INHIBITION OF GABA INITIATES RETINA REGENERATION IN THE ZEBRAFISH

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Dedicated to my family, friends, and loved ones

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LIST OF ABBREVIATIONS

AC	Amacrine Cell
AMD	Age Related Macular Degeneration
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apobec2b	Apolipoprotein B-editing catalytic subunit 2b
Ascl1a	Achaete-scute family bHLH transcription factor 1a
BAR	Bin, Amphiphysin, Rvs
BC	Bipolar Cell
BDNF	Brain Derived Neurotrophic Factor
BLBP	Brain Lipid Binding Protein
CGZ	Circumferential Germinal Zone
CMZ	Ciliary Marginal Zone
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CREB	cAMP Response Element-Binding Protein
Crx	Cone-Rod Homeobox
DA	Dopamine
DG	Dentate Gyrus
DIC	Dye Injected Control
Dkk	Dickkopf
DNγ2	Dominant Negative γ2 Subunit
dpf	Days Post Fertilization
ECM	Extracellular Matrix

ERG	Electroretinogram
FGF	Fibroblast Growth Factor
GABA	γ-aminobutyric acid
GABARAP	GABAA Receptor Associated Protein
GAD	Glutamic Acid Decarboxylase
GAT	GABA Transporter
GC	Ganglion Cell
GCL	Ganglion Cell Layer
GFAP	Glial Fibrillary Acidic Protein
GS	Glutamine Synthetase
HB-EGF	Heparin Binding-Epidermal Growth Factor
HC	Horizontal Cell
hpf	Hours Post Fertilization
hpi	Hours Post Injection
HPLC	High-Performance Liquid Chromatography
IKNM	Interkinetic Nuclear Migration
Il-6	Interleukin 6
ILM	Inner Limiting Membrane
INL	Inner Nuclear Layer
Insm1a	Insulinoma-Associated 1a
IPL	Inner Plexiform Layer
IRBP	Interphotoreceptor Retinoid Binding Protein
KLF4	Kruppel-Like Factor 4

MAK	Male Germ Cell-Associated Kinase
MG	Müller Glia
Mib	Mind bomb
MRE	miRNA Recognition Element
MTZ	Metronidazole
NBQX	nitro -2, 3-dioxobenzoquinoxaline-sulfonamide
NEI	National Eye Institute
NFATc4	Nuclear Factor of Activated T Cell c4
Nrl	Neural Retina Leucine Zipper
NTR	Nitroreductase
Oct4	Octamer-Binding Transcription Factor 4
OLM	Outer Limiting Membrane
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
Pax6	Paired Box Protein 6
PCNA	Proliferating Cell Nuclear Antigen
PDE	Phosphodiesterase
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PR	Photoreceptors
PX	Phox-Homology
RGL	Radial Glia like Stem Cells
RISC	RNA Induced Silencing Complex
RK	Rhodopsin Kinase

RLBP	Retinaldehyde Binding Protein
RP	Retinitis Pigmentosa
RPE	Retinal Pigmented Epithelium
SGZ	Subgranular Zone
SNX5	Sorting Nexin 5
Sox2	SRY (Sex Determining Region Y)-Box 2
SSRI	Selective Serotonin Reuptake Inhibitors
SVZ	Subventricular Zone
TNFα	Tumor Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor

Chapter I

INTRODUCTION

Vertebrate Retina

Structure and cell types

The retina is the main light-sensing tissue. It lines the back of the eye and is comprised of neurons and glia. It forms from the central nervous system (CNS) and develops into a three-layered structure consisting of seven main cell types (Figure 1) (Byerly and Blackshaw, 2009; Hitchcock and Raymond, 1992; Otteson et al., 2001; Stenkamp, 2007). The structure, function, cell types, and genes expressed are largely conserved among vertebrates, including zebrafish, mice, and humans (Bilotta and Saszik, 2001; Byerly and Blackshaw, 2009; Fleisch and Neuhauss, 2010; Stenkamp, 2007). This high conservation and simplified organization makes the retina a useful model to study.

The three main layers in the retina are the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) (Figure 1). The INL and ONL are separated by a thin synaptic layer called the outer plexiform layer (OPL), and the INL and GCL are separated by a thick synaptic layer called the inner plexiform layer (IPL). The ONL contains the two main types of photoreceptors (PRs) called rods and cones. These are the main light sensing cells responsible for vision. Rods and cones are stratified within the ONL, with cones most distal to the rest of the retina and rods more proximal, adjacent to the OPL. The INL contains three types of interneurons: bipolar cells (BCs), horizontal cells (HCs), and amacrine cells (ACs).

BCs connect PRs to cells in GCL, while HCs and ACs in general laterally connect PRs and BCs. BCs and HCs occupy the region closest to the OPL, with some organisms showing further stratification of these cells. ACs are typically adjacent to the IPL. Ganglion cells (GCs) occupy the last layer, along with displaced ACs, and collect information from BCs and send the signal to the brain for higher order visual processing. In the GCL, GCs and displaced ACs form a monolayer. In addition to these neuronal cell types there are also glia in the retina. The main glial cell type is the Müller glia (MG), which has its cell body in the INL between the ACs and BCs but sends processes to the ONL and GCL. The ONL is also in contact with an epithelial layer called the retinal pigmented epithelium (RPE) (Bilotta and Saszik, 2001; Goldman, 2014; Stenkamp, 2007).

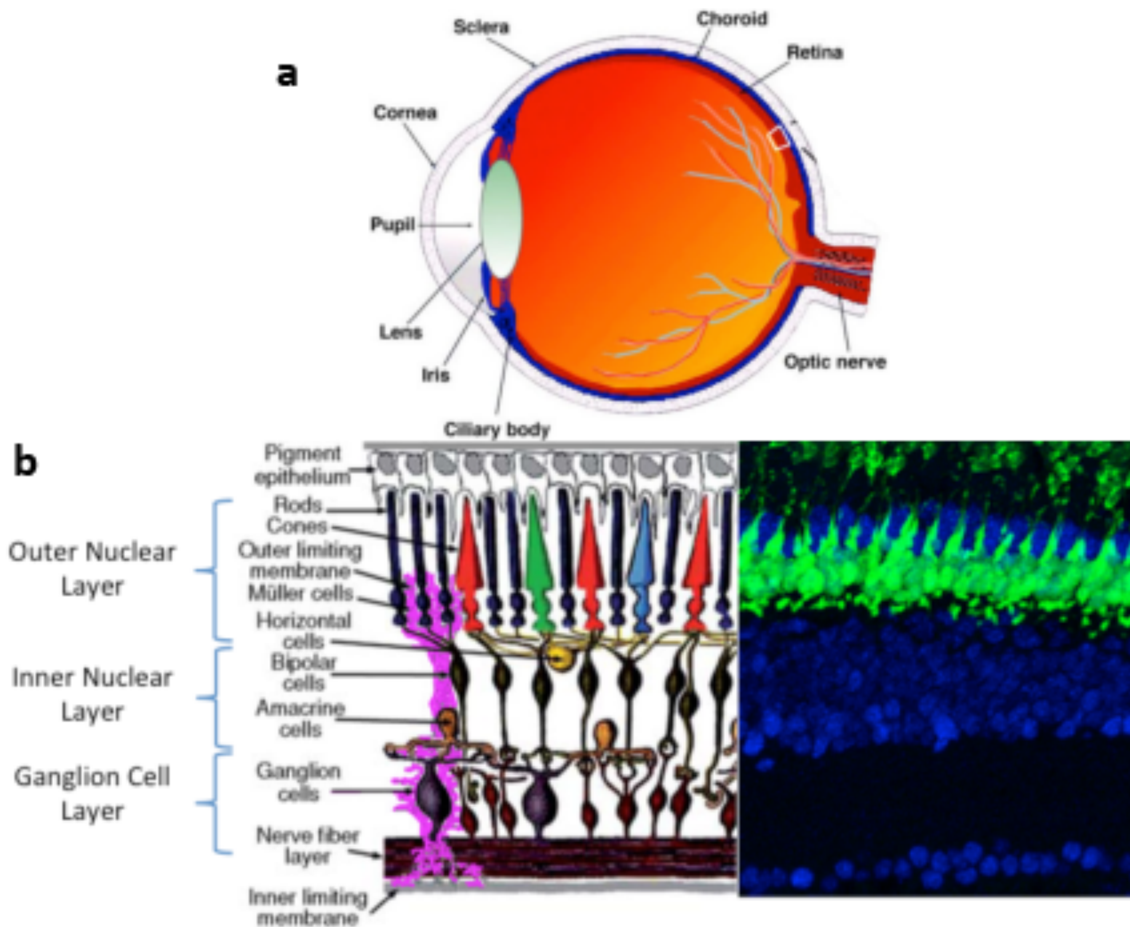

Figure 1. Retina Structure. Model cross-section of a human eye (a). Light enters through the pupil, passes through the vitreous and enters the retina that lines the back of the eye. The retina is divided into three layers, the Outer Nuclear Layer (ONL), Inner Nuclear Layer (INL), and Ganglion Cell Layer (GCL) (b). Rod and Cone photoreceptors are in the ONL. Bipolar, horizontal, and amacrine cells are in the INL, along with Müller glia. Ganglion cells are in the GCL. An actual cross section of a zebrafish retina appears to the right. Modified from http://webvision.med.utah.edu/

Development

During development of the CNS, bilaterally symmetric regions of the neural tube are specified as the optic primordium (Figure 2) (Bilotta and Saszik, 2001; Pei and Rhodin, 1970; Schmitt and Dowling, 1994). These regions grow outward from

the neural tube toward the overlying ectoderm. The neural tube outgrowth makes contact with the ectoderm, inducing the lens placode to form, as well as an invagination of the neural tube (Avanesov and Malicki, 2010; Jayakody et al., 2015; Lamb et al., 2007; Li and Guo, 2013). The lens placode thickens and extends inward. As the lens placode continues to grow it becomes spherical and pinches off from the ectoderm to form the lens. Invagination of the neural tube causes the formation of a two-layered cup of neuronal tissue. The cup separates from the rest of the developing CNS, with the outer layer becoming the RPE and in inner layer the neural retina (Moshiri et al., 2004). The neural retina is comprised of columnar neuroepithelial cells that extend the thickness of the tissue. As in the rest of the CNS, the nuclei of the cells migrate along the axis of the cell and divide at the apical region of the cell. Cell division can either be symmetric to produce new stem cells or asymmetric to produce one stem cell and one postmitotic daughter cell. The fate of the postmitotic daughter cell is highly dependent on when during development it forms. The earliest cells to form are GCs, followed by ACs, HCs, and cones. The last cells to be produced at BCs, rods, and MG (Bassett and Wallace, 2012; Cepko et al., 1996; Hu and Easter, 1999; Lamb et al., 2007; Livesey and Cepko, 2001; Stenkamp, 2007).

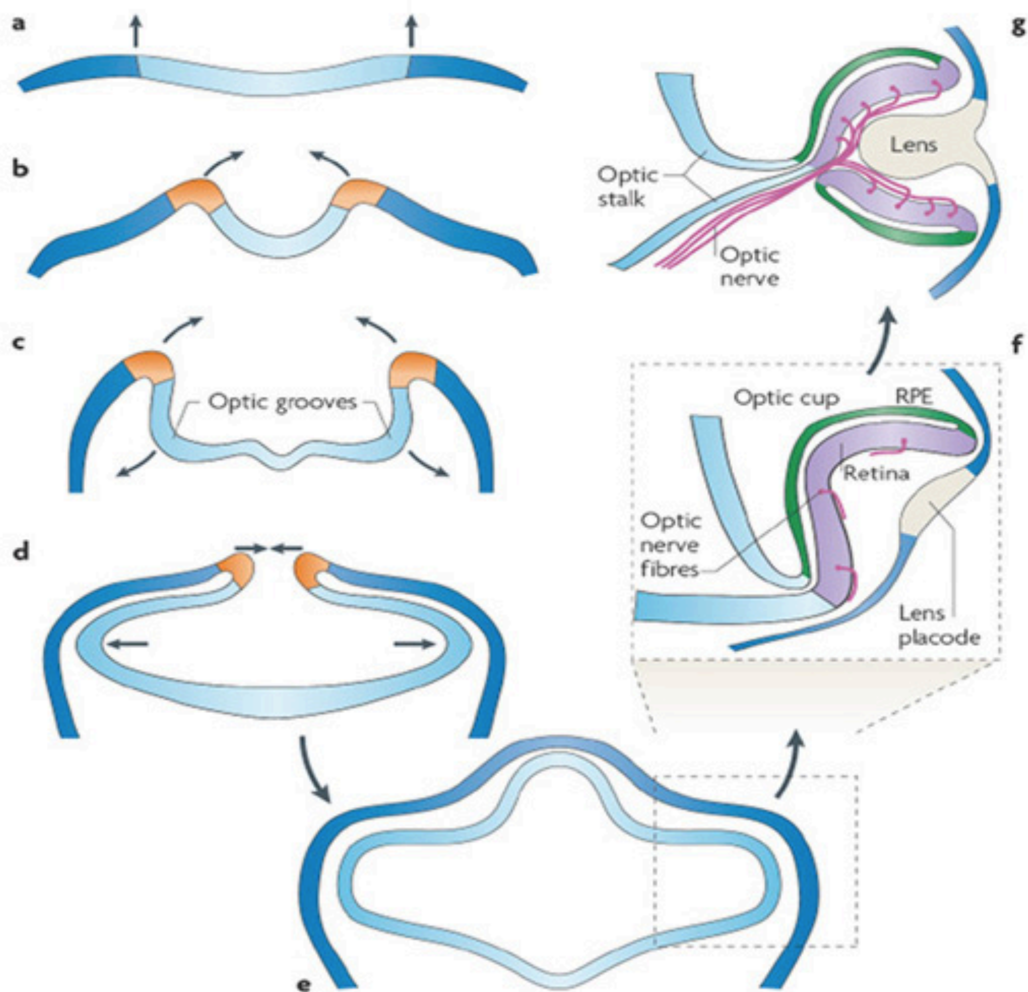

Figure 2. Development of the Retina. The neural plate develops from the ectoderm (a). The neural plate folds in to form the neural tube (b-d). Two regions of the neural tube develop as the optic primordium and grow out toward the overlying ectoderm (d-e). The neural tube makes contact with the ectoderm to induce the lens placode. The neural tube also invaginates to form a two layered structure where the outer layer is the RPE and the inner layer is the retina (f-g). (Lamb et al., 2007)

A broad decision during fate choice of postmitotic daughter cells is whether to follow a neuronal or glial fate. This decision is highly dependent on Notch signaling (Nelson et al., 2007; Scheer et al., 2001; Stenkamp, 2007). Notch is a

juxtacrine signaling mechanism where one cell expresses the transmembrane ligand Notch, and the other expresses the receptor Delta. Upon binding, Notch is cleaved within the membrane, liberating the Notch Intracellular Domain (NICD). The NICD then translocates to the nucleus to cause transcriptional changes. Delta activation also causes its own transcriptional changes, as well as undergoing endocytosis after binding. High Notch signaling results in a glial fate, while low Notch signaling results in a neuronal fate (Lasky and Wu, 2005). Therefore, correct temporal and spatial regulation of Notch factors greatly impacts the fate choice and timing of retinogenesis events.

Phototransduction

The cellular process of sensing light is called phototransduction and begins with a photon entering the retina. The photon must pass through the GCL and the INL before it encounters PRs in the ONL. Photons interact with the molecule retinal in the PRs (Yau and Hardie, 2009). In the absence of light, retinal is in the "11-cis" conformation, where the 11 and 12 carbons are in a cis orientation. When a photon of light interacts with 11-cis retinal, it isomerizes to "all trans" retinal, where the carbons are in a trans orientation (Kefalov, 2012; Wang and Kefalov, 2011; Yau and Hardie, 2009). This conformational change affects the seven transmembrane G-protein coupled receptor in which retinal is situated, called an opsin protein (Imai et al., 2007; Shen and Raymond, 2004b; Wang and Kefalov, 2011; Yau and Hardie, 2009).

The different opsin proteins are what differentiate PRs, in that they only allow retinal to isomerize with certain wavelengths of light. The most plentiful type of opsin is rhodopsin, which is found in rods. This opsin is maximally activated by wavelengths around 500 nm and is able to sense lower levels of light compared to other opsins (Rajamani et al., 2011). The other opsins in the retina correspond to the different types of cones. Mice, humans, and zebrafish all contain red and green cones. Humans also have blue cones, while fish have both blue and UV cones. Red cones are maximally excited by wavelengths around 560 nm, green cones are maximally excited by wavelengths around 530 nm, blue cones are maximally excited by wavelengths around 420 nm, and UV cones are maximally excited by wavelengths around 362 nm (Kefalov, 2012; Rajamani et al., 2011; Wang and Kefalov, 2011). In zebrafish there are approximately twice as many red and green cones than UV and blue cones, which can be explained by the stereotypical organization of cone cells. There is a pentameric repeat of green, red, blue, red, green with an adjacent UV cone (Allison et al., 2010; Stenkamp, 2007; Zou et al., 2012). The pentameric cells are closely opposed to each other. Red and green cones can become very tightly adhered to form double cones.

The change in retinal causes the opsin to initiate an intracellular cascade that results in hyperpolarization of the cell. Once activated, the α subunit of the G-protein dissociates from the βγ subunits and activates a cGMP Phosphodiesterase (PDE) (Collery and Kennedy, 2010; Fain et al., 2010; Kolandaivelu et al., 2011; Lamb, 2009; Yau and Hardie, 2009). The cGMP PDE catalyzes the linearization of cGMP to GMP(Christiansen et al., 2011; Fain et al., 2010). cGMP typically is binds and opens

Ca^{++}/Na^{+} permeable cation channels (Fain et al., 2010; Yau and Hardie, 2009). When the cGMP PDE is activated it reduces the amount of cGMP (Collery and Kennedy, 2010), reducing the influx of Ca^{++} and Na^{+}, resulting in a net decrease in membrane potential. The ultimate effect of this cascade is a decrease in the amount of neurotransmitter released (Fain et al., 2010; Yau and Hardie, 2009). As with most cells in the retina, a change in PR membrane potential does not lead to an action potential but rather a graded response. Furthermore, the effect of a single photon can produce a noticeable change in the cell because of amplification. In rods, one rhodopsin molecule can activate 500-1000 G-protein molecules, one activated PDE molecule can hydrolyze ~4000 cGMP molecules, and this closes enough cation channels to reduce the membrane voltage ~1mV.

With such a strong response to even one photon there must be a way to desensitize the cascade. Indeed the response lasts only seconds before it is silenced. Many mechanisms are in place to silence the response to light, beginning with the retinoid cycle in rods and cones (Fleisch and Neuhauss, 2010; Wang and Kefalov, 2011; Yau and Hardie, 2009). After retinal is isomerized from 11-cis to all-trans, it is dislodged from the opsin, diffuses into the cytoplasm, and is converted to all-trans retinol. Interphotoreceptor Retinoid Binding Protein (IRBP) transports retinol from the PR to the RPE where it is then converted to all-trans retinylester, 11-cis retinol, and finally 11-cis retinal. IRBP then transports 11-cis retinal back into the PR where it reassociates with the opsin (Fleisch and Neuhauss, 2010; Wang and Kefalov, 2011; Yau and Hardie, 2009). The opsin itself can be inactivated. Activated Rhodopsin in rods is phosphorylated by the serine/threonine GRK Rhodopsin Kinase (RK) (Wang

and Kefalov, 2011; Yau and Hardie, 2009). Arrestin then binds to Rhodopsin and prevents further interaction with the G-protein (Yau and Hardie, 2009). The G-protein can also be inactivated. Active G-proteins bind GTP molecules and are inactivated when the GTP is hydrolyzed to GDP. Hydrolysis is expedited by a GAP protein called a regulator of G-protein signaling (Yau and Hardie, 2009). The decrease in Ca^{2+} also inhibits phototransduction. Ca^{2+} inhibits RK and Guanylyl Cyclase as well as decreases the affinity of cGMP to cation channels. After activation by light these processes do not occur and photoransduction is attenuated. This is how light adaptation occurs, where in increasing light, rods become less sensitive(Yau and Hardie, 2009).

Cell types diversity and function

As mentioned previously activation of opsin by a photon results in hyperpolarization of the cell. This brings the PR further from threshold and less likely to release its cargo, the excitatory neurotransmitter glutamate (Connaughton et al., 1999). This is counter to most neurons where activation results in depolarization and increased likelihood of cargo release. This affects the synaptic partners of PRs, one of which are BCs. BCs connect to rods or cones and transmit their signal to GCs. Typically, one BC connects to one cone, while multiple rods can connect to a single BC. BCs will only connect to cones or rods, not both. BCs can be further divided into “on-center” and “off-center”, corresponding to if they are stimulated when light is entering the retina (on-center) or when light is absent from the retina (off-center). On-center BCs have mGluR6 receptors (Chalupa and Gunhan,

2004; Masu et al., 1995) while off-center BCs have AMPA or kainate receptors (DeVries, 2000). mGluR6 activation by glutamate results in hyperpolarization, while AMPA or kainate activation results in depolarization. When no light is present, the mGluR6 on-center cells are hyperpolarized because glutamate release from the PRs is high. This is the opposite for the AMPA/kainate off-center cells; high glutamate release from PRs causes depolarization. Therefore, when light activates PRs, on-center BCs are derepressed and can signal to the GCs, whereas the off-center BCs are inhibited and do not signal to the underlying GCs.

The other synaptic partners of PRs are HCs. HCs, PRs, and BCs all synapse together in the PR spherule (Song et al., 2008). HCs laterally inhibit adjacent rods or cones (Poché and Reese, 2009; Thoreson and Mangel, 2012). When PRs release glutamate they activate AMPA receptors on HCs. HCs then depolarize and release the inhibitory neurotransmitter γ-aminobutyric acid (GABA) onto adjacent PRs. By doing this, HCs execute a center-surround function, where glutamate release from one PR, inhibits glutamate release from adjacent PRs. This helps in edge detection and visual acuity (Matsuoka et al., 2012; Sonntag et al., 2012; Thoreson and Mangel, 2012).

ACs also laterally connect cells, and in doing so they integrate the rod and cone pathways (Chalupa and Gunhan, 2004), as well as execute lateral inhibition (Thoreson and Mangel, 2012). ACs are the most diverse cell type in the retina with as many as 33 subtypes (Balasubramanian and Gan, 2014; Masland, 2012; Zhang and McCall, 2012). The cells are spread through the INL and GCL as displaced ACs. ACs synapse with BCs, GCs, and other ACs. Their cargo is also diverse and includes

glutamate, GABA, dopamine (DA), serotonin, glycine, acetylcholine, and others (Balasubramanian and Gan, 2014; Masland, 2012). The plethora of ACs and their elaborate connections is why the IPL is so thick. The IPL can be divided based on which BP subtype projects into it. The top half of the IPL is the "off" layer, while the bottom half is the "on" layer, with further subdivisions of the IPL as well (Balasubramanian and Gan, 2014; Masland, 2012; Zhang and McCall, 2012). Broadly, ACs can be grouped into two subtypes based on neurotransmitter and dendrite arborization. Narrow-field ACs typically release glycine and have dendritic arbors that extend a small distance from the soma but penetrate multiple levels of the IPL. Wide-field ACs typically release GABA and have dendritic arbors that stretch far from the soma but only within a thin layer of the IPL (Hartveit and Veruki, 2012).

All of the complexity and division of signal information is for visual processing and the brain uses this information to synthesize an image of the visual field. GCs are responsible for collecting all the visual information and sending it to the brain. GCs are very diverse and their main synaptic partners are BCs, though that signal is modified by AC activity. GCs typically form a monolayer and each cell sends an axon to a single point in the retina where they all exit the eye via the optic nerve (Masland, 2012).

In order to keep the retina running smoothly there needs to be a strong support system. This task is fulfilled mainly by MG (Bringmann et al., 2006; Goldman, 2014; Reichenbach et al., 1993). MG are the most elaborate cell type in the retina and, as mentioned previously, extend projections from their cell bodies in

the INL to the ONL and GCL. Their projections encompass the whole retina. The plexus formed at the edge of the ONL is called the outer limiting membrane (OLM) and the plexus formed at the edge of the GCL is called the inner limiting membrane (ILM). MG are in close association with all cells in the retina and perform vital roles such as neurotransmitter buffering and recycling, ion buffering, free radical sequestration, and maintaining structural integrity that help to sustain the health of the retina (Bringmann et al., 2013; Bringmann et al., 2006; Giaume et al., 2007; Goldman, 2014; Reichenbach et al., 1993; Wurm et al., 2006). MG have also been suggested to behave like optic fibers that direct photons towards PRs (Reichenbach and Bringmann, 2013).

Retinal response to damage

Damage response in the mammalian retina

MG also play a vital role in maintaining retina health by responding to damage after cell death caused by damage or disease (Goldman, 2014). As opposed to previously mentioned functions of the retina, MG respond to damage differently in different organisms. In mice and other mammals retina damage results in reactive gliosis, which prevents regeneration (Bringmann et al., 2009; Bringmann et al., 2006; Giaume et al., 2007; Nieto-Sampedro et al., 1985; Pekny and Nilsson, 2005; Pekny et al., 2014; Wan et al., 2008). Reactive gliosis is a response that occurs throughout the mammalian central nervous system in response to injury (Bringmann et al., 2009; Bringmann et al., 2006). It begins with upregulation of factors such as Glial Fibrillary Acidic Protein (GFAP), Nestin, and Vimentin (Karl and

Reh, 2010; Pekny and Nilsson, 2005; Pekny et al., 2014; Wan et al., 2008) in addition to glial hypertrophy. Mammalian glia can dedifferentiate but in doing so, downregulate genes that help to support the retina, such as Glutamine Synthetase (GS), which processes glutamate to glutamine and prevents glutamate toxicity (Giaume et al., 2007). Mammalian glia can also reenter the cell cycle and divide, though rarely.

The proliferation is minimal and usually leads to the formation of a glial scar rather than formation of new functional neurons (Bringmann et al., 2009; Karl and Reh, 2010; Pekny and Nilsson, 2005; Pekny et al., 2014). A glial scar is the result of MG growth first into the area of lost cells, then into other areas outside the retina, including the subretinal space adjacent to the RPE and between the ILM and the vitreous. This aberrant growth disrupts vasculature and RPE function and creates an area where new cells cannot form and causes the death of existing ones. The glial scar creates a boundary around the damaged area, within which neurons cannot live. Glial scars are thought of as neuroprotective in the case of an infection. If neurons begin to die because they are infected, a glial scar will contain the infection and no allow it to spread. However, in the case where there is damage or disease that is not due to an infection, a glial scar would prevent the formation of new cells that would otherwise be able to repair the damage. Recent evidence, though, suggests that a glial scar is helpful in the case of CNS axon repair. Preventing glial scar formation reduces axon regrowth compared to when one is present and the addition of axon-specific growth factors results in axon regeneration when a glial

scar is present but not when it is absent (Anderson et al., 2016). Therefore, the function of glial scars may differ based on the type of neuronal damage.

Retinitis Pigmentosa

Since there is no way to initiate regeneration following damage in mammals many retina diseases go unchecked and lead to blindness. These include retinitis pigmentosa (RP), "wet" and "dry" age related macular degeneration (AMD), the most common retinal disease, and Leber's congenital amaurosis. In the case of RP, it is a hereditary disease that results in the progressive loss of rods, followed by the loss of cones, ultimately leading to blindness (Bringmann et al., 2006; Chang et al., 1993; Tucker et al., 2011; Wright et al., 2010). There are three major ways RP can be inherited: X-linked, autosomal dominant, or autosomal recessive, resulting in 22%, 24%, and 41% of cases, respectively (Wright et al., 2010). RP first manifests as night blindness, due to the loss of rods (Chang et al., 1993; Tucker et al., 2011; Wright et al., 2010). Holes in peripheral vision occur as rod loss increases. Because there are gap junctions between rods and cones, the health of cones deteriorates once the rods are absent, and they die as well, resulting in loss of central vision and total blindness. In addition, blood vessels narrow in the retina. Pigment from the RPE migrates into the retina, forming clumps around the vessels of various sizes.

Mutations in 30-40 genes have been associated with RP and more are likely to be discovered (Tucker et al., 2011; Wright et al., 2010). Most are PR genes associated with phototransduction and development such as PDE, Cone-Rod Homeobox (Crx), Retinaldehyde Binding Protein (RLBP, found in RPE and MG as

part of the retinoid cycle), Male Germ Cell-Associated Kinase (MAK), Neural Retina Leucine Zipper (Nrl) and Rhodopsin (Chang et al., 1993; Collery and Kennedy, 2010; Fleisch and Neuhauss, 2010; Tucker et al., 2011; Wright et al., 2010). Most genes are specific to rods, though RP where cone loss is first has been known to occur as well.

Current attempts at repairing vision and drawbacks

Current treatments to preserve or restore vision include the injection of antiapoptotic factors and neurotrophic factors to protect dying cells (Jayakody et al., 2015). This can be carried out by injection of molecules or cells that release the desired molecules. An example of this is the use of Vascular Endothelial Growth Factor (VEGF) inhibitors to treat "wet" AMD. Another treatment avenue is gene therapy, where viruses are used to add back correct versions of genes where disease is caused by a mutation, as in the case of RP. This requires knowledge of the mutation and treatment early enough where the compromised cell type is still present (Jayakody et al., 2015).

The mammalian retina does not readily produce new cells, so another, and the most promising, way to repair damaged retinas is the injection of stem cells or retinal progenitors into the eye, in the hopes that some of the cells will be able to differentiate appropriately and form the correct connections to restore vision (Barber et al., 2013; Jayakody et al., 2015; MacLaren et al., 2006). Unfortunately, this approach has been met with a number of issues that have proven difficult to surmount. Cells injected into the eye must enter the retina and migrate to the area of damage to replace lost cells. This means that cells injected into the vitreous of the

eye will need to migrate through the GCL and INL before arriving at the ONL to differentiate into PRs, as will be the case for most diseases. Subretinal injections place the cells closer to the ONL but this surgery is more invasive. Previous studies have shown that injected cells have a difficult time migrating to the appropriate area and sometimes remain at the site of injection (Jayakody et al., 2015; Zhao et al., 2014a).

One of the difficulties are the obstacles the cells need to traverse before they arrive at their desired destination. Intravitreal injections require cells to pass through the ILM, the thick, dense network of process in the IPL, the INL and the OPL before arriving at the ONL. Subretinal injections require passing through the RPE and the OLM before arriving at the ONL (Jayakody et al., 2015; Pearson, 2014). Experiments where the extracellular matrix (ECM) has been disrupted in order to ease the migration of cells has been successful, but disruption of the ECM leaves the retina less stable and also occurs in a number of diseases (Pearson et al., 2010; West et al., 2008).

Once the cells have migrated to the appropriate area they must differentiate into the appropriate cell type. This also has proven difficult, as the cells do not differentiate easily into any cell type other than rods (Barber et al., 2013; Jayakody et al., 2015; Santos-Ferreira et al., 2015; Singh et al., 2013; Zhao et al., 2014a). This may not be a problem in the case of diseases such as RP, where rod death is the initial step in the disease, but if the disease has progressed to cone cell death, formation of new cones has proven very difficult. In diseases such as AMD, cell transplantation may not be effective.

Once properly differentiated the cells must become part of the established circuitry to restore vision. Synapse formation occurs at a similar rate as differentiation but restoration of vision is not as reliable. Simple visual responses have been found to occur and electroretinogram (ERG) recordings show retinal function improves, but this requires a certain number of cells to reintegrate into the retina (Jayakody et al., 2015; Pearson et al., 2012a; Singh et al., 2013). This underscores one of the current shortcomings of cell transplantation: efficiency. The best reported efficiency with rod transplantation is 10%, though with the number of rods in the mouse eye approximately 6.4 million (Jeon et al., 1998) and the human eye much more than that, the number of cells needed to replace all those lost will be exceedingly high (Barber et al., 2013; Jayakody et al., 2015).

