Temporal differences in the recovery of visual function after the selective ablation of blue or UV cone photoreceptors in larval zebrafish

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology, Cell & Developmental Biology

Department of Biological Sciences

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Abstract

Retinal degenerative diseases are an irreversible cause of vision loss and blindness caused by the loss of photoreceptors, which are unable to regenerate in the adult human retina. Cone photoreceptor loss is especially debilitating as cones mediate high acuity vision and colour perception. Retinal stem cell therapies that reintroduce photoreceptor progenitors into the degenerate retina have the potential to restore lost cone-mediated vision. However, once in the host retina, these progenitors produce functional cones but fail to assume the cone photoreceptor fate and integrate in the host retina with a high enough efficiency to restore lost cone-mediated (daytime) visual function. These studies have been preformed in mice however; their retina may not be a suitable environment for cone neurogenesis and differentiation, as they possess a rod-dominated retina. Zebrafish are an excellent model to complement studies performed in mice. Zebrafish are a preeminent model of cone neurogenesis because they possess a cone dense retina capable of robust regeneration of lost retinal cells following retinal lesion, and in adulthood they continue to produce photoreceptors at the peripheral margin of the retina. In addition, this regenerative response has the capacity to functionally recover vision.

We have engineered two novel transgenic/chemical models of photoreceptor regeneration in zebrafish to conditionally ablate either ultraviolet (UV) or blue cones. We used the nitroreductase-metronidazole method of cell specific ablation in transgenic zebrafish in which the bacterial enzyme nitroreductase (NTR) is expressed in either UV or blue cones. In the presence of the prodrug, metronidazole (MTZ), NTR-expressing cone photoreceptors were ablated due to conversion of MTZ into a cell autonomous cytotoxin that induced cell death. Regeneration of the ablated cone subtype occurred after prodrug removal. To assess whether cone ablation detrimentally affected vision and whether cone regeneration functionally recovered the vision

deficit, we used a visually mediated behavioural assay known as the optomotor response (OMR), to detect changes in visual ability based on the visually mediated swimming behaviour. UV or blue cone loss immediately affected the visual ability of larval zebrafish, as determined by decreased OMR response. Following UV cone loss, recovery of visually mediated behaviour occurred gradually and was dependent on the generation of new UV cones suggesting that photoreceptors have integrated with the pre-existing circuitry and restored the OMR behavioural circuit. In contrast, the recovery of visually mediated behaviour occurred rapidly following ablation of blue cones and was independent of the blue cone generation. Recovery of OMR behaviour prior to blue cone photoreceptor regeneration was unexpected, which may suggest that the OMR behavioral circuit has been restored using an unforeseen mechanism and is not dependent on blue cone input. I speculate that the contrast in the timeframe for recovery of vision and the necessity of regenerated photoreceptors between UV and blue cone ablation models indicates that the retina reacts differently based on the subtype of cone photoreceptor lost. Previous research has shown that UV cone ablation triggers a robust regenerative response tailored to regenerating UV cones. In comparison, blue cone ablation triggers a proliferative response, yet few newly generated cones were observed assuming the blue cone fate. As a result of the limited blue cone regenerative response, I speculate that the retina may undergo rapid retinal remodelling to compensate for blue cone loss and restore visual ability. In conclusion, my work characterized: two novel models of conditional ablation targeting UV or blue cone photoreceptors, documented the recovery of visual function by observing visually mediated behaviour following cone loss, and regeneration of the ablated cone subtype. This study, which examined functional regeneration of cone photoreceptors in zebrafish, will contribute to the current body of knowledge surrounding the mechanisms driving cone photoreceptor fate, integration of new photoreceptors, and cone regeneration.

Preface

This thesis is an original work by Gordon F Hagerman. Approval for this study, including zebrafish maintenance and breeding, approved as protocol AUP00000077 by the Animal Care and Use Committee: BioSciences, which is an Institutional Animal Care and Use Committee at the University of Alberta, and which operates under auspices of the Canadian Council on Animal Care.

Chapter 2 and 3 of this thesis contain material that has been accepted pending minor reviews as G.F. Hagerman, N.C.L Noel, S.Y. Cao, M.G. Duval, A.P. Oel, W.T Allison, "Rapid recovery of visual function associated with blue cone ablation in zebrafish," PLOS ONE. I was responsible for designing and assembling a behavioural assay to test visual mediated behaviour. I preformed data collection and analysis regarding the optomotor response, and spontaneous behaviour assay, as well as characterizing our models of blue and UV cone photoreceptor ablation. Additionally I was responsible for manuscript composition. N.C.L Noel collected data and analyzed data regarding off-target effects of our novel model and analyzed transgene penetrance, as well as contributing to manuscript composition. S.Y. Cao collected and analyzed behavioural data. M.G. Duval, and A.P. Oel designed and established transgenic zebrafish lines and contributed to manuscript edits. W.T Allison was the supervisory author and was involved experimental design, data collection and manuscript composition.

Acknowledgement

Thank you to my supervisor, Dr. W. Ted Allison in the department of Biological Sciences at the University of Alberta, for giving me my start in research and the opportunity to join the Allison lab. Thank you for always being available to discuss my research and for your expert advice and mentorship, you taught me how to be a better scientist.

Thank you to my committee member Dr. Yves Sauvé and Dr. Keith Tierney for their expert feedback and guidance throughout my project. Thank you Dr. Yves Sauvé for your incredible effort and time spent helping me with the electroretinogram, and for teaching me about retina remodelling in the mammalian retina. Thank you Dr. Tierney for your advice and for lending me equipment for the ERG as well as access to your lab to preform behavioural analysis.

Thank you to my fellow members of the Allison Lab, their support to my research was invaluable as was their friendship. Thank you to Phil Oel for his support in my research and teaching me many of the techniques required for my project. Thank you to Nicole Noel for her support and collaborating on characterizing UV/blue cone ablation. Thank you to Sylvia Cao for her help performing behavioural analysis.

Thank you Department of Biological Sciences at the University of Alberta for the Teaching Assistantship. Thank you to the Faculty of Graduate Studies and Research at the University of Alberta for financial support.

Finally, thank you to my parents, girlfriend, friends and family for providing their continuous support and encouragement throughout the years. This would not have been possible without them. Thank you.

