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Retinal Proliferation Response in the Buphthalmic Zebrafish, *bugeye*

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Abstract

The zebrafish retina regenerates in response to acute retinal lesions, replacing damaged neurons with new neurons. In this study we test the hypothesis that chronic stress to inner retinal neurons also triggers a retinal regeneration response in the *bugeye* zebrafish. Mutations in the *lrp2* gene in zebrafish are associated with a progressive eye phenotype (*bugeye*) that models several risk factors for human glaucoma including buphthalmos (enlarged eyes), elevated intraocular pressure (IOP), and upregulation of genes related to retinal ganglion cell pathology. The retinas of adult *bugeye* zebrafish showed high rates of ongoing proliferation which resulted in the production of a small number of new retinal neurons, particularly photoreceptors. A marker of mechanical cell stress, Hsp27, was strongly expressed in inner retinal neurons and glia of *bugeye* retinas. The more enlarged eyes of individual *bugeye* zebrafish showed disrupted retinal lamination, and a persistent reduced density of neurons in the ganglion cell layer (GCL), although total numbers of GCL neurons were higher than in control eyes. Despite the presence of a proliferative response to damage, the adult *bugeye* zebrafish remained behaviorally blind. These findings suggest the existence of an unsuccessful regenerative response to a persistent pathological condition in the *bugeye* zebrafish.

Keywords

Zebrafish; Retina; Retinal Ganglion Cell; Regeneration; Glaucoma; Buphthalmos

Introduction

The teleost fish retina offers an interesting opportunity for studying retinal regeneration. It responds to acute retinal injury by robust cell proliferation that results in the production of new neurons (Stenkamp, 2007). Acute insults that trigger this response include surgical removal of retinal cells (Hitchcock *et al.*, 1992; Cameron and Easter, 1995; Cameron, 2000), light or laser-induced photoreceptor ablation (Braisted *et al.*, 1994; Vihtelic and Hyde, 2000;

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Wu et al., 2001), retinal puncture (Fausett et al., 2008), toxin injection (Maier and Wolburg, 1979; Negishi et al., 1988; Fimbel et al., 2007; Sherpa et al., 2008), or the loss of specific cell populations due to activation of a toxic transgene (Montgomery et al., 2010). Following acute damage, Müller glial cells re-enter the cell cycle and act as multipotent progenitors, which can generate all of the retinal cell types that were lost due to damage (Wu et al., 2001; Yurco and Cameron, 2005; Fausett and Goldman, 2006; Vihtelic et al., 2006; Kassen et al., 2007; Thummel et al., 2008; Qin et al., 2009). The regenerative process involves differential expression of many genes including those involved in retinal neurogenesis, retinal cell differentiation, and re-establishment of the optic nerve (Fimbel et al., 2007; Sherpa et al., 2008), resulting in functional recovery (Sherpa et al., 2008).

The response of the teleost retina to chronic damage is not as well understood, as there are very few fish models for retinal disease. In genetic models in which rod or cone photoreceptors selectively degenerate, prolonged proliferative responses continuously generate new photoreceptors that subsequently die due to the genetic defect (Morris et al., 2008). However, in an alternative genetic model for age-related cone loss, the *shh*^{+/-} zebrafish, proliferation is not extensive, although modification of the cone mosaic pattern provides indirect evidence for regenerative cone replacement (Stenkamp et al., 2008). The response of the zebrafish retina to chronic (genetic) damage to inner retinal neurons is not known.

Recently, an interesting zebrafish mutant has been reported that shows signs of chronic ganglion cell stress (Veth et al., 2011) and progressive visual impairment (Stujenske et al., in press). This phenotype results from a nonsense mutation in the gene encoding *low density lipoprotein receptor-related protein 2* (*lrp2*), and shows progressive onset and variable penetrance. The phenotype of *lrp2*^{-/-} zebrafish models several risk factors for human glaucoma (Quigley et al., 1988; Sommer et al., 1990), including elevated intraocular pressure (IOP), enlarged eye globes (buphthalmia), and overexpression of some genes related to ganglion cell pathology. However, the phenotype (referred to as *bugeye*) is distinct from human glaucoma in that ganglion cells do not die in large numbers and there are no signs of optic nerve pathology (Veth et al., 2011).

In the present study we use the *bugeye* zebrafish as a model to test the hypothesis that chronic damage to inner retinal neurons results in a retinal regeneration response. We found that *bugeye* retinas do show many characteristics common to teleost retinas that are in the process of regenerating following acute damage, including cell proliferation, the production of new neurons, and histological disorganization (Hitchcock et al., 1992; Stenkamp et al., 2000; Sherpa et al., 2008). A marker of mechanical cell stress, Hsp27 (O'Reilly et al., 2010) is localized to inner retinal neurons and glia, suggesting these cell populations experience chronic damage. However, the regenerative response generates only a small number of new inner retinal neurons, and the adult *bugeye* zebrafish is behaviorally blind. These results suggest that substantial retinal cell death may not be required to initiate a regeneration response, but also that this response is not successful at restoring vision.

Materials and methods

Animals

Zebrafish (*Danio rerio*) were raised at 28.5°C on a 14/10 h light/dark cycle in a recirculating aquatic housing system. Genotyped, mature *lrp2*^{-/-} zebrafish (Veth et al., 2011) were provided by Dr. Brian Link (Medical College of Wisconsin), and were maintained in-house for at least one month prior to any experiment. Experiments were performed using mature *lrp2*^{-/-} fish at ages 4, 10, 12, 13, 15, 20, 24 and 26 months. With the exception of some fish used for a behavioral assay, all displayed a buphthalmic phenotype in at least one eye, and

are referred to as *bugeye*. All control experiments were performed using adult wild-type (WT) zebrafish of 12 and 14 months of the background strain, TL (maintained in-house). In mature TL zebrafish, eye sizes do not vary significantly as a function of age, over a 4–26 month age range (data not shown). Prior to the harvesting of tissues, animals were anaesthetized one at a time using buffered 0.2% tricaine methanesulphonate (MS-222; Sigma, St. Louis, MO) and then they were sacrificed by decapitation. All procedures involving animals were approved by the Animal Care and Use Committee of the University of Idaho. A summary of experimental analyses performed on each age group of *lrp*^{-/-} and WT fish is provided as Table 1.