This leads to another difficulty, the source and type of cell injected into the retina. The best success has been found with injection of retinal precursors taken from embryos undergoing retinogenesis when the desired cells are being produced. Using induced pluripotent stem cells or differentiated cells was much less efficient. Using a renewable source of cells will be vital if these techniques will be used clinically and cultured cells are not as successful as embryonic cells, typically requiring the addition of growth factors (Gonzalez-Cordero et al., 2013; Jayakody et al., 2015; Li et al., 2013; Pearson, 2014).

Lastly, it is unknown how long the injected cells will survive. Most studies have been done in WT animals where retina degeneration does not happen readily. In a diseased retina when retina degeneration happens continuously, presumably multiple injections of cells will be required throughout the lifetime of the individual.

Additionally, injection of foreign cells activates the immune system in the retina. Microglia, the main immune cell in the retina, become activated and localize to the site of injection. Typically microglia, and MG, undergo phagocytosis to remove dead cells from the retina and perform similar functions with injected cells. Circumventing the microglia will prove difficult and may require prolonged immunosuppression (Jayakody et al., 2015; Pearson, 2014).

Zebrafish retina regeneration

Ideally, a treatment would only slightly perturb the retina to prompt it to regenerate endogenously, without the addition of new cells or growth factors. This would allow the retina to self regulate cell production and progenitors would migrate to the correct location and form appropriate connections. Any or all of the mentioned techniques may be used to treat retinal disease or damage, but considering the number of drawbacks to current efforts, developing new methods would be useful.

As opposed to mammals, zebrafish have the ability to regenerate a large array of tissues. Regeneration in many neuronal tissues such as the retina (Goldman, 2014; Stenkamp, 2007), spinal cord (Becker et al., 1997; Yu et al., 2011a; Yu et al., 2011b), lateral line neurons (Chitnis et al., 2012), and brain (Kizil et al., 2012a; Kizil et al., 2012b; Kizil et al., 2012c; Kyritsis et al., 2012) has been documented. Zebrafish can also regenerate a number of non-neuronal tissues such as the heart (Curado et al., 2007; Kang et al., 2016; Poss et al., 2002; Yin et al., 2012), pancreas (Curado et al., 2007), and complex tissues such as the fin, which requires

regeneration of multiple tissues simultaneously (Geurtzen et al., 2014; Kang et al., 2016; Knopf et al., 2011; Thatcher et al., 2008; Yin and Poss, 2008; Yin et al., 2008). Retina regeneration also occurs in other teleost fish and has historically been an area of great interest (Hitchcock and Raymond, 1992; Kurz-Isler and Wolburg, 1982; Maier and Wolburg, 1979; Raymond et al., 1988). The mechanisms, however, are still being elucidated. Considering how similar mammalian and zebrafish retinas are, it may prove useful to understand why zebrafish retinas regenerate to help inform why mammalian retinas do not, and possibly to help develop new therapies to treat retina disease in humans.

Sources of new cells

One difference between zebrafish and mammalian retinas is the amount of proliferation that occurs. In mammals little to no further proliferation occurs after development is complete. The zebrafish retina, however, continues to grow throughout the lifespan of the organism (Stenkamp, 2011). Since zebrafish continuously grow, their retinas also need to grow. This growth is accomplished in part by an increase in cell size but also by the addition of new cells to the periphery of the retina (Hitchcock et al., 2004; Johns, 1977; Moshiri et al., 2004; Raymond et al., 2006). The edge of the retina is called the ciliary marginal zone (CMZ). This area is proliferative and can produce all cell types found in the retina. The cells produced by the CMZ are only added to the periphery and new cells do not migrate further into the retina. Another type of proliferative cell is the rod precursor, found in the rod layer of the ONL. Rod precursors were first identified in regards to retina

development as a dedicated source of new rod cells (Johns and Fernald, 1981; Johns, 1977; Johns, 1982; Raymond, 1985; Raymond and Rivlin, 1987). It was observed that during development, the change in rod cell density was opposite that of other cell types in the retina. As the retina grows, the density of most cells decreases whereas rod density increases. Investigation of this phenomenon showed that there were proliferative cells scattered throughout the rod layer of the ONL. These cells proliferate even after normal retinogenesis is complete to produce new rod cells (Johns and Fernald, 1981; Johns, 1977; Johns, 1982; Raymond, 1985; Raymond and Rivlin, 1987). Rod precursors divide asymmetrically to produce new rods. Each rod precursor is only able to produce a limited number of rods, so the population of rod precursors also needs to be replenished.

The lineage of rod precursors was investigated further in order to determine where they come from during development (Raymond and Rivlin, 1987). Rod precursors arise from the migration of undifferentiated mitotic cells from the INL to the ONL (Julian et al., 1998; Otteson et al., 2001; Raymond and Rivlin, 1987). This phenomenon occurs in both developing retinas (Julian et al., 1998; Otteson et al., 2001; Raymond and Rivlin, 1987) and regenerating retinas (Vihtelic and Hyde, 2000; Wu et al., 2001). Furthermore, the number of neurogenic clusters in the INL as well as rod progenitors in the ONL increases following damage compared to undamaged retinas (Faillace et al., 2002). In both regeneration and development, the migrating nuclei have a characteristic spindle or elongated shape (Otteson et al., 2001; Wu et al., 2001). It was found that the antecedent to the rod precursors is a multipotent progenitor in the INL able to produce all cell types in the retina. Around

the same time, it was discovered that MG display a unique behavior in response to damage. In localized heat induced retina damage, MG upregulate GFAP and associate tightly with new progenitors in the ONL (Braisted et al., 1994). This led to the hypothesis that the proliferating cell bodies in the INL are MG.

Müller glia regeneration

The unique behavior and placement of MG following damage suggested they play an integral part in retina regeneration. This encouraged further investigation into MG involvement in retina regeneration. Three main lines of evidence strongly suggested MG as the source of retina progenitors after damage. First, new progenitors that form in the INL migrate along MG processes to the INL (Raymond et al., 2006; Raymond and Rivlin, 1987; Vihtelic and Hyde, 2000; Wu et al., 2001). Second, MG become mitotic after damage (Braisted et al., 1994; Faillace et al., 2002; Yurco and Cameron, 2005)}(Raymond et al., 2006; Vihtelic and Hyde, 2000; Wu et al., 2001). Last, gene expression profiles of MG and retina progenitor cells are very similar (Blackshaw et al., 2004). Together, these data suggest that MG are the source of progenitor cells following damage. Soon after, definitive evidence showed that MG produce rod precursors during development and that MG produce progenitors that can differentiate into any retina cell type following damage (Bernardos et al., 2007; Fausett and Goldman, 2006). The mechanism by which MG produce progenitor cells is suggested to be by dedifferentiation of the MG, owing to the fact that shortly after damage, MG begin to produce markers of neural progenitors such as Paired box protein 6 (Pax6), α tubulin, and Brain Lipid Binding

Protein (BLBP) (Fausett and Goldman, 2006; Raymond et al., 2006; Thummel et al., 2010).

Currently, it is understood that following damage, an as yet unknown signal is received by MG, which causes them to dedifferentiate to a stem cell-like state and reenter the cell cycle. This allows MG to divide once asymmetrically and produce a progenitor. The progenitor then goes on to divide multiple times (Nagashima et al., 2013). These divisions produce clusters of cells that divide, hypertrophy, and migrate along the MG to the site of damage where they exit the cell cycle and differentiate into new cells to replace those that were lost, integrating into the existing scaffolding (Figure 3) (Bernardos et al., 2007; Fausett and Goldman, 2006; Montgomery et al., 2010; Powell et al., 2012; Qin et al., 2011; Ramachandran et al., 2010a; Taylor et al., 2012; Thummel et al., 2010; Thummel et al., 2008). Rod precursors also proliferate to produce mature rod cells. This response, though, is short lived, and shortly after, MG take over and produce the majority of progenitors (Montgomery et al., 2010). Once the retina has fully regenerated, vision returns (Sherpa et al., 2008).

A limited number of genes involved in regeneration have been described and are involved in processes such as progenitor formation and maintenance (Fausett and Goldman, 2006; Hitchcock et al., 1996; Ramachandran et al., 2010a; Ramachandran et al., 2010b; Ramachandran et al., 2011; Shen and Raymond, 2004a; Thummel et al., 2010), epigenetic modification (Powell et al., 2012), cellular remodeling (Fausett and Goldman, 2006), as well as pathways such as Fibroblast Growth Factor (FGF) (Hochmann et al., 2012; Qin et al., 2011; Wan et al., 2014), Wnt

(Osakada et al., 2007; Ramachandran et al., 2011), Notch (Raymond et al., 2006; Yurco and Cameron, 2007), and EGF (Wan et al., 2012). The diversity of genes involved suggests dynamic cellular changes and a high amount of cross talk between pathways. It is likely, therefore, that a large number of genes involved in retina regeneration have yet to be identified.

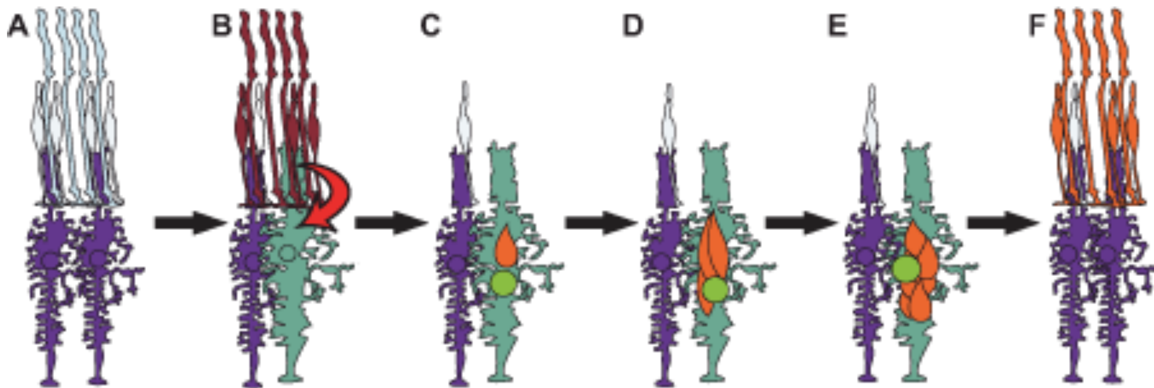

Figure 3. Retina Regeneration. Following an insult to the retina such as PR death (A-B) MG receive an as yet unknown signal (B). This causes MG to dedifferentiate, reenter the cell cycle, and divide once to produce a progenitor cell (B-C). The progenitor then goes on to divide a number of times to produce a cluster of progenitors (C-E). The progenitors then migrate to the area of damage and differentiate into the cell types that were lost (F). (Thummel et al., 2010)

Genes involved in regeneration

Understanding how zebrafish carry out retina regeneration can provide a number of new therapeutic targets to prompt the mammalian retina to regenerate. Though the current understanding of retina regeneration is incomplete, a number of factors have been identified that transition the retina through the various stages. The most important early marker of regeneration is the transcription factor

Achaete-scute family bHLH transcription factor 1a (Ascl1a) (Fausett et al., 2008; Ramachandran et al., 2010a; Ramachandran et al., 2011). Following damage it is upregulated in MG and has been shown to induce the expression of other downstream factors in regeneration as well as suppress expression of differentiation factors. Ascl1a can be induced by a number of signaling cascades including Wnt (Ramachandran et al., 2011), Mapk-Erk (Wan et al., 2012), and Jak-Stat (Zhao et al., 2014b). Though multiple pathways can be activated early in regeneration, they all appear to converge on upregulation of Ascl1a (Goldman, 2014). Ascl1a increases expression of epigenetic modifying enzymes, such as Apolipoprotein B-editing catalytic subunit 2b (Apobec2b) (Powell et al., 2012), and transcription factors, such as Insulinoma-associated 1a (Insm1a) (Ramachandran et al., 2012), as well as other factors known to contribute to proliferation of stem cells, such as lin28, *let7* (Ramachandran et al., 2010a), and Wnt signaling components (Ramachandran et al., 2011). Other factors in regeneration include microRNAs like *miR-203* (Rajaram et al., 2014b), which has been shown to regulate progenitor cell proliferation but not MG proliferation. Further downstream, additional signaling cascades are active, including Notch (Raymond et al., 2006; Yurco and Cameron, 2007) and FGF signaling (Hochmann et al., 2012; Qin et al., 2011; Wan et al., 2014).

A number of factors have been found to be upregulated in the retina or important for early stages in regeneration, including Leptin, Interleukin 6 (Il-6), Tumor Necrosis Factor α (TNFα), Ciliary Neurotrophic Factor (CNTF), and Insulin based on overexpression and knockdown studies (Conner et al., 2014; Nelson et al., 2013; Wan et al., 2014; Zhao et al., 2014b). Insulin and Il-6 have been found to be

important for early regeneration but do not originate in the retina based on *in situ* data, while Leptin and TNFα have been found to increase expression within the MG. Activation of these factors stimulates a number of intracellular cascades in MG or intercellular communication between MG. Jak-Stat signaling, specifically involving activated pStat3, is upregulated early by Leptin, TNFα, and CNTF (Nelson et al., 2013; Zhao et al., 2014b), and has been found to be very important in regeneration for intracellular cascades. Heparin Binding-Epidermal Growth Factor (HB-EGF) is also upregulated by Leptin and Il-6 and facilitates communication between MG via the EGF receptor and downstream cascades such as Mapk/Erk (Wan et al., 2012; Wan et al., 2014; Zhao et al., 2014b). Activation of Mapk/Erk via the EGF receptor is able to stabilize β-catenin and phosphorylate Stat3 to activate it. Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K) is also activated during this stage of regeneration, while Notch, which encourages glial differentiation and inhibits stemness and a neuronal fate, is downregulated (Conner et al., 2014; Wan et al., 2014).

All the activated pathways converge on upregulation of Ascl1a. Once activated, pStat3 is able to induce expression of Ascl1a (Zhao et al., 2014b). The HB-EGF signaling cascade also results in upregulation of Ascl1a (Wan et al., 2012). Knockdown of TNFα results in decreased levels of Ascl1a, indicating it too positively affects Ascl1a levels (Nelson et al., 2013). The DNA demethylases Apobec2a and -2b are activated as well to increase expression of Ascl1a (Powell et al., 2012).

Once activated, Ascl1a mediates increased expression of a number of stem cell associated factors and transcription factors. These include lin28, c-Myc$_a$ and $_{-b}$

SRY (Sex Determining Region Y)-Box 2 (Sox2), Nanog, Kruppel-Like Factor 4 (KLF4), Octamer-Binding Transcription Factor 4 (Oct4), Wnt signaling components, N-cadherin, and Insm1a (Nagashima et al., 2013; Ramachandran et al., 2010a; Ramachandran et al., 2011, 2012). Lin28 is an important stem cell factor and one of its functions is to repress the microRNA *let-7*, which is a negative regulator of stem cell genes such as Ascl1a, lin28, and other factors activated by Ascl1a. Factors involved in Wnt signaling, such as receptors and auxiliary proteins are upregulated, while inhibitors of Wnt, such as Dickkopf (Dkk) proteins, are downregulated by Ascl1a. Activation of these cascades by Ascl1a prompts MG to dedifferentiate and proliferate.

One of the first physical changes that occurs after dedifferentiation is an increase in GFAP as well as MG undergoing interkinetic nuclear migration (IKNM)(Lahne et al., 2015; Nagashima et al., 2013). Similar to radial glia during development, nuclei of dedifferentiated MG migrate to the top of the INL and into the ONL. Once there, they divide once to produce a single progenitor. The MG nucleus migrates back down to the INL and the progenitor divides a number of times to produce a cluster of progenitor cells that are adhered to the MG. The progenitors then migrate along the MG to the area of damage and differentiated into the required cell type. The adherence of the progenitors to the MG facilitates its migration and is established by N-cadherin (Nagashima et al., 2013). In heterozygote mutants for N-cadherin the progenitor clusters remain in the ONL, where the first cell division occurs. If the damage was in the ONL then the progenitors can differentiate normally, but if the damage was in another layer, such

as the GCL, then the cells fail to migrate and regeneration does not occur. The cell divisions of the progenitors are regulated by different factors. *miR-203* has been found to be involved progenitor cell proliferation but not MG proliferation (Rajaram et al., 2014b). It increases later in regeneration and functions to inhibit the activity of the transcription factor Pax6b. Pax6b has been found to be responsible for early divisions of progenitors, while Pax6a is responsible for proliferation after small clusters have already formed (Thummel et al., 2010). *miR-203*, therefore, may help to propagate progenitor cell proliferation by appropriately suppressing factors involved in early progenitor formation and allowing factors involved in late progenitor cell proliferation to take over.

Once the progenitors migrate there are additional signaling cascades that are involved in differentiation. FGF signaling, for example, is involved in progenitors following a rod fate, though it does not affect cone fate choice (Hochmann et al., 2012; Qin et al., 2011). All of these signaling factors work in concert to execute robust and reliable retina regeneration. There are likely other, as yet undiscovered, factors that also help to carry out retina regeneration and discovery of these new factors will help to develop new therapies to treat retina damage and disease in mammalian retinas.

Damage models

The precise timing of regeneration varies depending on how the retina is damaged. Broadly, there are three main techniques used to damage the retina: light, chemical, and physical. Light damage involves exposure of the dark adapted retina

to bright light and typically damages PRs in the ONL. One of the most common ways to employ this is to expose the retina to bright light (20,000 lux) continuously for four days (Vihtelic and Hyde, 2000; Vihtelic et al., 2006). This damages the dorsal and central retina efficiently, and to a lesser degree the ventral retina. Alternatively the retina can be exposed to very bright light (120,000 lux) for a shorter period of time (Bernardos et al., 2007). This damages a more restricted region of the central retina. The mechanism by which light kills PRs is currently not known but involves apoptosis, not necrosis.

Chemically damaging the retina involves injecting a chemical into the eye or bathing the fish in water containing a chemical (Fimbel et al., 2007; Fraser et al., 2013; Montgomery et al., 2010; Nagashima et al., 2013; Nelson et al., 2013; Sherpa et al., 2008). The first chemical used to damage the retina was ouabain, a neurotoxin (Maier and Wolburg, 1979). Injection of high concentrations of ouabain into the retina kills all neurons. MG, however, are spared. Since MG are still present, they are able to fully regenerate the retina. Lower doses of ouabain can also be injected to only destroy certain cell types, such as GCs, and ACs, since they are closest to the vitreous.

Newer methods that utilize chemical destruction of cells in the retina have also been developed. The bacterial enzyme Nitroreductase (NTR) has been used to make transgenic fish where the enzyme is expressed only in one cell type. Placing the fish in a tank containing the pro-drug Metronidazole (MTZ) allows MTZ to interact with NTR. NTR reduces MTZ, converting it to a toxin that causes DNA interstrand crosslinking, resulting in apoptosis. This underscores a major

advantage to using chemical methods of retina damage, which is that the chemical can be used to target specific cell types. Also the chemical can be titrated to produce different amounts of damage. In ouabain injections, for example, low dose injections that only kill the GCL do not elicit a regenerative response (Raymond et al., 1988). Likewise, low doses of MTZ can cause lower damage and sometimes does not cause a regenerative response.

Lastly, physical damage of the retina involves techniques such as poking the retina with a syringe needle (Fausett and Goldman, 2006; Fausett et al., 2008). The eye can be rotated and a needle inserted to destroy an entire column of the retina. This is beneficial because adjacent undamaged regions can be used as controls within the same eye. However, it has been shown that secreted factors can spread widely and affect the surrounding undamaged retina, some even affecting the whole retina. Additionally, more than just the neuroretina is destroyed, surrounding epithelial cells and vasculature are also disrupted. This potentially introduces other confounding variables including immune cell invasion.

Ascl1a has been studied in the mouse retina to determine its ability to initiate retina regeneration (Pollak et al., 2013; Ueki et al., 2015; Wohl and Reh, 2016). This has been met with limited success but shows that the underlying pathways that are active during zebrafish regeneration can also be activated. Understanding how Ascl1a influences retina regeneration, and how retina regeneration initiates in general, may prove vital to eliciting full regeneration in the mammalian retina. Various factors have been suggested as initiation factors in retina regeneration, and likely it is a combination of signals that work together to mount a robust

regenerative response. However, though the proposed factors may function very early in retina regeneration, they may not be true initiators of regeneration. HB-EGF was found to be induced as early as 1 hour post injury, indicating it is an early factor but likely not an initiator of regeneration (Wan et al., 2012). The inflammatory cytokines TNF-α (Conner et al., 2014; Nelson et al., 2013) and Il-6 (Zhao et al., 2014b) have been suggested as initiators of regeneration, though the source of Il-6 does not originate in the retina and TNF-α is likely affecting immune cells in the retina and may have only indirect effects on retina initiation. Furthermore, TNF-α has not been shown to directly affect MG. Leptin has also been suggested to induce regeneration (Zhao et al., 2014b) but, similar to HB-EGF, it is upregulated early on, indicating it is not an initiator of regeneration. Discovering true initiators of regeneration may prove vital to prompting the mammalian retina to regenerate endogenously.

Initiation of Neurogenesis

Mammalian adult neurogenesis

Other neuronal tissues undergo proliferation and may lend insight into mechanisms of retina regeneration initiation. In the mammalian brain the subventricular zone (SVZ) and the subgranular zone (SGZ) undergo neurogenesis even in adults (Braun and Jessberger, 2014; Lazarov et al., 2010; Lucassen et al., 2010; Pallotto and Deprez, 2014; van Wijngaarden and Franklin, 2013). The SGZ is found in the hippocampus and supplies new neurons to aid in memory formation. The SVZ is located around the lateral ventricle and supplies new neurons to the

olfactory bulb. Both areas contain stem cells that can proliferate to produce progenitor cells. The progenitor cells then migrate and differentiate into mature neurons and integrate into existing circuitry. The regulation of initiaton of adult neurogenesis in these areas is not well understood but neurotrophic factors such as Brain Derived Neurotrophic Factor (BDNF) or VEGF have been implicated in the process. Additionally, miRNAs and other factors involved in retina regeneration have also been found in stem cell proliferation in the SGZ and SVZ (Cheng et al., 2009; Pallotto and Deprez, 2014; Tong et al., 2014). Considering the similarity between mammalian adult neurogenesis and zebrafish retina regeneration, mechanisms found to regulate adult neurogenesis may also regulate zebrafish retina regeneration.

Neurotransmitters in regeneration

Neurotransmitters have been known to affect neuronal proliferation for some time, but recently the understanding and diversity of their functions have greatly increased (Berg et al., 2013). Selective serotonin reuptake inhibitors (SSRIs) have been used to treat symptoms of depression. An unexpected side effect is increased neurogenesis in the SGZ (Perez-Caballero et al., 2014). This suggests that serotonin may be involved in neuronal proliferation. In fact multiple neurotransmitters have been shown to be involved in various stages of neurogenesis. DA has been shown to suppress stem cell proliferation while also enhancing progenitor cell proliferation in the SVZ (Berg et al., 2013; Berg et al., 2011; Kippin et al., 2005). This suggests that DA may act differentially on

proliferating cells to provide directionality to the process. Glutamate appears to negatively affect proliferation and receptors have been found on both stem cells and progenitor cells in the SGZ (Berg et al., 2013). Acetylcholine has been found to affect proliferation only in the SGZ. Interestingly, activation of different receptors have differing effects, with nicotinic receptor activation suppressing proliferation and muscarinic receptor activation enhancing proliferation (Berg et al., 2013). Serotonin has been found to increase proliferation in the SGZ and progenitor proliferation in the SVZ (Brezun and Daszuta, 1999, 2000; Tong et al., 2014). This was shown using SSRIs, as mentioned previously, as well as specific serotonin receptor subunit agonists and antagonists. The receptor subunits shown to be involved in proliferation include 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2C, and 5-HT4 (Berg et al., 2013). GABA has also been found to affect proliferation (Pallotto and Deprez, 2014; Ramirez et al., 2012). In the SGZ, decreased GABA signaling promotes the proliferation of radial glia like stem cells (RGLs)(Song et al., 2012). Likewise, GABA signaling has been shown to inhibit progenitor proliferation in the SVZ (Berg et al., 2013). Conversely, GABA signaling has been shown to promote progenitor proliferation in the SGZ (Giachino et al., 2014; Quadrato et al., 2012; Quadrato et al., 2014; Song et al., 2013), suggesting again that GABA signaling may be providing a directionality to proliferation in the SGZ.

These same neurotransmitters are also present in the retina. Therefore, it is possible that they are playing similar roles in the retina as they are in the SGZ and SVZ. In particular, GABA appears to function in a circuit similar to one found in the retina and behaves in such a way as to possibly facilitate neurogenesis. In the

dentate gyrus (DG) of the mouse hippocamous, which contains the SGZ, glutamatergic granule cells synapse onto GABAergic, parvalbumin positive interneurons. The processes of the interneurons are in close proximity of the RGLs. When input from the granule cells is low, then GABA release from the interneurons also decreases. $GABA_A$ receptors on RGLs detect this decrease and cause the cells to proliferate (Figure 4) (Song et al., 2012).

A similar circuit exists in the retina. Glutamatergic PRs synapse onto GABAergic, parvalbumin positive HCs. In a disease such as RP, when PRs die they no longer stimulate HCs to release GABA. In this situation GABA levels would act as a damage sensor and when GABA levels decrease, MG detect that and begin to proliferate. This would act as a true initiator of retina regeneration as it acts as a direct signal to MG of damage and does not require any gene regulation or indirect signaling.

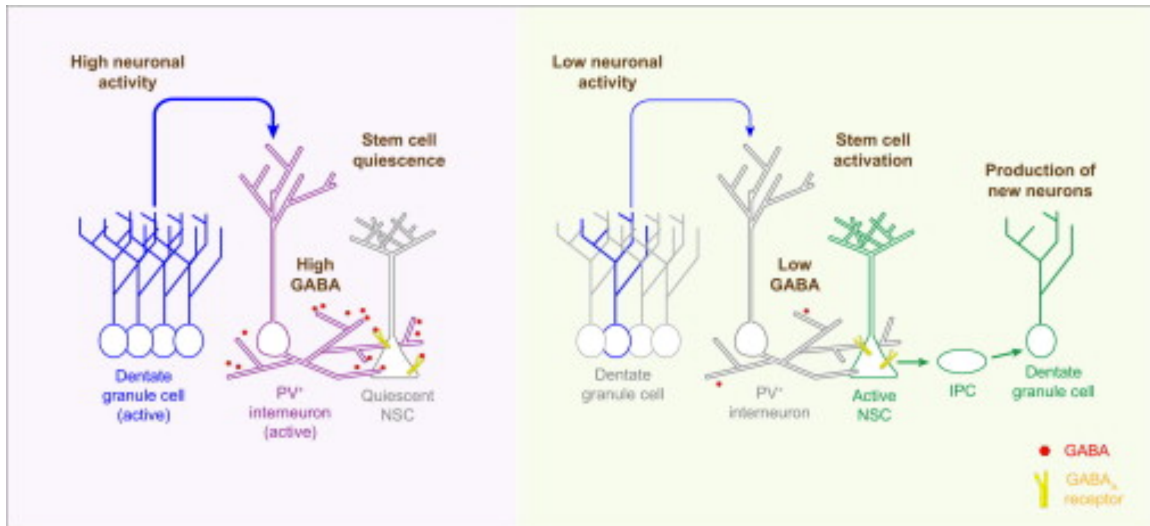

Figure 4. GABA mediated neurogenesis in the SGZ. Glutamatergic granule cells synapse with GABAergic interneurons. The processes of the interneurons are in close proximity to RGLs. When input from the granule cells is high, GABA release is high, and the RGLs sense the high levels of GABA and remain quiescent. When input from granule cells is low, GABA levels decrease, and RGLs detect this decrease and proliferate. (Chell and Frisen, 2012)

γ-aminobutyric acid

GABA function in neuronal communication

Synthesis, packaging, and release

GABA is the main inhibitory neurotransmitter in the CNS. Its typical function during neurotransmission is to lower the membrane potential of the post-synaptic cell (Rowley et al., 2012). The precursor of GABA is glutamate. Glutamate is converted to GABA by the action of Glutamic Acid Decarboxylase (GAD), the rate-limiting step in GABA synthesis (Owens and Kriegstein, 2002; Tritsch et al., 2016). GABA is only produced in cells that contain GAD. Once produced, GABA is packaged into vesicles via the vesicular GABA transporter.

Upon depolarization GABA is released into the synaptic cleft and binds to receptors on the postsynaptic cell. There are two main types of GABA receptors. $GABA_A$ receptors are ionotropic and become permeable to Cl^- ions following GABA binding (Chebib and Johnston, 1999; Farrant and Nusser, 2005; Jacob et al., 2008; Owens and Kriegstein, 2002; Sigel and Steinmann, 2012; Tretter et al., 2012). $GABA_B$ receptors are metabotropic and activate intracellular cascades that open K^+ and Ca^{2+} channels (Chebib and Johnston, 1999; Jacob et al., 2008; Owens and Kriegstein, 2002). Activation of either receptor decreases the postsynaptic cells membrane potential.

GABA is cleared from the synaptic cleft by the GABA Transporter (GAT), found on glial cells and presynaptic neurons. In zebrafish and other fish GAT is mainly found on BCs, HCs, and ACs, where as in mammals GAT is found mainly on MG, though they also exist on neurons (Bringmann et al., 2013; Ekstrom and Anzelius, 1998; Nelson et al., 2008). GAT is a Na^+ and Cl^- co-transporter, utilizing two Na^+ ions and one Cl^- ion for every GABA molecule, resulting in a net membrane depolarization (Bringmann et al., 2013). GABA is then prepared for release again (Farrant and Nusser, 2005; Owens and Kriegstein, 2002). Glial uptake of GABA, via GAT, causes GABA to be metabolized to glutamine, which is then transferred back to the presynaptic cell, where it is then processed back to GABA and repackaged.

Receptor subunits and function

$GABA_A$ receptors on the postsynaptic cell are located synaptically or extrasynaptically. Synaptic $GABA_A$ receptors are responsible for phasic inhibition,

which has high amplitude but a short wavelength. Extrasynaptic receptors mediate tonic inhibition, which has a lower amplitude but longer wavelength (Belelli et al., 2009; Farrant and Nusser, 2005; Jacob et al., 2008; Sigel and Steinmann, 2012). Different $GABA_A$ receptors are found in different places. The $GABA_A$ receptor is pentameric and can be composed of multiple subunits. Each subunit contains 4 transmembrane domains with intracellular loops between domain 1 and 2 and between 2 and 3.

In humans there are 6 α subunits (1-6), 3 β subunits (1-3), 3 γ subunits (1-3), 3 ε subunits (1-3), as well as one θ, δ, and π each. Zebrafish have the same types of subunits but more subunits within each type. Typically, the receptor is formed using two α subunits, 2 β subunits, and one of any subunit, including another α or β subunit. The most common receptor is composed of α1, β2, and γ2. This combination is typically found in the synapse. Synaptic $GABA_A$ receptors typically contain α1, 2, 3, or 5, any β, and any γ subunit. $GABA_A$ receptors containing α4, 5, or 6, β3, and the δ subunit are typically found extrasynaptically. Association of receptor subunits occurs through preferential binding based on sequences in the N-terminus. For example, δ preferentially binds with α4 and 6, though γ2 can take its place if it is not present. The different subunits also have different functionality. For example, δ containing receptors bind GABA for longer, have a higher affinity for GABA, and remain open longer.

Additionally, there are 3 ρ subunits (1-3) and these receptors homo-oligomerize. These were previously described as $GABA_C$ receptors but were later found to be a type of $GABA_A$ receptor. These receptors are most commonly found in

the retina. GABA molecules bind to receptors at the junction of the α and β subunits, meaning there are two binding sites for GABA (Chebib and Johnston, 1999; Farrant and Nusser, 2005; Jacob et al., 2008; Owens and Kriegstein, 2002; Sigel and Steinmann, 2012; Tretter et al., 2012).