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List of Abbreviations

AMD	age related macular degeneration
BC/ RBC	(rod) bipolar cell
CMZ	ciliary marginal zone
Dpf	days post fertilization
DMSO	dimethyl sulfoxide
EdU	ethynyl deoxyuridine
EFTFS	eye field transcription factors
ERG	electroretinogram
GFP	green fluorescent protein
INL	inner nuclear layer
IPL	inner plexiform layer
MTZ	metronidazole
NTR	nitroreductase
OKR	optokinetic Response
OMR	optomotor response
ONL	outer nuclear layer
OPL	outer plexiform layer
PTU	phenol thiourea
RGC	retinal ganglion cell layer
rh1	rhodopsin
RP	retinitis pigmentosa
RPE	retinal pigment epithelium

TG	transgene
TH	thyroid hormone
trβ2	thyroid receptor beta 2
UAS	upstream activating sequence
UV	ultraviolet
VBA	visually mediated background adaptation
opn1lw1, opn1lw2/ lws1, lws2	opsin 1; long-wave-sensitive 1, 2
opn1mw1, opn1mw2/ rh2-1, rh2-3, rh2-4	opsin 1; medium-wave-sensitive 1, 2
opn1sws1/sws1	opsin 1; short-wavelength sensitive 1
opn1sws2/sws2	opsin 1; short-wavelength-sensitive 2

Chapter 1. Introduction

Photoreceptor degeneration is an irreversible cause of vision loss and blindness because mammals cannot regenerate photoreceptors. Photoreceptors are the sensory neurons that receive light stimuli, which they convert into an electrochemical signal via phototransduction. There are two types of photoreceptors rods and cones; rods mediate high-sensitivity night vision, whereas cones are essential to high-acuity daytime vision. Zebrafish have a cone photoreceptor-dominated retina and unlike humans and other mammals, have a robust capacity to regenerate any cell in the retina lost to injury [1, 2]. This regenerative response following retinal injury can restore visual function in both larval and adult zebrafish [3, 4]. Currently, little is known about the mechanisms and biochemical signals necessary for the integration of regenerated photoreceptors into the pre-existing retinal circuitry.

1.1 Retinal degenerative diseases lead to permanent vision loss and retinal remodelling

Retinal degenerative diseases involving the death of the retinal photoreceptors are a major cause of vision loss and blindness. Retinitis pigmentosa (RP), a common inherited disease, and age-related macular degeneration (AMD), a multifactorial disease involving interplay between various genetic and environment factors, are the two most common photoreceptor degenerative diseases and are leading causes of blindness in the developed world [5, 6]. Loss of cone photoreceptors in the macula, which contains the densest population of cone photoreceptors, results in the loss of high-resolution central vision. RP initially results in peripheral vision loss and night blindness as the rods die in humans. Progressive rod loss leads to retinal degeneration and loss of cone photoreceptor in the macula.

Photoreceptor degenerative diseases such as RP and AMD do not solely affect the sensory photoreceptor layer of the retina. With the loss of photoreceptors, the downstream neurons respond to the lack of afferent input and begin to remodel, changing the retinal environment at molecular, cellular, and neural circuit levels [7-11]. This corruptive remodelling dramatically changes the retinal environment and poses a fundamental problem for therapies aiming to restore visual function due to the lack of synaptic targets for reintegrating photoreceptor progenitor cells in patients with advanced retinal degeneration [12].

1.2 Emerging therapies for photoreceptor degenerative diseases

Recently, several therapies have emerged with the potential to limit progression of photoreceptor degeneration and restore previously irreversible vision loss and blindness caused by photoreceptor degenerative diseases. The major steps for successful retinal repair and vision restoration are: generation and proliferation of stem cells, migration, differentiation, synapse formation, and cell survival. Each therapy has a unique approach, but they all attempt to restore the photoreceptor layer, so the photoreceptors can integrate with the remaining retina to restore vision. Stem cell based therapies aim to replace the lost photoreceptors via transplantation or endogenous regeneration, while gene therapy utilizes corrective gene-replacement to suppress and supplant a pathogenic mutation to prevent further photoreceptor loss or gene augmentation to insert a healthy gene. An alternative to cell-based therapies is retinal implants; these prostheses have the same goal as cell-based therapies, replacement of rod and cone photoreceptors. The retinal implant replaces photoreceptor function using an array of electrodes in conjunction with an external camera and image-processing device to control stimulation of the interneurons. Demonstrating that the underlying retinal circuitry driving image-processing stays relatively intact at certain stages of retinal degeneration [13]. A mouse model of eye transplantation has

been initiated, however there is yet no visual function in the transplanted eye due to the difficulty of regenerating the optic nerve [14].

1.2.1 Stem cell replacement therapy for photoreceptors

Retinal stem cell replacement therapies have already begun to transplant rod photoreceptors precursors into various mouse models of inherited photoreceptor degeneration [15-17]. Transplantation of post-mitotic photoreceptor precursor cells successfully restored rod-mediated vision to a mouse model of congenital stationary night blindness [17]. The degree of vision restoration was dependent on multiple factors: efficiency of migration to the site, proper integration with interneurons, and prolonged survival of the transplanted cells [18]. Progress towards developing a cone photoreceptor replacement therapy has been challenging: injected Crx expressing photoreceptor precursors favor the rod fate due to the recipient's adult environment, and the number of integrating mature cones in the retina is poor [19, 20]. The efficiency of cone photoreceptor precursor integration was greatest in a cone deficient retina [19]. Various steps have been taken to maximize the integration rate of transplanted cells, such as optimizing the transplanted cell type, enriching the number of putative cone photoreceptors [21, 22]. Thus far the limited integration of cone photoreceptors has provided proof of concept that cone replacement therapy is feasible; however further research is required to achieve robust integration of a significant number of transplanted cone photoreceptors before functional recovery of cone-mediated vision is possible.

1.2.2 Endogenous regeneration of photoreceptors

Stem cell therapy using endogenous retinal stem cells is a developing field in mammals with the potential for translation to human therapies. Endogenous regeneration has been well documented in fish and amphibians [23-25]. Stem cell populations that contribute to endogenous

regeneration include: Müller glial cells, which mediate a robust regenerative response in teleosts such as zebrafish (further discussed in zebrafish retinal regeneration) as well as in the chick retina, however in contrast to fish only a subset of neurons can be regenerated [24, 26], and the retinal pigment epithelium, which transdifferentiates to regenerate the retina in amphibians [27]. Endogenous regeneration in mammals is relatively limited; drug therapy is required to increase the number of functional regenerated neurons. Recently it was demonstrated that human Müller glia retain some morphological stem cell-like properties, and can differentiate and express rod photoreceptor markers such as *CRX, NR2E3*, and rhodopsin *in vitro* when stimulated with growth and differentiation factors (*bMP, FGF2*, taurine, retinoic acid, and *IGF-1*) [28]. When subretinally transplanted, these treated human Müller glia can migrate to the inner nuclear layer and rescue some of the scotopic photoreceptor response (observed using electroretinography) in a transgenic rat model of retinal degeneration (rhodopsin mutation: P23H-1) [28]. These cells did not adopt a rod-like morphology or develop outer segments. Further research demonstrated that forced expression of the proneural transcription factor *Ascl-1* was sufficient to reprogram mouse Müller glia cells giving them neurogenic properties [29]. *Ascl-1* was identified because it was, upregulated immediately in the Müller glia of the teleost retina following injury, but not upregulated in the mouse retina. Transgenic Müller glial cells expressing *Ascl-1* in the mouse retina can induce a proliferative response generating photoreceptors, bipolar cells, and amacrine cells following injury, reminiscent of teleost retinal regeneration [30]. Further evidence suggests that epigenetic changes in chromatin may be responsible for an attenuated regenerative response in mature mice [30]. Zebrafish retinal injury induces expression of DNA methylation/ demethylation genes in Müller glia; decreasing methylation of the promoters driving expression of pluripotency factors [31], nonetheless zebrafish also experience a decreased regenerative response with age.