Eye morphometry

Eye diameters were measured along the dorsal-ventral axis before eye extraction, using a dissecting microscope and calipers graded in 10 µm increments. However, since all of our histological analyses were performed on fixed sections, additional measurements were made following paraformaldehyde fixation (see below). In order to establish a relationship between the diameters of fixed and live eyes, we selected sections that contained the largest part of the optic nerve and that showed no obvious folding or distortion. Eye diameters of these sections were then measured and compared to the corresponding live eye diameters, and a consistent relationship was observed. All further calculations of retinal area, total proliferating cells, and total differentiated neuronal cells therefore assumed these relationships and utilized dimensions of fixed tissue. In order to calculate retinal surface area, we used a method similar to Raymond et al. (1988), based upon spherical geometry (Supplemental Material).

BrdU treatments

Bugeye and WT fish were allowed to swim in beakers containing 5 mM bromodeoxyuridine (BrdU; Sigma) in system water for 3 days. Fish were then rinsed with system water, returned to the fish facility and sacrificed after 4 or 8 additional days.

Behavioral assays

A place preference test was performed to assay visual function. The place preference test is based on captive zebrafish feeding behavior. In captivity, zebrafish respond to an approaching person by swimming to the front and top of their tank and waiting under the feeding hole (Oswald and Robison, 2008; Benner et al., 2010). We took advantage of this behavior to test whether fish could respond to visual stimulus. Five adult *bugeye* (26 months old) and five WT fish (14 months old) were kept individually in 1 liter tanks. Fish were randomly assigned to tanks and coded so that genotype was not immediately obvious. Sheets of paper were placed between each tank blocking the view of adjacent tanks. Behavioral data were collected three times per day (10:00 am, 1:00 pm and 4:00 pm) and 3 days per week for 4 weeks. Each tank was demarcated into six, 2-cm vertical zones using string tied in front of the tanks. At each observation time, the vertical and horizontal locations of each fish were recorded three times over a short period of time (3–5 seconds) using scan sampling. Vertical location was coded with an integer (1 through 6, where 1 = top, 6 = bottom). Horizontal location was recorded as a binary variable, with 1 indicating the fish was in the front half of the tank nearest to the observer, and 0 indicating that the fish was in the back half of the tank. These data were then used to calculate an approximate distance from the feeding area. A linear mixed effects model was fit in order to estimate average distance from feeding area and to test whether this distance was significantly different between genotypes.

Retinal cryosections

For immunocytochemistry, tissue was processed according to Barthel and Raymond (Barthel and Raymond, 1990). Briefly, after removal of the lens, whole eyes were placed in 4% paraformaldehyde in buffered, 5% sucrose solution for one hour. Eyes were washed in buffered 5% sucrose solution, and then in increasing concentrations of sucrose prior to cryoprotection overnight at 4°C in buffered 20% sucrose solution. The following day, eyes were embedded in a solution of 1 part OCT embedding medium (Sakura Finetek, Torrance, CA) and 2 parts buffered 20% sucrose and were sectioned at a thickness of 8 μ m on a cryostat.

Measurement of optic nerve heads

Sections containing the optic nerve head (ONH) were stained with 0.1% methylene blue and slides were mounted using glycerol. Brightfield images were collected using a SPOT camera and software (Diagnostic Instruments, Sterling Heights, MI). Three sections per eye were evaluated, and for each section, three linear measurements of ONH diameter were obtained using Scion Image software, and measurements were averaged. Only the highest value for each eye was considered as representing the diameter of the ONH (Sherpa et al., 2008).

Immunocytochemistry

Immunocytochemistry was performed on retinal sections using various cell specific markers for different retina cell types. Retinal sections were blocked in 20% goat serum, and then one of the following monoclonal antibodies was used: anti-HuC/D (1:200) from Molecular Probes (Eugene, OR); Zpr-1 (1:200) and Zrf-1(1:200) from the Zebrafish International Resource Center (University of Oregon, Eugene, OR); rat anti-BrdU (1:20) purchased from Chemicon (Temecula, CA) and anti-zebrafish Hsp27 (1:250) provided by Dr. Eric Shelden (Washington State University) (Tucker et al., 2009). For BrdU staining, sections were washed in a 1:1 solution of 4N HCl:PBST (phosphate-buffered saline + 0.05% Triton X-100) for 30 minutes. Blocked sections were incubated with primary antibody overnight at 4°C. Sections were then washed in PBST, and were incubated for 2 hours at room temperature with Cy3-conjugated secondary antibody, either donkey anti-rabbit or donkey anti-mouse (either at 1:200) (Jackson ImmunoResearch, West Grove, PA). For dual immunocytochemistry involving BrdU, sections were processed for anti-BrdU indirect immunofluorescence following acid treatment, using goat anti-rat conjugated to FITC (Jackson Immunoscientific). Tissues were then washed, mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and viewed under epifluorescence and Nomarski optics.

Cell counting

Three cryosections per eye were immunolabeled with anti-HuC/D monoclonal antibody, to detect differentiated amacrine and ganglion cells. HuC/D+ cells in the ganglion cell layer were counted on these sections over a distance of 172 μ m. The dimensions, 172 μ m * 8 μ m section thickness, were used to calculate the density of HuC/D+ cells per mm² of retina. Using our measurements of retinal length and eye diameter to calculate total retinal surface area (Supplemental Material), we estimated the total number of HuC/D+ neurons in the GCL. The Spearman rank test was performed to test for a significant relationship between a) neuronal density vs. age, b) neuronal density vs. eye diameter, c) total number of GCL neurons vs. eye diameter, and d) total GCL neurons vs. eye diameter. The Welch two sample t-test was used to detect differences in neuronal densities and total number of GCL neurons of different identified sets of eyes.

Densities of BrdU+ cells were calculated using the same methods as those described for HuC/D+ cells. Paired t-tests were performed to determine whether significant differences in densities of BrdU+ cells were present between layers of the retina. The Spearman rank test was performed to determine whether a significant relationship exists between BrdU+ cell density and age or between BrdU+ cell density and age or between BrdU+ cell density and eye size.