Trafficking and recycling

$GABA_A$ receptors are inserted into the membrane by a number of interacting proteins. One such protein is $GABA_A$ Receptor Associated Protein (GABARAP), which binds to the second intracellular loop of the γ2 subunit and facilitates trafficking and insertion of γ2 containing receptors into the membrane. Once in the membrane, clustering of receptors in the synapse is maintained by Geyphrin, while extrasynaptic receptor localization is maintained by Radixin. Endocytosis of receptors is via Clathrin and receptors are typically endocytosed within 30 minutes of insertion into the membrane. Once endocytosed, receptors can be recycled or degraded (Farrant and Nusser, 2005; Jacob et al., 2008; Tretter et al., 2012).

Intracellular effects of GABA signaling

$GABA_A$ receptor activation is typically thought to bring the postsynaptic cell away from threshold and make action potentials less likely. There are, however, a few molecules that have been suggested to function intracellularly following $GABA_A$ receptor activation. Few studies have investigated GABA signaling during neurogenesis either from neurogenic niches or during development and have suggested that $GABA_A$ receptor activation can induce Ca^{2+} transients (Owens and

Kriegstein, 2002; Pallotto and Deprez, 2014; Quadrato et al., 2014; Schwirtlich et al., 2011; Tozuka et al., 2005). This, however, has only been studied in the maturation of progenitor cells. Downstream of Ca^{2+}, the cAMP Response Element-Binding Protein (CREB), a transcription factor, has been shown to be upregulated (Jagasia et al., 2009; Pallotto and Deprez, 2014; Ramirez et al., 2012). Another transcription factor, Nuclear Factor of Activated T Cell c4 (NFATc4) has been shown to be upregulated by $GABA_A$ receptor activity as well (Quadrato et al., 2012; Quadrato et al., 2014). At the level of chromatin, histone H4 acetylation and γH2AX levels have been found to be increased by GABA signaling (Andang et al., 2008; Fernando et al., 2011). It seems probable that there is a signaling cascade where activation of the $GABA_A$ receptor at the cell membrane could alter gene expression, prompting such changes as increased neuronal proliferation. A caveat to these findings is that they were observed in neuronal progenitors and are shown to affect progenitor cell survival and maturation.

Summary

The retina is a neuronal structure that is highly conserved across vertebrates. In some vertebrates, such as mammals, damage and disease in the retina results in cell death that cannot be repaired. In other vertebrates, such as zebrafish, retina damage is met with a robust regeneration response. By understanding how zebrafish regenerate, new therapies and treatments can be developed to help repair damaged or diseased retinas in mammals. Retina regeneration in zebrafish begins with MG proliferation to produce progenitors, which migrate to the area of damage

and replace the lost cells. It is unknown what initiates MG to regenerate. Based on literature from the mouse SGZ, one possible signal is the neurotransmitter GABA. I hypothesize that GABA levels decrease after PR death and the decrease is sensed by MG via γ2-containing $GABA_A$ receptors, causing them to proliferate (Figure 5).

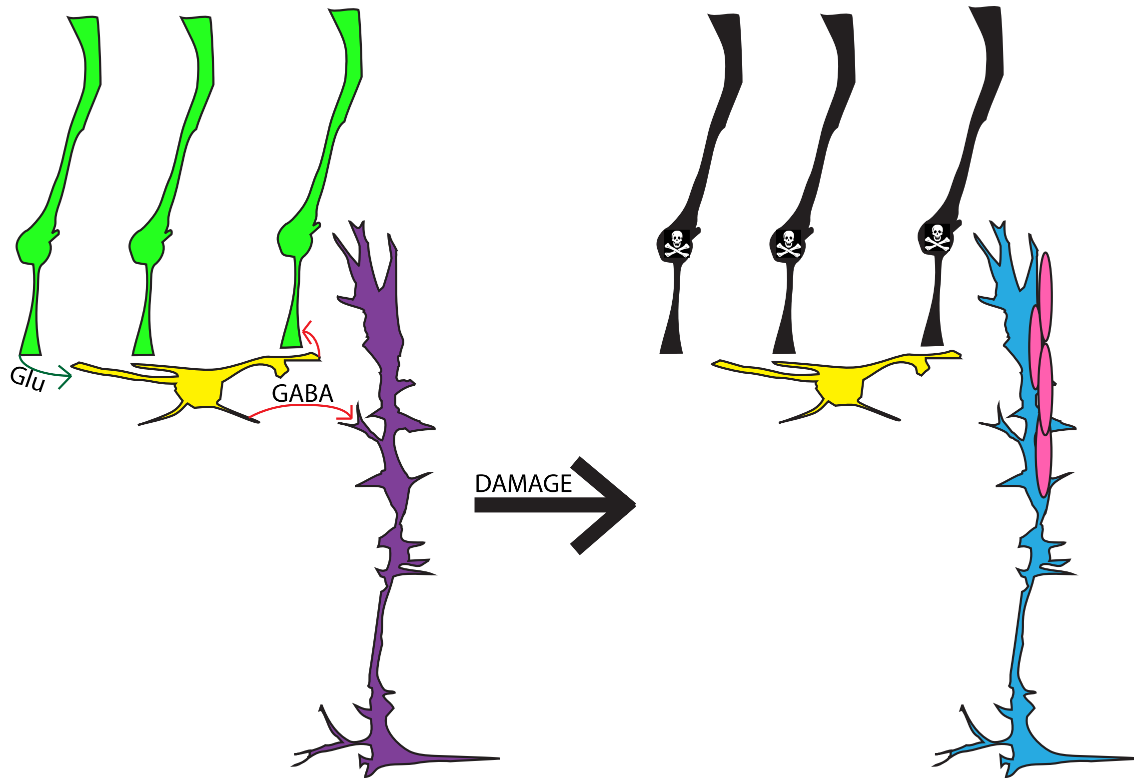

Figure 5. Model of how GABA regulates retina regeneration. Glutamatergic rods synapse with GABAergic HCs. When PRs are alive, GABA release from HCs is high. Upon damage, PRs no longer release glutamate and HCs are no longer stimulated to release GABA. MG detect the decrease in GABA and initiate regeneration

Chapter II

miR-216a REGULATES *snx5*, A NOVEL NOTCH SIGNALING COMPONENT, DURING ZEBRAFISH RETINAL DEVELOPMENT

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Abstract

Precise regulation of Notch signaling is essential for normal vertebrate development. Mind bomb (Mib) is a ubiquitin ligase that is required for activation of Notch by Notch's ligand, Delta. Sorting Nexin 5 (SNX5) co-localizes with Mib and Delta complexes and has been shown to directly bind to Mib. We show that *microRNA-216a* (*miR-216a*) is expressed in the retina during early development and regulates *snx5* to precisely regulate Notch signaling. *miR-216a* and *snx5* have complementary expression patterns. Knocking down *miR-216a* and/or overexpression of *snx5* resulted in increased Notch activation. Conversely, knocking down *snx5* and/or *miR-216a* overexpression caused a decrease in Notch activation. We propose a model in which SNX5, precisely controlled by *miR-216a*, is a vital partner of Mib in promoting endocytosis of Delta and subsequent activation of Notch signaling.

Introduction

Since their discovery as regulators of *C. elegans* developmental timing in 1993 (Lee et al., 1993; Wightman et al., 1993), miRNAs have been shown to be involved in diverse aspects of development. miRNAs are 21-23 nucleotide (nt) non-coding RNAs that regulate gene expression by binding to complementary sequences in the 3'UTR of messenger RNAs (Bartel, 2004; Fabian et al., 2010; He and Hannon, 2004; Huntzinger and Izaurralde, 2011; Liu et al., 2012). This results in the recruitment of the RNA Induced Silencing Complex (RISC), the effector complex that mediates translation repression, deadenylation, and decay of target mRNAs (Bazzini

et al., 2012; Djuranovic et al., 2012; Giraldez et al., 2006). We and others have identified developmental roles for several individual miRNAs in zebrafish (Flynt et al., 2007; Flynt et al., 2009; Giraldez et al., 2005; Li et al., 2008; Li et al., 2011; Mishima et al., 2009; Stahlhut et al., 2012; Wei et al., 2013). However, the exact roles and mRNA targets for most miRNAs that function during development are still unknown.

Notch signaling regulates many processes during vertebrate development, from vasculogenesis to segmentation (Fortini, 2009; Lawson et al., 2001; Wright et al., 2011). It is especially important during neurogenesis (Louvi and Artavanis-Tsakonas, 2006), is instructive for gliogenesis in the zebrafish retina (Scheer et al., 2001), and has been shown to be essential for zebrafish retinal development (Bernardos et al., 2005). Notch is a transmembrane receptor that mediates interaction with adjacent cells through membrane bound ligands, such as Delta, that trigger proteolytic cleavage of Notch and release of an intracellular domain that travels to the nucleus to alter gene expression (Louvi and Artavanis-Tsakonas, 2006). Mind bomb is a ubiquitin ligase that ubiquitylates Delta, thereby facilitating its endocytosis, which is essential for cleavage of Notch and subsequent activation of signaling (Itoh et al., 2003). Mutants in Mind bomb have disorganized retinal architecture and do not have Müller glia (Bernardos et al., 2005).

Sorting Nexin 5 (SNX5) is part of the large sorting nexin protein family, members of which have been previously shown to bind phosphoinositides through a specialized phox-homology (PX) domain (Cullen, 2008; Cullen and Korswagen, 2012). SNX5 is part of a select group of sorting nexins that also contain a carboxy-

terminal BAR (Bin, amphiphysin, Rvs) domain, thought to facilitate binding to and/or induce membrane curvature, possibly functioning in endocytosis or vesicle budding (Cullen, 2008). The sorting nexins function in diverse cellular trafficking processes, including developmental signaling cascades as in the case of SNX3, which has been shown to be required for Wnt secretion (Harterink et al., 2011) and SNX17 which functions in integrin recycling (Steinberg et al., 2012). SNX5 was previously shown to co-localize with Mib and Delta (Yoo et al., 2006). Knockdown of SNX5 using morpholinos in zebrafish causes defects in vascular development (Eckfeldt et al., 2005; Yoo et al., 2006). Accumulating evidence, therefore, suggests that SNX5 could play a role in modulating Notch signaling.

In this study, we show for the first time that *miR-216a*, a miRNA that is expressed in the developing zebrafish retina, regulates *snx5*. Results using reporter fish show that *miR-216a* regulates *snx5* to modulate Notch signaling during eye development.

Materials and Methods

Zebrafish Lines and Maintenance

Wildtype (AB) (Walker, 1999), *albino* (University of Oregon, Eugene, OR), *Tg(gfap:GFP)* (Bernardos and Raymond, 2006), *Tg(her4:dRFP)* (Yeo et al., 2007) *Tg(flk1:GFP)* (Choi et al., 2007) and *Tg(Tp1bglob:eGFP)* (Parsons et al., 2009) lines were maintained at 28.5°C on a 14:10 hour light:dark cycle. Embryos were raised in egg water (0.03% Instant Ocean) at 28.5°C and staged according to morphology (Kimmel et al., 1995) and hours post fertilization (hpf). All experiments were

performed with the approval of the Vanderbilt University Institutional Animal Care and Use Committee (M/09/398).

Microarrays of developing eyes

Developing eyes were dissected at 2 and 5 days post fertilization (dpf), homogenized in Trizol and total RNA was extracted. Small RNAs were enriched and arrays were performed and normalized as previously described (Thatcher et al., 2007). Fold changes were calculated compared to a negative control consisting of probes for *Pseudomonas aeruginosa* dehydrogenase (Thatcher et al., 2007). Microarray data was analyzed using GeneSpring software, and paired t-tests were performed using Prism (GraphPad) to determine *p* values.

Molecular Cloning

Potential target mRNA 3'UTRs were amplified by RT-PCR using the primers below. Each 3' UTR was cloned into pCS2+ downstream of the coding sequence of GFP (Table 2) (Flynt et al., 2007).

Table 1. Primers used to amplify predicted *miR-216a* targets

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
snx5 (NM_214769)	ACCTGATCGAGATGACTGAG	TTATCTTCGCTGAGTTGCAC
her4.2 (NM_131090.3)	AGTCACATCTGGAGACCCTG	GCTTCAACACACAAACAAGTCC
notch1b (NM_131302.2)	GTCACAAATCGGACACATGC	CACAAATCGTTTCAATCGGATG
heyl (NM_181736.1)	GGGCTTTGAGTTCCTCCAG	TCTCCTCAAGCACTTCAATCTC
numb (NM_001040406.1)	CGCTCCATCACCCACAAACC	GACGAGTCGTTCCCTGTATGG
hey2 (NM_131622.2)	AGTAAACCATACCGACCGTG	GGTTACATCTTACAGAGGGTGG

miRNA recognition elements (MREs) were deleted from the *snx5* 3'UTR with PCR. For MRE 1, forward (5'-TGCAGACACATAAAGTACCACTATG-3') and reverse (5'-GCTAATATTTGCATAACTTGGAATATG-3') primers and for MRE 2, forward (5'-GTCCGAATGCATTACTCTGCATTACAGAT-3') and reverse (5'-TATTAGGAGGAAAGATATCTGAAGCATTACA-3') primers were designed to exclude each MRE. *snx5* mRNA was amplified by RT-PCR using forward (5'-GCCGAGGGATCCTGAGGAACGAGCTTGCTGCTGGAA-3') and reverse (5'-GCCGAGCTCGAGCAACTGGGGACATCAGTCAGTCCTT-3') primers and cloned into pCS2+ (Rupp et al., 1994). *snx5* mRNA without its 3'UTR was amplified by RT-PCR using forward (5'-GCCGAGGGATCCTGAGGAACGAGCTTGCTGCTGGAA-3') and reverse (5'-GCCGAGCTCGAGGTCATCATCGTGTGGGTC-3') primers and cloned into pCS2+. All clones and MRE deletions were verified by Sanger sequencing in the Vanderbilt DNA Sequencing Core.

Microinjection

All injections were performed in fertilized 1-cell zebrafish embryos. Phenol red dye (0.05%) was used in each injection solution and alone as an injection control. Capped *snx5* RNA (from the pCS2+ vector containing the *snx5* mRNA without 3'UTR) or GFP-*snx5* 3'UTR RNA (from the pCS2+ vector containing the coding sequence of GFP and either the full length *snx5* 3'UTR or the *snx5* 3'UTR with both MREs deleted) were prepared using an Sp6 mMessage Machine Kit (Ambion). *snx5* RNA was injected at 100 pg/embryo concentration for functional experiments and 50 pg/embryo for rescue experiments. GFP RNA was injected at 25 pg/embryo concentration. Synthetic *miR-216a* duplexes (Dharmacon) were injected at 50 pg/embryo concentration in functional experiments and 25 pg/embryo in GFP reporter experiments. Two different morpholinos against *miR-216a* (one against the mature *miR-216a*: 5'-TCACAGTTCCCAGCTGAGATTA-3' and a second against the loop of pre-miR-216a: 5'-GCAGCGCCTGTGAGAGGGATGAAAA-3'), a morpholino against the *snx5* start site: 5'-ACGTCATGTTCAGGAGATATTTCGC-3' (Eckfeldt et al., 2005), and an exon 4 splice donor morpholino: 5'-CAGAGTTAGACTCACGCCTCAAGTT-3' (Yoo et al., 2006), and a *p53* morpholino (5'-GCGCCATTGCTTTGCAAGAATTG-3') were from Gene Tools. Two different *miR-216a* morpholinos were injected together at 150 pg each/embryo for functional experiments and a morpholino targeting just the mature form of *miR-216a* was used at 100 pg/embryo for GFP reporter experiments. *snx5* morpholinos were injected together at 100 pg each/embryo for all experiments. The *p53* morpholino was

injected at 150 pg/embryo. All injection amounts were experimentally determined to be the lowest effective dose.

In situ hybridization

Staged *albino* zebrafish embryos were fixed in 4% paraformaldehyde (PFA) in 1X PBS (pH 7.4) at 4°C overnight on a 3D rocker. Whole-mount mRNA *in situ* hybridization was performed as described (Thisse and Thisse, 2008) using a digoxigenin (DIG)-labeled *snx5* RNA probe generated with Roche Applied Science reagents and pCS2+ vector containing the full length *snx5* mRNA sequence. Whole-mount miRNA *in situ* hybridization was performed as described (Lagendijk et al., 2012) using a miRCURY 5'- and 3'-DIG labeled *hsa-miR-216a* LNA probe (Exiqon).

Immunoblotting

Embryos were deyolked at 1 dpf (day post fertilization) and placed in lysis buffer [25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 300 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10% glycerol, 1.0% Triton X-100, 1 mM PMSF] for protein extraction. Total proteins were separated on 10% SDS-PAGE gels and transferred to PVDF-plus membranes (GE Osmonics). Membranes were incubated with rabbit polyclonal antibodies against SNX5 (1:2000, Aviva Systems Biology) and a-tubulin (1:500, Abcam). Anti-rabbit HRP-conjugated secondary antibodies (1:5000, GE Healthcare) were used for visualization with ECL reagents (Perkin Elmer). Using ImageJ, SNX5 levels were normalized to α-tubulin control levels, after which the ratio of SNX5 under varying injection conditions was determined. One-way ANOVA using

Bonferroni's correction to adjust for multiple comparisons was performed using StatPlus (AnalystSoft).

Staining and Imaging

Live embryos, either *Tg(flk1:GFP)* at 3-4 dpf or those injected with GFP reporter transcripts were briefly anesthetized with 0.02% tricaine for imaging on a Zeiss Discovery V8 stereo microscope and photographed using an Axiocam MRM black and white camera and Axiovision software (Zeiss). Live embryos that were staged and fixed in 4% PFA in 1X PBS (pH 7.4) at room temperature for 2-3 hours or embryos upon which *in situ* hybridization had been performed were embedded in 1.5% agarose/5% sucrose in egg water. The resulting blocks were cryoprotected in 30% sucrose overnight, frozen, and sectioned on a Leica CM1850 cryostat (10-15μm sections). The resulting transverse sections were mounted on VistaVision Histobond slides (VWR). *Tg(her4:dRFP*) sections were stained with Alexa Fluor 488-conjugated phalloidin (1:100, Molecular Probes) and Hoescht (1:3000, Molecular Probes), and *Tg(gfap:GFP)*sections were stained with the mouse monoclonal antibody zpr-1 (1:1000, Zebrafish International Research Center), HuC/D (1:1000, Invitrogen), and/or TOPRO-3 (1:1000, Molecular Probes). TUNEL labeling was performed using an *in situ* Cell Death Detection Kit, TMR red (Roche). Fluorescent sample slides were mounted with Vectashield (Vector Laboratories) and *in situ* sample slides were mounted in 100% glycerol. *In situ* and *Tg(her4:dRFP*) samples were imaged on a Leica DM6000B microscope or Leica LSM 510 confocal (inverted) microscope with a 40× objective. *Tg(gfap:GFP*) samples were imaged on

a Leica LSM 510 confocal (inverted) microscope with a 20x or 40x objective in the Vanderbilt Cell Imaging Shared Resource. Images were processed using ImageJ and Adobe Photoshop, and one-way ANOVA was calculated as described for immunoblotting.

Results

miRNA expression analysis in developing eyes

In order to examine the role of miRNAs during vertebrate eye development, we dissected developing eyes from zebrafish at 2 and 5 dpf and isolated RNA for miRNA expression profiling. We detected 12 miRNAs expressed at levels above background at 2 dpf and 23 miRNAs detected at 5 dpf (Table 2). From *in situ* localization experiments, only three of these miRNAs (*miR-9*, *miR-124*, and *miR-216a*) are expressed specifically in the developing eye at these times, the remainder are expressed ubiquitously (Ason et al., 2006; Kapsimali et al., 2007; Wienholds et al., 2005; Wienholds and Plasterk, 2005). Because *miR-9* and *miR-124* have been extensively studied during neural development (Gao, 2010), we decided to focus on the role of *miR-216a* in zebrafish eye development.

2 dpf	Fold Difference	p-value
miR-9	4.1791	0.0002
miR-17-5p	7.7904	0.0002
miR-19a	3.6866	0.0069
miR-20	4.6253	0.0018
miR-25	2.6127	< 0.0001
miR-31	3.3801	0.0008
miR-93	3.7530	0.0002
miR-108	4.1121	0.0033
miR-124a	7.0932	< 0.0001
miR-152	4.9246	0.0017
miR-210	2.8556	0.0076
miR-216	3.9684	0.0016
5 dpf	**Fold Difference**	**p-value**
miR-9	5.4529	< 0.0001
miR-17-5p	7.1188	< 0.0001
miR-18	3.8517	0.0002
miR-19a	6.6342	< 0.0001
miR-19b	3.9508	< 0.0001
miR-20	6.0296	< 0.0001
miR-22	5.6745	< 0.0001
miR-25	5.3233	< 0.0001
miR-31	3.4258	0.0001
miR-93	5.2311	< 0.0001
miR-108	5.1436	0.001
miR-124a	7.3811	< 0.0001
miR-125b	6.0221	< 0.0001
miR-152	4.0968	< 0.0001
miR-181a	4.4360	0.0001
miR-181b	4.6483	< 0.0001
miR-182	5.4448	< 0.0001
miR-183	6.3569	< 0.0001
miR-204	7.9874	< 0.0001
miR-210	8.0629	< 0.0001
miR-213	3.8558	< 0.0001
miR-216	8.8500	< 0.0001
miR-217	6.8886	0.0002

*Fold difference is calculated by dividing the normalized miRNA expression by the negative control

**All p-values are calculated based on a paired t-test

Expression of miR-216a in developing eyes is temporally and spatially specific

To determine the expression of *miR-216a* over the course of eye development, we performed whole mount LNA *in situ* hybridization for *miR-216a* on zebrafish embryos, which were then sectioned and visualized (Figures 6 & 7). *miR-216a* is robustly and widely expressed throughout the eye cup at 22 hpf (Figure 6), but its localization then progressively changes as development proceeds (Figure 7 A-C). From 26 to 48 hpf, *miR-216a* expression shifts from the central retina to an increasingly restricted marginal region that will become the Circumferential Germinal Zone (CGZ) or Ciliary Marginal Zone (CMZ)(Hitchcock and Raymond, 2004). Given the role that miRNAs play in regulating the expression of target mRNAs, we conclude that the temporal and spatial specificity of the expression of *miR-216a* suggest that it plays a role in patterning the developing retina.

Table 2. miRNA expression profiling in developing zebrafish eyes. Microarrays containing probes for 346 zebrafish miRNAs were performed on tissue from developing retinas at 2 and 5 days post fertilization (dpf) zebrafish. Fold differences were calculated by dividing the normalized expression values by negative control signals derived from probes against a *Pseudomonas aeruginsa* dehydrogenase. All p-values were calculated based on paired t-tests.

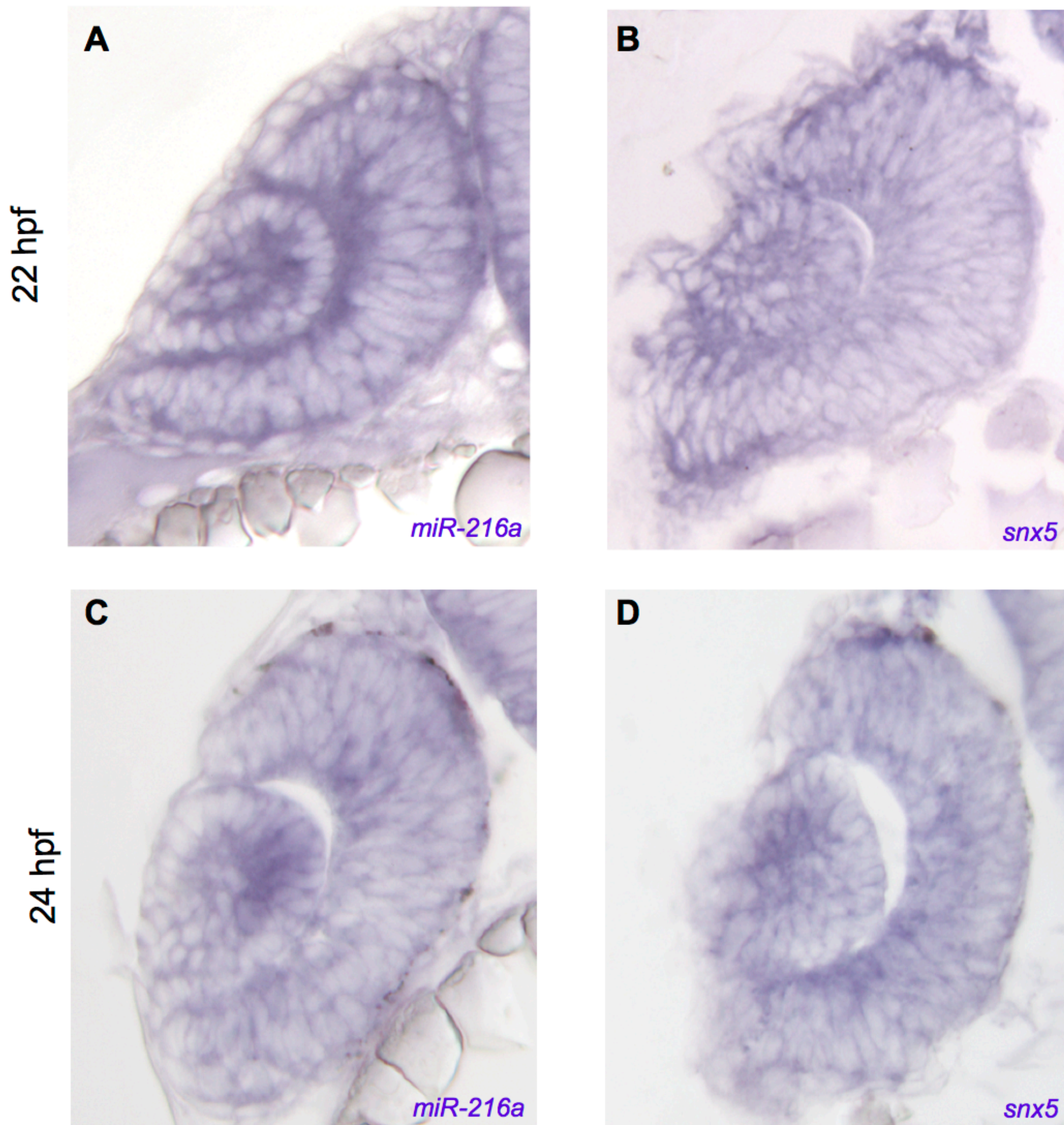


Figure 6. *miR-216a* and *snx5* are widely expressed in the developing eye at 22 and 24 hpf. In situ hybridizations were performed for *miR-216a* (A,C) and *snx5* (B,D) at 22 hpf (A,B) and 24 hpf (C,D). Embryos were sectioned and sections were imaged on an epifluorescence scope. At 22 hpf, both *miR-216a* and *snx5* are expressed throughout the developing eye. At 24 hpf, these expression patterns become slighty more restricted; however, expression of both *miR-216a* and *snx5* can still be found throughout the developing eye.

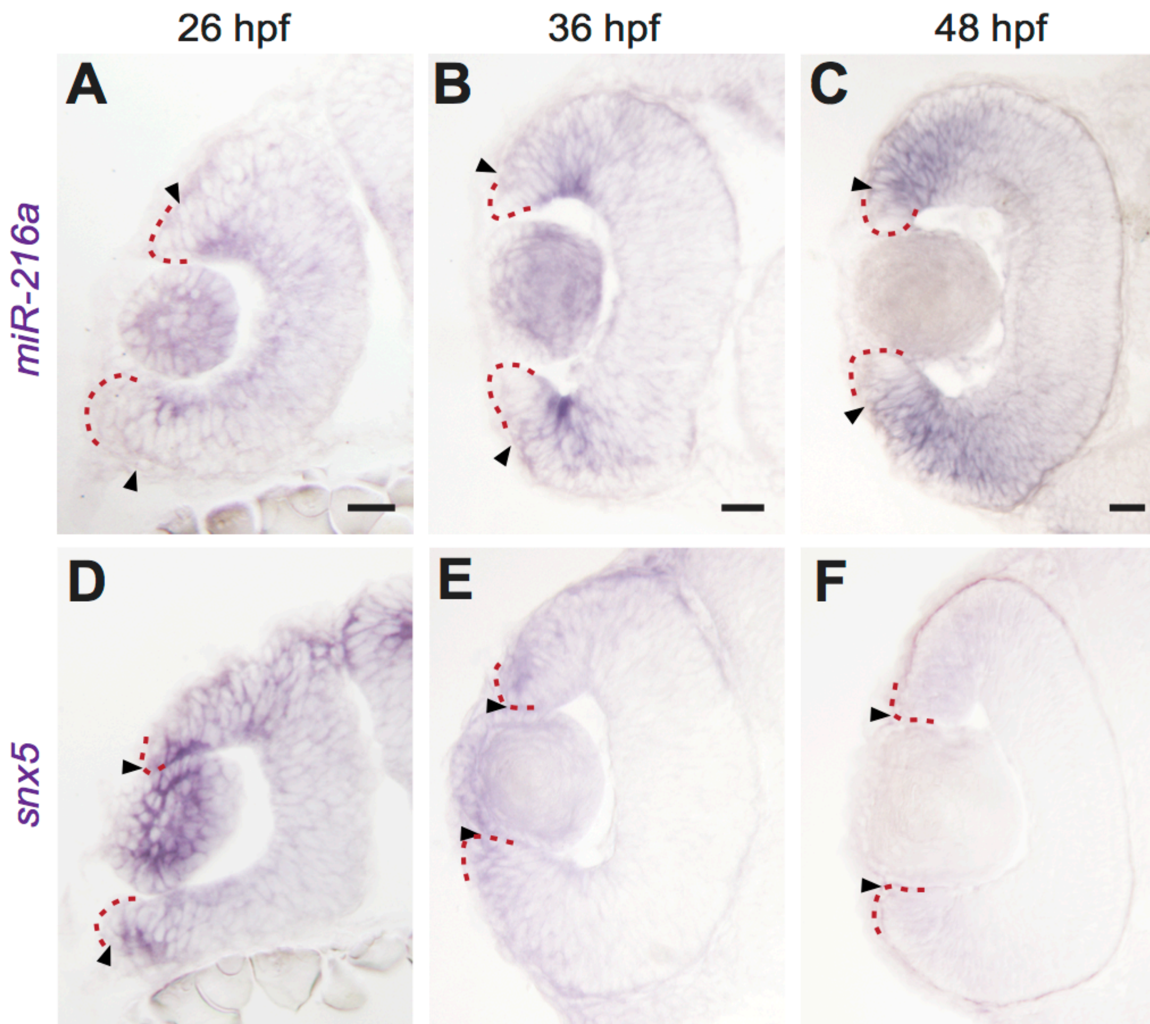


Figure 7. miR-216a and snx5 have complementary expression patterns during development. Transverse sections of whole mount in situ hybridizations for miR-216a and snx5 at 26 (A, D), 36 (B, E), and 48 h (C, F) post-fertilization (hpf). miR-216a expression spreads from the center of the developing retina toward the periphery. snx5 is detected in a complementary pattern becoming increasingly restricted over time to a small number of cells at the far periphery of the developing retina. Arrowheads indicate the extent of signal, the red dashed line indicates the lateral edge of the optic cup. Scale bar: 20µm.

miR-216a targets snx5

MicroCosm and TargetScan online target prediction algorithms (Griffiths-Jones et al., 2008; Lewis et al., 2005) were used to identify potential targets of *miR-216a*. Concurrently, we conducted a series of *miR-216a* gain- and loss-of-function

experiments in developing zebrafish embryos. We observed vascular defects upon altered expression of *miR-216a* that were remarkably similar to previous reports demonstrating an involvement of Notch signaling and a requirement for SNX5 in vascular development (Figures 8 & 9)(Lawson et al., 2001; Yoo et al., 2006). Thus, we focused our target search on Notch pathway related genes and SNX5. Several Notch related genes contain one predicted miRNA recognition element (MRE) for *miR-216a* in their 3' UTRs, including *her4.2*, *heyl*, *notch1b*, *hey2*, and *numb*. In contrast, *snx5* contains two MREs in its 3' UTR. Based on the involvement of Notch signaling in retinogenesis (Bernardos et al., 2005; Scheer et al., 2001), we assessed whether these predicted targets of *miR-216a* were true targets using GFP reporter assays.