1.2.3 Photoreceptor gene therapy

Gene therapy has emerged as a potential technique for replacing pathogenic genes in affected retinal neurons to restore vision. Preliminary gene therapy using recombinant adeno-associated virus vector to transfect retinal pigment epithelium and investigate the effect of *RPE65* gene therapy in Leber's congenital amaurosis or *CHM* in choroideremia are promising, as they have shown a limited recovery of visual acuity [32, 33]. However, treatment of photoreceptor degenerative diseases caused by a genetic flaw in the photoreceptors has proven to be more challenging, due to the diverse number of genetic mutations in diseases such as retinitis pigmentosa [34]. Gene augmentation therapy has been performed for X-linked retinitis pigmentosa in dogs; therapy preserved retinal function and prevented photoreceptor death in treated regions. X-linked RP caused retinal remodelling, however in treated regions remodelling was reversed or prevented based on the time of disease onset and treatment [35]. Gene therapy may not be a viable option for advanced photoreceptor degeneration where photoreceptor survivorship is low.

1.3 Zebrafish as a model of cone neurogenesis

The zebrafish is an ideal model organism to study cone regeneration in the vertebrate retina. Zebrafish are a diurnal teleost that have a cone dense retina with four different cone subtypes (tetrachromatic vision) each with their respective visual pigments called opsins: red-sensitive cone opsin (opn1lw1, opn1lw2 also called lws 1, lws2), green-sensitive cone opsin (opn1mw1-4 or mws1-4), blue-sensitive cone opsin (opn1sws2 or sws2), and UV-sensitive cone opsin (opn1sws1 or sws1) as well as rod photoreceptors (rh1) [36, 37]. Studying cone neurogenesis in zebrafish effectively complements the mouse model, which is well-adapted to studying rod photoreceptor neurogenesis because of their rod-dominated retina adapted for a

nocturnal lifestyle [37]. Structurally, the vertebrate retina is highly conserved, except that humans possess a fovea, which mediates high acuity central vision. The fovea is absent in zebrafish; nonetheless the zebrafish retina remains a relevant model for cone studies because their retina is effectively fovea-like with a high cone density.

As a model organism zebrafish are advantageous because of their small size, ease of maintenance, rapid development, and high fecundity. In addition, there is a wealth of genetic tools available making zebrafish an ideal genetic model. Furthermore, visual monitoring of development is simple due to external fertilization and a transparent egg and embryo. There are a numerous established behavioural tests that can be evaluated in very young fish, and it is simple to administer pharmaceuticals into their tank water.

1.4 Zebrafish retinal development

The neural retina develops from a bilayered optic cup formed from the invagination of the neural epithelium. The inner layer of the optic cup develops into the neural retina, while the outer layer becomes the retinal pigment epithelium. The peripheral margin of the optic cup develops into the ciliary epithelium. The early embryonic retina is composed of proliferating progenitor cells that are responsible for the expansion of the retina to achieve the correct size. These progenitor cells are multipotent retinal stem cells that express a unique set of eye field transcription factors (EFTFS) such as Rax/Rx3 (zebrafish homolog: *rx3*), Pax6 (*pax6a* and *pax6b*), Six3 (*six3b*), Lhx2 (*lhx2b*), Otx2 (*otx2*), and Ath5 (*atoh7*) to establish the fate of the neural epithelium as presumptive retina [38]. These retinal progenitors will subsequently generate the five types of neurons present in the retina: retinal ganglion cells, (RGCs), amacrine cells, horizontal cells, bipolar cells and photoreceptor cells, as well as a glial cell: Müller glia (Fig. 1). These cells stratify to three laminar layers: the ganglion cell layer (retinal ganglion cells), inner

nuclear layer (amacrine, bipolar, and horizontal cells), and the outer nuclear layer (photoreceptors), while Müller glial cells span all three layers (Fig. 1)[39].

Retinal progenitor cells generate the various cell types in a fixed chronological order, which is conserved across various vertebrate species. The first wave of neurogenesis produces retinal ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells, followed by a second wave of rod photoreceptors, bipolar cells, and lastly Müller glia. The order of cell appearance suggests there may be a temporal mechanism that defines progenitor cell competence. Recent studies have suggested that this mechanism is intrinsic as *atoh7* is required for the proliferative to neurogenic state switch in early retinoblasts independent of external factors, yet environmental modulation could still be occurring [40]. The seven cell types can be further subdivided into approximately 80 anatomically and physiologically distinct retinal cell types, which make up at least 20 separate circuits in the retina [41].

Retina cell differentiation occurs in waves that originate in the central retina and spread to the periphery; retinal lamination quickly follows as neurons migrate to the correct layer, extend axons and dendrites and integrate into the retinal circuitry. Layers of neuronal synapses border the 3 neuronal layers forming the inner plexiform layer, and the outer plexiform layer. Establishment of retinal circuitry is thought to follow the paradigm that dendritic overgrowth first occurs, which is subsequently followed up by pruning of inappropriate synapses [42]. This is true for the development of zebrafish retinal circuitry, but it also displays targeted-lamination of retinal neurons with minimal pruning [43]. Retinal remodelling occurs naturally during development and is not solely a symptom of retinal degeneration. Retinal ganglion cells utilize different strategies to develop the proper stratification of their dendrites. For example, some develop a multistratified dendritic arbour spanning the ON and OFF sublayers of the inner plexiform layer, and undergo arbour trimming in the mature retina to become mono-stratified to

one of the sub-layers [43, 44]. Other retinal ganglion cells grow in a biased manner and do not require remodelling, as they integrate precisely with pre-existing amacrine cell processes in a mature stratified pattern [43]. ON bipolar cells in zebrafish demonstrate a trial-and-error process wherein axonal projections extend and retract throughout the IPL before concentrating at a single point [45].

Proper development of the retina depends on the continuous generation of retinal progenitor at the ciliary marginal zone (CMZ), these stem cells recapitulate the development order and differentiate sequentially. Therein, cone photoreceptors are continuously generated in zebrafish due to persistent neurogenesis in fish ([46]) [47]. Rod photoreceptors are generated from a separate cell lineage and follow a different developmental pathway than that of cones. As the retina grows there is gradual stretching resulting in greater spacing between photoreceptors, rods are then generated from a distinct pool of rod precursors derived from Müller glia to maintain visual sensitivity [2, 48, 49]. Zebrafish are tetrachromats, which utilize four cone subtypes: UV (*opn1sw1* or *sws1*), blue (*opn1sw2* or *sws2*), green (*opn1mw1*, *opn1mw2*, or *rh2-1*, *rh2-3*, and *rh2-4*) and red-sensitive (*opn1lw1* and *opn1lw2 or lws1* and *lws2*) cones. Recent studies have focused on the signaling pathways and gene regulatory networks that determine photoreceptor cell fate as well as the fate of the various subtypes. *Otx2* and *crx* are two key factors required for photoreceptor cell fate and differentiation [50]. *Otx2* is upstream of *trβ2*, a critical transcription factor in red cone fate in zebrafish [51]. *Tbx2b* has an important role in promoting UV cone fate, while repressing the rod differentiation pathway [9]. *Gdf6a* is an upstream regulator of *tbx2b*, and is involved in the development and or maintenance of blue cone fate [52]. *Six7* is essential for development and potentially maintenance of green cones [53]. Additionally, *trβ2* has been used to show that red cone precursors undergo symmetric terminal

divisions generating a pair of L-cones [54]. UV, blue and green cones each have their own dedicated retinal precursors.