Quantification of doubly-labeled cells was performed using retinal sections processed for BrdU immunofluorescence along with immunofluorescence for either zpr-1 or HuC/D. Numbers of colabeled cells per section were generated as averages for 6–8 sections, derived from n=35 eyes for the 4 day chase experiment and n=6–8 eyes for the 8 day chase experiment.

Terminal dUTP Nick-End labeling

Cell death was assayed on cryosections of *bugeye* and wild-type retina using Terminal dUTP Nick-End Labeling (TUNEL), an *in situ* cell death detection kit from Roche Applied Science (Indianapolis, IN). Experiments were carried out according to the manufacturer's instructions, with a peroxidase-based signal amplification step (Stenkamp et al., 2002). For each set of reactions, sections obtained from ethanol-treated zebrafish embryos (Kashyap et al., 2007) were used as positive controls. Tissues were mounted in glycerol and viewed under Nomarski optics using a Leica DMR compound microscope.

Photography and statistics

Images were collected using a SPOT camera and associated software (Diagnostic Instruments). In some cases, images obtained under different optical conditions were combined using the “apply image” function in AdobePhotoshopCS. All statistics were performed in the R statistical environment (R Core Development Team, 2005). The Welch (paired) two-sample t-test was used for comparing data obtained from the larger vs. the smaller eyes of *bugeyes* with asymmetric eyes.

Results

Buphthalmic (*bugeye*) *lrp2*^{-/-} zebrafish have large and asymmetric eyes with disorganized retinas

The *lrp2*^{-/-} zebrafish displays an enlarged eye phenotype (buphthalmia) that may become evident as early as 4 months but varies in time of onset, and which is likely related to high IOP (Veth et al., 2011; Stujenske et al., in press). We wished to understand the consequences of buphthalmia as a chronic condition in mature fish; therefore we sampled buphthalmic *lrp2*^{-/-} zebrafish sacrificed at 4, 10, 12, 13, 15, 23, 24, and 26 months of age. We used, as our experimental population, only *lrp2*^{-/-} fish with one or both eyes at least 20% larger than average TL control eyes (Fig. 1A–D). These phenotypically buphthalmic *lrp2*^{-/-} zebrafish will be referred to as *bugeyes* (see also Veth et al., 2011; Stujenske et al., in press). We measured eye diameters in live as well as paraformaldehyde-fixed eyes, the latter of which were reliably found to be 48% of the corresponding diameter of a freshly excised eye (data not shown). The eyes of *bugeye* fish were significantly larger than those of control fish (Fig. 1A–D). Most *bugeyes* showed pronounced asymmetry in eye size, with one eye visibly larger than the other. When sorted according to relative size, bigger eyes (those at least 10% larger than the contralateral eye) obtained from *bugeye* fish were significantly larger than control eyes ($p < 0.001$); smaller eyes obtained from *bugeye* fish were also significantly larger than controls ($p < 0.01$), and bigger eyes were significantly larger than smaller eyes obtained from *bugeye* fish ($p < 0.05$; Fig. 1D). When considered as a function of animal age, the bigger eyes, as well as the smaller eyes did not vary in size as a

function of age (Fig. 1E). These data indicate that the eyes of *bugeye* zebrafish reach a terminal size and do not continue to grow with organismal age.

To determine whether retinal surface area was also enlarged in mature *bugeye* zebrafish, we selected radial cryosections that bisected (or nearly bisected) the optic nerve head, and measured the linear distance of the retina from the inferior margin to the superior margin (Fig. 1F–H). Retinal length in these sections was highly correlated with eye diameter ($p < 0.001$; $\rho = 0.92$), indicating that retinal area in *bugeye* remains greatly expanded as compared to control eyes (Fig. 1I).

We next evaluated the histological appearance of the retinas in mature *bugeyes* as compared to those of control zebrafish, using sections traversing the optic nerve head (Fig. 1J–M and accompanying insets in J and K). The smaller eyes of *bugeyes* showed markedly thinner retinas, but with generally normal histological organization (Fig. 1K; see also Veth et al., 2011; Stujenske et al., in press). Interestingly, retinas of the larger eyes of *bugeyes* more frequently showed an unusual histological organization, with some regions that were thinned and similar in appearance to the smaller *bugeye* eyes, and other regions that were profoundly disorganized (Fig. 1L, M). The disorganized regions had extensive lamination defects with ectopic cell bodies residing within plexiform layers, and occasionally, radial columns of cell bodies spanning the thickness of the retina (Fig. 1M). These histological abnormalities were not observed in *lrp2*^{-/-} mutants at the onset of the buphthalmic phenotype (Veth et al., 2011; Stujenske et al., in press). To some extent, the lamination defects of bigger *bugeye* eyes resemble the histological abnormalities seen in teleost retinas that have regenerated in response to acute damage (Hitchcock et al., 1992; Stenkamp et al., 2001; Fimbel et al., 2007; Sherpa et al., 2008).

Bugeye zebrafish retinas show robust cell proliferation

The histological appearance of *bugeye* retinas (Fig. 1K–M), together with the documented increases in expression of some genes related to ganglion cell stress (Veth et al., 2011), suggests a condition of chronic retinal injury. The WT zebrafish retina responds to acute retinal injury with a regenerative response such that Müller glia re-enter the cell cycle and become pluripotent (Wu et al., 2001; Yurco and Cameron, 2005; Fausett and Goldman, 2006; Qin et al., 2009). We therefore wished to determine whether the adult *bugeye* mutant retina displayed evidence of a regenerative response, and to understand the extent of this response. Adult *bugeyes* and wildtype zebrafish of various ages were immersed in 5 mM BrdU for three days and were returned to system water for an additional four days before sacrifice. Eyes were removed and sectioned, then processed for BrdU indirect immunofluorescence. *Bugeye* zebrafish showed extensive cell proliferation in all retinal layers, throughout the entire retina (Fig. 2B). By comparison, WT adult zebrafish retinas generally showed very few or no BrdU+ cells (Fig. 2A), and these were restricted to the circumferential germinal zone (data not shown), and the outer nuclear layer (ONL), regions that show persistent neurogenesis, albeit at a very slow rate, in adult WT zebrafish (Marcus et al., 1999). We counted BrdU+ cells in three sections per *bugeye* eye, using sections sampled from central retina. Densities were calculated based on section thickness and the distance over which cells were counted. Densities of BrdU+ cells were significantly greater in bigger vs. smaller eyes (Fig. 2C, $p < 0.05$; matched-pairs Wilcoxon signed rank test). In addition, the density of BrdU+ cells in the ONL was higher compared to that of the inner nuclear layer (INL; Fig. 2D; $p < 0.001$) and the GCL (Fig. 2D; $p < 0.01$). The densities of BrdU+ cells in each layer did not vary significantly as a function of age (Fig. 2D, Spearman's rank correlation test), but densities of BrdU+ cells in the INL and GCL did vary as a function of eye diameter, with the larger eyes showing higher rates of BrdU incorporation (Fig. 2E, $p < 0.01$, $\rho = 0.40$ for INL; $p < 0.001$, $\rho = 0.50$ for GCL; Spearman's rank correlation test). These data suggest that pathological features within the *bugeye* eye