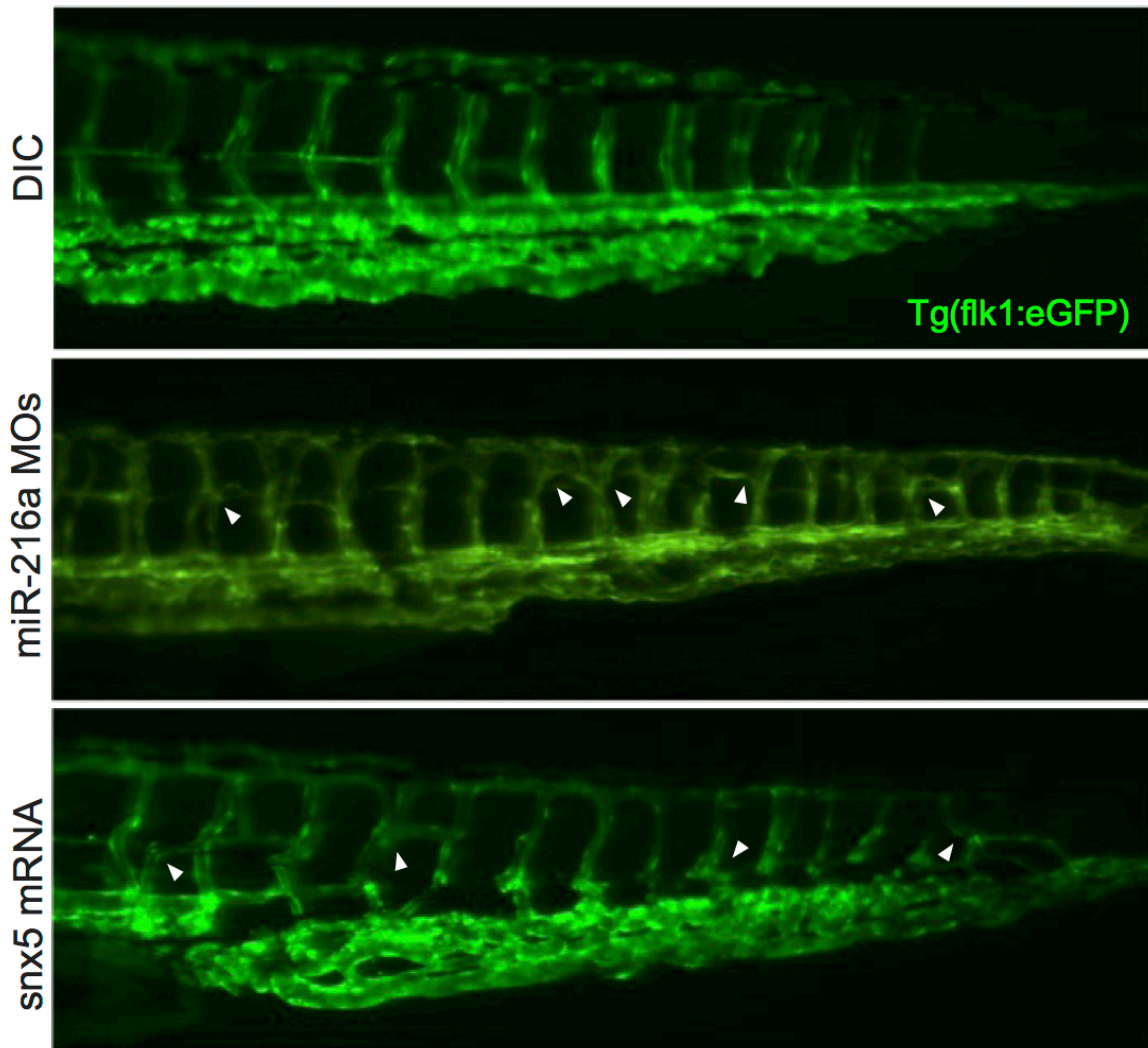


Figure 8. Knockdownof*snx5*andoverexpressionof*miR-216a*resultin vascular patterning defects. *Tg(flk1:eGFP)* embryos were injected at the 1-cell stage with dye control, *snx5*MOs, or *miR-216a*. Embryos were grown in egg water and vascular development was monitored. Intersegmental vessels were missing in the trunks of *snx5*MOs- and *miR-216a*-injected larvae at 4 dpf, but not from dye control- injected larvae. Left column contains representative images, and right column shows vessels outlined with dotted white lines in order to highlight the absence of some vessels.

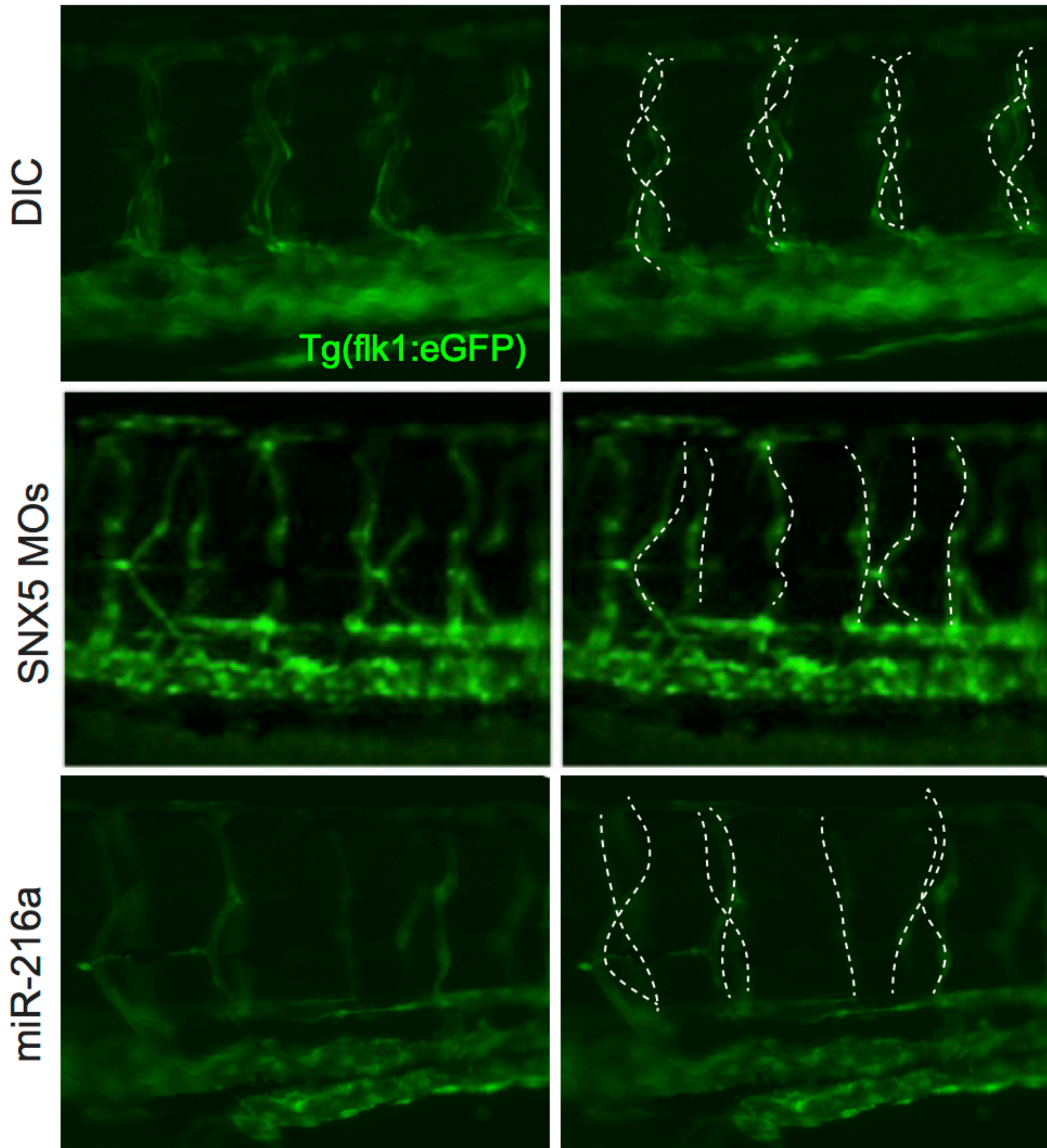
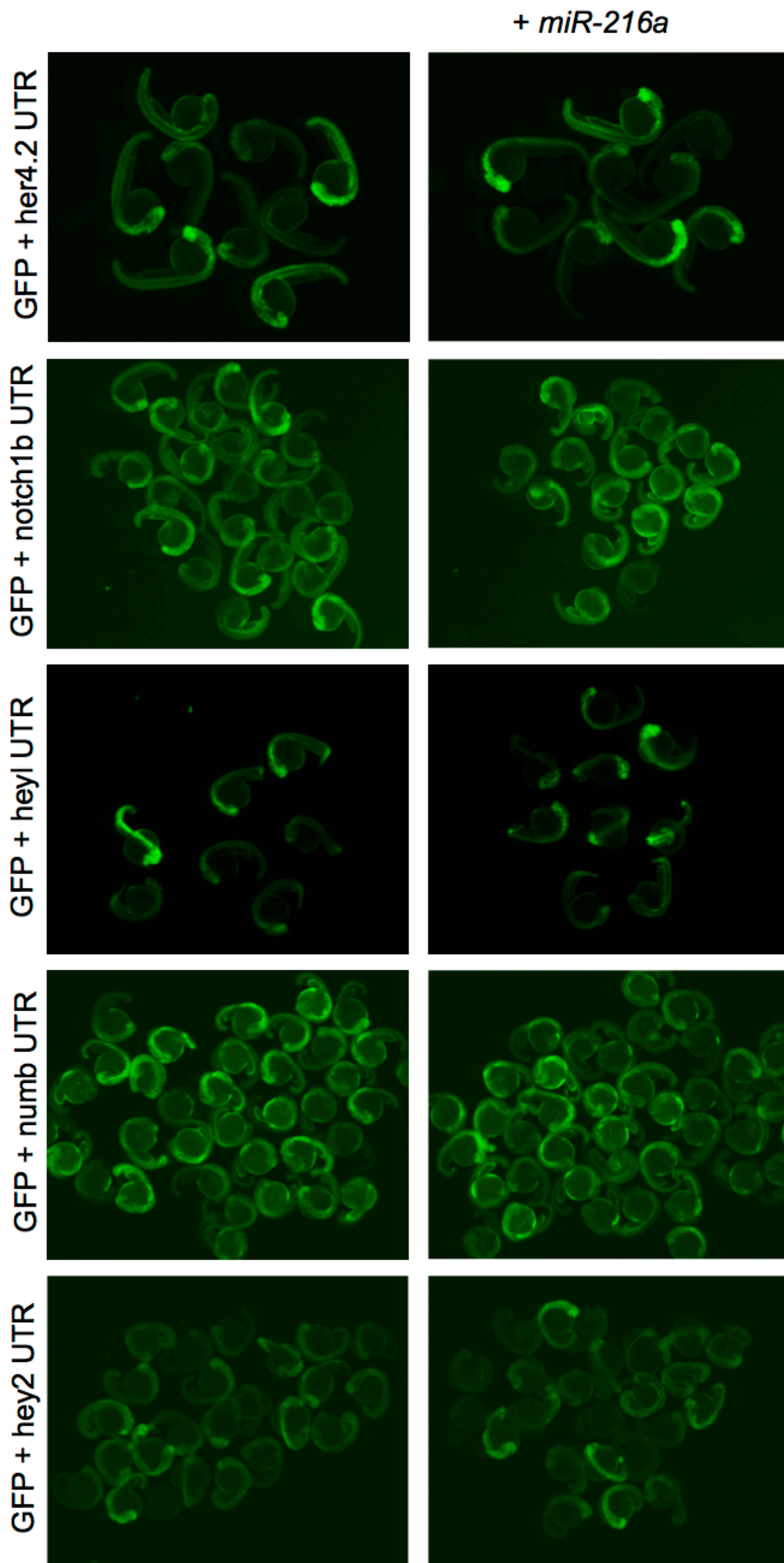


Figure 9. Knockdown of *miR-216a* and overexpression of *snx5* result in vascular patterning defects. *Tg(flk1:eGFP)* embryos were injected at the 1-cell stage with dye control, *miR- 216a*MOs, or *snx5* mRNA. Embryos were grown in egg water and vascular development was carefully monitored. Branching defects in the intersegmental vessels were observed in the tails of 4 dpf larvae (indicated by white arrowheads) and imaged using a fluorescence stereoscope.

The full-length 3' UTR of each of these predicted targets was fused to the coding sequence of GFP. mRNA transcripts were then generated from these reporter constructs and injected into single cell zebrafish embryos in the presence or absence of co-injected, exogenous *miR-216a*. The effect of *miR-216a* was determined by measuring GFP fluorescence at 24 hpf. Fluorescence levels of the *her4.2*, *heyl*, *notch1b*, *hey2*, and *numb* 3'UTR reporters were comparable with or without co-injection of *miR-216a*, suggesting that these genes are not targeted by *miR-216a* (Figure 10). However, for *snx5*, we observed a robust decrease in GFP fluorescence upon co-injection with *miR-216a* (Figure 11 B,C,E). Importantly, the effect of *miR-216a* could be partially suppressed by co-injection of a morpholino targeting the mature sequence of *miR-216a,* indicating specific suppression of *snx*5 by *miR-216a* (Figure 11 D,E). To further test for specificity, we deleted each of the two predicted MREs in the *snx5* 3'UTR. No differences were observed in GFP fluorescence among fish injected with the mutated reporter transcripts compared to co-injection with *miR-216a* (Figure 11 F,G,I). As an additional test of specificity, co-injection of both *miR-216a* and *miR-216a*^{MO} with the GFP reporter containing a mutated *snx5* 3' UTR resulted in no change in fluorescence (Figure 11 H,I). These results indicate that *miR-216a* can regulate *snx5* via two MREs located in its 3' UTR.


+ miR-216a
GFP + her4.2 UTR
GFP + notch1b UTR
GFP + heyl UTR
GFP + numb UTR
GFP + hey2 UTR

To address whether endogenous *snx5* is targeted by *miR-216a*, we isolated protein from 1 dpf embryos injected at the one cell stage with either a dye control (DIC), *miR-216a*, or two morpholinos targeted to *miR-216a*, one complementary to the mature sequence of *miR-216a* and one targeted to the Dicer cleavage site of the *miR-216a* precursor (*miR-216a*^{MOs}). We initially performed the experiments with just one of the morpholinos but combining the two allowed us to use a lower dose of each, reducing the chances of off target effects. We then performed western blots using an antibody against SNX5 protein and α-tubulin as a control. Injection of *miR-216a* significantly decreased endogenous levels of SNX5, while injection of *miR-216a*^{MOs} led to a significant increase in endogenous SNX5 (Figure 11 J,K). Taken together, these results indicate that *miR-216a* targets endogenous *snx5* via two MREs in its 3'UTR.

Figure 10. *miR-216a* does not target *her4.2, notch1b, heyl, numb,* or *hey2.* Embryos were injected at the 1-cell stage with GFP reporters containing the 3'UTRs of *her4.2, notch1b, heyl, numb,* or *hey2* alone or in combination with *miR-216a*. Embryos were grown in egg water until 24 hpf, at which point they were photographed using a fluorescence dissecting scope. We did not detect changes in GFP fluorescence with co-injection of *miR-216a.*

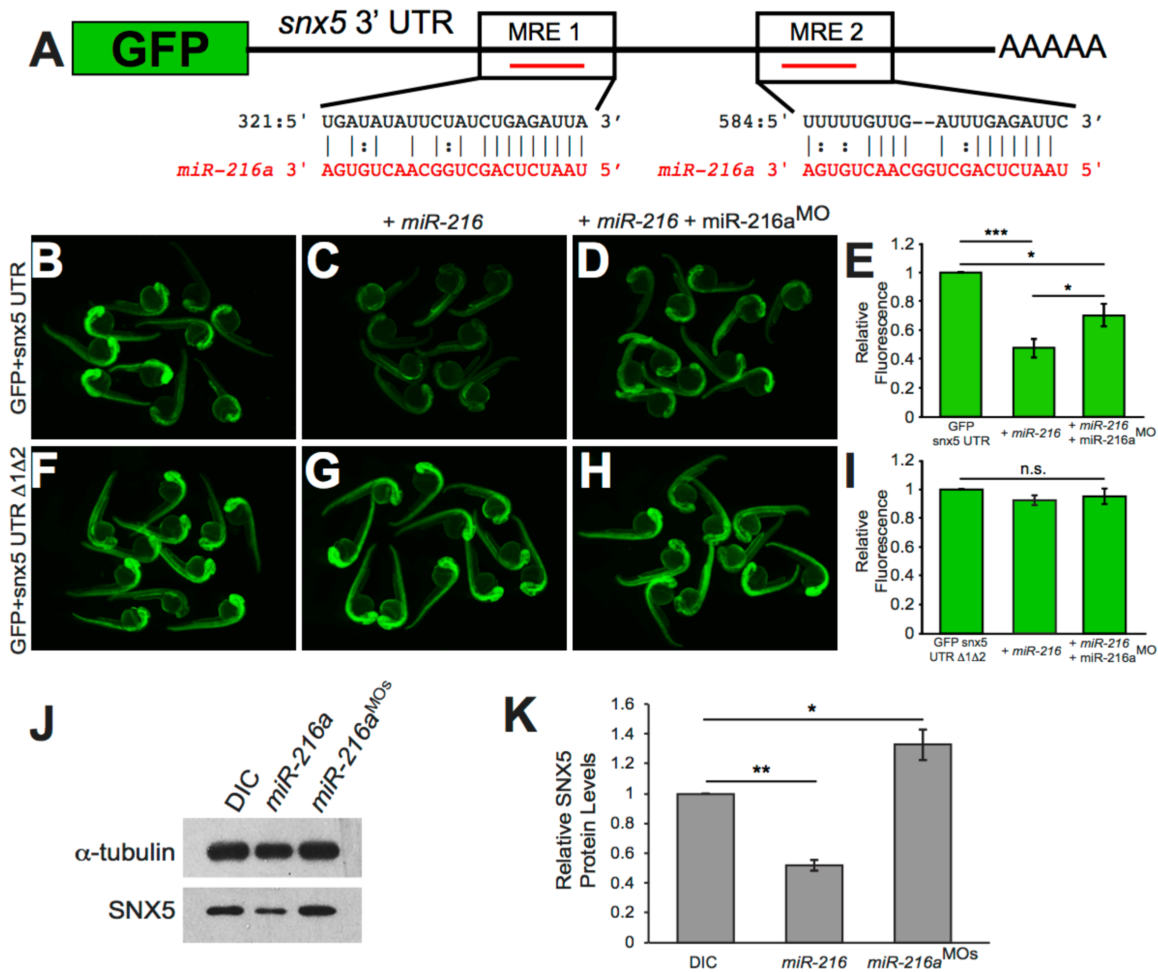


Figure 11. *snx5* is a target of *miR-216a*. The coding sequence of GFP was fused to the 3'UTR of *snx5*. (A) Predicted pairing between microRNA Recognition Elements (MREs) in the *snx5* 3' UTR (black) with *miR-216a* (red). (B) 1-cell stage embryos were injected with GFP- *snx5* 3' UTR reporter mRNAs alone, or co-injected with *miR-216a* (C), or the combination of *miR-216a* and *miR-216a*MO (D) and imaged at 1 dpf. (F-H) Silencing is MRE Dependent. 1 dpf embryos were injected with mRNAs lacking both MREs alone, or co-injected with *miR-216a* (G), or the combination of *miR- 216a* and *miR-216a*MO (H). (E, I) Relative fluorescence levels from the representative embryos shown in B-H were quantified using ImageJ, and comparisons were made using one-way ANOVA with Bonferroni's correction. (J) Western blots for endogenous SNX5 and a-tubulin were performed on protein lysates from 1 dpf zebrafish injected with dye control (DIC), *miR-216a*, or *miR- 216a*MOs. (K) Western signals were quantified using ImageJ, and comparisons were made using one-way ANOVA with Bonferroni's correction. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Error bars show SEM.

miR-216a spatially and temporally restricts expression of snx5 in the eye

Because we observed specific spatial and temporal expression of *miR-216a* over the course of early eye development (Figure 7 A-C), we were interested to examine the expression of *snx5* at corresponding time points. We thus performed *in situ* hybridization using *snx5* riboprobes on whole mount zebrafish embryos, which were then sectioned and imaged (Figure 7 D-F). Expression of *miR-216a* was largely complementary to that observed for *snx5*. As *miR-216a* expression moved toward the future CGZ at 36 and 48 hpf (Figure 7 B,C), localization of *snx5* became increasingly restricted (Figure 7 E,F) until *snx5* expression was virtually undetectable from all cells of the developing retina except for a limited number of cells at the very margins of the future CGZ. The complementary expression patterns of *miR-216a* and *snx5* suggest that *miR-216a* restricts temporal and spatial expression of *snx5* in the developing eye.

Notch-Delta signaling and miR-216a-snx5 interaction

Previous experiments have demonstrated interaction between SNX5 with MIB, co-localization with MIB and Delta (Yoo et al., 2006), and a role for MIB and Notch-Delta signaling in gliogenesis (Bernardos et al., 2005; Scheer et al., 2001). However, the exact effects of *snx5* on Notch-Delta signaling have not been characterized nor has there been any previous work investigating the regulation of *snx5* during early retina development. We therefore used a Notch reporter zebrafish line (*Tg(her4:dRFP)*) which expresses dRFP under the control of the *her4* Notch-responsive element (Takke et al., 1999; Yeo et al., 2007). We injected

Tg(her4:dRFP) single cell embryos with either dye control, synthetic *miR-216a* duplexes, *miR-216a*^{MOs}, *snx5*^{MOs}, or *snx5* mRNA, and then fixed the embryos at 30 hpf and sectioned to examine Notch activation in the developing retina. Strikingly, overexpression of *miR-216a,* or knockdown of *snx5,* resulted in a marked decrease in Notch activation compared to DICs, as reported by the loss of *Tg(her4:dRFP*) fluorescent protein expression (Figure 12 A,B,E). Conversely, knockdown of *miR-216a,* or overexpression of *snx5,* mRNA resulted in expansion of the zone of *Tg(her4:dRFP*) fluorescence and presumptive Notch activation compared to DICs (Figure 12 C,F). Co-injection of *snx5* lacking its 3'UTR with *miR-216a* restored the zone of *Tg(her4:dRFP*) activation (Figure 12 D), as did co-injection of *snx5*^{MOs} and *miR-216a*^{MOs} (Figure 12 G). These data indicate that *snx5* is a positive regulator of Notch-Delta signaling and that *miR-216a* negatively regulates Notch-Delta signaling via its interaction with *snx5*. Consistent with this hypothesis, we used a second zebrafish Notch reporter line (*Tg(Tp1bglob:eGFP)*) and observed repression of Notch activation by increasing amounts of *miR-216a* or knockdown of *snx5* (Figure 13)(Parsons et al., 2009).

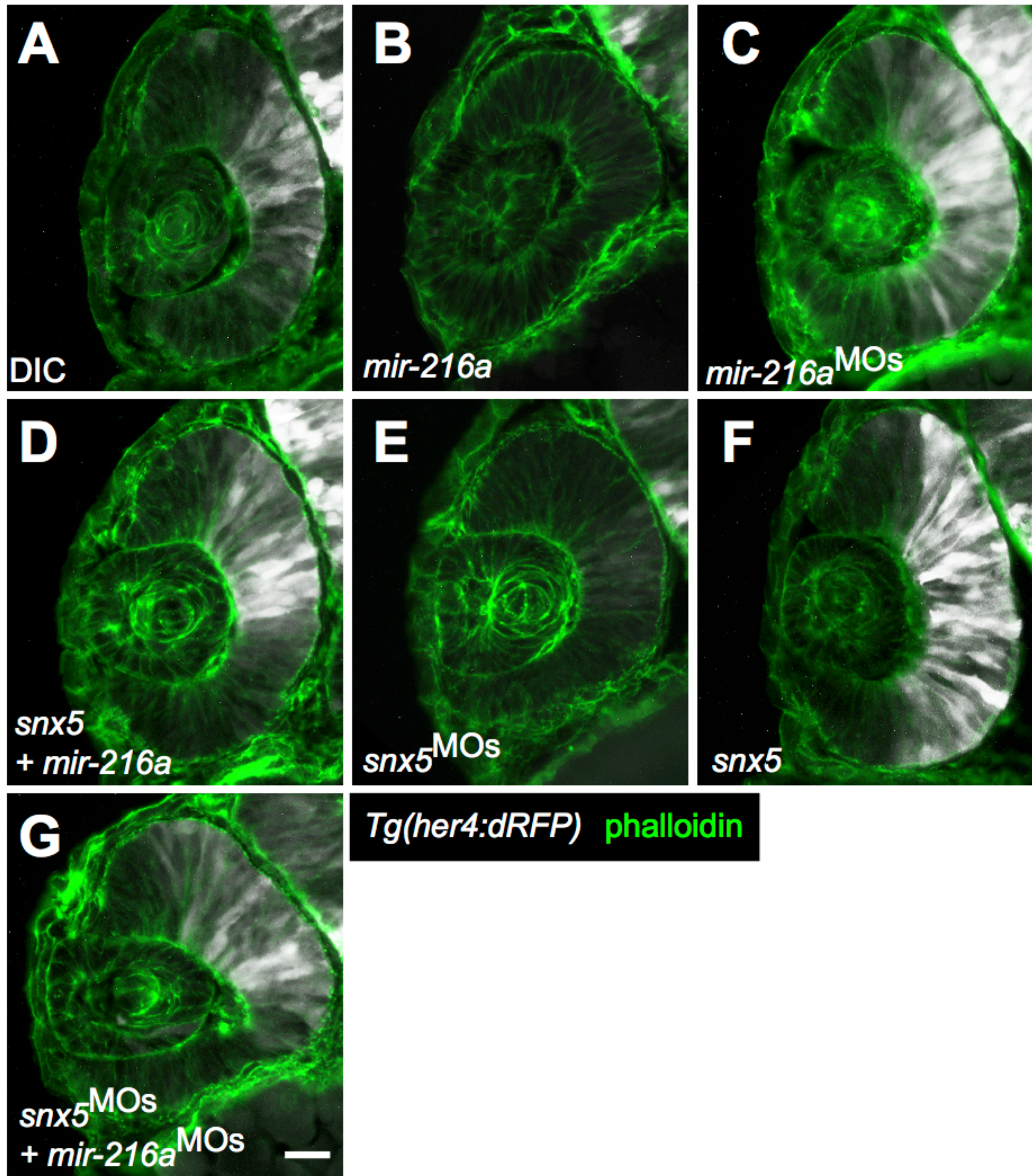


Figure 12. *miR-216a* and *snx5* regulate Notch activation. Transverse sections of developing retinas from 30 hours post fertilization (hpf) *Tg(her4:dRFP)* embryos were injected with dye control (DIC; A), *miR-216a* (B), *miR-216a*MOs (C), *snx5*MOs (E), or *snx5* mRNA (F). Reporter expression (white) indicates changes in the zone of Notch activation. Partial rescue of Notch activity is shown in (D) and (G) where embryos were co-injected with combinations of either *snx5* and *miR-216a* (D) or *snx5MOs* and *miR-216aMOs* (G). Sections were stained with Alexa Fluor 488-conjugated phalloidin (green) to visualize cell boundaries. Scale bar: 20μm.

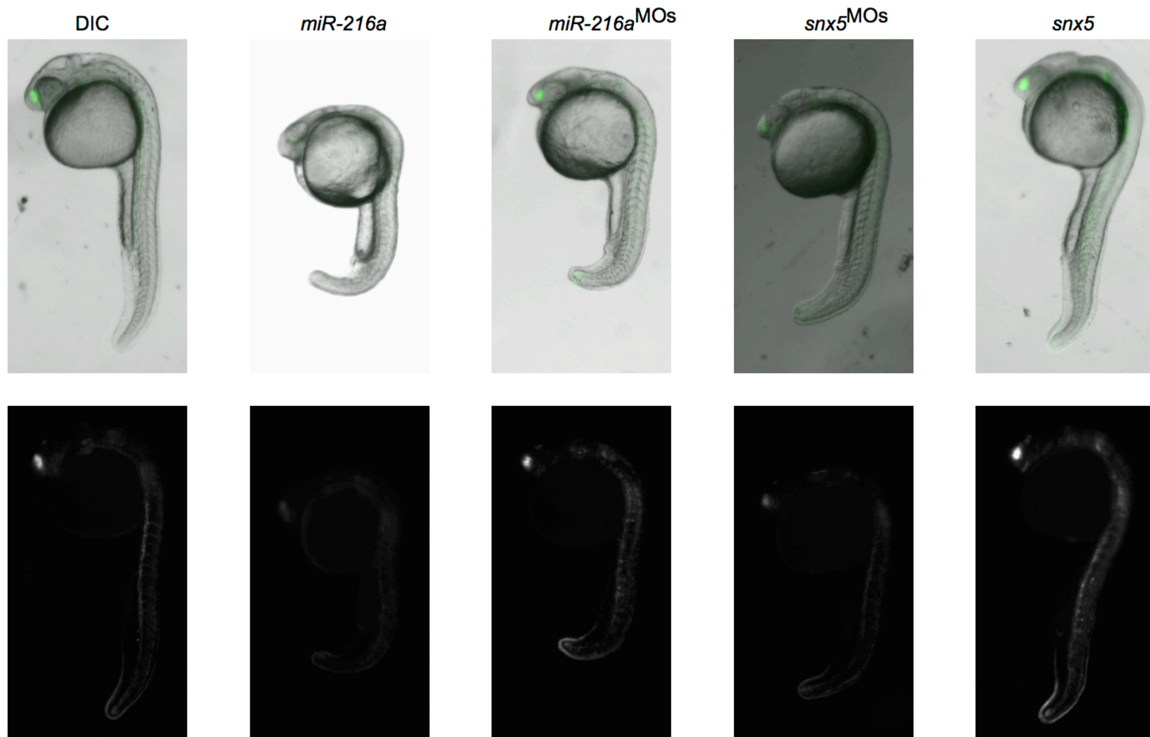


Figure 13. *Tg(Tp1:GFP)* reporter reveals changes in Notch signaling upon perturbation of *miR-216a* and *snx5.* Embryos *(Tg(Tp1:GFP))* were injected at the 1-cell stage with *miR-216a, miR- 216a*MOs, *snx5*MOs, *snx5,* or dye control (DIC). Embryos were grown in egg water until 24 hpf, at which point they were photographed using a fluorescence dissecting scope. Top panel shows Tp1 reporter fluorescence in green superimposed onto images of fish; bottom panel shows Tp1 reporter fluorescence in white. We observed a decrease in reporter fluorescence in *miR- 216a* and *snx5*MOs injected embryos, suggestive of lower Notch activation, and an increase in reporter fluorescence in *miR-216a*MOs and *snx5* injected embryos, suggestive of higher Notch activation, as compared to DIC.

Because it was formally possible that the effects we observed might be due to morpholino-induced apoptosis as opposed to regulation of *snx5* by *miR-216a*, we conducted TUNEL staining. Previous work has illustrated potential pitfalls with the use of morpholinos, including increased levels of apoptosis due to activation of p53 (Gerety and Wilkinson, 2011). To ensure that the effects we observed were specific to knockdown of *miR-216a or snx5*, we injected morpholinos in the presence and absence of p53 and found no change in the levels of TdT-mediated incorporation of

dUTP (Figure 14). Combined with our suppression/rescue experiments, these results demonstrate that the effects of *miR-216a and snx5* knockdown are specific and that the changes in Notch activation we observe are due to regulation of *snx5* by *miR-216a*.

