) in the overall retina, (F) in the GCL (ganglion cells) and (G) in the INL (amacrine cells). (H) Quantifications of the number of PKC-positive neurons in the INL (bipolar cells). (B', C', E-H) Error bars represent the SEM. N=3-4. The quantifications were performed per area of the retina (100 000 µm^2). Differences between genotypes are indicated above each timepoint, whereas differences over-time in each genotype are indicated in the end of the lines, in black WT, in red *tert*$^{-/-}$. P-value: * <0.05; ** <0.01; *** <0.001.

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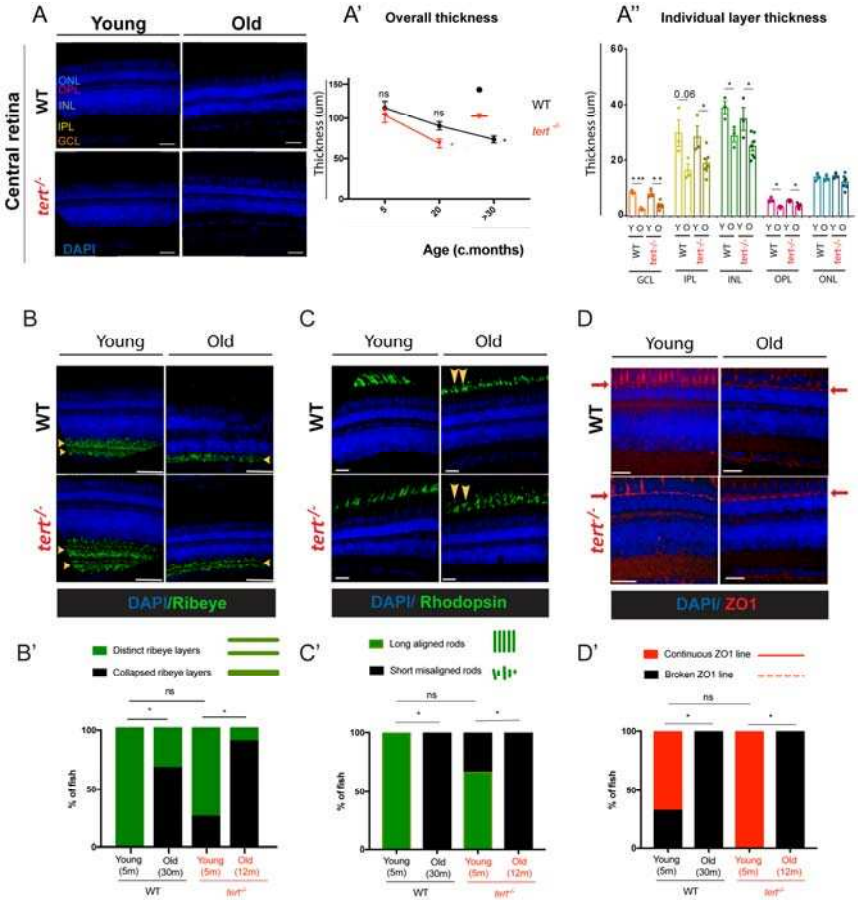

Supplementary Figure 1: Hallmarks of ageing in the zebrafish retina. (A) Central retina thinning in both WT and *tert*$^{-/-}$, at young (5 months) and old ages (>30 months in WT and 20 months in *tert*$^{-/-}$). Scale bars: 20µm. (A') Quantifications of the central retina thickness, (A') in the overall retina and (A'') per layer of the retina. Error bars represent the SEM. N=3. (B-D) The central retina immunolabeled with (B) rhodopsin (photoreceptors outer segments, in green), (C) ZO1 (outer limiting membrane, in red) and (D) ribeye (pre-synaptic ribbons, in green), in both WT and *tert*$^{-/-}$, at young (5 months) and old ages (>30 months in WT and 12 months in *tert*$^{-/-}$). Scale bars: 20µm. (B'-D') Quantification of the percentage of fish presenting defects in (B') rhodopsin (short and misaligned outer segments), (C') ZO1 (broken outer limiting membrane) and (D') ribeye (collapsed ribeye layers). Error bars represent the SEM. (B'-C') N=3-6; (D') N=4-9. Differences between genotypes are indicated above each time-point, whereas differences over-time in each genotype are indicated in the end of the lines, in black WT, in red *tert*$^{-/-}$. (B'-D') Qui-square. P-value: * <0.05; ** <0.01; *** <0.001.