result in a widespread retinal proliferation response, with large eyes engaging in higher rates of proliferation, that generates cells of all retinal layers. However, this robust proliferation does not result in increased eye size as the animal ages (Fig. 1E).

Adult bugeye zebrafish generate some new retinal neurons

In order to determine whether new retinal cells were generated in adult *bugeye*, we carried out co-labeling studies using BrdU together with three different retinal cell markers. Anti-HuC/D labels postmitotic neurons (Kim et al., 1996), and in embryonic and adult zebrafish retina it stains predominantly RGCs and amacrine cells, in the GCL and the INL (Fig. 3A). In sections obtained from adult *bugeye* mutant eyes, we occasionally observed colabeled cells (Fig. 3B–D), suggesting that some new amacrine cells and/or RGCs were generated over the BrdU exposure and chase period. The Zpr-1 antibody labels an unknown cell surface epitope on red- and green-sensitive cone photoreceptors (Larison and Bremiller, 1990) (Fig. 3E). In the adult *bugeye* retinas, we again occasionally observed colabeled cells (Fig. 3F–H), indicating that new photoreceptors were also generated. However, we note that these photoreceptors were less densely distributed (see also Veth et al., 2011; Stujenske et al., in press), and appeared disorganized. Finally, the Zrf-1 antibody detects glial fibrillary acidic protein (GFAP), characteristic of differentiated Müller glia; Zrf-1 most strongly stains the glial processes and end feet in the inner plexiform layer and GCL (Trevarrow et al., 1990) (Fig. 3I). Adult *bugeye* retinas contained BrdU+ profiles that were associated with glial processes; however, unambiguous colabeling was not detected (Fig. 3J–L), likely due to the different subcellular localization of the two antigens (BrdU is nuclear and GFAP is cytoplasmic).

Quantification of colabeling suggested that the fate of some proliferating cells in adult *bugeye* retinas was a photoreceptor fate, with fewer cells fated to become HuC/D+ inner retinal neurons (Fig. 4A). To further evaluate the fate of proliferating cells in the *bugeye* retinas, we performed an additional BrdU exposure experiment, but with an 8 day post-BrdU survival period (8 day chase), rather than 4 days (4 day chase). In the 8 day chase experiment, BrdU labeling was far less extensive than in the 4 day chase experiment, although BrdU+ cells were found in all retinal layers (Fig. 4B). The densities of BrdU+ cells following the 8 day chase were significantly reduced as compared to the densities following the 4 day chase (Fig. 4C). When evaluated as a function of eye diameter and laminar position, BrdU+ cell density within the GCL was weakly correlated with eye diameter (Fig. 4C; $p < 0.05$; $\rho = 0.77$), but no other correlations were statistically significant. In the 8 day chase experiment, we observed BrdU+/Zpr-1+ photoreceptors (Fig. 4A; data not shown) at densities that were statistically indistinguishable from those observed following a 4 day chase. Therefore, new cone photoreceptors generated in *bugeye* retinas are likely maintained and potentially integrated into the retina. However, we did not observe any BrdU+/HuC/D+ cells in the 8 day chase experiment (Fig. 4A; data not shown). It is possible that the colabeled HuC/D+ cells observed in the 4 day chase experiment did not continue to express this marker (i.e. were fated to differentiate as a neuronal cell type that does not express HuC/D), or that these cells re-entered the cell cycle, or they did not survive. Collectively these results suggest that proliferation had continued during the 8 day chase period, predominantly resulting in dilution of label rather than the generation and differentiation of additional retinal neurons.

Inner retinal neurons and glia of bugeyes express a marker of mechanical cell stress

To evaluate an alternative fate of proliferating cells in buphthalmic *bugeye* retinas, we performed cell death (TUNEL) assays on 10 sections per eye from adult *bugeye* eyes at 12, 23 and 26 months ($n=9$ small eyes, 9 big eyes, and 8 'equal' eyes). We were unable to locate any apoptotic cells (not shown). This finding is consistent with that of Veth et al. (2011),

who documented extremely low levels of cell death – less than 1 dying cell per whole retina – in *lrp2*^{-/-} (*bugeye*) zebrafish earlier in their lifespan, at the onset of the buphthalmic phenotype. The current results in older *bugeye* zebrafish suggest that retinal cell death remains a rare occurrence despite the presence of a chronic injury condition.

In zebrafish models for genetic forms of retinal damage, the rate and location of retinal proliferation is tightly correlated with the extent of cell death and the retinal cell types that are lost (Morris et al., 2008; Montgomery et al., 2010). Because retinal cell death in *bugeye* is rare (current study; Veth et al., 2011), we tested the hypothesis that proliferation in *bugeye* is a response to cell stress rather than cell death. Molecular signatures of general cell stress were reported for *bugeye* (Veth et al., 2011), but the expression patterns were not. Therefore we probed retinal cryosections obtained from adult *bugeye* and WT fish with an anti-Hsp27 (heat shock protein 27) polyclonal antibody. The small heat shock protein 27 is a marker of mechanical cell stress (O'Reilly et al., 2010), and is a common feature of several animal models of glaucoma, as well as human glaucoma (Tezel et al., 2000; Huang et al., 2007; Kalesnykas et al., 2007). Control retinas showed very weak or no labeling (Fig. 5A,G); however, *bugeye* retinas showed intense and widespread labeling, predominantly in the GCL and in a radial pattern suggestive of Müller glial morphology (Fig. 5D, J). Dual immunolabeling experiments revealed colocalization of Hsp27 in Hu+ neurons (Fig. 5D–F), and in Zrf+ Müller glia (Fig. 5J–L). These results suggest that inner retinal neurons and glia in mature *bugeye* retinas experience and respond to considerable cell stress.