1.4.1 Zebrafish retinal mosaic

As previously mentioned the zebrafish retina is organized into a precisely stratified laminar structure. This organization is complemented by a nonrandom mosaic of neurons observed in each layer, and is most dramatically illustrated by the precise cone photoreceptor mosaic observed in the adult zebrafish retina [48, 55, 56]. The photoreceptor mosaic is composed of alternating UV cones and blue cones in parallel to a row of red and green double cones. The double cones are precisely aligned so that the red cones flank blue cones, while green cones flank UV cones (see Fig. 3). The larval photoreceptor mosaic is not as precise as is observed in adulthood, but the larval cones subtypes possess a heterotypic spatial relationship such that they are not randomly distributed relative to other cone subtypes [55]. The precise spatial arrangement of cone photoreceptors makes zebrafish an ideal model to study cone regeneration, as the ratios of photoreceptors are known and any disruption is easily evident. Recent studies have suggested the Crumbs (crb) complex may mediate homophilic and heterophilic cell-cell adhesion between cone photoreceptors and Müller glia, and could be involved in cell signaling and patterning of the cone photoreceptor mosaic [57].

1.5 Zebrafish retinal regeneration

1.5.1 Ciliary marginal zone and indeterminate growth of the retina

The ciliary marginal zone (CMZ) is a ring of cells located at the periphery of the maturing retina composed of retinal stem cells and progenitor cells. Retinal cells are continuously added to the margin of the maturing retina as retinal stem cells and progenitor cells differentiate [58]. All

vertebrates possess stem cells at the CMZ, but these proliferating cells are typically depleted by adulthood [59]. However, in amphibians and fish that grow indeterminately this stem cell population continuously self-renews as the retina expands. Recent studies have investigated the genetic factors involved in the balance between cell proliferation and differentiation and found that the Wnt signaling is essential to maintain the proliferative progenitor cell population and inhibit differentiation [60]. Conversely sonic hedgehog signaling is involved in arresting proliferation and promoting cell cycle exit [61].

The role of the CMZ in regeneration is contentious; in *Xenopus* retinal injury stimulates proliferation of the CMZ, but regeneration is primarily mediated by transdifferentiation of the retinal pigment epithelium [62-64]. In zebrafish the CMZ may be responsible for regeneration at the periphery of the retina, as there is a difference in the density and pattern of regenerated photoreceptors in the peripheral and central retina following cytotoxic treatment. This suggests zebrafish have two separate regenerative sources [65]. The ciliary epithelium, a mammalian equivalence of the CMZ, retains some neurogenic properties that make it an attractive candidate for the induction of endogenous regeneration. Recent attempts at producing differentiated cells from this source have been unsuccessful, but the potential given the correct environment remains [64, 66].

1.5.2 Role of Müller Glia in the regenerative response

Müller glia span the entire neural retina and function as support cells providing homeostatic, metabolic support as well as structural integrity to the retina [67]. It was recently shown that Müller glia have an important neurogenic role in the developing retina as they continuously proliferate, producing retinal progenitors that migrate radially from the inner nuclear layer to the outer nuclear layer [49]. Additionally, Müller glia have an important

regenerative role in response to retinal injury. Müller glia undergo an asymmetric self-renewing division, generating a dedifferentiated retinal progenitor, which then proliferates to form neurogenic clusters of multipotent retinal progenitor cells that are closely associated with the Müller glia in the inner nuclear layer, thereby providing a stem cell niche [23, 24]. These retinal progenitors then migrate along the radial fibers of the Müller glia to reach the correct layer and regenerate missing neurons. This regenerative response is sufficient to restore functional vision following cytotoxic lesioning or light ablation to the zebrafish retina [3, 4]. Interestingly, Müller glia "reprogramming" and dedifferentiation suggests that regeneration may be epimorphic regeneration, as it involves many of the same switches for proliferation and differentiation as development [67].

1.6 Retinal regeneration following various methods of retinal neuron death

Various methods have been utilized to successfully induce regeneration in zebrafish. The first experiments successfully induced retinal regeneration in zebrafish using surgical excision of the retina [4, 68]. This method was used to characterize the regenerative response, but lacked the specificity of lesioning necessary to address questions regarding gene regulatory networks and mechanisms involved in cell fate determination, or functional integration of newly regenerated cells. Methods such as neuron ablation using the neurotoxin ouabain or light lesioning give a certain measure of control of the specificity of cell ablation, but these methods are time and labor intensive, limiting the feasibility of large-scale analysis [69]. Novel techniques utilizing transgenic approaches to target and induce cell death provide specificity of lesioning with unparalleled target fidelity, permitting the study of the regeneration of a single cell type in an intact retina.

1.6.1 Surgical lesioning

Surgical lesioning of the retina successfully stimulates a proliferative response at the lesion site and in the surrounding tissue. Following the injury the retinal neurons regenerate in approximately the correct proportions and restore the retinal circuitry [68, 70]. However, regenerated photoreceptors fail to reposition in the spatial organization of the photoreceptor mosaic [56]. Ultimately, retinal regeneration following surgical lesioning in teleosts is sufficient to recover the photoreceptor and interneuron electroretinogram response as well as the dorsal light behavioural response, thereby demonstrating recovery of visual function [4].

1.6.2 Neurotoxin mediated cell death

Intravitreal injection of neurotoxin ouabain (Na-K ATPase inhibitor) can be used to kill retinal neurons. The concentration of the neurotoxin can be manipulated to provide a relative measure of tissue specificity. At high doses the neurotoxin penetrates further into the neural retina causing death in all layers, while at low doses it only affects the inner retinal layers leaving the photoreceptors relatively intact [71, 72]. Ouabain treatment triggers asymmetric division of the Müller glia to self-renew and generate a retinal progenitor cell [73]. Regeneration is functional as the visually mediated behaviour recovers; as well, the electroretinogram (ERG) recording recovers. A photoreceptor response (a-wave) and interneuron response (b-wave) were detectable 50 days post-injury, demonstrating that regenerated cells have integrated into the retina [3, 74].

1.6.3 Light lesioning

Light lesions specifically induce photoreceptor apoptosis by increasing the generation of reactive oxygen species and heat. In fish, this induces robust photoreceptor regeneration, specifically from Müller glia [75]. There are two separate paradigms for light ablation: chronic

bright light exposure and acute UV light exposure. The chronic or constant light treatment predominately affects rod photoreceptors leaving cones largely unaffected, while acute UV exposure affects both rods and cones. These light mediated photoreceptor ablation treatments do not affect all regions of the retina, and leave the ventral posterior retina relatively intact [76, 77]. Acute light exposure followed by constant light treatment results in significant cone and rod loss in the dorsal and ventral retina [76].