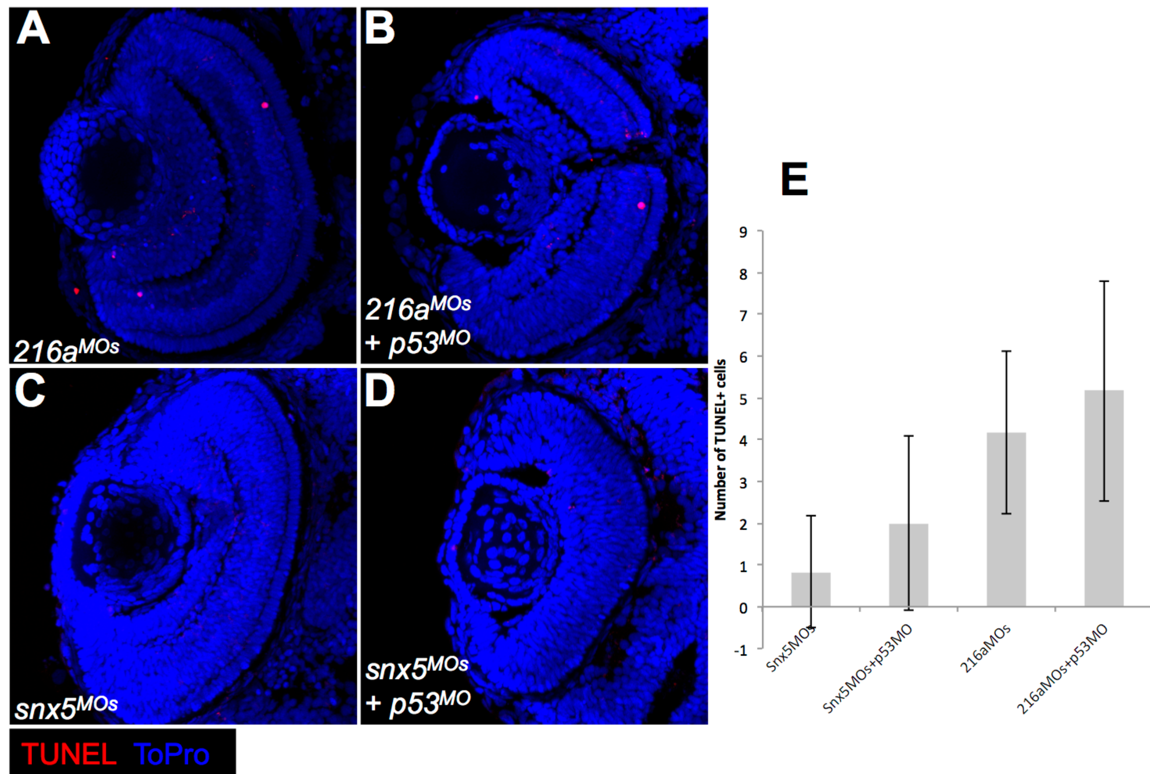


Figure 14. No differences in programmed cell death observed with morpholino injections. We injected morpholinos into one cell stage embryos in the presence and absence of p53 and found no change in the levels of TdT-mediated incorporation of dUTP (red) in sectioned eyes at 36-48 hpf, quantified on right.

<u>*Disruption of Müller glia*</u>

Notch signaling is required for gliogenesis (Bernardos et al., 2005; Scheer et al., 2001) and the prediction is that early alteration in Notch signaling by *miR-216a* and *snx5* should affect the subsequent number of Müller glia during retinal development. To assess the functional consequences of disrupting *miR-216a* and

snx5 expression, we injected *miR-216a*, *miR-216a*^{MOs}, *snx5*^{MOs}, or *snx5* mRNA into single cell *Tg(gfap:GFP)* zebrafish embryos and examined fluorescence levels during early development. These animals express GFP under the control of the glial-specific GFAP promoter (Bernardos and Raymond, 2006). We initially examined retinas from embryos at 30 hpf to coincide with the *her4* reporter experiments. Fluoresence was detectable at this time but the levels were not robust, consistent with the timing of Müller glia specification (Easter and Malicki, 2002). Since it has been reported that Müller glia are specified by 65 hpf (Bernardos et al., 2005), and because we observed Notch activation in Müller glia at 65 hours using the *her4* reporter fish (Figure 15), we counted GFP+ cells at this time. Upon overexpression of *miR-216a*, a significant decrease in GFP+ cells was observed compared to DICs (Figure 16). In contrast, knocking down *miR-216a* with morpholinos resulted in an increase in GFP+ numbers (Figure 16). Correspondingly, knockdown of *snx5* resulted in significantly decreased numbers of GFP+ cells whereas overexpression of *snx5* led to an increase in GFP+ cells (Figure 16). These results are consistent with regulation of *snx5* by *miR-216a*. To further test this hypothesis, we conducted co-injection rescue/suppression experiments. The prediction is that the decreased numbers of GFP+ cells caused by knockdown of *snx5* should be suppressed by co-injection of *miR-216a*^{MOs}. Similarly, the effects of overexpression of *miR-216a* should be suppressed by co-injection of *snx5.* In both cases, we observed rescue of GFP+ cell numbers indicating that Müller glia numbers were largely restored (Figure 16). Taken together, these data are consistent with the hypothesis that *miR-216a* modulates gliogenesis via its interaction with *snx5*.

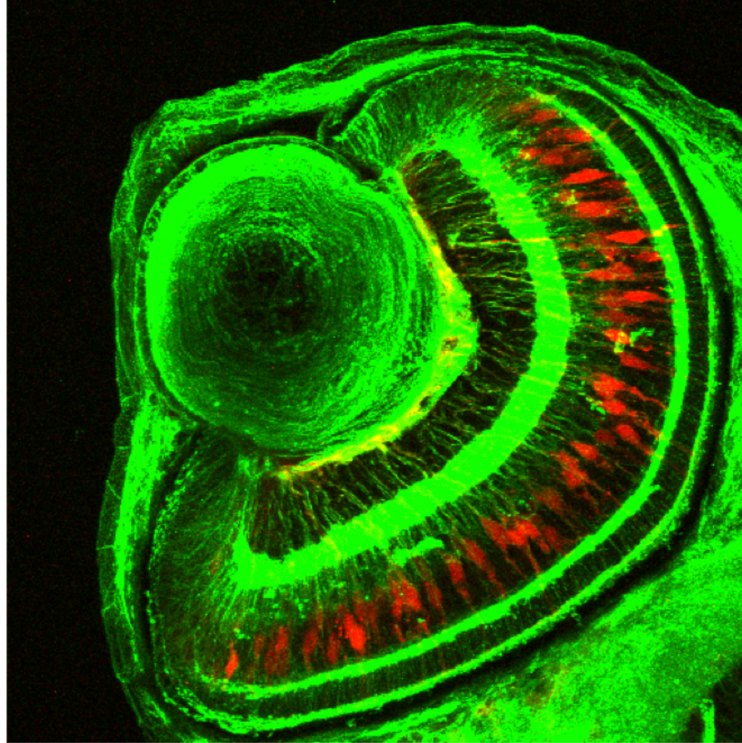


Figure 15. Notch is activated in Müller glia at 65 hpf. In a cross section of *Tg(her4:dRFP)* fish at 65 hpf, Notch activation (in red) was detected primarily in Müller glia. Cell membranes are labeled with phalloidin, here visualized in green.

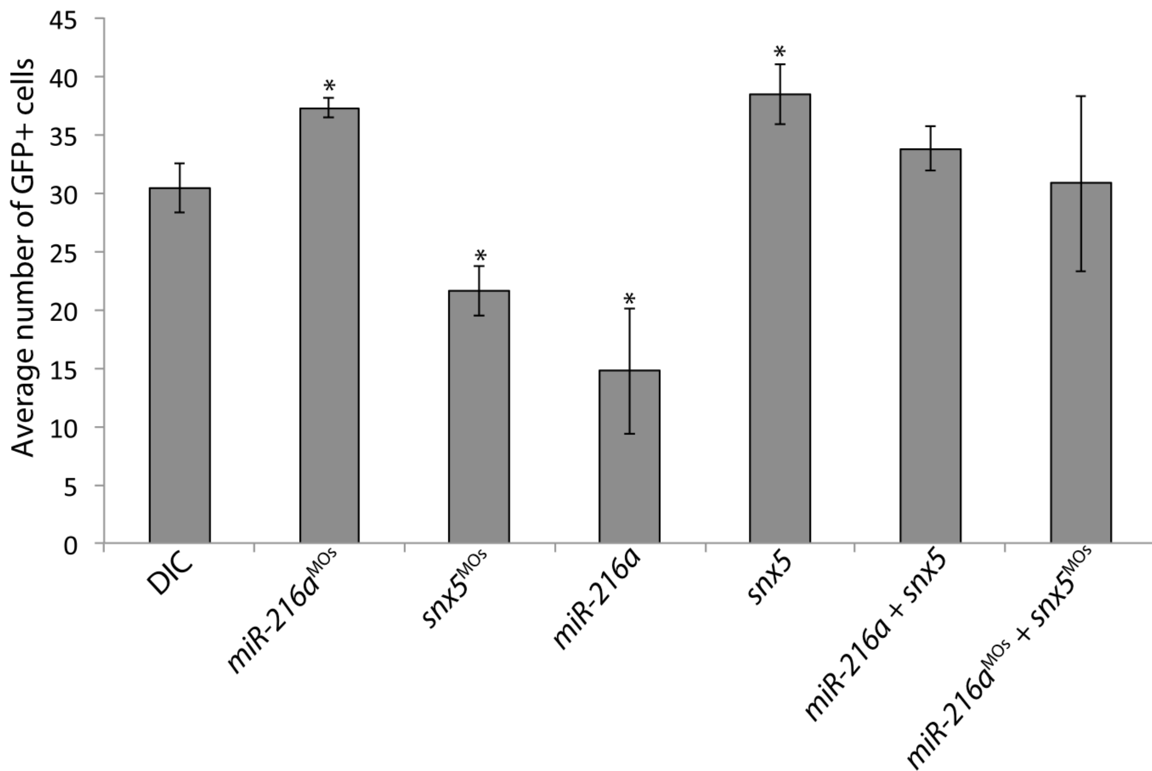
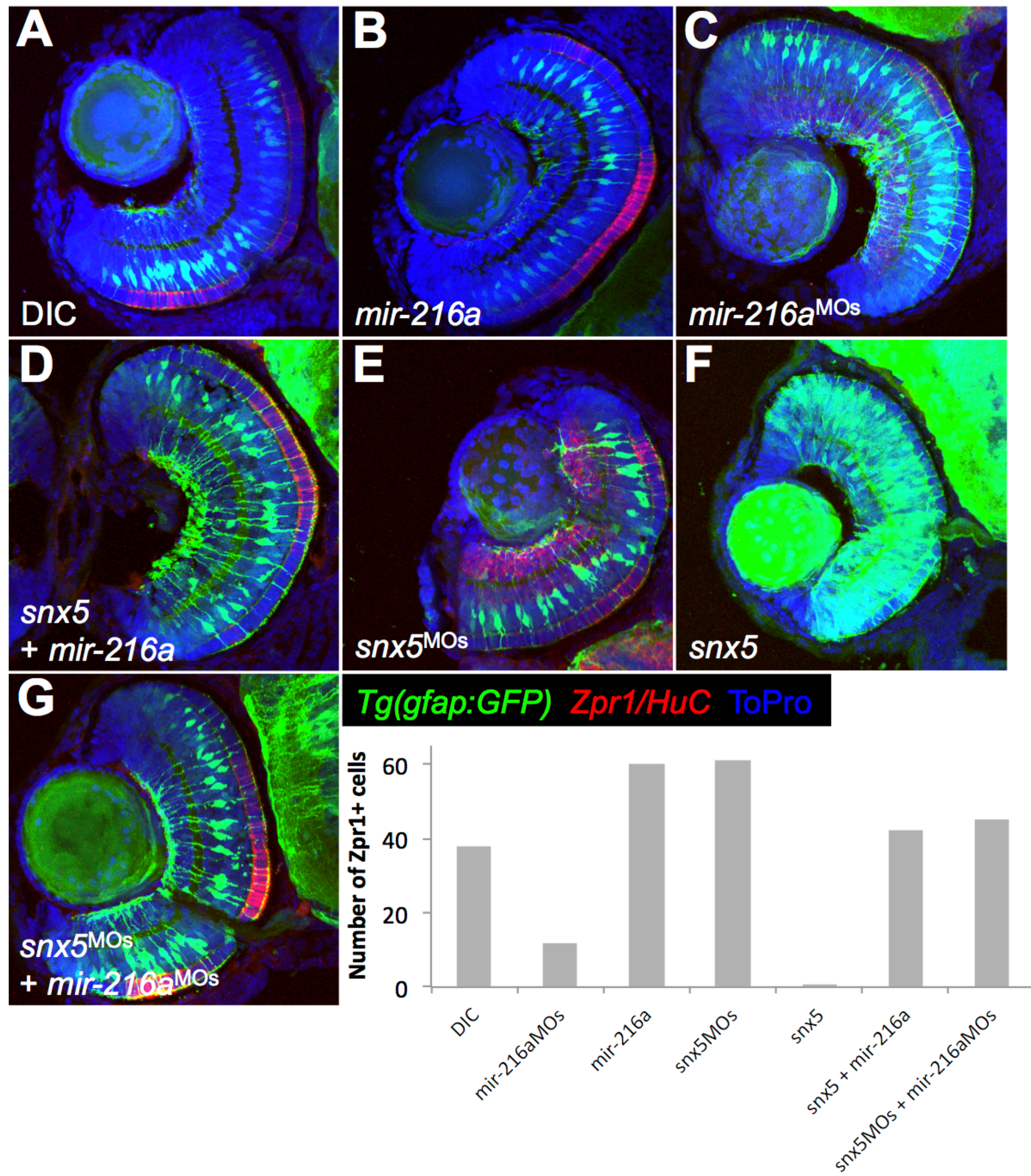


Figure 16. *miR-216a* and *snx5* regulate Müller glia cell numbers. *Tg(gfap:GFP)* transgenic zebrafish were injected as indicated, grown to 65 hpf, and GFP+ cell numbers were counted. Compared to DICs, injection of *miR- 216a*MOs or *snx5* caused a significant increase in GFP+ cells ($p<0.05$). Injections with *miR-216a* or *snx5*MOs caused a significant decrease in GFP+ cells ($p<0.05$). Partial rescue of GFP+ cell counts was observed in embryos co-injected with combinations of either *snx5* and *miR-216a,* or *snx5MOs* and *miR-216aMOs*. Error bars=SEM.

Effects of Müller glia specification on cone photoreceptor differentiation

A prediction of the effects of altered gliogenesis is that other retinal neuronal cell types would be altered after either loss or gain of Müller glia. For these experiments we used *Tg(gfap:GFP)* embryos fixed at 65 hpf and stained transverse retinal sections using antibodies that mark cone photoreceptors (Zpr-1). As shown in Figure 17, alteration in Müller glia number was accompanied by complementary changes in the extent of Zpr-1 staining in the outer nuclear layer. Overexpression of

snx5 or knockdown of *miR-216a* led to increased Müller glia and decreased Zpr-1 staining while overexpression of *miR-216a* or knockdown of *snx5* led to decreased Müller glia and increased Zpr-1 staining. These results are consistent with the model that altered gliogenesis can in turn affect neuronal differentiation.



Discussion

We used expression profiling experiments to identify several candidate miRNA regulators of zebrafish eye development. As demonstrated by *snx5* and *miR-216a* expression, GFP reporter assays, and SNX5 immunoblotting, we show that *miR-216a* regulates *snx5*. Based on the expression of *miR-216a* and *snx5* in the retinal neuroepithelium, it appears that *miR-216a* plays a role in both spatial and temporal control of *snx5* expression and, in turn, Notch signaling.

Figure 17. Inverse correlation between Müller glia numbers and cone photoreceptor staining. *Tg(gfap:gfp)* embryos were injected with dye control (DIC; A), *miR-216a* (B), *miR-216a*MOs (C), *snx5*MOs (E), or *snx5* mRNA (F) at the 1-cell stage, fixed at 65 hpf, and transverse sections of developing retinas were obtained. Immunohistochemistry was performed using antibodies to identify cone photoreceptors in the outer nuclear layer (Zpr-1) or amacrine/ganglion cells in the inner nuclear layer and the ganglion cell layer (HuC). Changes in Müller glia cell numbers led to consistent changes in cone photoreceptor numbers. Zpr-1 staining increased in embryos injected with mir-216a or snx5MOs and decreased in embryos injected with *miR-216a*MOs or *snx5* compared to embryos injected with dye. Partial rescue of Zpr-1 levels is shown in (D) and (G) where embryos were co-injected with combinations of either *snx5* and *miR-216a* (D) or *snx5*MOs and *miR-216a*MOs (G). Amacrine and ganglion cell numbers demonstrated similar, though less striking and less consistent changes compared to cone photoreceptors. Nuclei were marked by staining with To-Pro.

miR-216a *regulates Notch signaling via* snx5

SNX5 binds Mib and knocking down SNX5 leads to vascular defects (Eckfeldt et al., 2005; Yoo et al., 2006). The role of Notch signaling in vascular development is also well established (Lawson et al., 2001). In addition to changes in fluorescent protein expression in *Tg(her4:dRFP*) fish, we also observed defects in vascular patterning upon knockdown and overexpression of *miR-216a* and *snx5* (Figures 8 & 9). This suggests that *miR-216a* and *snx5* also play a role in Notch signaling in zebrafish vascular development. We also show that perturbing expression of *miR-216a* and *snx5* causes changes in Notch activation, as reported by altered zones of fluorescent protein expression in the retinas of *Tg(her4:dRFP*) embryos.

Based on prior work about SNX5 and Mib and our experiments, we propose a model where *miR-216a* regulates Notch-Delta signaling via regulation of *snx5* (Figure 18). We hypothesize that SNX5 (bound to Mib) moves to the site of Delta activation where it binds to the membrane as Mib ubiquitylates Delta. SNX5 then facilitates membrane curvature through its BAR domain with subsequent Delta endocytosis, which is required for Notch activation and neuronal development (Parks et al., 2000) (Louvi and Artavanis-Tsakonas, 2006).

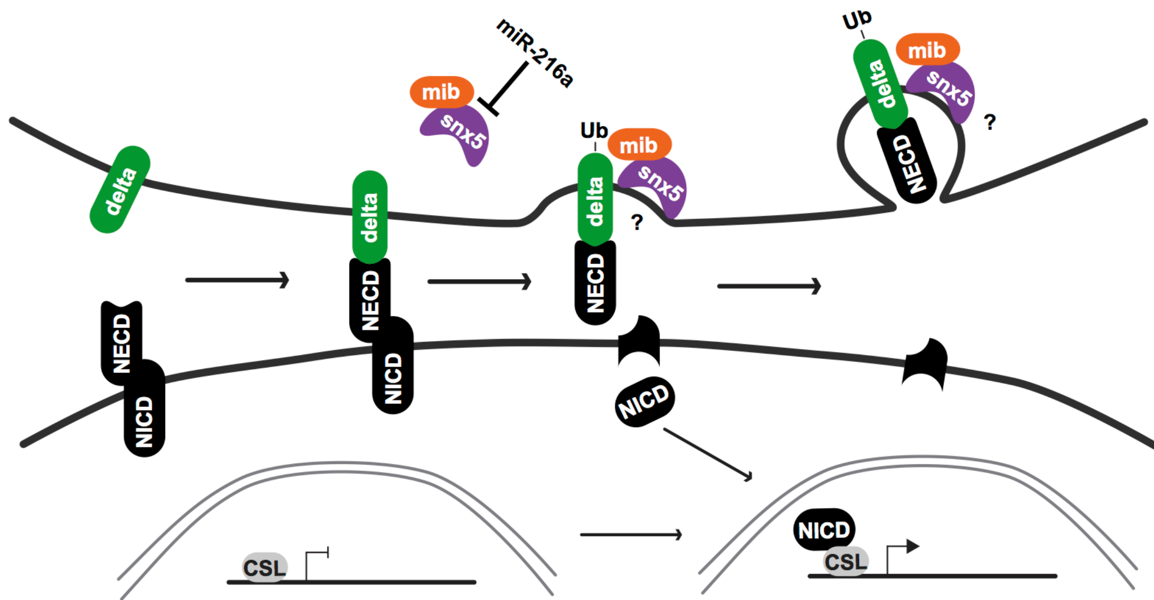


Figure 18. Model for the role of SNX5 and *miR-216a* in Notch signaling. SNX5 (bound to Mib) moves to the site of Delta activation, where it binds to the membrane as Mib ubiquitylates Delta. SNX5 then facilitates membrane curvature and Delta endocytosis, which is required for cleavage of the Notch extracellular domain (NECD). Cleavage of the NECD frees the Notch intracellular domain (NICD), which is translocated to the nucleus to co-activate downstream target genes with the CSL transcription factor.

While our experiments show a role for *snx5/miR-216a* in controlling Notch activity during retinal development, it is likely that overall control of Notch involves multiple factors and control points during cell fate specification and development. Focusing just on the retina, we show that early changes in Notch signaling manifest themselves at later time points by altering neuronal cell fate. However, several other Notch components, including Delta, are likely to be subject to additional temporal regulation as the wave of differentiation spreads from the central retinal to the periphery. Despite the fact that our morpholino knockdown experiments of *miR-216a* allow sufficient Notch activity to affect changes in cell fate, our experiments cannot preclude the role of additional Notch components and/or

regulators during the dynamic processes occurring during retina development. This likely includes other miRNAs that might regulate other components of the Notch pathway.

miR-216a *and* snx5 *modulate Müller glia cell numbers*

The changes in Notch signaling in response to perturbation of *snx5* and *miR-216a* expression that we observed are striking and consistent with previous experiments. Scheer et al. (2001) showed that expressing a constitutively active version of Notch1a resulted in a disruption of neurogenesis and an increase in gliogenesis (Scheer et al., 2001). Additionally, differentiation of Müller glia does not occur in *mib* mutant fish (Bernardos et al., 2005). These results suggest that Notch signaling is instructive for gliogenesis in the zebrafish retina. We observed that high Notch activation at 30 hpf, as reported by fluorescent protein expression in the *Tg(her4:dRFP*) zebrafish and induced by either *miR-216a* knockdown or *snx5* overexpression, caused increased numbers of Müller glia at 65 dpf, as reported by *Tg(gfap:GFP)* fluorescence. Because high Notch signaling at 30 hpf, in the case of *miR-216a* knockdown or *snx5* overexpression, translates to increased numbers of Müller glia, we hypothesize that the *snx5-miR-216a* interaction may directly impact Notch signaling, and therefore gliogenesis, in the developing retina. Of note, we observed Notch activation in Müller glia at 65hpf (detected by *Tg(her4:dRFP*; Fig. 4).

It has been suggested that SNX5 is localized to a distinct domain of the early endosome, a cellular location where it could be playing multiple, as yet unknown, roles in cellular trafficking (Yoo et al., 2006). Furthermore, *miR-216a* and *snx5* are

each expressed throughout the developing optic cup and retinal neuroepithelium in early development (Figure 6). By knocking down or overexpressing both *miR-216a* and *snx5* globally at early stages, we have likely disrupted functions that manifest themselves later in development leading to a disruption in Notch activation and correspondingly, specification of Müller glia. It has been shown that the interaction of different Delta ligands with Notch can result in different outcomes for Delta activation in neural tissue (Matsuda and Chitnis, 2009).

We also found that altered gliogenesis impacts neuronal differentiation. We show that MG numbers show an inverse correlation with the staining of a marker of cone photoreceptor differentiation. This suggests that overall specification of cell types in the developing retina are coordinately regulated.

miRNAs regulate developmental signaling

We have previously shown that miRNAs play regulatory roles in Hedgehog signaling (Flynt et al., 2007), the development of endoderm and left-right asymmetry (Li et al., 2011), and synaptogenesis (Wei et al., 2013). miRNA regulation of Notch signaling is important during *Drosophila* follicle development (Poulton et al., 2011) and bone development in mice (Bae et al., 2012). Additionally, Notch signaling has been shown to regulate the expression of *miR-9*, a miRNA that we detected in our eye-field microarray and is involved in multiple aspects of neural development (Coolen et al., 2012). The finding that *miR-216a* regulates *snx5* adds to the mounting evidence for the importance of miRNAs in regulating developmental processes in vertebrates.

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Competing Interests

The authors declare no financial or non-financial competing interests.

Chapter III

INITIATION OF RETINA REGENERATION BY A CONSERVED MECHANISM OF ADULT NEUROGENESIS

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Abstract

Retinal damage or disease in humans often leads to reactive gliosis, preventing the formation of new cells and resulting in visual impairment or blindness. Current efforts to repair damaged retinas are inefficient and not capable of fully restoring vision. Conversely, the zebrafish retina is capable of spontaneous regeneration upon damage, using Müller glia (MG) derived progenitors. Understanding how zebrafish MG initiate regeneration may help develop new treatments that prompt mammalian retinas to regenerate. Here we show that inhibition of GABA signaling facilitates initiation of MG proliferation. GABA levels decrease following damage, and MG are positioned to detect the decrease. Using pharmacological and genetic approaches we demonstrate that $GABA_A$ receptor inhibition stimulates regeneration in undamaged retinas while activation inhibits regeneration in damaged retinas. GABA induced proliferation causes upregulation of regeneration associated genes. This is the first evidence that neurotransmitters control retinal regeneration in zebrafish through an evolutionarily conserved mechanism of neurogenesis.

Introduction

Retinal damage or diseases, such as retinitis pigmentosa, result in a loss of retinal cells and most often lead to blindness. A current effort to mitigate these effects involves intravitreal injections of stem cells or retinal precursors, hoping for successful integration and connection to existing neuronal circuits (Barber et al., 2013; Hanus et al., 2016; MacLaren et al., 2006; Pearson et al., 2012b; Santos-

Ferreira et al., 2015). Though improving, these therapies, are very inefficient and not yet capable of restoring vision (Barber et al., 2013; Bringmann et al., 2006; Pearson, 2014; Pearson et al., 2010). An alternative method would be to prompt the retina to endogenously regenerate and replace lost cells.

Mammalian retinas do not possess the ability to regenerate following disease or damage. Instead, damage commonly results in reactive gliosis, preventing regeneration (Bringmann et al., 2006; Pearson, 2014). Zebrafish, however, mount a robust spontaneous regeneration response upon damage (Goldman, 2014). When damaged, Müller glia (MG) dedifferentiate, divide asymmetrically, and produce progenitor cells that are capable of restoring all lost cell types (Bernardos et al., 2007; Fausett and Goldman, 2006; Nagashima et al., 2013; Rajaram et al., 2014a; Rajaram et al., 2014b; Ramachandran et al., 2012; Thummel et al., 2008; Vihtelic and Hyde, 2000; Wan et al., 2012; Zhao et al., 2014b). A number of key regulators that transition the retina through the stages of regeneration have been determined, but very little is known about what initiates the regeneration process. Because overall retinal architecture and cells are largely conserved between fish and mammals, understanding how zebrafish initiate retina regeneration will help develop novel treatments or therapeutic targets for retinal damage or diseases, especially treatments that target or induce regeneration from mammalian Müller glia.

Select regions of the mammalian central nervous system (CNS) are capable of adult neurogenesis, particularly the subgranular zone (SGZ) of the mouse hippocampus. Recently, the inhibitory neurotransmitter γ-aminobutyric acid (GABA) was shown to play an important role in regulating the proliferation of radial

glia-like stem cells (RGLs) in the mouse hippocampus (Song et al., 2012). Synaptic input from glutamatergic granule cells regulates activity of GABAergic interneurons in the SGZ. When input from granule cells is low, decreases in extracellular GABA are detected by $GABA_A$ receptors on RGLs, resulting in elevated proliferation. We sought to test whether this could be a mechanism that regulates regeneration in the damaged zebrafish retina. In the retina, photoreceptors (PRs) release glutamate onto GABAergic Horizontal cells (HCs). When PRs die they no longer stimulate HCs to release GABA. We hypothesize that MG detect the decrease in GABA and initiate regeneration in a response similar to activation of glial-like stem cells in the mouse hippocampus. We show here that disrupting GABA signaling causes spontaneous proliferation in undamaged zebrafish retinas and that increasing GABA signaling in damaged retinas suppresses regeneration. Our results indicate that GABA signaling is directly detected by MG, supporting an evolutionarily conserved mechanism of adult neurogenesis.

Results

For MG to detect GABA released from HCs, their processes must be in close proximity. To test this, cross sections of retinas from two lines of zebrafish that express GFP in either MG (*Tg(gfap:GFP)^{mi2001}*) or HCs (*Tg(lhx1a:EGFP)^{pt303}*), were immunostained with antibodies against GABA, glutamic acid decarboxylase 65/67 (GAD65/67), or glutamine synthetase (GS). We found that HC and MG processes co-localize in the inner nuclear layer (INL) (Figure 19 A-I). Closer examination of the INL in flat mounted *Tg(lhx1a:EGFP)^{pt303}* retinas stained with GS showed that MG

processes appear to wrap around HCs (Figure 20). Additionally, the subunit of the GABA$_A$ receptor (GABRG2) that is essential for neurogenesis in the SGZ (Song et al., 2012) was detected on MG processes (Figure 19 J-M). This was validated by RT/PCR of sorted MG (data not shown) and by previous findings (Ramachandran et al., 2012).

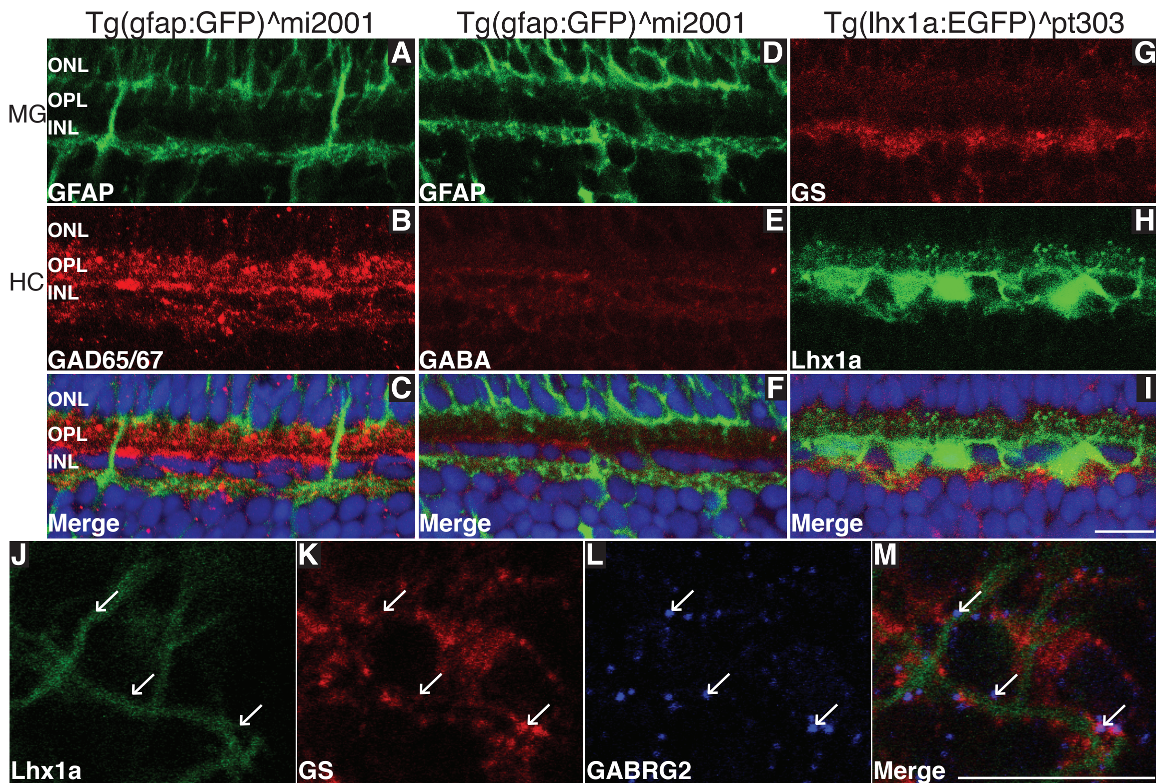

Figure 19. Close association of MG and HC processes in the INL.
Tg(gfap:GFP)^{mi2001} and *Tg(lhx1a:EGFP)^{pt303}* retina sections were stained for GAD 65/67 (A-B), GABA (D-F), or GS (G-I). Co-localization of MG and HC markers was observed in the INL. *Tg(lhx1a:EGFP)^{pt303}* retinas were removed, stained for GS and GABRG2, and the area of co-localization imaged in flat mount (J-M). Arrows indicate GABRG2 puncta. Scale bar is 100µm.