Reduced neuronal densities are found in the GCL of adult bugeyes

The *bugeye* zebrafish shows reduced cell densities in the GCL at 6 months (Veth et al., 2011). Given that extensive proliferation in adult *bugeye* results in the production of some new neurons, we wished to specifically determine the densities of HuC/D+ neurons in the GCL of *bugeye* zebrafish of a variety of adult ages and eye sizes. Sections were stained with the anti-HuC/D antibody and neuronal densities were calculated. The general distribution of HuC/D+ neurons in control eyes, and in the bigger and smaller eyes of *bugeye* fish was similar (Fig. 6A–C), though *bugeye* retinas occasionally showed clumps of HuC/D+ cells (Fig. 6B), and/or laminar fusions (HuC/D+ cells residing within the inner plexiform layer; Fig. 5E; see also Fig. 1K–M). A comparison of GCL neuron densities of all *bugeye* eyes compared to those of all control eyes evaluated found no significant differences (data not shown). However, when sorted by bigger vs. smaller eye of *bugeyes* with asymmetric eye sizes, the bigger eyes of *bugeye* zebrafish showed significantly reduced GCL neuron densities as compared to control eyes (Fig. 4D; $p < 0.001$; Welch two sample t-test). Using calculations derived from spherical geometry (see Methods and Supplemental Material), we estimated retinal surface area for each eye, and then calculated the total number of neurons in the GCL per eye (Fig. 6E). Perhaps not surprisingly, the eyes of *bugeye* zebrafish (both the bigger eyes and the smaller eyes in cases of eye asymmetry) contained significantly more neurons in the GCL than WT control eyes (Fig. 6E; $p < 0.001$ for bigger *bugeye* eyes vs. control and $p < 0.05$ for smaller *bugeye* eyes vs. control; Welch two sample t-test).

We wished to further establish the relationships of neuronal density in the GCL with respect to animal age and eye size. There was no correlation between neuronal densities in the GCL of bigger or smaller *bugeye* eyes with animal age (Fig. 6F), or with eye diameter (Fig. 6G). Therefore, although the larger eyes in *bugeyes* showing eye asymmetry have reduced neuronal density in the GCL as compared to control eyes, neuronal density is not strictly correlated with eye size.

The total number of neurons in the *bugeye* GCL did not vary significantly with animal age (Fig. 6H). However, for both bigger and smaller *bugeye* eyes, a significant positive correlation was detected for total GCL neurons vs. eye diameter (Fig. 6I; $p < 0.001$,

$\rho=0.69$ for bigger eyes and $p<0.001$, $\rho=0.85$ for smaller eyes), indicating that larger eyes contain more RGCs.

Optic nerve heads are enlarged in bugeyes

At the time of onset of the *bugeye* phenotype, some *lrp*^{-/-} zebrafish display “wandering axons” in the nerve fiber layer, but also histologically normal optic nerve heads (ONHs; Veth et al., 2011). To gain insights into whether the high total number of GCL neurons in *bugeye* mutants included RGCs with axons traversing the ONH, we measured ONH diameters in adult *bugeye* and WT zebrafish. In agreement with the results of Veth et al. (2011), the ONHs of adult *bugeye* mutants appeared grossly normal upon dissection, and in methylene blue-stained cryosections contained pigment as well as lightly-stained tissue, similar to the appearance of control ONHs (Fig. 7A–C). However, regions of retinal disorganization (as in Fig. 1L, M) were often seen near the ONH. Diameters of ONHs in adult *bugeye* were significantly larger than those of WT eyes (Fig. 7D). In *bugeyes* with asymmetric eyes, ONHs of the smaller eyes ($p<0.05$) and those of the bigger eyes ($p<0.001$) were significantly larger in diameter than control ONHs (Fig. 7D; Welch two sample t-test). There was no significant relationship between ONH size and animal age (Fig. 7E); however, a highly positive correlation was detected, in the bigger *bugeye* eyes, of ONH size with eye diameter (Fig. 7F; $p<0.001$, $\rho=0.955$, Spearman rank correlation test).

GCL neuronal density was not correlated with size of the ONH in the large or small eyes of *bugeye* zebrafish (Fig. 8A). However, we observed a significant positive correlation between total numbers of GCL neurons and ONH diameter when the bigger *bugeye* eyes were considered (Fig. 8B; $p<0.01$, $\rho=0.67$). There was no correlation between total numbers of GCL neurons and ONH size in the smaller *bugeye* eyes (Fig. 8B). Because the large number of GCL neurons in the bigger eyes of the *bugeye* mutant predicts the size of the ONH, many of the HuC/D+ GCL neurons are likely to be RGCs extending axons through the ONH. However, this may not be the case in the smaller eyes of the *bugeye* mutant. These results suggest that the mechanisms for populating the GCL, and therefore for maintenance and growth of the ONH within the big eyes may be distinct from those of small eyes in the *bugeye* zebrafish.

Buphthalmic *lrp2*^{-/-} zebrafish are visually impaired

The *lrp2*^{-/-} zebrafish displays visual deficits including myopia (Veth et al., 2001; Stujenske et al., in press), abnormalities in the electroretinogram (ERG), and reduced contrast sensitivity as revealed by the optomotor response (OMR) (Stujenske et al., in press). The OMR deficits are mild but progressive over 3–12 months, and not strictly associated with myopia, suggesting that retinal defects, rather than refractive error, underlie the abnormalities in visual function. Here we evaluate visual function in an older cohort of *bugeye* zebrafish (26 months old; 22% greater eye diameter as compared to WT, $p<0.05$; see Fig. 1E) using a place-preference assay. The place preference test is based on captive zebrafish feeding behavior and is an indirect measurement of visual function (see Methods; Benner et al., 2010). Distance from feeding area, in response to a visual stimulus (a waving hand) was recorded against observation days for 27 days (Fig. 9A). Visual stimuli generated significantly different responses in adult *bugeye* as compared to WT fish at all observation times ($p<0.05$; Simultaneous Tests for General Linear Hypotheses (Hothorn et al., 2008)). The lack of movement toward the feeding area was not likely due to nonvisual differences, such as differences in hunger or swimming ability, because the *bugeye* zebrafish responded to the actual presence of food in the water, by moving to the feeding area (data not shown).