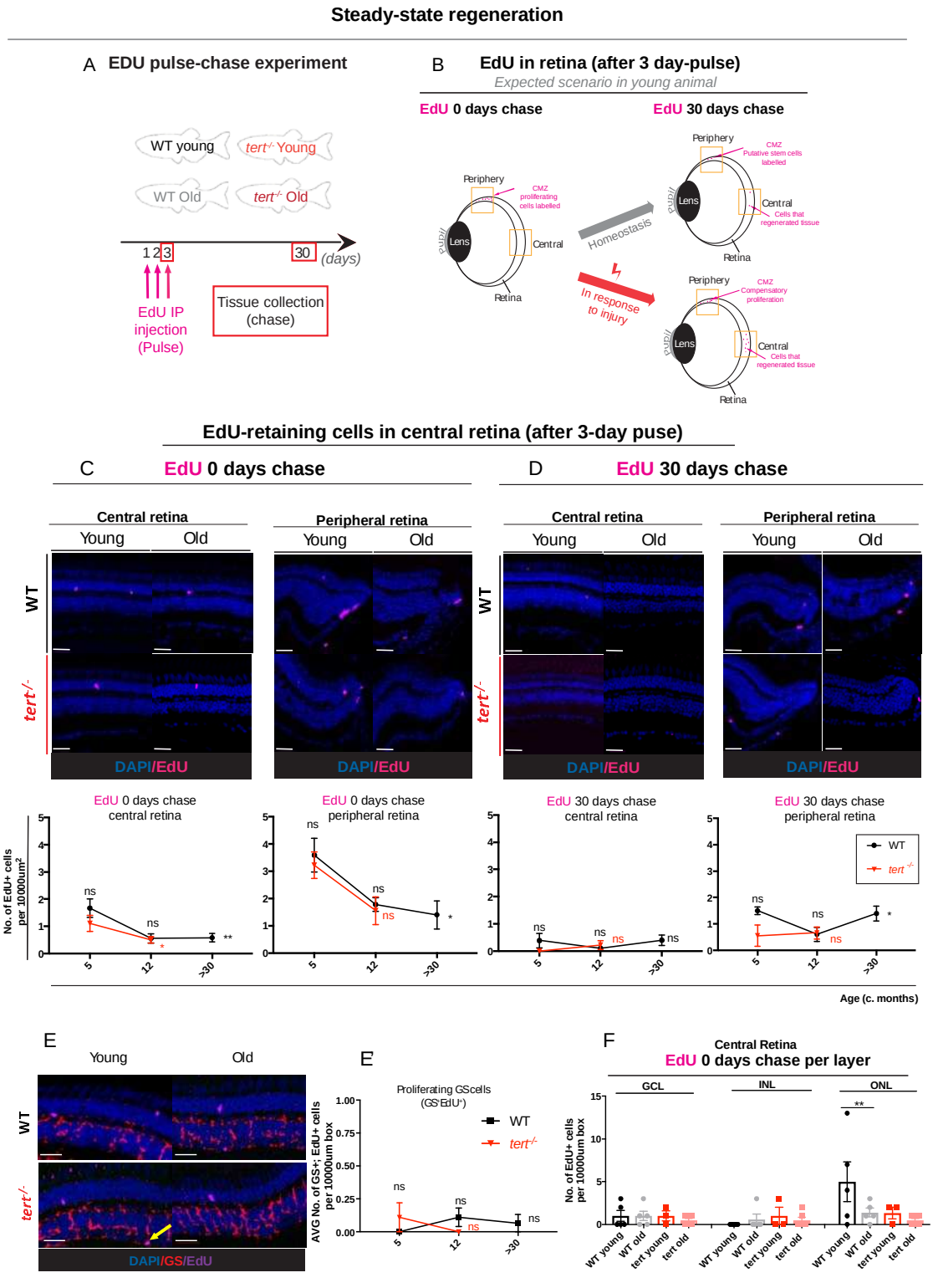

Figure 3. Aged zebrafish retina does not show signs of regeneration in response to spontaneous cell death and neuronal loss. (A) Schematic figure of the experimental