1.6.4 Photoreceptor degeneration

Retinal regeneration has also been studied using genetic models of chronic cone or rod photoreceptor degeneration in zebrafish. Rod degeneration in the XOPS-mCFP line occurs as a result of the toxic effect of a fluorescent protein expressed specifically in rods [78]. Rod degeneration in XOPS-mCFP does not stimulate a significant proliferative response from Müller glia, but increases proliferation of the rod progenitor cell population in the ONL. By comparison, the cone degeneration model, caused by a mutation in the cone specific phosphodiesterase gene *pde6c*, stimulates proliferation from Müller glia in the INL [49]. Demonstrating that these two distinct types of photoreceptors are regenerated using separate pathways.

Genetically inducing rod or cones photoreceptor loss is an effective method to specifically study regeneration of the respective photoreceptor types. However, genetic degenerative photoreceptor diseases such as mammalian RP have indirect effects such as the secondary loss of cones photoreceptors when the predominant rod photoreceptors die off [6]. In comparison, zebrafish do not experience secondary cone degeneration when rods degenerate using the XOPS-mCFP transgenic zebrafish line [49, 78]. This is most likely attributed to the cone rich retina of the zebrafish; because degeneration of cones in the *pde6c* zebrafish model can cause secondary rod degeneration in areas of low rod density. Areas with high rod density are resistant to

secondary rod loss [78]. Larval zebrafish with an increased number of rod photoreceptors, using the *tbx2b lots-of-rods* (*lor*) phenotype, do not experience secondary rod loss during cone photoreceptor degeneration [79]. Altogether this suggests that the density of surviving photoreceptors is neuroprotective.

1.7 Retinal remodelling during retinal degeneration

Retinal remodelling occurs as a result of the loss of afferent activity from photoreceptors and has been a major focus of murine studies [7, 8, 80]. Remodelling of the retina results in a cascade of changes at the molecular, cell and tissue level, and has serious implications limiting the potential success of regenerative therapies such as stem cell replacement therapy and even gene therapy. Research on retinal remodelling has focused on therapies to slow and attenuate negative plasticity to minimize the potential corruptive changes so that rescue can be successful. However, retinal remodelling cannot be classified solely as a negative symptom of retinal degeneration. As mentioned previously, retinal remodelling occurs naturally during development [43-45], and occurs throughout the day with light and dark adaptation [81], however the functional importance of retinal remodelling poorly understood especially in the context of regeneration. Therefore, it is important to improve our understanding of the significance of retinal remodelling in facilitating regeneration and recovery of vision in zebrafish.

1.7.1 Stereotypical characterization of neural remodelling

Photoreceptor degeneration, regardless of the initializing method, results in retinal remodelling [8]. Retinal remodelling is progressive, and in mammals retinal remodelling follows a stereotypical pattern that can be broken down into three phases. First, there are changes in the types of neurotransmitter receptors expressed accompanied by shifts in expression of synaptic proteins, as well as mislocalization of opsins, which indicates photoreceptor stress prior to

photoreceptor cell death [7, 82, 83]. Second, photoreceptor synaptic terminals degenerate, photoreceptors die, outer nuclear layer thins, and deafferentation occurs through the retraction of dendrites [8, 84]. Anomalous neurite sprouting of second order neurons to novel targets in the outer nuclear layer or inner plexiform layer is another commonly observed phenotype present during late stage remodelling. Sprouts often form ectopic synapses with novel partners [85, 86]. The final stage of retinal remodelling results in a massive reorganization of the retina as surviving neurons migrate and aggregate causing the retinal layers to become disorganized and forming neuropils. In summary, retinal remodelling dramatically changes the retinal environment at the molecular, synaptic, cell, and tissue levels.

1.7.2 Retinal remodelling during regeneration

Degeneration of photoreceptors is especially problematic in mammals because photoreceptor loss is permanent, and it leads to progressive pan-retinal degeneration with remodelling of the retinal interneurons. Recent studies have shown that retinal remodelling during development and in diseases varies across species and even zebrafish, a species able to regenerate the retina, experience remodelling of secondary interneurons following loss of cone photoreceptors [9, 10, 87]. However, zebrafish are able to restore the original circuitry pattern of horizontal cells following regeneration of the primary afferent input [88]. H3 Horizontal cells normally synapse with UV or blue cones, but when blue cones are selectively ablated the H3 horizontal cells form sprouts reminiscent of mammalian sprouting. These sprouts extend to novel synaptic partners following loss of the major afferent input; however these sprouts are transient and the dendritic tips subsequently retract [85]. Formation of non transient ectopic synapses to novel synaptic partners (red and green cones) did occur if blue cones failed to regenerate or if regeneration was delayed, suggesting that signals present during regeneration inhibit anomalous

sprouting and maintain circuit connectivity patterns [88]. These observations signify that time constraints could be a problem for retinal stem cell replacement therapies to successfully integrate and restore the retinal circuitry prior to remodelling as the disease progresses. Future studies are required to determine if regenerated photoreceptors integrate with the existing retinal circuits and reverse remodelling. Regenerated photoreceptors fail to recapitulate the spatial organization of the adult photoreceptor mosaic following retinal lesion [56], which may impact the regeneration of the visual circuitry and recovery of vision.

1.8 Retinal plasticity following metamorphosis in salmonids

Some salmonids undergo dramatic remodelling of the retina during metamorphosis as the visual system adapts to a different photic environment. Atlantic salmon lose their UV cones during a metamorphosis-like event called smoltification when they prepare to move to deeper and/or marine waters [89]. The loss of UV cones may be accompanied by an increase in the number of rod photoreceptors. These UV cones reappear in the retina as the salmonid fish reaches sexual maturity and return to their natal streams [90]. In rainbow trout, application of exogenous thyroid hormone (TH) is sufficient to induce UV cone death reminiscent of natural metamorphosis, which has a corresponding surge in serum TH [91-93]. Rainbow trout regenerate UV cones following cessation of TH treatment, and the ERG recordings support that these regenerated UV cones are functional and integrate as the interneuron response recovers (b-wave) [93]. Ontogenetic changes in circuitry due to dramatic events such as metamorphosis and changes in retinal circuitry due to TH mediated photoreceptor death are poorly characterized, yet provide an ideal system to address questions regarding the importance of retinal remodelling for proper integration of new neurons.

1.9 Methods of assessing functional vision recovery in zebrafish

Functional recovery of vision requires regeneration and differentiation of the lost neuron, migration to the proper retinal layer, and integration into the pre-existing neural circuitry. Functional regeneration of vision can be assessed using electroretinography (ERG) to directly measure the electrical activity of various cell types in the eye, or by using innate behavioural responses. The loss of behaviour due to neuron death and subsequent regeneration of the neuron with recovery of the behaviour is an established method of demonstrating functional regeneration [3, 4]. Below are some commonly used visually mediated behaviours use to assess visual ability and vision recovery, some additional assays include the visual startle / escape response, phototaxis, dorsal light response, and locomotor activity during different light states [69, 94-96]. Many of these visually mediated behaviours utilize motor activity as a behavioural output, it is important to utilize behavioural controls to screen for motor deficits that could affect behavioral outputs independent of visual ability. Some common assays testing movement behavior are: spontaneous behaviour, touched evoked escape response, and swim performance [94, 96].