We next independently tested a younger adult cohort (15 months old) of four, *lrp2*^{-/-} zebrafish. Three of these did not show significant buphthalmia, while the fourth displayed

one slightly enlarged eye (10% greater eye diameter than WT). These younger *lrp2*^{-/-} zebrafish responded to visual stimuli in a manner resembling those of WT fish, with average distances from the feeding area of 2.3 ± 1.3 cm (based upon eight weeks of twice-weekly observations). These data indicate that the *lrp2*^{-/-} genotype can be conditioned for this visually-mediated behavior. Collectively our behavioral results corroborate that visual impairment in the *lrp2*^{-/-} zebrafish is progressive and associated with buphthalmia rather than strictly to the *lrp2*^{-/-} genotype, and further indicate that deficits in visually-mediated behavior become quite severe in older *bugeyes*.

Discussion

The major findings of this study are: 1) the eyes of adult *bugeye* zebrafish are enlarged and often asymmetric, and in some cases the retinas are highly disorganized; 2) the retinas of adult *bugeye* zebrafish show large numbers of ectopically proliferating cells, and this proliferation results in the production of some new retinal neurons, particularly photoreceptors; 3) retinal cell death was not observed in the adult *bugeye* zebrafish, although a marker of cell stress, Hsp27, was strongly expressed in Müller glia and neurons; 4) the more enlarged eyes of *bugeye* zebrafish show reduced densities of GCL neurons, although total numbers of GCL neurons are very high and ONHs are enlarged; and 5) older *bugeye* zebrafish are behaviorally blind. Together our findings suggest the existence of a proliferative response to an ongoing pathological condition in the *bugeye* mutant, which provides insights into the retinal response to chronic cellular stress, as well as insights into the factors that control eye size.

Proliferative response to retinal pathology in bugeye

In adult *bugeye*, BrdU is robustly incorporated into all retinal layers at levels far beyond those seen in mature WT fish. This level of BrdU incorporation is reminiscent of that seen following acute retinal damage (Sherpa et al., 2008; Vihtelic and Hyde, 2000), and therefore suggests a regenerative response to retinal damage. In the acute damage models, the newly-generated cells (those that incorporated BrdU) express markers consistent with their differentiation as specific retinal neurons (Wu et al., 2001; Fimbel et al., 2007). In *bugeye*, however, only a very small number of newly-generated neurons could be confirmed through dual immunocytochemistry. Unlike the response to acute damage, *bugeye* mutants show a progressive phenotype which develops over a longer period of time (Veth et al., 2011; Stujenske et al., in press). For this reason, it is possible that cell differentiation events are rarer in adult *bugeye* than one would expect following acute damage.

Regeneration following acute retinal damage in the zebrafish is preceded by a wave of cell death (Fimbel et al., 2007; Sherpa et al., 2008). Interestingly, the frequency of cell death in *bugeye* was not detectably higher than in WT retinas, and even when whole retinas were evaluated, the rate of cell death was surprisingly small – less than one cell per entire retina in 12 month old *bugeyes* (Veth et al., 2011). However, robust expression of the cell stress marker, Hsp27, was localized to inner retinal neurons and Müller glia. It is possible, therefore, that the extensive level of retinal proliferation observed in the *bugeye* zebrafish is a response to cell stress, rather than to cell death as seen in regenerative responses to acute retinal damage. These findings suggest that widespread retinal cell death may not be a prerequisite for a proliferative response in teleost fish.

This response to chronic mechanical cell stress generates new neurons, although very few of the neuronal types showing evidence of this stress – HuC/D+ cells of the INL and GCL – are generated, and none appear to persist. Rather, the proliferative response generates photoreceptors, and is also non-regenerative in that many proliferative cells may not exit the cell cycle to produce post-mitotic neurons. In other models for retinal damage in zebrafish in

which specific cell populations die, the regenerative response selectively replaces these cell populations (Morris et al., 2008; Montgomery et al., 2010). This is clearly not the case in *bugeye*. It is tempting to speculate that in *bugeye*, although mechanical cell stress promotes some aspects of a regenerative response, in the absence of substantial cell death the critical signals are missing that are required to determine fates of replacement retinal cells.

Bugeye as a model for human ocular disorders

The *bugeye* zebrafish displays several features considered risk factors for human glaucoma; these include elevated IOP and buphthalmia (Veth et al., 2011). Additionally, retinal neurons and glia in *bugeye* zebrafish show upregulation of the cell stress factor Hsp27, similar to the situation in rodent models of glaucoma (Huang et al., 2007; Kalesnykas et al., 2007), suggesting a conserved stress response to elevated IOP across species. However, *bugeye* is capable of maintaining a population of GCL neurons even under elevated IOP, and may therefore provide a system in which to study resistance to stress in retinal neurons.

A critical feature of human glaucoma is progressive loss of visual function. Our data, together with those of Stujenske et al. (in press) demonstrate progressive visual deficits in *bugeyes* resulting in behavioral blindness at 26 months. In human glaucoma and mammalian glaucoma models, blindness has been linked to RGC loss and damage to the optic nerve (Vrabec and Levin, 2007; McKinnon et al., 2009; Quigley et al., 1995). In the *bugeye* zebrafish, the level of RGC death was either not detected (present study; fish aged 4–26 months), or detected at less than one cell per entire retina Veth et al., 2011; 12 month old fish). Nor does the ONH atrophy, suggesting that the mechanisms leading to blindness are different in *bugeye* as compared to mammalian glaucoma models. One possibility is that, although new neurons are generated continuously in adult *bugeye*, these cells may not be generated in sufficient numbers, or fail to become functionally integrated into the retina due to high IOP and/or elevated levels of cell stress. The reduced neuronal densities reported here and elsewhere (Veth et al., 2011; Stujenske et al., in press), together with retinal disorganization, support this hypothesis, and may underlie the a- and b-wave ERG deficits recorded from *bugeye* eyes (Stujenske et al., in press).