design: 3-day pulse of EdU, by IP injection, followed by 0- or 30-day chase; (B) and respective hypothesised result. We anticipated that in a healthy young fish, the retina has some cells proliferating in the CMZ, which, over-time, will replace older cells in the central retina. In the case of injury, we anticipate that there will be elevated levels of proliferation in peripheral and central retina in order to replace the death cells. (C-D) The central and the peripheral retina immunolabeled with EdU (in purple), at (C) 0- and (D) 30-days chase, in WT at young (5 months), middle (12 months) and old ages (>30 months). Scale bars: 20µm. Graphs below show quantifications of the number of EdU-retaining cells per area (100 000µm^2), in the overall central and peripheral retina at 0-days chase (C) and in the overall central and peripheral retina at 30-days chase (D). (E) The central retina immunolabeled with GS (müller glia, in red) after a 3-day pulse of EdU, by IP injection, at 0-days chase, in WT at young (5 months) and old ages (>30 months). Scale bars: 20µm. (E') Quantifications of the number of GS$^+$; EdU$^+$ cells per area (100 000µm^2). Error bars represent SEM. N=3-6. CMZ: ciliary marginal zone. Statistics: Two-way ANOVA (WT vs *tert*$^{-/-}$ at young versus old ages) and one-way ANOVA (WT and *tert*$^{-/-}$ over-time). F) Quantifications of the number of EdU-retaining cells per area (100 000µm^2), per layer of the retina, at 0-days chase. Error bars represent SEM. N=3-6. Differences between genotypes are indicated above each timepoint, whereas differences over-time in each genotype are indicated in the end of the lines, in black WT, in red *tert*$^{-/-}$. P-value: * <0.05; ** <0.01; *** <0.001.