1.9.1 The optomotor response (OMR)

The optomotor response (OMR) is an innate behaviour in zebrafish; zebrafish perceive a whole-field moving stimulus and swim with the direction of perceived motion (Fig. 2A). The behaviour is thought to be a stabilizing behaviour to maintain a stable position in a flowing stream relative to substrate level visual cues [97]. The OMR is present as early as 5 days post-fertilization (dpf) and persists into adulthood. Previous evidence suggests that the OMR is principally mediated by the red and green cones, but potentially incorporates inhibitory input from the blue and UV cones [98-100].

1.9.2 The optokinetic response (OKR)

The optokinetic response (OKR) is an innate behaviour involved in stabilizing the eyes relative to a visual field (Fig. 2B) [97]. The OKR tracks eye movements that are evoked using a series of moving bars in the visual field of the subject. The behaviour develops early in zebrafish, between 73 and 80 hours post-fertilization, and persists in adulthood [101]. The behaviour consists of two movements a smooth pursuit phase, which tracks the visual stimulus and a rapid saccade or reset that moves the eye to a new position once the moving stimulus has left the visual field. The stimulus is typically generated using a rotating drum to create a series of moving illuminated stripes and presented to an immobilized zebrafish [97, 98, 102]. The early presence of the behaviour prior to rod maturity suggests the behaviour is a cone-mediated visual response [36], and previous studies support that the OKR is mediated by the ON-pathway [103].

1.9.3 Visually mediated background adaptation (VBA)

The zebrafish visually mediated background adaptation (VBA) is a neuroendocrine response to ambient light levels. This light mediated endocrine response controls melanophores in the skin, adapting to the light environment by either dispersing or aggregating the melanosomes to make the larvae appear darker or lighter respectively (Fig. 2C) [95, 104]. This innate response causes the larvae to match the light environment to better camouflage itself.

The VBA employs a distinct retinal circuit that is unaffected by OMR/OKR mutants supporting that it is functionally distinct from motion detection [105]. The retinal ganglion cells are required for VBA behaviour, however photoreceptor-defective mutants, have an intact VBA, suggesting that another light sensitive cell in the neural retina may be responsible for neuroendocrine control of melanophores [105].

UV light exposure does not elicit the same background adaption in larval zebrafish, but rather triggers melanosome dispersal providing a protective effect against potentially harmful UV radiation (Fig. 2D) [106]. This response is present at 2 days post-fertilization, which is prior to opsin development, supporting that the response is mediated by photoreceptors intrinsic to the melanophores. However, as the larvae develops, UV cone photoreceptors in the retina may become involved in mediating the UV radiation protective behaviour [100].

1.9.4 Prey capture

Prey capture in zebrafish is a complex behaviour that is primarily guided by the visual system as it perceives and tracks the prey. Prey capture is also reliant on fine motor control for prey pursuit, and capture [107]. Prey tracking involves distinct behavioral events such as the J-turn, which orients zebrafish towards prey. Prey capture also involves a series of distinct swim motions leading to biting the prey [108]. Larval zebrafish begin hunting live prey at 5 days post-fertilization. This behaviour usually examines the time until prey capture, but has not been as frequently used to assess visual function as other visually mediated behaviors [98].

1.9.5 Assessing integration of regenerated photoreceptors using electrophysiology (Zebrafish Electroretinogram)

The electroretinogram (ERG) is a noninvasive electrophysiological tool for assessing outer retinal function in both larval and adult zebrafish [94, 109]. Retinal function is assessed in response to a light stimulus, which stimulates a full field potential from retina neurons. Corneal surface electrodes record these extracellular currents as the light disrupts the dark current. This characteristic waveform response can be broken down into 4 different components: the leading a –wave derived from the photoreceptor response, the b-wave generated by interneurons (primarily

the ON-center bipolar cells), and the c-wave and d-wave which are generated by the retinal pigment epithelium and the OFF-bipolar cells respectively [110].

The ERG can be used in various states of light adaptation to assess the rod (scotopic) and cone (diurnal) photoreceptor response. The rod system is sensitive to dim light in a dark-adapted state, whereas dim background illumination saturates the rod system and bright light stimuli reflect activity are detected by the cone system. The wavelength of the light utilized in the stimulus can be controlled to determine the sum spectral sensitivity of the cone-mediated vision. The frequency of the stimulation can also be utilized to separate cone and rod responses. This critical flicker fusion takes advantage of the different temporal properties of the rod and cone systems. Only the cone system responds to flickering frequencies higher than 28Hz [110]. Additional information about the different wave components in regards to the kinetics of the response can be gained by recording the amplitude of the waveform in response to various intensities as well as the implicit time, which reflects the temporal properties of the ERG response [110]. Electrophysiological experimentation can be complemented by pharmacology to isolate specific ERG contributions.

1.10 Utilizing the metronidazole-nitroreductase method of cell ablation to induce conditional targeted blue or UV cone photoreceptor death

The KalTA4-UAS system is a zebrafish-optimized version of the Gal4/UAS system that we engineered to drive expression of bacterial nitroreductase (NTR). NTR is fused to a fluorescent marker gene, mCherry, for visualization. A cone specific promoter provides spatial control, restricting expression of NTR to the target cone photoreceptor subtype. In juvenile zebrafish, the cones survive and function normally, until the application of the prodrug metronidazole (MTZ). Metronidazole is reduced by nitroreductase, converting the prodrug into a

cell autonomous DNA cross-linking agent, which is recognized by the cell; initiating apoptosis [111, 112]. Additionally, MTZ reduction causes nitro radical formation, and axonal degeneration [113]. The toxic form of MTZ is confined to NTR-positive cells leaving neighboring cell unaffected by the cytotoxin. Ablation in zebrafish is temporally controlled by the conditional addition of the prodrug to the tankwater. Ablation ceases once the prodrug has been removed, permitting regeneration of the ablated cell type. Observation and quantification of the fluorescent marker were made at various time points to assess ablation and subsequent regeneration of the ablated cell type following prodrug exposure.

1.10.1 Nitroreductase and metronidazole use

Escherichia coli nitroreductase (NTR) is a flavin mononucleotide-containing flavoprotein homodimer. The NTR enzyme is first reduced by NADH or NADPH, after which NTR can then bind to MTZ. Metronidazole (MTZ) [1-(2-hydroxythyl)-2-methly-5-nitroimidazole] is a nitroimidazole antibiotic and is commonly prescribed to treat anaerobic bacteria and protozoal infections [113]. Metronidazole is administered in its inactive form, and is converted to its active cytotoxic form in the presence of NTR, which binds and reduces it. Limiting off-target cell death or bystander effects (i.e. death or other deleterious effects on cells other than the target) is crucial for retinal regenerative studies of photoreceptors. In our design, neither MTZ in its active cytotoxic form or its metabolites have a toxic bystander effect when used at concentrations required for targeted cell ablation [10mM] in zebrafish larvae. The toxic bystander effect is a problem with another nitroimidazole derived prodrug: CB1954, previously used in NTR-mediated ablation [114]. Conversely NTR and the *nfsB* transgene does not have any deleterious effects in transgene expressing cells in the absence of the prodrug.