The *lrp2* gene encodes a transmembrane protein with functions in receptor-mediated endocytosis of a variety of signaling molecules (Fisher and Howie, 2006), and is expressed in the retinal pigmented epithelium in zebrafish (Veth et al., 2011; Kur et al., 2011). In humans, mutations in *LRP2* (also known as *Megalin*) cause Donnai-Barrow syndrome (DBS), a rare disorder with a complex phenotype that includes high myopia and buphthalmia, along with sensorineural deafness, hypertelorism, and agenesis of the corpus callosum (Donnai and Barrow, 1993; Kantarci et al., 2007). Curiously, the mouse *Lrp2*^{-/-} mutant displays microphthalmia or anophthalmia (Blake et al., 2011), which is likely secondary to holoprosencephaly (Willnow et al., 1996), suggesting that the zebrafish may more appropriately model the ocular phenotype of the human disorder.

Insights into factors controlling eye size

The presence of enlarged and asymmetric eyes in the *bugeye* zebrafish provides an opportunity to explore mechanisms that regulate eye size. The adult eye phenotype of the *bugeye* mutant zebrafish bears some resemblance to that of the black moor goldfish. A mechanism suggested by (Raymond et al., 1988), to account for increased cell division in the black moor, is that mechanical stretch of the retina may be accelerated when IOP is higher, and a decrease in cell density due to stretching activates a signaling mechanism initiating proliferation. This mechanism is a possibility in *bugeye*, which shows an increase in proliferation in the INL and the GCL as a function of eye size (Fig. 2E), sufficient to maintain GCL neuron density in small eyes, but not in big eyes (Fig. 6D).

Despite the existence of robust retinal proliferation in adult *bugeye* zebrafish, the eyes, retinas, densities of GCL neurons, total numbers of GCL neurons, and ONH diameters all reach terminal sizes (Figs. 1, 6, 7). Furthermore, at the circumferential germinal zone (CGZ), a region containing retinal stem cells and contributing to retinal growth in larval and juvenile WT fish (Stenkamp, 2007), proliferation is reduced in *lrp2*^{-/-} retinas at the onset of the buphthalmic (*bugeye*) phenotype (Veth et al., 2011). Ongoing proliferation is therefore not likely a direct mechanism underlying eye enlargement in the adult *bugeye* mutant, but is more likely a response to a retinal pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Retinas of the buphthalmic, adult *bugeye* zebrafish show high rates of ongoing proliferation
- Adult *bugeye* retinas regenerate some new neurons
- Some adult *bugeye* retinas show profound disorganization
- Adult *bugeye* retinas show evidence of mechanical cell stress to inner retinal neurons
- Regenerative responses to pathology in adult *bugeye* do not restore visual function

Figure 1. Eye morphometry of adult buphthalmic (*bugeye*) *lrp2*^{-/-} zebrafish

A.–C. Dorsal views of 10 month-old WT (TL strain) (A), and *bugeye* (B, C) zebrafish. *Bugeyes* may have symmetric (B) or asymmetric (C) eye sizes. Image in B contains a discontinuity (*) as this fish was not placed as close to the scale. D. Boxplot of diameters of *bugeye* and WT zebrafish eyes; diameters are those obtained after paraformaldehyde fixation. In the boxplots, lines depict the medians, boxes delineate the 25th to 75th percentiles, and whiskers indicate the limits. a=significantly different from WT eyes; b=significantly different from big *bugeye* eyes; c=significantly different from small *bugeye* eyes. E. Fixed eye diameter as a function of age in *bugeye* zebrafish. Trendlines for small *bugeye* eyes (blue) and large *bugeye* eyes (black) are not statistically significant. F–H. Brightfield images of representative radial cryosections of WT (F) and *bugeye* (G,H) eyes. G shows the smaller eye of a pair of asymmetric eyes; H shows the larger eye. I. Retinal length vs. eye diameter in *bugeye* (open diamonds) and WT (closed red circles). The trendline depicts a significant correlation ($p < 0.001$; $\rho = 0.92$). J–M. Methylene blue-stained cryosections of retinas from 10-month old WT (J), and *bugeye* (K–M) zebrafish; K shows a retina from the smaller eye of a pair of asymmetric eyes; M shows a retina from a larger eye; L was derived from a *bugeye* with symmetric (and very large) eyes. Insets in J and K show overviews of entire section from which the corresponding histological images (in red boxes) were derived; red asterisks (*) indicate position of optic nerve head. Regions of some *bugeye* retinas displayed profound disorganization (L,M); arrow in M shows an ectopic cell body and asterisk (*) shows a radial array of cell bodies spanning the thickness of the retina. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in A (applies to A–C) = 1 mm; scale bar in F (applies to F–H) = 250 μ m; scale bar in K (applies to J–M) = 50 μ m.

Figure 2. Cell proliferation in *bugeye* retinas

A–B. Indirect immunofluorescence image of BrdU staining in 12 month old WT (**A**) and *bugeye* (**B**) zebrafish retinas; the fish were exposed to BrdU for 3 days and returned to system water for 4 days before sacrifice. In WT fish, BrdU+ cells are occasionally seen in the outer nuclear layer (ONL). In *bugeye*, BrdU+ cells are abundant in the ONL, inner nuclear layer (INL), and ganglion cell layer (GCL). **C.** Boxplots showing densities of BrdU+ cells in big vs. small eyes of *bugeye* fish having asymmetric eyes; asterisk (*) indicates a significant difference ($p < 0.05$). In the boxplots, lines depict the medians, boxes delineate the 25th to 75th percentiles, and whiskers indicate the limits. **D.** Boxplots showing densities of BrdU+ cells in each retinal layer (small circle indicates an outlier); density of BrdU+ cells in the ONL is higher than in the other two layers ($p < 0.001$ vs. INL; $p < 0.01$ vs. GCL). **E.** Densities of BrdU+ cells in each retinal layer as a function of age; trendlines do not depict significant correlations. **F.** Densities of BrdU+ cells in each retinal layer as a function of eye diameter; a significantly positive correlation was detected for cells of the INL ($p < 0.01$; $\rho = 0.40$), and for cells of the GCL ($p < 0.001$; $\rho = 0.48$). Scale bar in **A** (applies to **A,B**) = 50 μm .