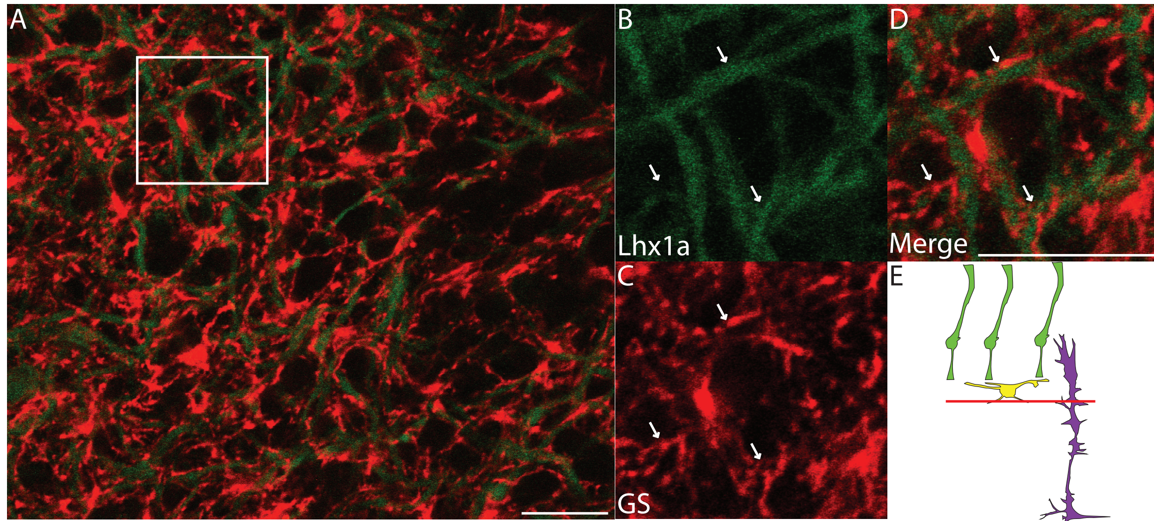

Figure 20. MG processes surround HC processes in INL. *Tg(lhx1a:EGFP)*^{pt303} retinas were removed and stained for GS and prepared for flat mount imaging. The region of the INL where MG and HCs overlap (red line in E) was imaged. Arrows indicate regions of close association between MG and HCs. Scale bar is 100μm.

To determine if GABA levels decrease after damage, *Tg(zop:nfsb-EGFP)*^{nt19} fish were treated with metronidazole (MTZ) to destroy rod photoreceptors and retinas were harvested at different times of recovery (i.e. regeneration). It was found that MG begin to proliferate 52 hours after the end of MTZ treatment (Figure 21). Whole retinas were then subjected to HPLC and the levels of GABA were measured. GABA levels were also significantly reduced 52 hours after MTZ treatment (Figure 22). Together, these results support the idea that MG are poised to detect the decrease in GABA released from HCs that occurs after damage, causing them to regenerate. These findings prompted us to directly test whether altered GABA signaling initiates retina regeneration.

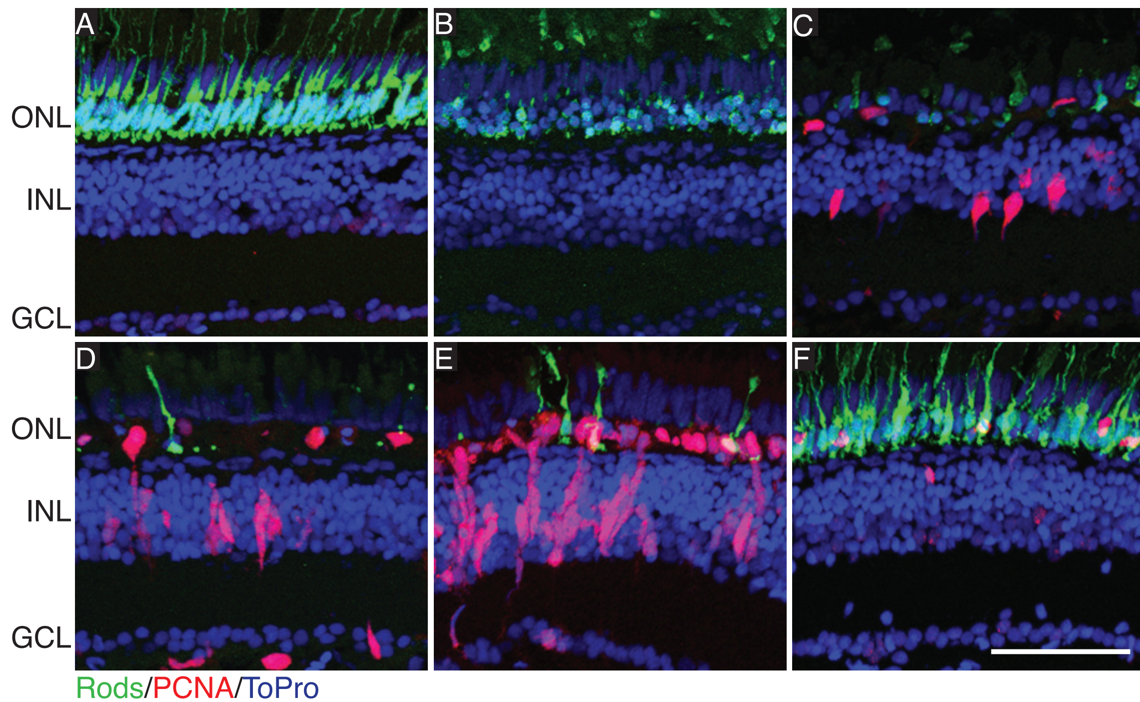

Figure 21. Timeline of regeneration in *Tg(zop:nfsb-EGFP)^{nt19}* after MTZ treatment. *Tg(zop:nfsb-EGFP)^{nt19}* fish were placed in egg water containing 10mM Metronidazole and treated for 24 hours, then returned to normal egg water to recover. Eyes were removed and proliferation assessed by PCNA staining. Times of recovery observed were pretreatment (A), 0h. recovery (B), 52h. recovery (C), 72h. recovery (D), 96h. recovery (E), and 28 days recovery (F). Scale bar is 100μm.

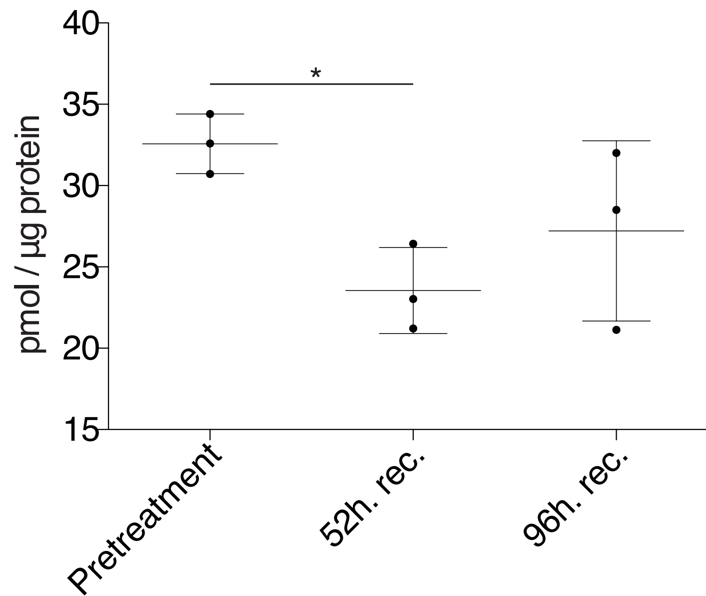

Figure 22. Whole retina GABA levels decrease following rod ablation. *Tg(zop:nfsb-EGFP)^{nt19}* fish were treated with MTZ and allowed to recover. Whole retinas were removed at indicated time points and subjected to HPLC. Levels of GABA were measured. A one-way ANOVA was used; Error bars = SD; * = $p<0.05$.

As a first test of the model, we injected a $GABA_A$ antagonist (gabazine), into undamaged retinas and determined whether inhibition of GABA signaling would cause spontaneous proliferation (Figure 23 A). As early as 48 hours post injection (hpi) the number of proliferating cells, as detected by Proliferating Cell Nuclear Antigen (PCNA) expression, was significantly greater than both uninjected and PBS injected controls (Figure 23 B-D, Figure 24). The increase in proliferation was not a result of an increase in apoptosis (Figure 25). Proliferating cells were found in the INL and co-labeled with GS, indicating that MG were proliferating. Proliferating cells were found in clusters, which indicates a robust regenerative response with multiple divisions of MG-derived progenitor cells. The increase in spontaneous proliferation was dose dependent (Figure 26) suggesting a specific effect via the $GABA_A$ receptor.

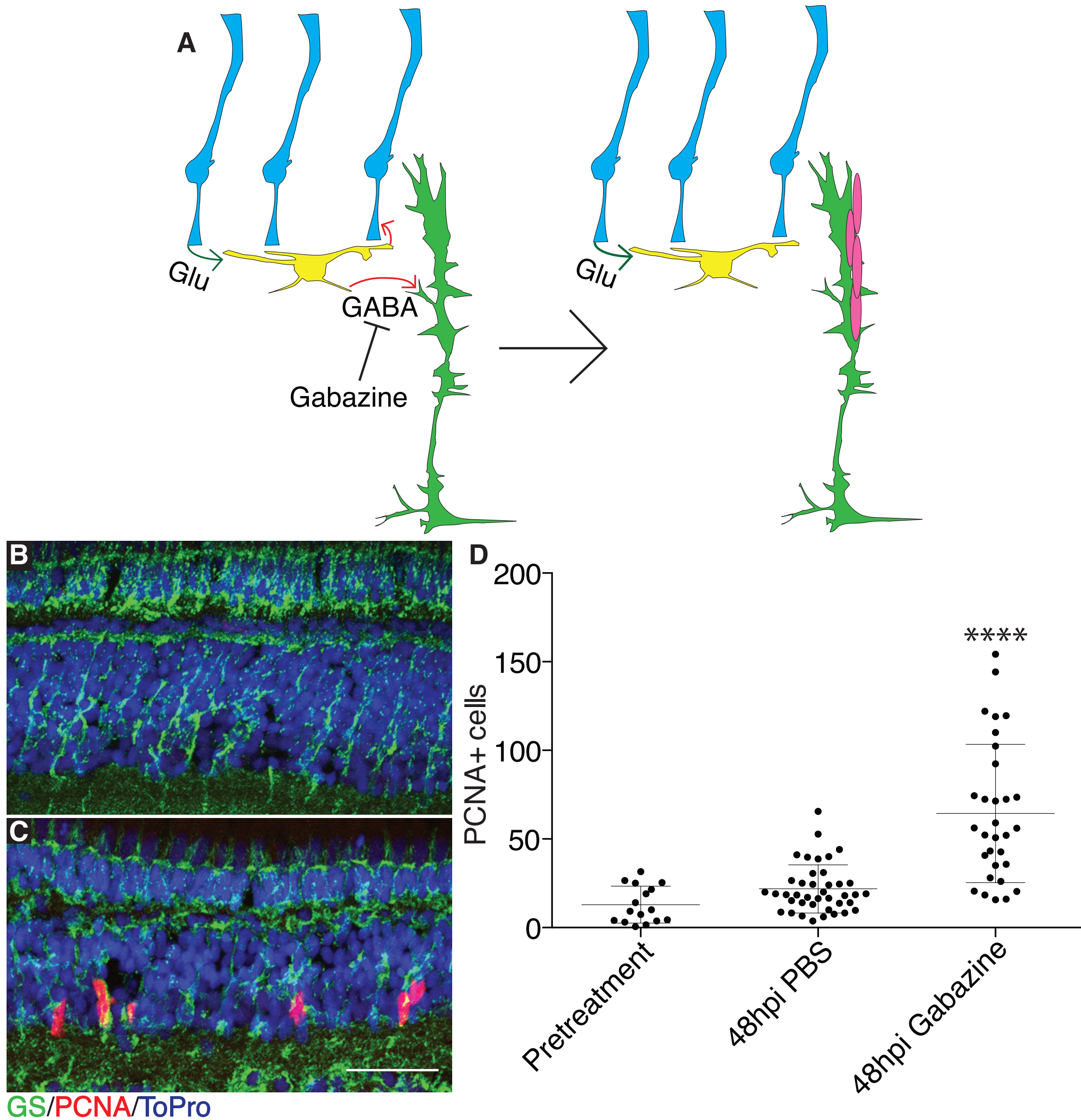

Figure 23. Gabazine injections cause time dependent spontaneous proliferation in undamaged retinas. Model illustrating effects of gabazine injection on MG proliferation (A). WT eyes were injected with PBS (B) or 12.5 nmol gabazine (C) into one eye. Fish recovered for 48 hours after gabazine injections (B, C) before proliferation was measured. Representative images are small portions of entire retina. Proliferating cells were counted across whole sections by PCNA staining (D). Scale bar is 100μm. A one-way ANOVA was used; Error bars = SD; **** = p<0.0001.

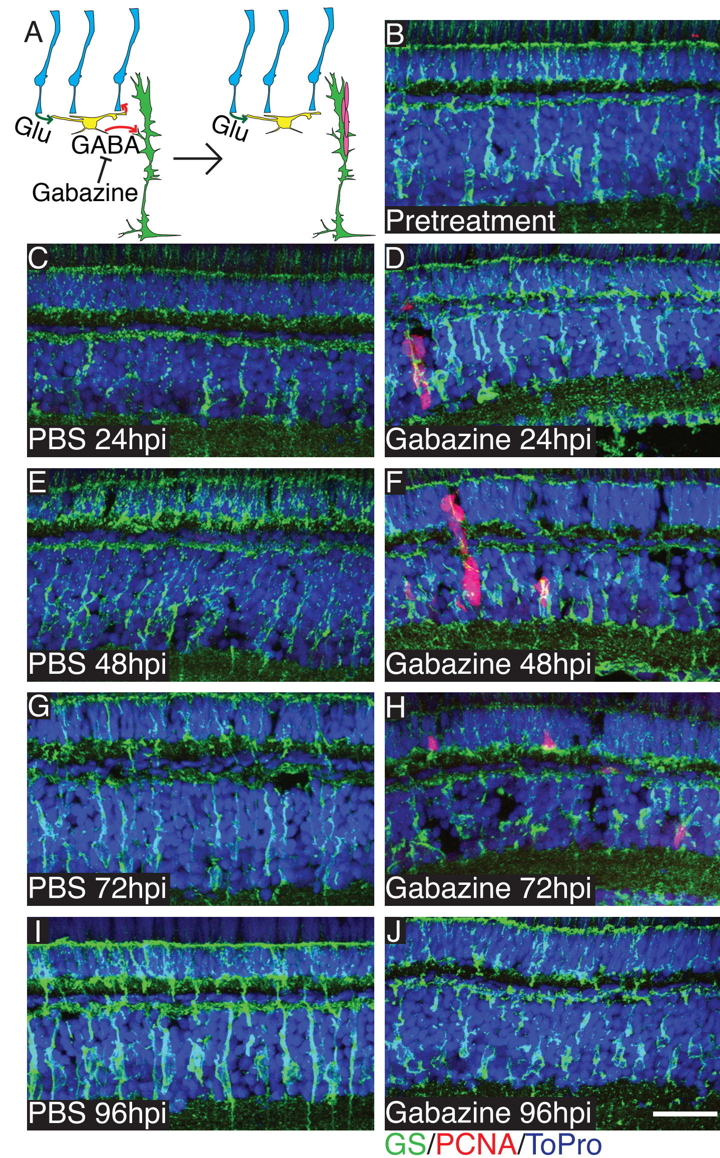

Figure 24. Gabazine injection causes time dependent spontaneous proliferation in undamaged retinas. Model illustrating predicted effects of gabazine on MG proliferation (A). Eyes were injected with 12.5 nmol of gabazine or PBS and allowed to recover. Eyes were removed before injection (B) as well as at 24hpi (C,D), 48hpi (E,F), 72hpi (G,H), or 96hpi (I,J) and proliferation assessed by PCNA staining. Scale bar is 100μm**.**

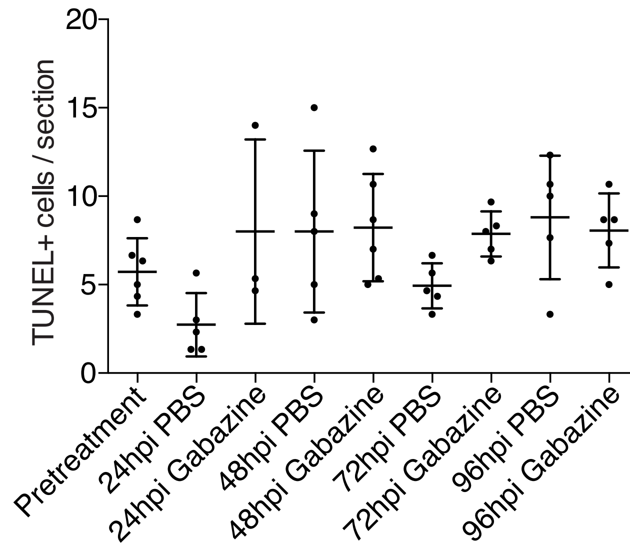

Figure 25. Gabazine injection does not cause increase in apoptosis. Eyes were injected with 12.5 nmol of gabazine or PBS and allowed to recover. Eyes were removed before injection as well as at 24hpi, 48hpi, 72hpi, or 96hpi. At each time point apoptosis was measured by TUNEL labeling. A one-way ANOVA was used; Error bars = SD.

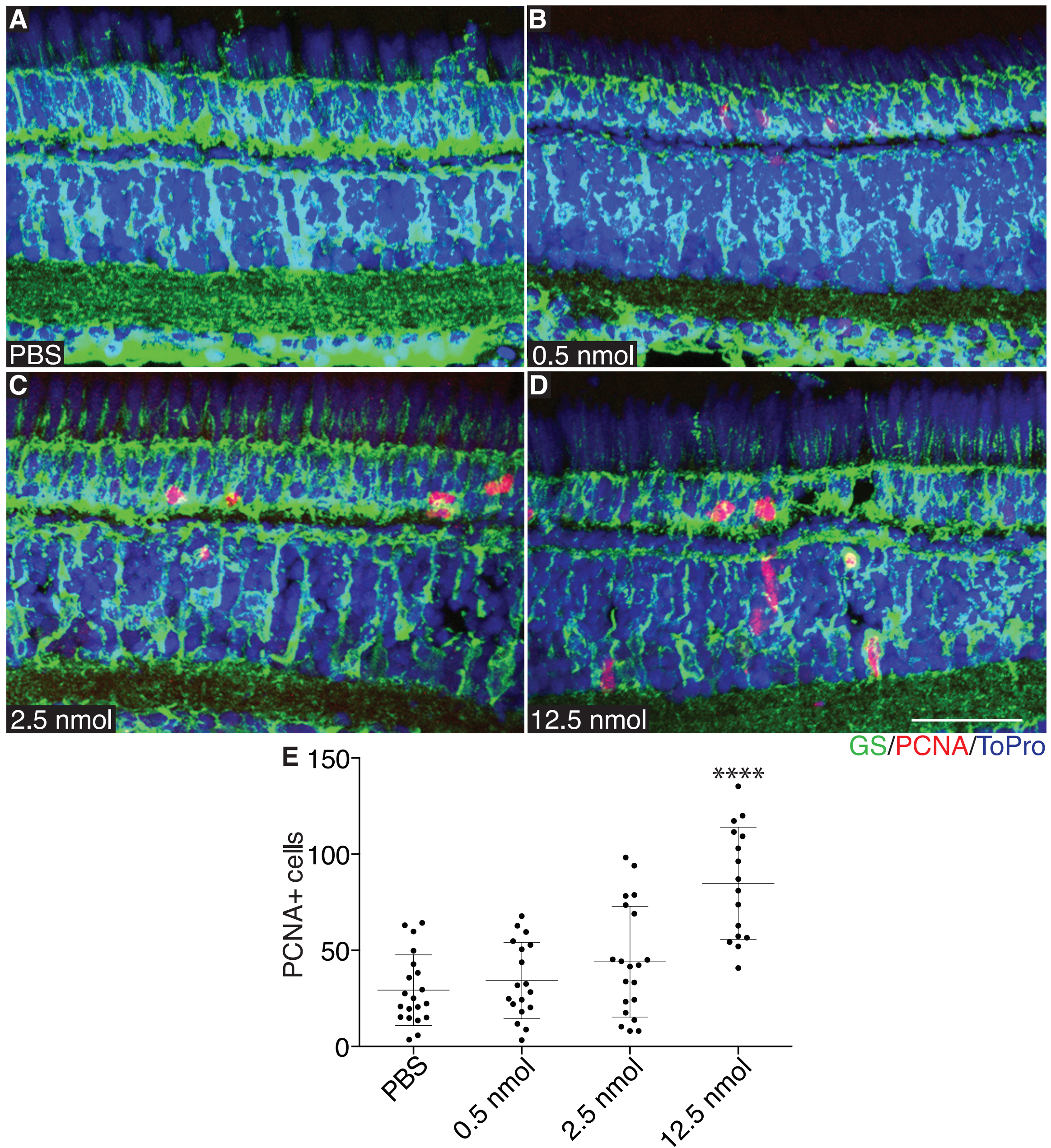

Figure 26. Gabazine induced spontaneous proliferation is dose dependent. WT fish were injected with PBS (A), 0.5 nmol (B), 2.5 nmol (C), or 12.5 nmol (D) and proliferation measured at 48hpi by PCNA staining. Representative images are small portions of entire retina. Proliferating cells were counted across whole sections by PCNA staining (E). Representative images are small portions of total retina sections. Scale bar is 100μm. A one-way ANOVA was used; Error bars = SD; **** = $p<0.0001$.

Upstream of GABA signaling, a second prediction of the model is that inhibiting glutamate signaling should produce similar effects as inhibiting GABA (Figure 27 A). To test this, the AMPA receptor antagonist NBQX was injected into undamaged eyes and proliferation measured by PCNA staining. Proliferation was significantly greater at 48hpi but reached a maximum at 72hpi (Figure 27 B-D, Figure 28). As with gabazine injections, proliferation was not the result of increased apoptosis (Figure 29). Clusters of proliferating cells in the INL that co-labeled with GS were observed at 72hpi, indicating a robust regenerative response with multiple divisions of MG-derived progenitor cells. Furthermore, a dose dependent decrease in proliferation was also observed with NBQX injections (Figure 30).

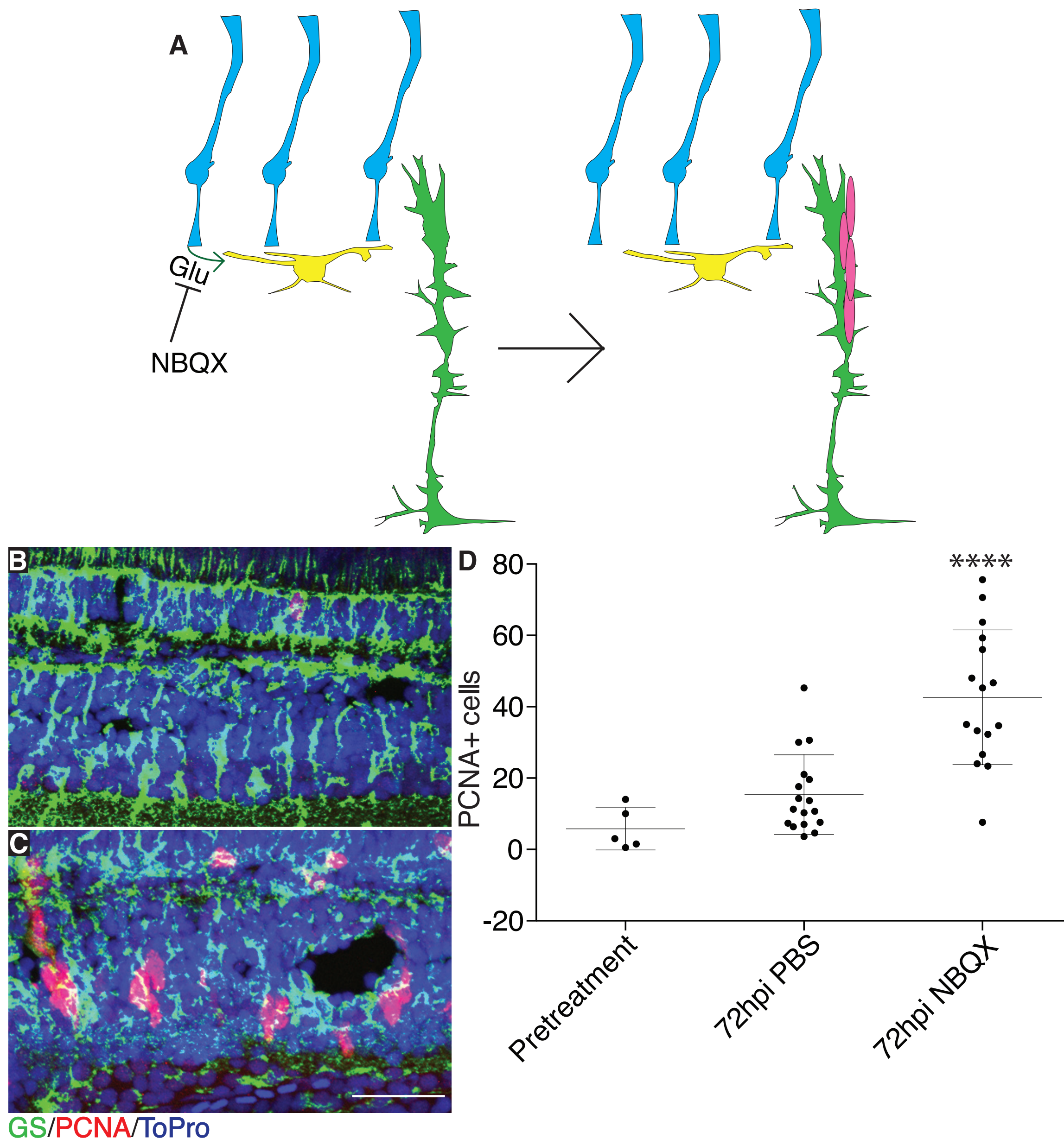

Figure 27. NBQX injections cause time dependent spontaneous proliferation in undamaged retinas. Model illustrating effects of NBQX injections on MG proliferation (A). WT eyes were injected with PBS (B) or 25 nmol NBQX (C) into one eye. Fish recovered for 72 hours after NBQX injections (B, C) before proliferation was measured. Representative images are small portions of entire retina. Proliferating cells were counted across whole sections by PCNA staining (D). Scale bar is 100μm. A one-way ANOVA was used; Error bars = SD; ** = p<0.01, **** = p<0.0001.

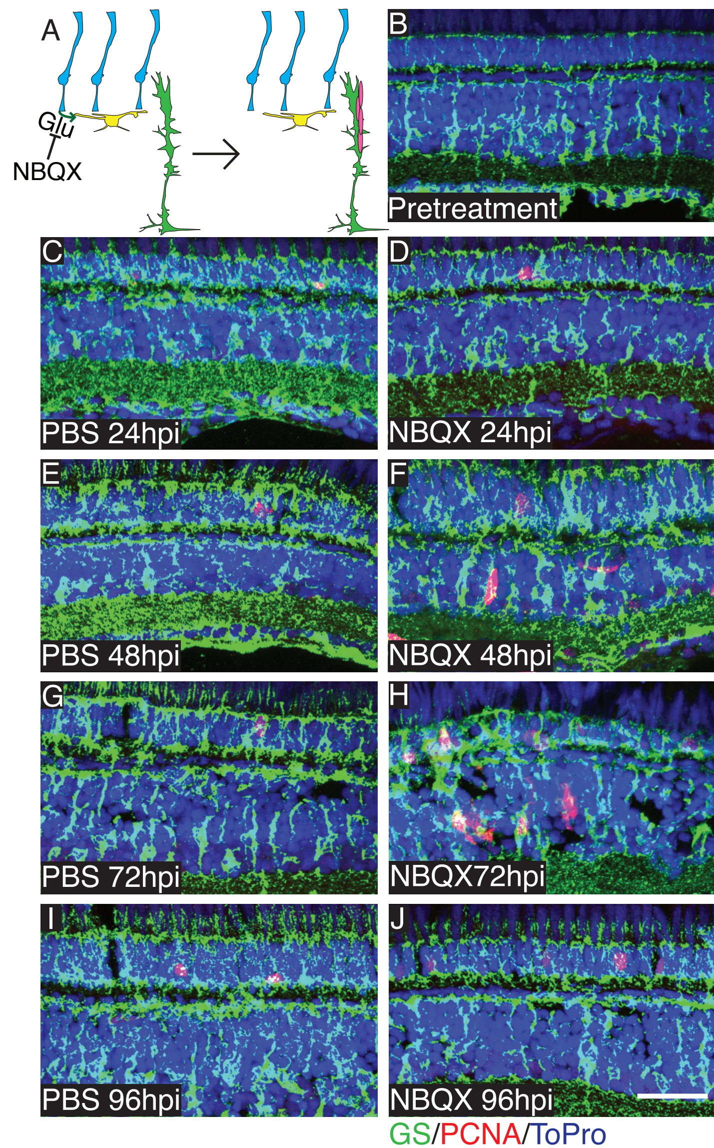

Figure 28. NBQX injection causes time dependent spontaneous proliferation in undamaged retinas. Model illustrating predicted effects of NBQX injections on MG proliferation (A). Eyes were injected with 25 nmol of NBQX or PBS and allowed to recover. Eyes were removed before injection (B) as well as at 24hpi (C,D), 48hpi (E,F), 72hpi (G,H), or 96hpi (I,J) and proliferation assessed by PCNA staining. Scale bar is 100μm.

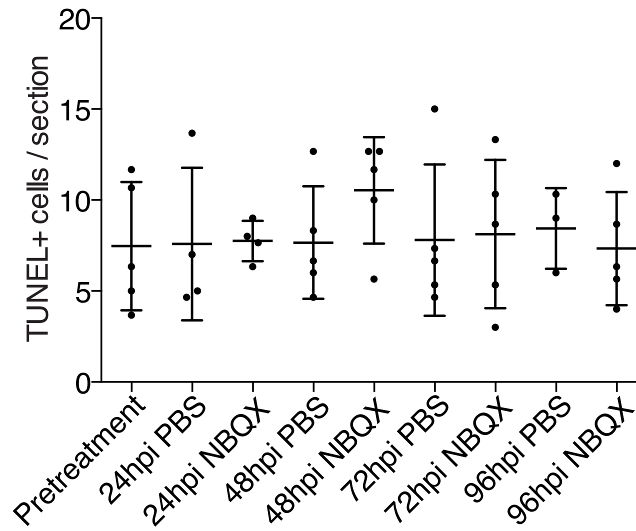

Figure 29. NBQX injection does not causes increase in apoptosis. Eyes were injected with 25 nmol of NBQX or PBS and allowed to recover. Eyes were removed before injection as well as at 24hpi, 48hpi, 72hpi, or 96hpi. At each time point apoptosis was measured by TUNEL labeling. A one-way ANOVA was used; Error bars = SD.