1.10.2 The KalTA4/UAS system of cell specific expression

The KalTA4-UAS system is a zebrafish-optimized variant of the Gal4-UAS system, a widely used system in Drosophila [115, 116]. It utilizes two transgenic lines: an activator line and an effector line. The activator line expresses the transcriptional activator (KalTA4) using a cell specific promoter. The effector line expression of the gene of interest is controlled by the upstream activating sequence (UAS). Each line when expressed is alone innocuous, but when different stable transgenic lines are crossed, the transcriptional activator binds to the UAS in the offspring, activating expression of the effector line in a specific cell type. This system was engineered to express the nitroreductase gene, *nfsB,* in specific subtypes of cone photoreceptors.

1.11 Objectives

My Master's thesis research project utilizes zebrafish as a model of cone neurogenesis to demonstrate the regeneration of UV or blue cones following specific ablation of the respective photoreceptor cell types. The regenerative capacity of the zebrafish is ideal to identify the gene regulatory pathways involved in cone photoreceptor fate determination as well as the mechanisms involved in integration of the newly generated photoreceptors into the retina.

The first aim of my thesis was to selectively ablate blue cones or UV cones using our lab's novel model of selective cone photoreceptor ablation, and to compare and contrast the regenerative responses of two different cone subtypes without non-specific cell death in the retina. Past work in our lab using a previous iteration of our UV cone targeted cell ablation model, demonstrated that UV cone ablation is sufficient to induce a biased regenerative response, generating an increased number of UV cones at the expense of rod photoreceptor generation. My second aim was to determine whether regenerated UV or blue cone photoreceptors were functional and capable of restoring visual ability lost due to ablation of the respective cone

photoreceptor. In order to determine whether regeneration was functional I had to identify a deficit in a visually mediated behaviour or in the electroretinogram response following cone-specific ablation and determine whether regeneration of the ablated photoreceptors functionally recovered the loss visual ability.

The final aim of my project was to identify the mechanism responsible for the novel rapid recovery of visually mediated behaviour following blue cone ablation, because the recovery occurred independently from blue cone new generation.

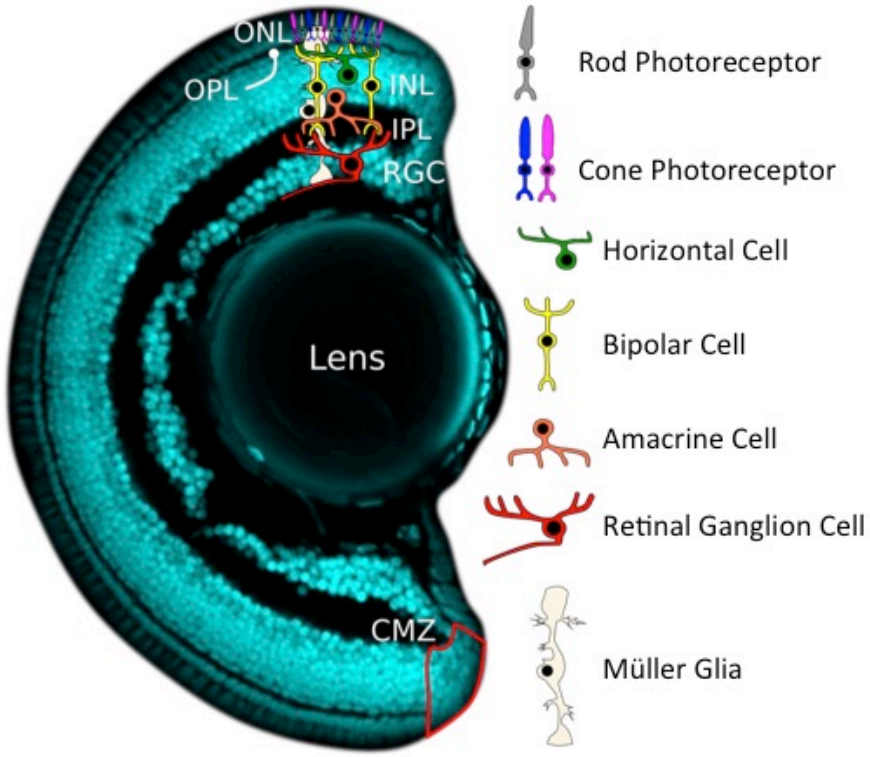


Figure 1. Anatomy of the larval zebrafish neural retina.

The neural retina contains the photosensitive rod (grey) and cone photoreceptors (violet/blue) in the outer nuclear layer (ONL). These photoreceptors synapse with the secondary interneurons: the horizontal cells (green) and the bipolar cells (yellow) at the outer plexiform layer (OPL). Horizontal cells integrate and provide inhibitory feedback to photoreceptors, while bipolar cells collect photoreceptor input from their receptive field and transmit input to the retinal ganglion cells (red). The cell bodies of the horizontal, bipolar, and amacrine cells (orange) are located in the inner nuclear layer (INL). Bipolar cells synapse with retinal ganglion cells at the inner plexiform layer (IPL). Amacrine cells also synapse with bipolar cells and retinal ganglion cells at the IPL to integrate and modulate the visual input for the retinal ganglion cells. Retinal ganglion cells serve to transmit visual information to the brain by axons leaving the eye via the optic nerve cord. Their cells bodies are located within the retinal ganglion cell layer (RGC). Müller glia cells (white) are support cells that also have an important role in retinal regeneration. The ciliary marginal zone (outlined in red) consists of a population of retinal stem cells and progenitor cells that continuously add new retinal neurons to the periphery of the neural retina.

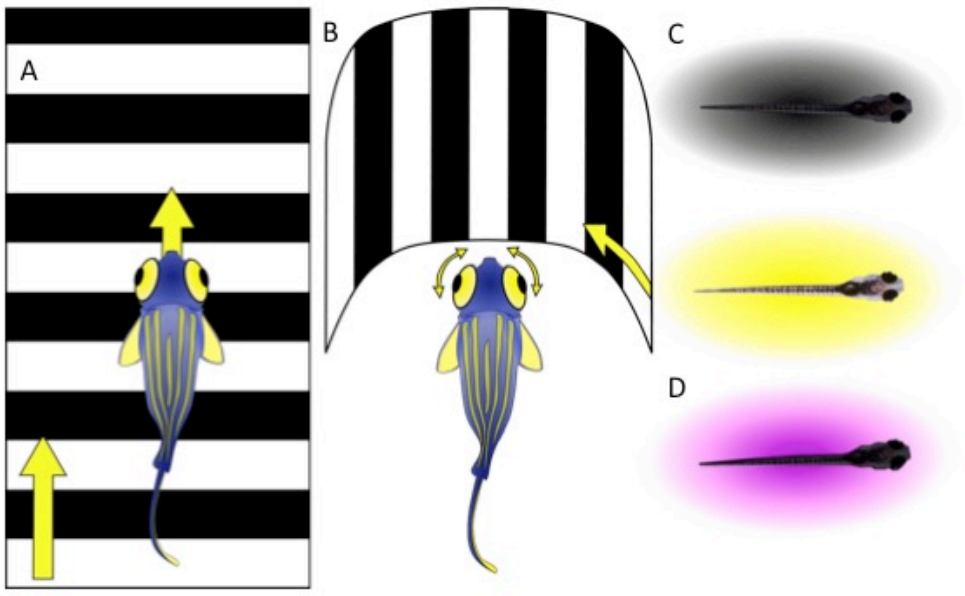


Figure 2. Common visually mediated behaviours of zebrafish.