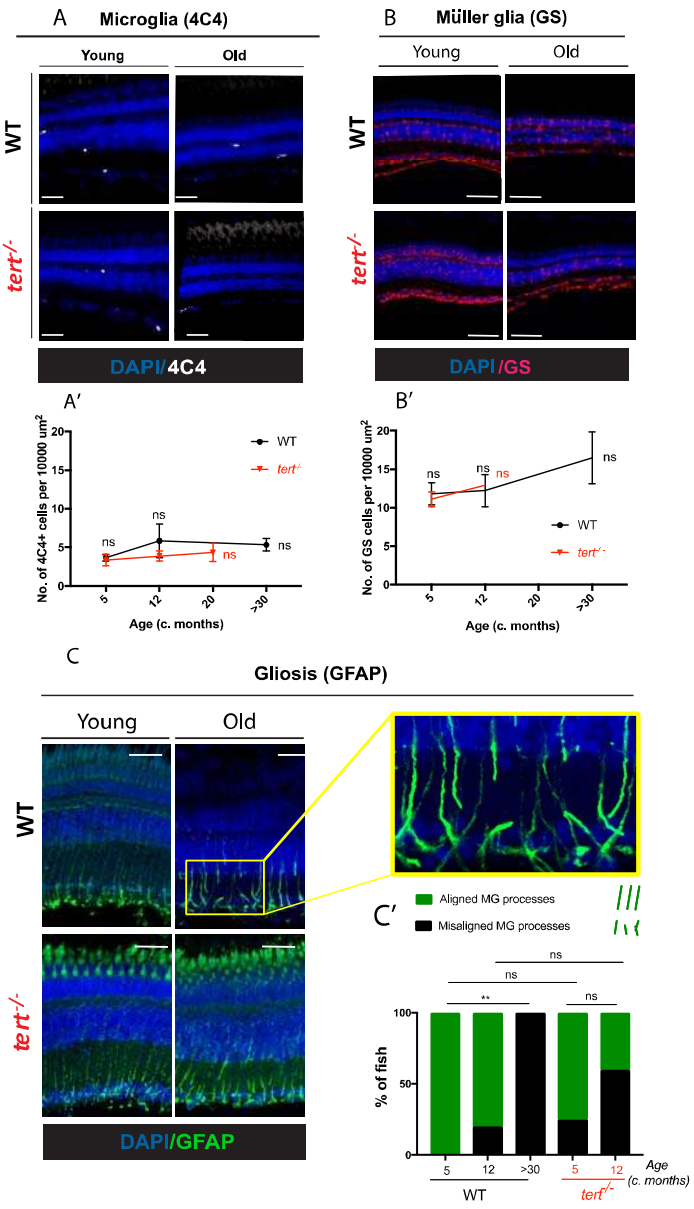

Figure 4. The zebrafish retina shows signs of gliosis with ageing, independently of telomerase. (A-B) The central retina immunolabeled with (A) 4C4 (microglia, in white) and (B) GS (Müller glia, in red), in WT, at young (5 months) and old ages (>30 months). Bars: 20µm. (A'-B') Quantification of the number of (A') 4C4-positive cells (microglia) and (B') GS-positive cells (MG), per area (100 000 µm2). Error bars represent the SEM. N=3. (C) The central retina immunolabeled with GFAP (reactive MG, in green), in WT at young (5 months), middle (12 months) and old ages (>30 months). Bars: 20µm. (C') Quantifications of the percentage of fish presenting disorganised MG processes (gliosis-phenotype). N= 3-6. Differences between genotypes are indicated above each time-point, whereas

differences over-time in each genotype are indicated in the end of the lines, in black WT, in red *tert*$^{-/-}$. (C') chi-square. P-value: * <0.05; ** <0.01; *** <0.001.

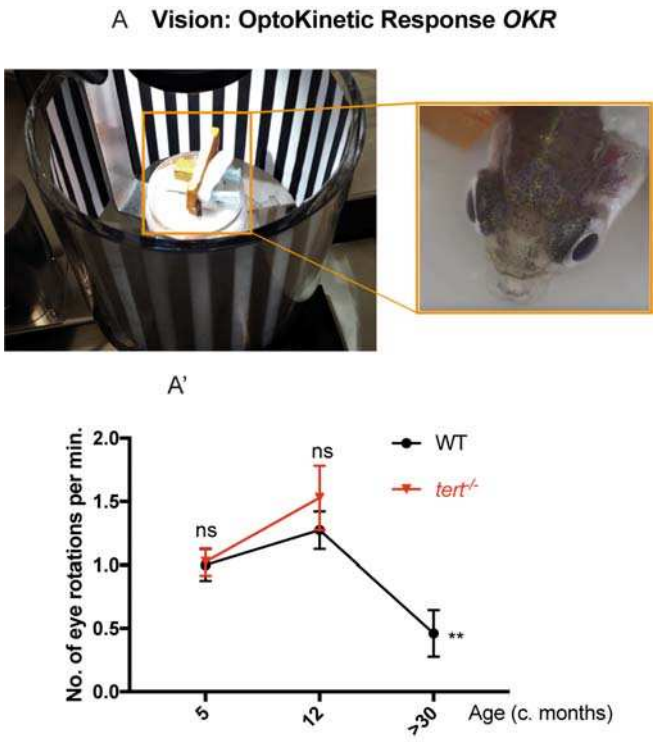

Figure 5: Zebrafish vision declines with ageing, independently of telomerase. (A-B) OKR assay was performed by immobilising the fish in between soft sponges, inside a petri dish containing water, placed in the centre of a rotation chamber. The walls of the rotation chamber had 0.8 mm-thick black and white stripes and the chamber was maintained at a constant velocity of 12 rpm throughout the experiment. (B') The number of eye rotations per minute was manually quantified by video observation. Error bars represent the SEM. N=5-8. Differences between genotypes are indicated above each timepoint, whereas differences over-time in each genotype are indicated in the end of the lines, in black WT, in red *tert*$^{-/-}$. P-value: * <0.05; ** <0.01; *** <0.001.

Phenotype of WT aged		Accelerated in tert$^{-/-}$?		Likely to be telomerase dependent?
		At 5 months	At 12 months (end life for tert$^{-/-}$)	
Visual impairment		No	No	No
Thinning of the central retina	GCL	No	N/A	No
	IPL	No	N/A	No
	INL	No	N/A	No
	OPL	No	N/A	No
	ONL	No	N/A	No
Neuronal degeneration in central retina	Rods morphology	No	N/A	No
	ZO-1 membrane	No	N/A	No
	Pre-synaptic ribbons (Ribeye)	No	N/A	No
	BCs (PKC in INL)	Yes	No	No
	ACs (HuCD in INL)	No	No	No
	GCs (HuCD in GCL)	No	No	No
Hallmarks of cellular ageing In central retina	Apoptosis	No	No	No
	DNA damage/ senescence	No	No	No
	Proliferation	No	No	No

Supplementary Table 1: Summary of the phenotypes observed in the aged zebrafish retina, and which phenotypes are telomerase-dependent or independent.