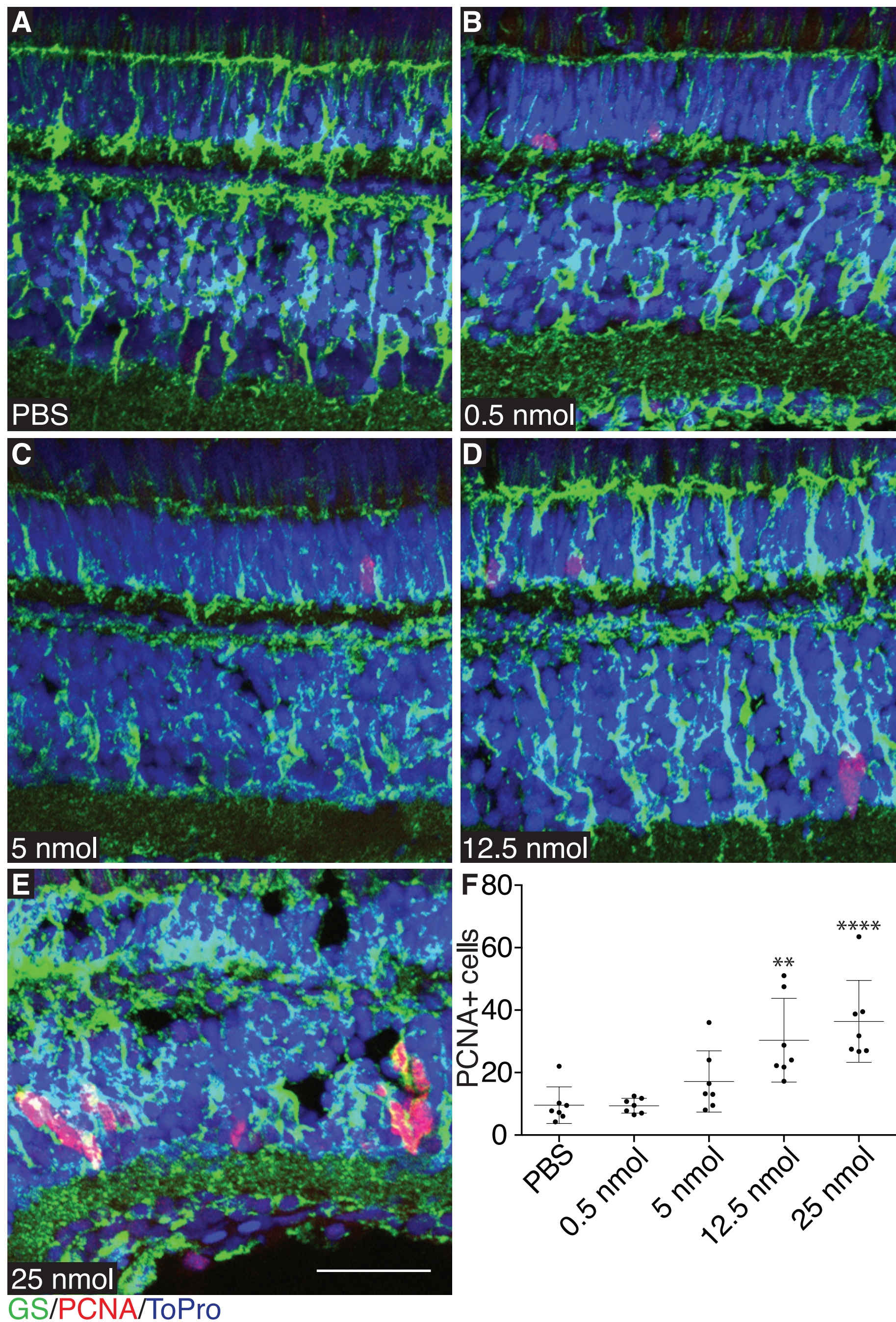

Figure 30. NBQX induced spontaneous proliferation is dose dependent. WT fish were injected with PBS (A), 0.5 nmol (B), 5 nmol (C), 12.5 nmol (D), or 25 nmol (E) and proliferation measured at 72hpi by PCNA staining. Representative images are small portions of total retina sections. Proliferating cells were counted across whole sections by PCNA staining (F). Representative images are small portions of total retina sections. Scale bar is 100μm. A one-way ANOVA was used; Error bars = SD; ** = $p<0.01$, **** = $p<0.0001$.

In order to verify that proliferation resulting from gabazine or NBQX injections accurately replicates regeneration, markers of regeneration were analyzed following injections. Drugs were injected into *Tg(tuba1a:GFP)*, and *Tg(her4:dRFP)* fish, marking activation of α-tubulin 1a and Notch signaling, respectively. Both genes have been shown to be upregulated during regeneration (Fausett and Goldman, 2006; Hayes et al., 2007; Ramachandran et al., 2010b; Wan et al., 2012). After injection, both were found to be upregulated and associated with PCNA expressing cells (Figure 31) suggesting that drug induced proliferation is accurately replicating what is observed during damaged-induced retinal regeneration.

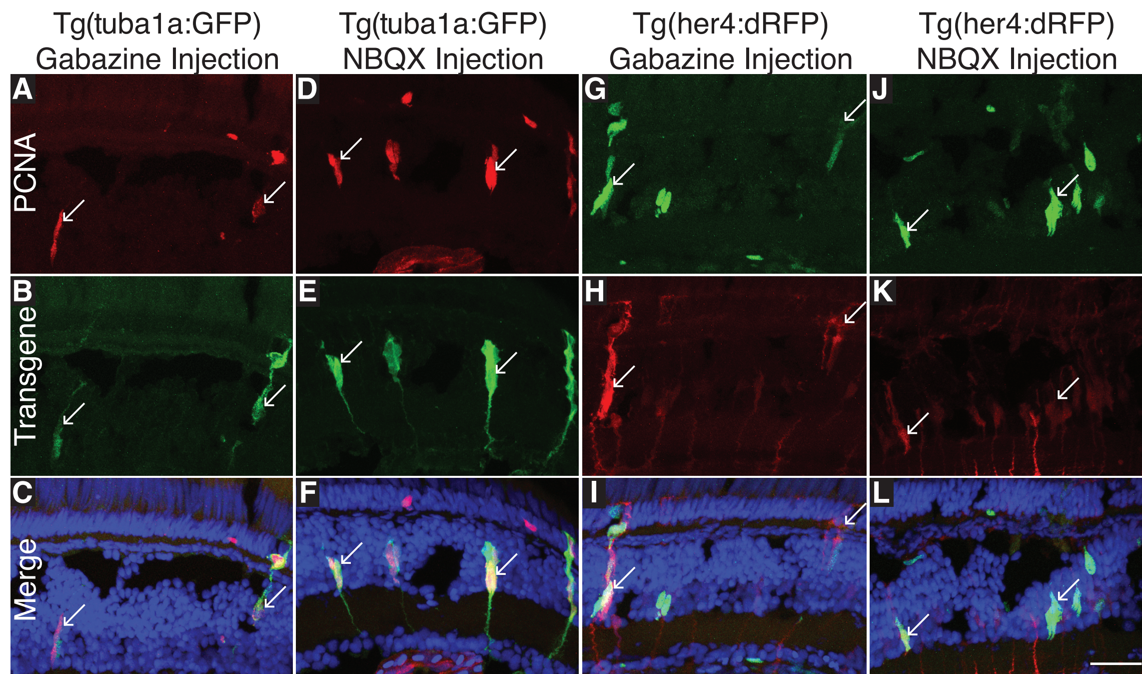

Figure 31. Injection of gabazine or NBQX into undamaged eyes causes upregulation of factors associated with regeneration. Gabazine or NBQX was injected into one eye of *Tg(tuba1a:GFP)* or *Tg(her4:dRFP)* fish. Fish were allowed to recover for 72 hours, after which retinas were removed and stained for PCNA. Both GFP and dRFP expression colabeled with PCNA. Arrows indicate colocalization of transgene and PCNA. Scale bar is 100μm.

The above experiments showed an increase in proliferation without damage. A converse set of experiments was devised to determine whether activating the $GABA_A$ receptor or the AMPA receptor would suppress regeneration after damage (Figure 32 A). To test this, *Tg(zop:nfsb-EGFP)*^{nt19} fish were damaged with MTZ and eyes injected with either muscimol, a $GABA_A$ receptor agonist, or AMPA, a glutamate receptor agonist. Injected retinas were collected at 52 hours after MTZ treatment, when MG proliferation begins (Figure 21). Both injections showed a significant decrease in proliferating cells compared to PBS injections, as measured by PCNA expression (Figure 32 B-F).

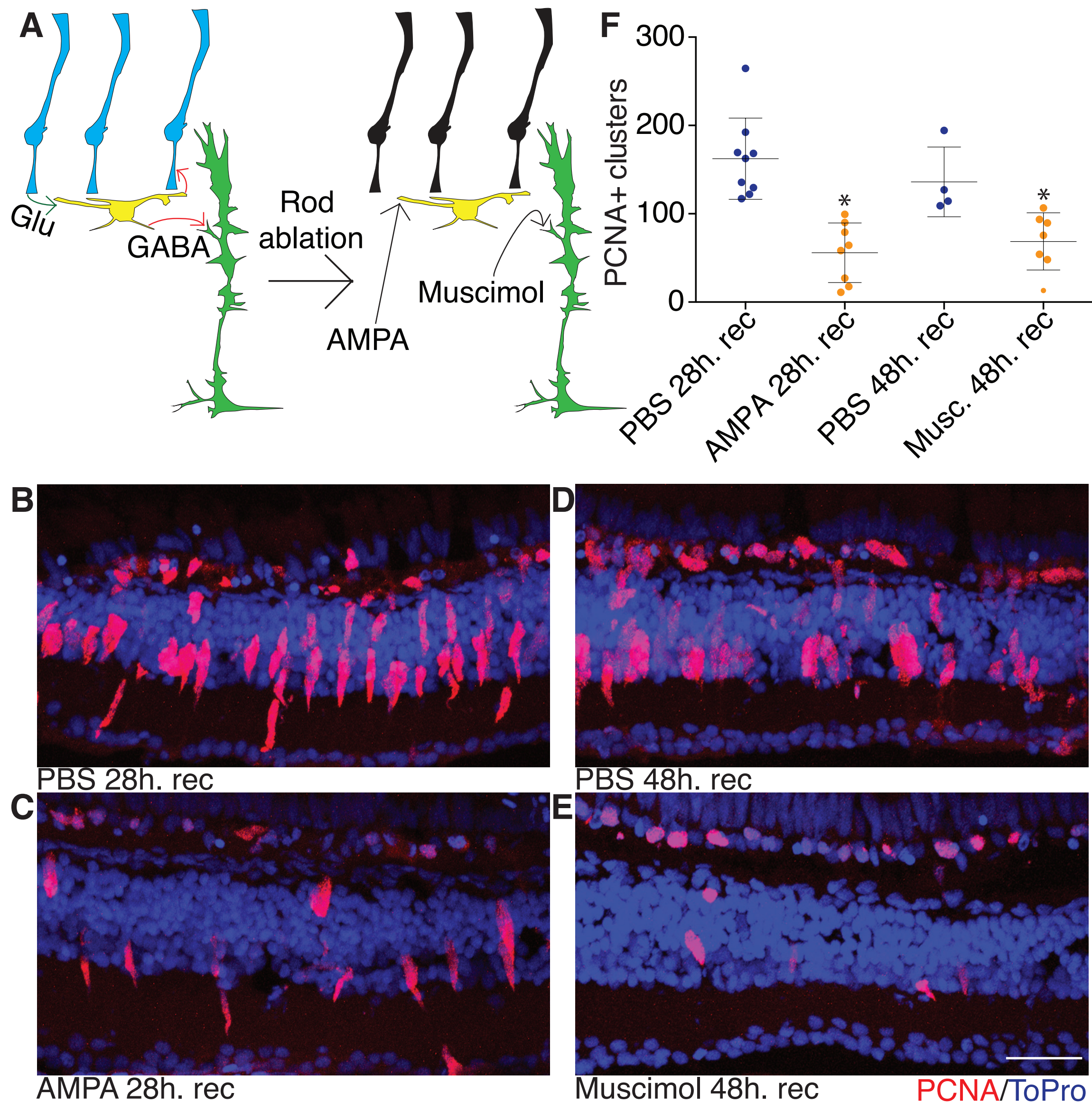

Figure 32. AMPA and Muscimol injections suppress regeneration in damaged retinas. Model illustrating effects of muscimol and AMPA injections on MG proliferation (A). *Tg(zop:nfsb-EGFP)*^{nt19} fish were treated with 10mM metronidazole for 24 hours, then allowed to recover. Fish were then anesthetized and injected with either AMPA/PBS control at 28h. recovery or muscimol/PBS control at 48h. recovery. Injected eyes were removed at 52h. recovery. Proliferation was assessed by PCNA staining (B-E). Representative images are small portions of the entire retina. Clusters of proliferating cells were measured across entire sections (F). Scale bar is 100μm. A Students t-test was used; Error bars = SD; * = $p<0.05$.

One caveat to the above experiments is that the various agonists and antagonists could be acting indirectly to cause MG proliferation. To address this, we used a genetic approach by creating a construct expressing a dominant negative version of the zebrafish γ2 subunit of the $GABA_A$ receptor (DNγ2) following an identical human mutation that underlies an inherited form of epilepsy (Harkin et al., 2002; Kang et al., 2009). The zebrafish glial fibrillary acidic protein (GFAP) promoter was used to drive expression of an mCherry tagged version of the DNγ2 isoform in MG. Injection and electroporation of this construct into *Tg(gfap:GFP)^mi2001^* fish was performed followed by analysis of proliferating cells that co-localize with mCherry, GFP, and PCNA. There was a significant increase in the total number of proliferating cells, marked by PCNA and co-localizing with GFP and mCherry (Figure 33, Figure 34). This indicates that MG directly respond to GABA.

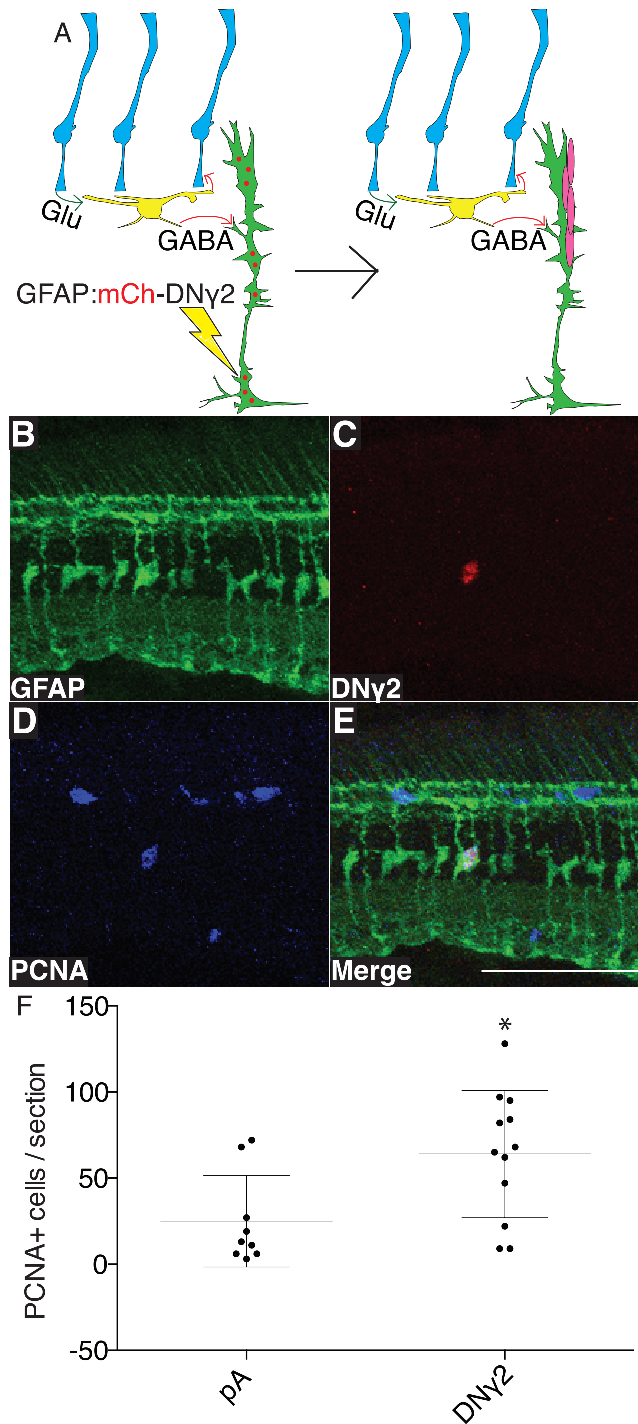

Figure 33. Expression of DNγ2 in MG of undamaged retina causes increased proliferation. Model illustrating effects of electroporation of DNγ2 into MG on proliferation (A). A GFAP:mCh-DNγ2 construct was electroporated into one retina of undamaged *Tg(gfap:GFP)*^{mi2001} fish. GFP expression (B), mCh expression (C), and staining for PCNA (D) all co-labeled in the same cell (E). Total number of PCNA expressing cells was measured (F). Scale bar is 100µm. A Students t-test was used; Error bars = SD; * = $p<0.05$.

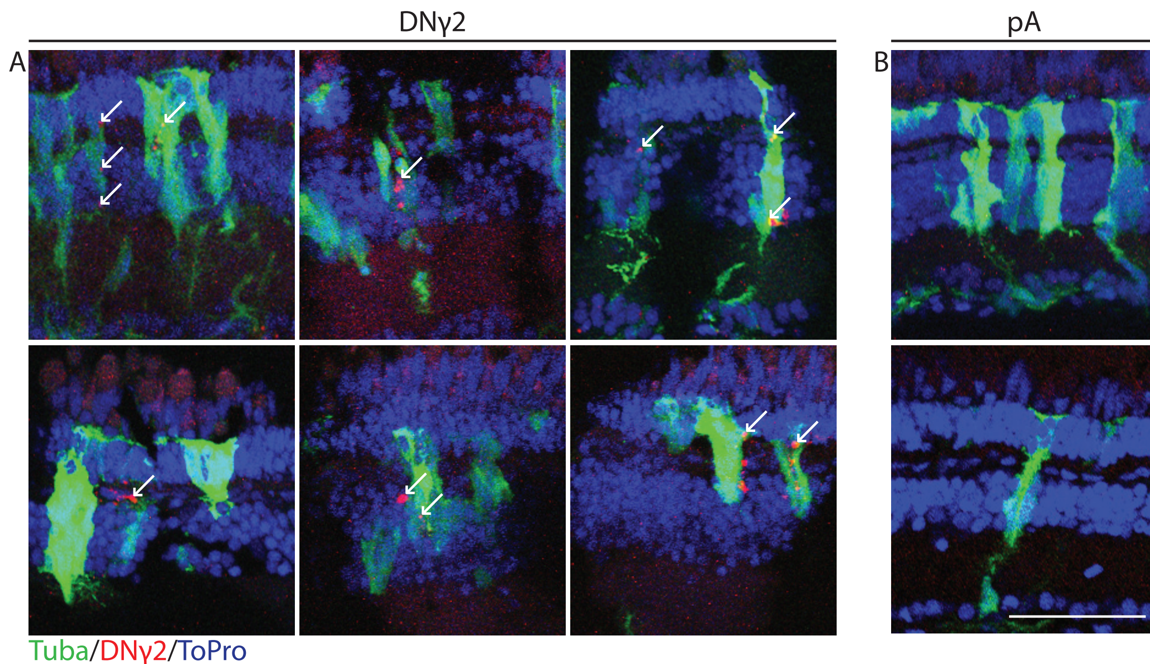

Figure 34. Expression of DNγ2 in proliferating cells. *Tg(tuba1a:GFP)* fish were electroporated with a construct containing either a GFAP:mCh-DNγ2 or GFAP:mCh-pA and allowed to recover for 96 hours, after which retinas were stained for GFP and mCherry to measure co-localization. Representative images of DNγ2 (A) and pA (B) are small portions of entire retinas. Arrows indicate co-localization of mCh and GFP. Scale bar is 100μm.

Discussion

Our data support a novel mechanism in which decreased GABA is sensed by MG to initiate retinal regeneration. Photoreceptor death results in a decrease in GABA, and MG are poised to detect this decrease by their close association with HCs and their expression of $GABA_A$ receptors. These findings indicate that regulation of neurogenesis in the mouse hippocampus is a conserved mechanism across structures and organisms and strongly suggests this is a basic archetype for eliciting neurogenesis. GABA has been found to be involved in neurogenesis in other areas of the CNS, specifically the SGZ and subventricular zone (SVZ). Multiple reports have indicated an importance for GABA in proliferation of progenitor cells (Braun and

Jessberger, 2014; Giachino et al., 2014; Liu et al., 2005; Pallotto and Deprez, 2014; Ramirez et al., 2012; Song et al., 2013; Tozuka et al., 2005). This may also be true in the retina, where elevated GABA signaling may promote progenitor cell proliferation or differentiation but suppress MG proliferation, providing directionality for the regeneration process.

Our data suggest that PR damage is communicated via GABA, based on the timing of events in different experiments. Nevertheless, the possibility exists that MG directly sense changes in glutamate as well. Maximum proliferation for gabazine injections was observed at 48hpi while maximum proliferation for NBQX injections was observed at 72hpi. This suggests that GABA affects MG more proximally than glutamate. Furthermore, muscimol injection into damaged retinas only produced an effect when injected at 48 hours after MTZ treatment, while AMPA injections only produced an effect when injected at 28 hours after MTZ treatment. Injecting muscimol earlier or AMPA at later times did not cause significant changes in proliferation (data not shown). The overall timing best supports the idea that GABA acts directly on MG and glutamate is upstream.

Previous studies have suggested that TNFα (Nelson et al., 2013), Notch(Conner et al., 2014), leptin, and interleukin 6 (IL-6)(Zhao et al., 2014b), are involved in initiating retina regeneration in zebrafish. It is possible that these and other, as yet undiscovered, signals act synergistically to mount a full, robust regenerative response. However, even though TNFα, Notch, leptin, and IL-6 are all relatively early markers of regeneration, it is not clear what signals induce their expression, especially because numerous gene expression changes accompany

differential expression of these factors. An attractive hypothesis based on our data is that decreased GABA is the primary signal for retinal regeneration initiation and other signals follow to act synergistically. This is supported by the fact that injection of gabazine or NBQX causes an upregulation of the Notch reporter Her4, suggesting that GABA is upstream of Notch signaling. This is also in line with earlier studies that showed that Notch signaling is important for later stages of regeneration and development (Hayes et al., 2007; Karl et al., 2008; Kassen et al., 2007; Olena et al., 2015; Raymond et al., 2006). In addition, leptin mRNA was observed to increase following injury, suggesting that it is induced by some other signal. Interestingly, Il-6 mRNA was not detected in regenerating retinas, indicating that its source originates from outside the retina and may be prompted to increase only after damage occurs (Zhao et al., 2014b). Inflammatory signals like TNFα and Il-6 may be released by endogenous immune cells (e.g. microglia(Fischer et al., 2014)) or those invading from the vasculature, after damage.

Retinitis pigmentosa and age related macular degeneration arise from dysfunction and death of photoreceptors. We have focused on photoreceptor regeneration but the questions remains how bipolar, amacrine, or ganglion cells regenerate. It may be that feedback mechanisms are in place where HC activity would be affected by the death of other cells. For example, dopaminergic amacrine cells have been found to have processes that project to the HC layer and may also affect HC activity (Herrmann et al., 2011). There are likely other mechanisms by which the retina senses bipolar, amacrine, or ganglion cell death as well. MG processes appear to surround the cell bodies of amacrine and ganglion cells (data

not shown), suggesting a different mechanism to sense cell death, such as juxtacrine or paracrine signaling. These mechanisms may also be involved in sensation of PR and HC death. Identifying a method to induce spontaneous MG proliferation and the production of progenitor cells may be sufficient if the new progenitors can then follow endogenous cues to differentiate into whatever cell is needed. New therapies that activate MG in this manner could lead to robust endogenous regeneration and counteract many retinal diseases. These might include both agonists and antagonists of neurotransmitter signaling but could also be targeted at intracellular cascades downstream of GABA binding as well as other factors (Jagasia et al., 2009; Quadrato et al., 2012; Quadrato et al., 2014; Ramirez et al., 2012) (Andang et al., 2008; Fernando et al., 2011)

Lastly, the question remains why teleost retinas have maintained a robust regenerative response while mammalian retinas are largely incapable of repair. The cellular organization found in zebrafish may be a key difference. In zebrafish, the HCs form a monolayer that is separated from the rest of the INL by the network of HC and MG processes, observed in the current study (Fig. 19). A consequence of this organization is that the HCs contain processes that project into the INL as well as into the outer plexiform layer (OPL). In mice, however, the HCs are comingled with other cells in the INL and only contain projections into the OPL (Matsuoka et al., 2012; Poche et al., 2007). Investigating HC development and potential interactions between HCs and MG could greatly inform about how MG respond to damage in the mammalian retina. It may be that MG are inefficient or blocked from detecting

changes in GABA after damage but perhaps alteration of signaling by pharmacological agents such as gabazine could push MG down a regenerative path.

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Author Contributions: MBR, DD, and JGP conceived, designed, and performed all experiments and wrote the paper.

Competing Financial Interests: The authors declare no competing financial interests.

Materials and Methods

Zebrafish lines and maintenance

Zebrafish lines used in this study include *Tg(gfap:GFP)*^{mi2001} (Bernardos and Raymond, 2006), which marks differentiated MG, *Tg(zop:nfsb-EGFP)*^{nt19} (Montgomery et al., 2010), which marks rods and is used for rod ablation, *Tg(lhx1a:EGFP)*^{pt303} (Swanhart et al., 2010), which marks HCs, *Tg(tuba1a:GFP*) (Fausett and Goldman, 2006), which marks dedifferentiated MG and progenitor cells, and *Tg(her4:dRFP)* (Yeo et al., 2007), which marks notch activated cells. All fish were maintained in a 14:10 light:dark (L:D) cycle at 28^{0}C unless otherwise noted.

Metronidazole induced rod damage

Rod ablation was induced similar to previously established protocols (Montgomery et al., 2010). Briefly, *Tg(zop:nfsb-EGFP)*^{nt19} transgenic zebrafish were transferred to egg water containing 10mM metronidazole (MTZ) for 24 hours in darkness at 28^{0}C. Fish were then transferred to normal egg water and returned a 14:10 L:D cycle for recovery. The extent of regeneration was assayed at the indicated times post recovery after MTZ treatment.

HPLC analysis of GABA

Whole retinas were dissected from *Tg(zop:nfsb-EGFP)*^{nt19} transgenic zebrafish following recovery from MTZ treatment. Protein was extracted from 10 whole retinas for each time point and subjected to High-Performance Liquid

Chromatography (HPLC). The levels of each amino acid and derivations were quantified.

Drug injections

Different neurotoxins were injected into the vitreous using a protocol adapted from previous studies (Rajaram et al., 2014a; Rajaram et al., 2014b; Thummel et al., 2008). The drugs included gabazine (25mM; Sigma S106), nitro -2, 3-dioxobenzoquinoxaline-sulfonamide (NBQX) (50mM; Abcam ab210046), muscimol (5mM; Sigma M1523), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (10μM; Sigma A9111). Briefly, zebrafish were anesthetized in 0.016% tricaine, an incision was made in the sclera with a sapphire knife, and a blunt end 30 gauge needle inserted. 0.5μL were injected into one eye of adult zebrafish. Fish were immediately placed into a recovery tank, times indicated are hours of recovery.

Immunohistochemistry and TUNEL labeling

Zebrafish were euthanized in 0.08% tricane and whole eyes were removed and fixed in 9:1 ethanolic formaldehyde (PCNA staining) or 4% paraformaldehyde (all other staining) overnight. Eyes were then washed in PBS and cryoprotected in 30% sucrose for 4 hours at room temperature. Eyes were then transferred to a solution containing 2 parts OCT and 1 part 30% sucrose overnight followed by transfer to 100% OCT for 2 hours and then embedded in OCT for cryosectioning. Antibodies used were PCNA (Sigma, P8825; Abcam, ab2426), Glutamine Synthetase (Millipore,

mab302), GABA (Sigma, A0310), $GABA_A$ receptor gamma 2 subunit (Novus Biologicals, NB300-151), GAD65+GAD67 (Abcam, ab11070), GFP (Torrey Pines BioLabs, TP401), mCherry (Novus Biologicals, NBP1-96752). TUNEL labeling was performed following IHC. The In Situ Cell Death Detection Kit, TMR red (Roche Applied Sciences, 12156792910) was used to detect apoptotic cells.

Design of Dominant Negative γ2 and electroporation

A dominant negative form of the γ2 subunit of the $GABA_AR$ was previously characterized in humans (Harkin et al., 2002; Kang et al., 2009). The mutation is in a conserved position in zebrafish and generates a premature codon. A plasmid containing the mRNA sequence of γ2 until the premature stop codon was created by GeneArt®. The sequence was cloned into a Tol2 backbone to create the vector GFAP:mCh(no-stop)-DNγ2. A control vector GFAP:mCh(no-stop)-pA was also created. Both constructs were electroporated into retinas following a protocol adapted from previous studies (Rajaram et al., 2014a; Rajaram et al., 2014b; Thummel et al., 2008). Briefly, fish were anesthetized, the outer cornea was removed, an incision was made in the sclera with a sapphire knife, and a blunt end 30 gauge needle was inserted into the vitreous. 0.5µL of plasmid DNA at a concentration of 2 ng/µL was injected into the vitreous of one eye. Anesthetized fish were allowed to recover, re-anesthetized, and then injected eyes were electroporated (50 V/pulse, 4 pulses, 1 second intervals between pulses). Treated fish were placed into recovery tanks for the times indicated.

Statistical Analysis

A two-tailed Students t-test analysis was performed when comparing two means and a one-way ANOVA analysis was performed when comparing 3 or more means. The test used is indicated in each figure as well as the number of eyes measured. If possible, experiments contained a single clutch of fish that were divided among different treatment groups. When multiple clutches were required, fish were mixed, then distributed among treatment groups to reduce bias. 3 sections from one eye were measured and the resulting values averaged to arrive at the reported value.

Chapter IV

SUMMARY AND CONCLUSIONS

Significance

Vision is the main sense we use to interpret and interact with the world around us. Losing this sense drastically changes how we live as well as greatly decreases quality of life and exacts a large economic toll. In 2014 the estimated economic burden caused by blindness in the US was $139 billion, as estimated by the National Eye Institute (NEI), a number that is expected to rise. The NEI has also put forth an Audacious Goals Initiative to develop new therapeutic approaches for regeneration of the retina, underscoring the severity of retina diseases and the dire need for new treatments.

The reason diseases in the retina are so devastating is because the mammalian retina, much like the rest of the CNS, has no way to repair itself if it incurs damage. Devising a strategy to replace lost cells in the retina is the main way vision can be restored to those suffering from blindness. Current attempts aim to add back new cells by injecting them into the retina and allowing them to reintegrate into existing circuits. Though progressing, these methods have many drawbacks and are far from being applied readily as a treatment. These techniques can also become quite invasive.

An alternative approach would be to prompt the retina to regenerate itself. Zebrafish have the ability to undergo damage induced retina regeneration. As fellow vertebrates, the retinas of zebrafish and mammals are similar, with the same cell types, structure, function, and gene expression patterns. However, zebrafish

mount a robust regenerative response following retina damage. Understanding how zebrafish initiate regeneration may highlight key therapeutic targets for treating retina diseases and damage.