Figure 3. Cell types generated in adult *bugeye* retinas

A.–D. Indirect immunofluorescence images of staining for the ganglion cell/amacrine cell marker HuC/D in 12 month old WT retina (**A**), and in *bugeye* retina (**B**) together with staining for BrdU (**C**; **D** shows merged image); the *bugeye* fish was exposed to BrdU for 4 days and returned to system water for 4 days before sacrifice. Arrows indicate colabeled cell. **E.–H.** Indirect immunofluorescence images of staining for the cone photoreceptor cell marker Zpr-1 in control retina (**E**), and in *bugeye* retina (**F**) together with staining for BrdU (**G**; **H** shows merged image). Arrows indicate colabeled cell. **I.–L.** Indirect immunofluorescence images of staining for the Müller glia marker Zrf-1 in control retina (**I**), and in *bugeye* retina (**J**) together with staining for BrdU (**K**; **L** shows merged image); arrowheads indicate possible colabeling. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; scale bar in **A** (applies to all) = 50 μ m.

Figure 4. Fates of proliferating cells in adult *bugeye* retinas

A. Quantification of colabeling of BrdU with zpr-1 (cone photoreceptors), and HuC/D (ganglion cells and amacrine cells), in the 4 day chase and 8 day chase experiments. In the boxplots, lines depict the medians, boxes delineate the 25th to 75th percentiles, and whiskers indicate the limits. **B.** Indirect immunofluorescence image of BrdU staining in 10 month old *bugeye* zebrafish retina; the fish were exposed to BrdU for 3 days and returned to system water for 8 days before sacrifice. BrdU+ cells are seen in the ONL, and occasionally in the inner nuclear layer (INL) and ganglion cell layer (GCL); scale bar = 50 μ m. **C.** Boxplots showing densities of BrdU+ cells in *bugeye* retinas following a 4 day or 8 day chase period; ***, $p < 0.001$. **D.** Densities of BrdU+ cells in each retinal layer as a function of eye diameter in the 8 day chase experiment; a significantly positive correlation was detected for cells of the GCL ($p < 0.05$; $\rho = 0.77$).

Figure 5. Mechanical cell stress in adult *bugeye* retinas

A.–F. Indirect immunofluorescence images of staining for the cell stress marker Hsp27 (**A,D**), together with staining for the ganglion cell/amacrine cell marker HuC/D (**B,E**; **C** and **F** show merged images) in control (**A–C**) and *bugeye* (**D–F**) retinas. Colabeled cells are indicated by arrows. **G.–L.** Indirect immunofluorescence images of staining for the cell stress marker Hsp27 (**G,J**), together with staining for the Müller glia marker zrf-1 (panels **H,K**; **I** and **L** show merged images) in control (**G–I**) and *bugeye* (**J–L**) retinas. Colabeling is indicated by arrowheads. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; scale bar in A (applies to all) = 50 μ m.

Figure 6. Neuronal densities and numbers in the retinal ganglion cell layer (GCL) of *bugeye* zebrafish

A.–C. Indirect immunofluorescence of HuC/D expression in 12 month old WT (**A**) and *bugeye* (**B,C**) eyes. **B** represents the smaller eye of a pair and **C** represents the larger eye. The HuC/D antigen is present in neurons of the GCL and some neurons of the inner nuclear layer (INL). Scale bar in **A** (applies to **A–C**) = 50 μ m. ONL, outer nuclear layer. **D.** Boxplots showing HuC/D+ GCL neuron densities in *bugeye* and control eyes. In the boxplots, lines depict the medians, boxes delineate the 25th to 75th percentiles, whiskers indicate the limits, and small circles depict outliers. **, significantly different from WT eyes ($p < 0.01$; Welch two sample T-test). **E.** Boxplots showing total numbers of Hu+ GCL neurons in *bugeye* and control eyes; asterisks indicate significantly different from WT eyes (*, $p < 0.05$; ***, $p < 0.001$). **F.** HuC/D+ GCL neuron density as a function of age in *bugeye* zebrafish; trendlines do not depict significant correlations. **G.** HuC/D+ GCL neuron density as a function of eye diameter in *bugeye* zebrafish; no significant correlations were detected. **H.** The total number of neurons in *bugeye* GCL did not vary significantly with animal age. **I.** For both bigger and smaller *bugeye* eyes, a significant positive correlation was detected for total GCL neurons vs. eye diameter ($p < 0.001$, $\rho = 0.70$ for bigger eyes and $p < 0.001$, $\rho = 0.85$ for smaller eyes).

Figure 7. Optic nerve head (ONH) diameters of *bugeye* zebrafish

A.–C. Brightfield images of methylene-blue stained ONHs of 14 month old WT (**A**) and 15 month old *bugeye* (**B,C**) eyes. **B** shows the smaller eye of a pair of asymmetric eyes; **C** shows the larger eye. In each panel, the outside of the eye is on the top, arrows span the ONH distance measured. **D.** Boxplots showing ONH diameters in *bugeye* and WT eyes. In the boxplots, lines depict the medians, boxes delineate the 25th to 75th percentiles, whiskers indicate the limits, and small circles depict outliers. Asterisks indicate significant differences from WT eyes (*, $p < 0.05$; ***, $p < 0.001$). **E.** ONH diameter as a function of age in *bugeye* zebrafish; no significant relationships were detected. **F.** ONH diameter as a function of eye diameter; a positive correlation was detected for the bigger eyes (black trendline; $p < 0.001$; $\rho = 0.79$), though not for the smaller eyes. Scale bar in **A** (applies to **A–C**) = 100 μm .

Figure 8. GCL neurons as a function of ONH diameter in *bugeye*

A. Ganglion cell layer neuronal density vs. optic nerve head diameter; no correlations were observed. **B.** Total number of GCL neurons vs. ONH diameter; a significant positive correlation was observed for this relationship when the bigger *bugeye* eyes were considered (black trendline; $p < 0.01$, $\rho = 0.67$).

Figure 9. Visual function of adult *bugeye* mutants

Figure shows the positions of five, 26-month old *bugeye* and five WT fish in the tank in response to a hand waved near the feeding area. The positions of *bugeye* fish (circles) were significantly different from the positions of WT fish (triangles) at all observation times, suggesting that adult *bugeye* fish are blind ($p < 0.05$; Simultaneous Tests for General Linear Hypotheses (Hothorn et al., 2008)).