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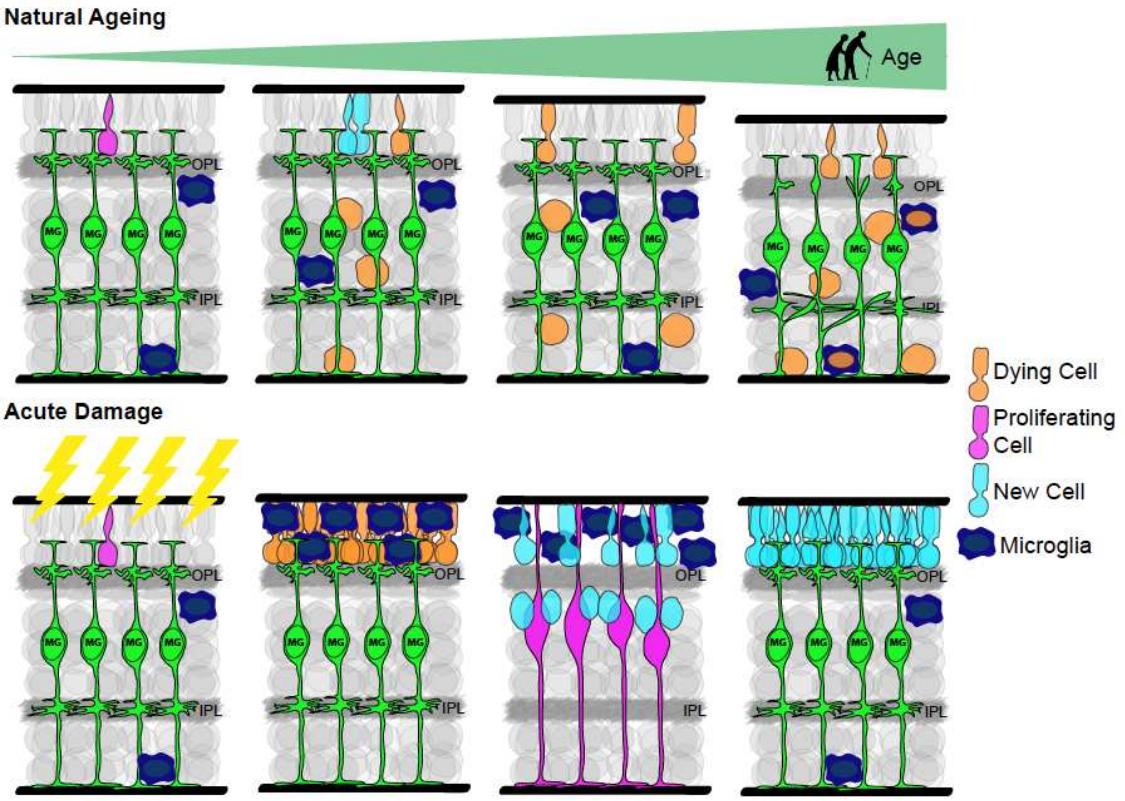

Figure 6: Our proposed model: A molecular "tipping point" required to stimulate regeneration in ageing. While zebrafish can regenerate the retina upon acute damage until late ages, its regenerative capacities do not prevent cell death, degeneration and consequent retina thinning with natural ageing. Schematic figure with the working model. In natural ageing, zebrafish retina undergoes degeneration characterised by increased cell death and neuronal loss. Our current findings show that the proliferation levels are low in young ages and decrease even more with advancing age (represented in magenta). Moreover, the number of microglia, a key play in retina regeneration, is maintained stable throughout lifespan, with an increased number of microglia dying. The lack of MG proliferation in response to chronic, age-related damage seems to lead to retina thinning with ageing, where death cells are not replaced. Middle: In contrast, in response to acute damage where there is a great number of dying cells followed by recruitment of microglia, MG proliferates, generating new neuronal cells, which replace the dying ones. Our current findings show that zebrafish is able to regenerate its retina in response to acute damage until late ages (>30 months), suggesting that the lack of regeneration in response to chronic damage is not due to MG's inability of proliferate.

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