In this work I have presented a novel mechanism of retina regeneration initiation in zebrafish. By understanding how neurogenesis occurs in other tissues and organisms I was able to integrate the information to gain a better understanding of the signaling mechanisms that exist in the zebrafish retina. In doing so, I have expanded the knowledge of what regulates retina regeneration. These findings will hopefully expand investigation of possible initiators of retina regeneration as well as allow for more precise investigation of downstream changes that occur during retina regeneration.

Summary of findings

GABA is an important signaling molecule in the retina and has been shown previously to affect neurogenesis in the mouse SGZ (Song et al., 2012). In the SGZ, glutamatergic granule cells synapse with GABAergic interneurons. When input to the interneurons is low, then the resulting decrease in GABA levels is sensed by γ2-containing $GABA_A$ receptors on RGLs, resulting in their proliferation. In the retina a similar circuit may exist. Glutamatergic PRs synapse with GABAergic HCs. When PRs die, HCs would no longer be stimulated to release GABA. It is possible that MG, the glial stem cells of the retina, may detect the decrease in GABA. Considering this, I investigated the effect of GABA on regeneration in the zebrafish retina. I hypothesized that GABA levels decrease after PR death and the decrease is sensed

by MG, via γ2-containing $GABA_A$ receptors, causing them to proliferate. Here I have shown data supporting this hypothesis.

MG and HCs project to a specialized layer of the INL, forming intimate associations where MG processes appear to wrap around HC processes. Furthermore, the $GABA_A$ receptor subunit γ2 was found on MG processes in this region. Additionally, using HPLC I found that GABA levels significantly decrease in retinas damaged with MTZ at the time when MG begin to proliferate. To test the hypothesis, I inhibited the $GABA_A$ receptor in undamaged retinas using gabazine and observed spontaneous MG proliferation, as measured by PCNA immunostaining. In addition, inhibition of AMPA receptors in undamaged retinas by NBQX also resulted in spontaneous MG proliferation. In order to verify these findings were faithfully replicating regeneration, two other markers of regeneration were assayed: the Notch reporter Her4, and α-Tubulin1a, which have both been shown to be upregulated in regenerating MG (Fausett and Goldman, 2006; Hayes et al., 2007; Ramachandran et al., 2010b; Wan et al., 2012). Both of these were found to be upregulated following gabazine or NBQX injection into undamaged retinas. Together these findings suggest that $GABA_A$ or AMPA receptor inhibition can cause MG proliferation reminiscent of regeneration.

The converse of these experiments was to elicit damage in the retina and subsequently activate GABA signaling. To this end retinas were damaged with MTZ and injected with either muscimol, a $GABA_A$ receptor agonist, or AMPA, an AMPA receptor agonist. Following injection of these drugs into damaged retinas, a significant decrease in proliferation was detected. Together these findings indicate

that activation of the $GABA_A$ receptor or AMPA receptor can suppress regeneration in a damaged retina.

Lastly, it was tested if GABA directly binds to MG. A DNγ2 subunit of the $GABA_A$ receptor was expressed only in MG in undamaged retinas. The DNγ2 prevents trafficking of whole $GABA_A$ receptors to the cell surface. Expression of the DNγ2 caused significantly more spontaneous MG proliferation compared to controls. This suggests that inhibiting $GABA_A$ signaling specifically in MG can cause them to proliferate. These findings outline a novel mechanism of retina regeneration initiation in zebrafish where GABA levels are used as a damage sensor. When GABA levels decrease, $GABA_A$ receptors on MG directly sense this and initiate regeneration.

Discussion and Future Directions

Impact of glutamate on MG proliferation

My data suggest GABA signaling can directly affect MG proliferation. Based on these data, though, it is possible that glutamate can also directly affect MG proliferation. Injecting NBQX into undamaged retinas or AMPA into damaged retinas shows that glutamate signaling affects MG proliferation. The hypothesis is that glutamate acts via GABAergic HCs, though this was never directly tested. It is still possible, therefore, that glutamate can act directly on MG to affect proliferation. There are pieces of data presented here that suggest it is a stepwise process. Maximum proliferation following gabazine injection was observed at 48hpi while maximum proliferation following NBQX injections was observed at 72hpi. This

suggests that GABA affects MG proliferation more rapidly than glutamate. Furthermore, muscimol injection into damaged retinas only reduced regeneration when injected 48 hours after MTZ treatment, while AMPA injections only reduced regeneration when injected at 28 hours after MTZ treatment. Injecting muscimol earlier or AMPA at later times did not cause significant changes in proliferation (data not shown). The overall timing best supports the idea that GABA acts directly on MG and glutamate is upstream. Furthermore, previous findings suggest that glutamate has an inhibitory effect on neurogenesis (Berg et al., 2013) and that metabatropic glutamate receptors are specifically not involved in the regeneration response (Bailey et al., 2010).

This possibility, though, needs to be evaluated. One possibility is to inject drugs into the eye to affect both glutamate and GABA receptors to attempt to determine the signaling order. One set of experiments that would strongly suggest, though not definitively show, that glutamate is upstream of GABA is as follows. Based on the hypothesis, injecting NBQX to inhibit glutamate signaling and muscimol to activate downstream GABA signaling should result in reduced MG proliferation compared to injecting NBQX alone, as muscimol would mask the decreased GABA release from HC caused by NBQX. However, the same result could still occur if both act on MG directly and the effect of each drug is additive. In order to solve this issue the reverse experiment could also be performed. Based on the hypothesis, injecting both AMPA and gabazine would be predicted to have the same amount of proliferation as gabazine alone, since GABA is downstream. If the amount of proliferation following the combined injection is less than the amount by

gabazine alone, it would suggest that both glutamate and GABA are acting on MG independently and their effect is summed in the MG. A further caveat may be that that AMPA injection will cause enough GABA to be released from HCs that it reduces the affinity of gabazine to the $GABA_A$ receptor and suppresses proliferation, further confounding the results. To account for this possibility, injection of NBQX and gabazine together could be carried out. The prediction of this experiment is that it would produce a similar amount of proliferation to gabazine injection alone since GABA is downstream. If this result is observed then it is quite possible that glutamate is upstream of GABA and not acting on MG directly. If this result is not observed, however, then the possibility of glutamate acting directly on MG cannot be ruled out.

A superior approach would be to carry out an experiment similar to expressing the DNγ2 in MG. Expressing a dominant negative version of either the metabotropic or ionotropic glutamate receptor in MG would be a way to determine if MG can directly sense glutamate. If inhibition of glutamate signaling in MG results in proliferation then they may be able to sense glutamate. If glutamate does regulate MG proliferation, that does not lessen the findings here but rather would show that multiple neurotransmitters can affect MG proliferation. Further investigation will be required to see how glutamate signaling is involved in retina regeneration.

Impact of GABA signaling on proliferation

Another consideration after this study is how quickly changes in GABA signaling can affect transcription in MG. When muscimol is injected into damaged eyes, a drastic reduction in PCNA staining is observed within four hours of injection. It may be, therefore, that transcriptional changes occur within the MG very rapidly following $GABA_A$ receptor inhibition. By understanding more clearly the timing of when transcriptional changes occur following a decrease in GABA signaling it may become possible to identify gene expression changes that are first to occur following damage. This has thus far been difficult to pinpoint, though identifying initial gene expression changes could provide a number of important therapeutic targets.

The impact of GABA on regeneration has only been hypothesized and studied in the context of PR death. Indeed this is relevant for the vast majority of retina diseases, including AMD and RP. The same changes in GABA levels would occur with HC death, but beyond that PR death may not result in decreases in GABA release from all GABAergic cells in the retina. This is because PR death may not result in a decrease in stimulation for all cell types. For example, on-center BCs are depolarized when light enters the retina, while off-center BCs depolarize when light is absent from the retina. Theoretically, this would mean that off-center BCs would activate downstream components when PRs die, while on-center BCs would not. Therefore, ACs and GCs that synapse with these cells would behave differently. Furthermore, ACs and GCs would add additional layers of complexity as their response to BC depolarization are diverse. GABA levels may still act as an initiator

of regeneration if other specific GABAergic cells die, though it would not be used to indicate PR death.

It may be that simply initiating regeneration is sufficient for the retina to repair itself. Therefore, perhaps by simply blocking GABA signaling in a damaged retina to produce progenitor cells, they will then go on to proliferate, migrate, and differentiate appropriately following endogenous cues. This is making a few assumptions, one being that the cues to initiate retina regeneration are independent from those regulating progenitor cell proliferation, migration, and differentiation. If, however, the regeneration initiation cues also inform MG about the type of damage and thus MG produce a progenitor cell fated to become a certain cell type, then progenitor cell populations would have to be heterogeneous in order to produce specific cell types depending on initiation cues.

This, however, is not supported by previous findings. Nagashima et al. (Nagashima et al., 2013) showed that differentiation occurs only if progenitors are in the correct position. In this study, N-cadherin was required for progenitor cell migration. The initial cell division by MG occurred in the ONL and progenitor cells used N-cadherin to migrate back down along the MG to continue to proliferate in the INL. Without N-cadherin, progenitor cells remained in the ONL. If PRs are damaged then regeneration proceeds as expected since the progenitors are in the correct place to differentiate, despite the defect in migration. However, if the GCL is damaged then progenitors fail to migrate to the damaged area and simply remain in the ONL/INL, undifferentiated. This suggests that cues to differentiate reside in the damaged area. Considering this, the more likely scenario is that there are distinct

stages of regeneration and progenitors that are produced can differentiate into any cells in the retina. Differentiation is dependent on where the progenitors migrate. This implies that damaged areas in the retina contain signals that direct progenitors to differentiate. These may be factors that are released or components that are exposed when cells are removed.

Sufficient damage is required to initiate regeneration

Another consideration based on the literature is the amount of damage that must occur to trigger regeneration. Mild damage paradigms do not activate MG to regenerate. Mild damage of rod cells only activates proliferation of rod precursors, not MG (Montgomery et al., 2010). Mild damage of the retina by ouabain injection also does not activate MG (Nagashima et al., 2013; Raymond et al., 1988). Therefore, there may be a threshold where damage must be severe enough for MG to become activated. An interesting question then is if damage is mild enough to avoid MG activation, but MG are stimulated to proliferate by injection of gabazine, do the resulting progenitors replace the lost cells? If they do, it suggests that MG activation is a limiting step to regeneration and that other signaling mechanisms in the retina are in place earlier. If not, then this suggests that sufficient damage is required for more steps than just MG proliferation. This could have an impact on inhibiting GABA in damaged mammalian retinas. Since mammalian retinas do not regenerate on their own, the assumption is that MG are not being activated correctly and if they were, then retinas would be able to regenerate. However, if progenitors do not migrate or differentiate correctly without sufficient damage, then this could mean

that other signaling patterns in the mammalian retina also need to be modified to utilize newly produced progenitors.

Conserved mechanism of neurogenesis

One interesting comparison is between neurogenesis in the mouse SGZ and regeneration in the zebrafish retina. In both systems, an apical glutamatergic cell synapses onto a GABAergic parvalbumin positive interneuron that has processes in close proximity to a glial stem cell that possess γ2 containing $GABA_A$ receptors. In both areas, decreased GABA results in proliferation of the glial stem cells (Song et al., 2012). Previous findings and those presented here are consistent with an evolutionarily conserved mechanism of initiation of neurogenesis. The inhibition of proliferation by GABA may also be found in other areas of the nervous system and should be further investigated.

GABA induced regeneration in the mammalian retina

The effect of $GABA_A$ receptor inhibition on MG proliferation in mammals should also be tested. As mentioned previously the mammalian and zebrafish retinas are very similar. However, the zebrafish retina can regenerate following damage while mammalian retinas cannot (Goldman, 2014). Shown here is a mechanism by which a decrease in GABA signaling initiates retina regeneration in zebrafish. It is possible that this neuronal communication pathway is not found in mammals. In favor of this is a finding reported here concerning the organization of the INL. In zebrafish, HCs form a monolayer at the top of the INL, adjacent to the

OPL. Further into the INL there is a tiny acellular area, followed by BCs. In contrast, mammalian INLs do not possess this gap in the INL, but rather have HCs and BCs co-mingled (Poche et al., 2007; Poché and Reese, 2009). This specialized layer in the zebrafish INL contains HC processes, indicating that HCs in zebrafish have processes that go up into the OPL to synapse with PRs, as well as down into the INL. This has also been shown previously (Connaughton et al., 1999). In mammals, HC processes only go into the OPL. Lastly, this specialized layer of the INL is a possibility of where MG and HCs communicate and how MG detect changes in GABA. All these findings combined suggest that GABA signaling in the mammalian retina may occur differently than in zebrafish, and may be a reason why mammals are not able to undergo retina regeneration, as MG cannot as easily detect changes in GABA levels. It is possible, therefore, that by antagonizing the $GABA_A$ receptor in the mouse retina, MG proliferation can occur. This experiment has been attempted by injecting gabazine into the mouse retina with limited success (data not shown). Proliferation increased in the mouse retina at the same time it increased in the zebrafish retina, though the change was modest. Further testing will need to be done to see if GABA inhibition in the mouse retina can result in MG proliferation.

Impact of HC activity on initiation retina regeneration

There are unanswered questions that need to be addressed to fully investigate this mechanism of retina regeneration initiation, specifically concerning the involvement of HCs. I have shown here that whole retina GABA levels decrease after retina injury. However, HCs are not the only cells that release GABA. Indeed,

GABAergic ACs are more plentiful than HCs. Therefore, it will be necessary to show that GABA levels decrease specifically in HCs following injury. To accomplish this, retinas can be injured and at different times during regeneration immunostained for GABA or GAD65/67, the rate limiting step in GABA synthesis. Based on my hypothesis, I expect that GABA levels will decrease in HCs following injury, possibly reaching the lowest point when MG begin to proliferate, the same time when GABA levels were significantly decreased, as measured by HPLC.

I have also shown here that HCs and MG are in close association with each other, and that a decrease in GABA signaling causes MG proliferation. However, it is still unclear if a decrease in HC GABA release causes MG proliferation. To test this, HC activity could be modulated using optogenetics. For example, halorhodopsin could be expressed specifically in HCs of undamaged retinas using the Lhx1a promoter. When activated, halorhodopsin will hyperpolarize the HCs, reducing GABA release. Reduced GABA release will be sensed by MG, causing them to spontaneously proliferate. As a second test, channelrhodopsin could also be expressed in HCs under the control of the Lhx1a promoter and the retina damaged. When activated, channelrhodopsin will depolarize the HC, resulting in increased GABA release. The elevated GABA levels will be sensed by MG, causing a reduction in proliferation. As the retina is a light sensing tissue, using a light based technique presents obvious and immediate confounds. The proper controls will need to be in place to verify that simply exposing the retina to a specific wavelength of light does not cause or inhibit proliferation, that the optogenetics proteins are working as expected, and that GABA levels are changing as expected.

$GABA_B$ receptor

Another consideration that has not been explored is the possible involvement of $GABA_B$ receptors. In the mouse hippocampus it was found that $GABA_A$ receptors are what convey the signal to the RGLs in order for them to proliferate (Song et al., 2012). In the current work I have shown that $GABA_A$ receptors are also involved in zebrafish retina regeneration. However, I have not considered the action of $GABA_B$ receptors in retina regeneration. $GABA_B$ receptors have been detected in MG of bullfrogs (Zhang and Yang, 1999) and, therefore, may also be involved in zebrafish retina regeneration. To assess the involvement of $GABA_B$, a set of experiments similar to those carried out for $GABA_A$ receptors could be attempted. Initially, immunostaining should be done to determine if $GABA_B$ receptors are found on zebrafish MG. Following, $GABA_B$ specific antagonists could be injected into undamaged eyes and proliferation assessed. Additionally, $GABA_B$ specific agonists could be injected into damaged eyes and proliferation assessed. If $GABA_B$ is found to affect MG proliferation that does not lessen the results presented here, but rather indicates that GABA signaling can influence initiation of retina regeneration in multiple ways.

Fate of newly produced progenitors

Another future consideration would be the fate of the progenitor cells produced after gabazine injection. Simply saying that GABA inhibition can produce progenitor cells is insufficient, the potency of the progenitors must be determined if

the goal is to help develop new treatments or targets for retinal diseases. Findings presented here suggest that the progenitors produced follow a similar path to regeneration. Furthermore, previous findings suggest that additional signals are required for differentiation of progenitors once they are produced (Nagashima et al., 2013). In order to test the potency of progenitors resulting from GABA inhibition by gabazine, a lineage tracing paradigm could be used to follow newly produced progenitors. Observing migration patterns and using immunohistochemistry to identify expression of cell specific markers would help determine what cells can be produced from the progenitors. It is possible that the progenitors will fail to differentiate and instead undergo apoptosis, which suggests that differentiation requires proper placement or cues for progenitor cells. If specific differentiation signals only occur after damage, then the produced progenitors will not receive any differentiation signal. To address this issue, gabazine injection could be performed after mild retina damage that is below the threshold to induce MG regeneration. This may provide the signals required for the progenitors to differentiate. If progenitors still do not differentiate then perhaps they do not possess the ability to differentiate or require further cues.

Intracellular changes downstream of GABA signaling

How GABA influences other factors involved in retina regeneration is an important future direction of this research. RNA levels of factors such as Leptin (Zhao et al., 2014b) and HB-EGF (Wan et al., 2012) have been shown to be upregulated as early as 1 hour post injury. However, HB-EGF upregulation may only

be specific to certain types of damage (Nelson et al., 2013). GABA may be involved in increasing transcription of these factors after damage. How $GABA_A$ inhibition affects transcription, though, is uncertain. As mentioned previously, $GABA_A$ signaling can cause activation of transcription factors such as CREB (Jagasia et al., 2009; Pallotto and Deprez, 2014; Ramirez et al., 2012) and affect epigenetic modifications such as γH2AX (Andang et al., 2008; Fernando et al., 2011) or other acetylation marks on enhancer elements (Kang et al., 2016). By inhibiting the $GABA_A$ receptor perhaps transcription of inhibitors of regeneration will be shut off, allowing activators to become upregulated. More directly related to the $GABA_A$ receptor, Ca^{2+} regulation may be altered. Ca^{2+} is an important second messenger and it is sensitive to intracellular voltage and can cause rapid changes within the cell. Inhibition of the $GABA_A$ receptor can increase membrane potential enough to modify Ca^{2+} levels. Downstream effects could include increased activity of kinases or phosphatases, which could go on to affect activity of transcription factors, epigenetic modifying enzymes, post-transcriptional or post-translational modifying enzymes, as well as any other molecules sensitive to phosphorylation status. These transcriptional changes would constitute a "first wave" of regeneration specific gene expression. Indeed the existence of enhancers only activated during regeneration has been shown (Kang et al., 2016) and GABA signaling may be involved in establishing open chromatin marks in these enhancers to initiate regeneration specific transcription.

Identifying second messengers and the "first wave" of regeneration specific transcription hold many possibilities for identifying novel therapeutic targets. In

regards to transcriptional changes, the best way to unbiasedly identify all transcriptional changes would be to utilize next generation sequencing. The most difficult task for this would be to identify the correct timepoints at which to prepare sequencing libraries. Based on injections of muscimol into damaged retinas, changes in PCNA can occur within 4 hours. Based on gabazine injections, PCNA levels increase by 48 hours. Depending on the way GABA signaling is manipulated may change the rate at which it affects transcription. As muscimol injection into a damaged retina results in the most rapid change in PCNA levels, this paradigm should be assessed first. To verify that muscimol injection results in a transcriptional change in PCNA, PCR could be performed to quantify levels of PCNA mRNA with and without muscimol injection. If mRNA levels are lower in muscimol injected retinas then the minimum amount of time for muscimol to be present could be more closely defined. Muscimol could be injected into retinas from 0-4 hours prior to enucleation in various intervals to determine the minimum amount of time required to elicit a transcriptional change in PCNA. At that point, sequencing libraries could be prepared and compared to identify genes that are differentially regulated with increased $GABA_A$ receptor activation after damage. Using that list, rescue experiments could be performed after gabazine injections. Gabazine could be injected into undamaged eyes coupled with up- or downregulation of $GABA_A$ sensitive genes and proliferation status assessed. If modification of downstream genes suppresses gabazine induced proliferation then it may be regulated by $GABA_A$ receptor activity. To further identify intracellular cascades, the mechanisms regulating expression of these genes should be explored.

Additional functions of GABA in retina regeneration

Beyond gaining a better understanding of how GABA affects initiation of retina regeneration, additional roles of GABA in retina regeneration may exist. Previously, GABA has been shown to affect progenitor cell proliferation in both the SVZ and SGZ (Berg et al., 2013; Giachino et al., 2014; Pallotto and Deprez, 2014; Quadrato et al., 2012; Quadrato et al., 2014; Ramirez et al., 2012; Song et al., 2013; Song et al., 2012). Signaling between progenitors or between GABAergic cells and progenitors has been found to facilitate progenitor cell survival, proliferation, and maturation. In fact, Song et al. (Song et al., 2013) followed their first finding of showing that a decrease in GABA signaling results in RGL proliferation by showing that increased GABA signaling from parvalbumin positive interneurons is important for progenitor cell maturation and survival. This two step process, where GABA first inhibits stem cell proliferation and then encourages progenitor cell survival and maturation could provide important directionality for the proliferation process. Once GABA levels decrease then RGLs become activated to proliferate. The resulting progenitors differentiate and become new glutamatergic granule cells. The new granule cells increase GABA release from parvalbumin positive interneurons that simultaneously suppress RGL proliferation and encourage progenitor cell survival and maturation. The secondary increase in GABA that originates from new granule cells indicates to the RGLs that sufficient proliferation has occurred and should now discontinue.

The same mechanism of a secondary GABA increase may also occur in the regenerating zebrafish retina and may be carried out by parvalbumin positive HCs.

Decreased GABA release, possibly from HCs, results in MG activation and proliferation. It has been shown previously that activated MG undergo IKNM and migrate to the ONL to divide (Lahne et al., 2015; Nagashima et al., 2013). Once produced, progenitors are in close proximity to HCs and could be influenced by GABA release from HCs. Further studies suggest voltage sensitive transcription factors that could be acting in progenitors. NFATc4 has been shown to be involved in progenitor cell survival in the SGZ (Quadrato et al., 2012; Quadrato et al., 2014). NFATc4 activity is voltage dependent and its activity was found to be modulated by $GABA_A$ receptor signaling where increased $GABA_A$ activity results in increased NFATc4 function. Considering the discovered similarities between the mouse SGZ and the zebrafish retina it is possible that NFATc4 is also acting in progenitors to facilitate survival and maturation downstream of $GABA_A$ receptor activation.

Additional mechanisms of initiating retina regeneration

Signaling through HCs

An additional consideration is other factors that may be involved in initiating retina regeneration. As mentioned previously, other neurotransmitters have been shown to influence neurogenesis, such as serotonin, DA, glutamate, acetylcholine, and glycine (Berg et al., 2013). All of these neurotransmitters are found in the retina, mainly utilized by ACs (Balasubramanian and Gan, 2014; Masland, 2012). It is possible that these neurotransmitters or cells affect GABA signaling as well. In fact, dopaminergic ACs have been suggested to regulate HC activity (Herrmann et al.,

2011). Perhaps, death of these cells would result in a decrease in GABA release, prompting the MG to regenerate.

Other neurotransmitters

Additionally, neurotransmitters utilized by other cells in the retina, such as DA, serotonin, and acetylcholine found in ACs, could act directly on MG to initiate retina regeneration. This could result in a few different outcomes, including activation of a central regeneration pathway or activation of a neurotransmitter specific pathway. The latter possibility is not supported by previous findings, as discussed earlier. Therefore, it is more likely that any neurotransmitter acting directly on MG would stimulate a central regeneration pathway. However, this could present difficulties later in regeneration, best demonstrated when considering AC death. The diversity of ACs could present a potential challenge to differentiation in that the progenitors must differentiate into the correct AC subtype. As mentioned previously, there may be cues present in the ECM or on adjacent cells directing the correct differentiation decision. Perhaps the ECM directs progenitors into areas where cells have since died. Once there, progenitors can sense the types of cells surrounding them. Subtypes of ACs display homotypic repulsion, where they do not overlap with cells of the same subtype (Kay et al., 2012). Considering this, ACs would differentiate into the correct cell type as processes from that cell type would not be in the vicinity due to previously established cellular location regulated by homotypic repulsion. Therefore, loss of a single neurotransmitter could still

activate a central regeneration pathway and also result in replacement of the correct cell types.

Juxtacrine, paracrine, or integrin signaling

Beyond neurotransmitters functioning to communicate damage in the retina, other signaling mechanisms may be used to communicate the message. As mentioned above, ACs display homotypic repulsion with cells of similar subtype. This can be accomplished using transmembrane proteins such as Megf10 and 11, as is the case with starburst ACs (Kay et al., 2012). Additionally, cells possess other transmembrane signaling proteins important for proliferation, such as Notch and N-cadherin, mentioned earlier. These signaling molecules could also be used to maintain contact with MG, and in their absence to inform MG of damage prompting them to regenerate. This could represent another, activity independent, method to communicate damage in the retina and initiate regeneration. MG are ideally placed to have such a mechanism in place, as they appear to be in contact will all cells (data not shown). Additionally, most cells in the retina display homotypic repulsion to maintain even distribution across the retina. As such, it is possible that these or other juxtacrine signaling mechanisms could be utilized by MG to survey the health of all cell types in the retina and respond promptly to damage. In addition to communicating directly with MG, cells could communicate with MG via integrins and the ECM. Indeed, the ECM has been shown previously to be important for health of GCs (Zhang et al., 2004), though the involvement of the ECM in retina regeneration has been vastly understudied.

Contact with other cells, such as endothelial cells of the vasculature, could affect neurogenesis. Previously it has been shown that contact with endothelial cells maintains quiescence of stem cells taken from the SVZ (Ottone et al., 2014). This is established by interaction of such proteins as Ephrin2b and Jagged1. It is possible, therefore, that in order for proliferation to occur, MG interaction with vasculature must be reduced. This brings up an interesting possibility concerning the effect of different damage paradigms on regeneration. Of the three damage paradigms mentioned previously (chemical, light, and physical), only physical damage to the retina disrupts vasculature tissue as well as neuronal tissue. Vasculature is present on both the inner and outer layers of the retina and in order for damage to occur by poking the retina at least one, and most likely both, layers of vasculature must be disrupted. This introduces additional complexity not present in the other two damage models. By damaging the vasculature there could be manual disruption of the interaction of MG with endothelial cells. Additionally, by disrupting vasculature any number of cells could be entering the retina via the blood stream. Importantly, these could include immune cells. As some of the findings, including the finding showing that Il-6 levels increase in the retina despite not finding a source of the Il-6, could be explained by immune cells or signals entering the retina via the vasculature (Zhao et al., 2014b). This may not be a problem if similar results were found after other methods of damage, but that does not appear to be the case. An additional finding after physical damage to the retina was increased levels of HB-EGF. HB-EGF was also sufficient to stimulate MG to proliferate in undamaged retinas (Wan et al., 2012). This finding was not replicated by other labs (Nelson et al., 2013). Lastly,

relevance of this damage model for finding mechanisms important to treat retinal disease in mammals is limited due to the vast difference in how the retina is damaged compared to what occurs in other damage models. Damage models involving light or chemicals are more likely to produce findings that are more directly relevant to mammalian systems.

Immune response to damage

Lastly, the immune system may be involved in initiation of regeneration in the retina. The main immune cells in the retina are microglia. Following damage, these cells function to phagocytose dead cells and debris in order to facilitate regeneration (Goldman, 2014). An important signaling molecule for microglia is the proinflammatory cytokine TNFα (Fischer et al., 2014), which has been found to act early in retina regeneration (Nelson et al., 2013).

In other tissues that undergo regeneration, components of the innate immune system have been found to be important for early stages of regeneration. In the zebrafish brain damage induced by a stab results in robust and complete regeneration. Early in regeneration leukocytes and microglia invade the damaged area, proliferate, and then exit in later stages of regeneration (Kroehne et al., 2011). Additionally, levels of the inflammatory cytokines Il-8, Il-1β, and TNFα were found to be elevated (Kyritsis et al., 2012). Inflammation was also found to be necessary and sufficient for brain regeneration (Kyritsis et al., 2012).

In the salamander axolotl, amputation of limbs result in upregulation of the same cytokines as well as Il-6, CCL2 and many other pro- and anti-inflammatory

cytokines (Godwin et al., 2013). Amputation of limbs in animals depleted of macrophages results in wound closure but a complete failure to regenerate. However, reamputation after macrophages have been added back results in complete regeneration (Godwin et al., 2013).

Microglia are activated by injections of Il-6 into chick eyes, where retinas display MG driven regeneration. In damaged retinas where microglia were ablated, levels of Ascl1a, TNFα, and Il-1β were reduced, indicating the importance of microglia increasing expression of these factors. Furthermore, retinas depleted of microglia failed to produce MG derived progenitors (Fischer et al., 2014).

In regenerating hair follicles in mice, a sufficient number of plucked hairs are required to initiate a regeneration response in the entire area. Once regeneration is induced, levels of CCL2, TNFα, Il-1β, and other inflammatory cytokines are upregulated. CCL2 increases early after plucking then decreases shortly after, while TNFα increases once CCL2 levels reach a maximum then is maintained at a high level. It was found that CCL2 is released from plucked hair follicles and acts to attract TNFα releasing macrophages. Once present, macrophages signal to each other and adjacent hair follicles to propagate the regeneration signal for a full response (Chen et al., 2015).

In diabetes, chronic wounds can occur that fail to heal, driven by macrophages that reside at the wound sight too long and prevent a pro-healing environment (Wicks et al., 2014). Based on this and earlier mentioned findings it is possible that once immune cells, such as microglia, act to stimulate regeneration, they then need to exit the area to allow regeneration to continue to later stages.

Considering the documented involvement of TNFα and Il-6 in retina regeneration, as well as the observation that microglia are present at the site of damage early and that sufficient damage is required to elicit MG proliferation, it is possible that the findings from other regenerating tissues could also hold true in regenerating retinas (Conner et al., 2014; Nelson et al., 2013; Zhao et al., 2014b). In this case, retina damage may release pro-inflammatory cues, such as CCL2 or Il-6, which are attractive to microglia. When the amount of damage and pro-inflammatory cues are high enough, microglia will invade the damaged area, phagocytose debris and dead cells, and release TNFα. TNFα will signal to adjacent MG that damage has occurred, initiating regeneration. Microglia will then exit the area and stop releasing pro-inflammatory cytokines, which allows retina regeneration to proceed.

Previous findings are consistent with this hypothesis. TNFα was found to be upregulated following damage, and knocking down TNFα suppressed regeneration (Nelson et al., 2013). The interpretation of this data was that TNFα was released from dying cells and sensed by MG. Based on the findings summarized here it may be that damage is sensed by microglia and they are the source of TNFα that stimulates retina regeneration. This is similar to findings in the chick retina that show the involvement of microglia in forming MG derived progenitors (Fischer et al., 2014).

Il-6 was shown to be upregulated following damage, though a source of Il-6 was not detected. It is possible that Il-6 is coming from dying cells or from invading cells from the vasculature, since the observation with Il-6 was after physical damage

to the retina. The involvement of microglia in retina regeneration is not very well understood but more attention should be given to these cells in regeneration as it is likely that innate immune cells are involved in many different types of regeneration. This could represent a parallel and important pathway to GABA signaling that initiates retina regeneration.

Summary

I have shown a novel mechanism regulating initiation of retina regeneration in zebrafish. Levels of the inhibitory neurotransmitter GABA are detected by MG in the retina. When levels decrease, such as following PR death, MG sense the decrease in GABA via $GABA_A$ receptors and initiate regeneration. I found that $GABA_A$ receptor inhibition is necessary and sufficient for retina regeneration and that MG directly sense changes in GABA. These findings inform about what regulates regeneration in the zebrafish retina and could be used to develop new therapies to treat retina diseases and damage in humans. Much more work still needs to be done to fully investigate the intricacies of these findings as well as other regulatory mechanisms involved in zebrafish retina regeneration. Together with previous studies, these findings have helped identify an important step in retina regeneration.

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