Visually mediated behaviours are used to assess the visual ability of zebrafish. **(A)** The optomotor response (OMR), zebrafish swim in the direction of perceived movement based on a substrate level moving bar stimulus. **(B)** Optokinetic response (OKR): immobilized zebrafish utilize eye movements to track a moving bar stimulus. **(C)** Visually mediated behavioural adaptation (VBA) is a camouflage behaviour wherein the light conditions stimulate melanosome dispersal to darken the fish in a dark environment or aggregation to lighten the zebrafish. **(D)** When larval zebrafish are exposed to UV light it stimulates melanosome dispersal as a UV radiation protective behaviour.

Chapter 2. Conditional targeted ablation of either UV or blue photoreceptors and functional regeneration of vision in larval zebrafish

Our lab previously demonstrated UV cone specific ablation induces a regenerative response; this response is tailored generating an increased number of UV cones at the expense of rods [117]. Cone specific ablation provides the opportunity to assess retinal remodelling in an intact retina, and the mechanisms involved in biasing stem cell fate. However, it remains unknown whether these regenerated cones are capable of integrating into the retina and synapsing with the correct partners to re-establish the retinal circuitry and functionally restore vision. In order to determine whether the visual ability of the zebrafish recovered following cone photoreceptor ablation, I assessed the visually mediated behavioural response using the optomotor behaviour (OMR).

2.1 Methods

2.1.1 Animal ethics statement

Approval for this study, including zebrafish maintenance, anesthesia, and breeding, under protocol AUP00000077, was granted by the Animal Care and Use Committee: BioSciences, which is an Institutional Animal Care and Use Committee at the University of Alberta, and which operates under the auspices of the Canadian Council on Animal Care.

2.1.2 Zebrafish maintenance

Larval zebrafish were raised in E3 media as embryos and larvae and maintained according to standard procedure [118]. Select larvae were treated with 1- phenol-2-thiourea (PTU) 6-8

hours after fertilization to block synthesis of melanin pigment. Adult zebrafish were maintained at 28°C in brackish water (1250±50 μS) and fed brine shrimp or trout chow twice daily.

2.1.3 Constructs for transgenesis and characterization of transgenics

Zebrafish were engineered to express a nitroreductase-mCherry fusion protein using either the UV opsin (sws1) or the blue opsin (sws2) promoter driving expression of the transcriptional activator KalTA4 to achieve expression of the transgene in either UV or blue sensitive cone photoreceptors respectively. Constructs for transgenesis were generated using multisite Gateway Cloning into Tol2 systems amenable vectors to recombine plasmids (pDestTol2CR2) following standard procedures [119]. A construct driving expression of KalTA4 under the control of sws1 or sws2 was generated using an LR reaction to recombine UV opsin promoter (p5E-sws1) and blue opsin (p5E-sws2) with pME-KalTA4, p3E-pA and pDestTol2CG2 to generate pDestTol2CG2.sws1:KalTA4 and pDestTol2CG2.sws2:KalTA4 respectively [100, 116, 117, 119]. The destination vector, pDestTol2CG2, expresses GFP in heart muscles as a marker of successful transgenesis.

The transcriptional activator KalTA4 binds to a specific UAS (upstream activating sequence) recognition sequence, which activates transcription of the target genes. The KalTA4-UAS system is a zebrafish-optimized variant of the Gal4-UAS method of cell specific expression [116]. Our second effector construct used the destination vector, pDestTol2CR2, expressed mCherry in heart muscle as a marker of successful transgenesis. A kaloop vector enabled transgene expression into adulthood, inspired by the kaloop system previously validated by Distel et al [116], was recombined into pDestTol2CR2. A middle entry vector, pME-nfsB-mCherryΔSTOP, was recombined with vectors p5E-4X-uas and a p3E-P2a-KalTA4 vector created by BP reaction amplifying off existing plasmids [116, 117, 120]. The resulting plasmid

from the LR reaction, pDestTol2CR2.4UAS:kozak-nfsB-mCherry^DAV-T2A.KalTA4.pA, expresses an mCherry fusion protein and a secondary KalTA4 when transcribed by activation with KalTA4. This second copy of KalTA4 creates a positive feedback loop to maintain expression of the construct. These constructs were delivered to zebrafish embryos, which were screened for mCherry and GFP fluorescence in the heart muscles, as well as mCherry expression in the retina. Zebrafish were grown to adulthood and screened for somatic insertions, through the generation of stable transgenic carriers. Carriers were identified by fluorescent expression in appropriate cells.

Our UAS sequence drives expression of a nitroreductase-mCherry fusion protein. Nitroreductase is a bacterial enzyme that reduces the prodrug/ antibiotic metronidazole (MTZ) converting it into a cell autonomous DNA crosslinking agent. DNA crosslinking signals to the cell to initiate apoptosis resulting in targeted ablation of transgene expressing cells. The fluorescent reporter mCherry allows for visualization of cells expressing the transgene. Expression of the transgene is maintained using a "kaloop" system inspired by Distel et al., wherein a second copy of the KalTA4 sequence is expressed as part of the UAS cassette creating a positive feedback loop [116]. This was put in place to combat gene silencing due to DNA methylation of the UAS site, thereby extending expression into adulthood [121].

Expression of the transgene was mosaic; only individuals with detectably robust mCherry expression in the retina were selected for experimentation using florescent microscopy. Off-target expression of the Tg(SWS2:KalTA4;UAS:nfsB-mCh-2A-KalTA) in vasculature was observed in certain alleles of the blue cone ablation model and was easily selected against (see Table 1 for various alleles). Exposure to the prodrug was lethal for zebrafish that had off-target expression.

2.1.4 Metronidazole-nitroreductase method of conditional targeted ablation of UV or Blue cone photoreceptors

Transgenic zebrafish (*SWS1:KalTA4;UAS:nfsB-mCh-P2A-KalTA4* and *SWS2:KalTA4;UAS:nfsB-mCh-P2A-KalTA4)* were placed in an E3 solution containing 10mM metronidazole (MTZ) with 0.2% DMSO for 24 hours at 7 days post fertilization (dpf) to induce targeted ablation of nitroreductase expressing cells. Following treatment the larvae were washed thrice with E3 to remove MTZ and cease ablation. Select larvae were exposed to 10mM MTZ with 0.2% DMSO in E3 solution for 48 hours at 7 dpf or a second treatment of 10mM MTZ with 0.2% DMSO in E3 solution at 10 dpf. Select transgenic zebrafish utilized for the drug vehicle control were placed in an E3 solution containing 0.2% DMSO at the same time intervals as the experimental MTZ treatment. Non-transgene expressing siblings were placed in an E3 solution containing 10mM MTZ with 0.2% DMSO for the same time intervals as the experimental MTZ treatment group to induce MTZ toxicity/ off target effects on larval swimming behaviour.

2.1.5 Quantification of photoreceptor ablation

To determine the effectiveness of photoreceptor ablation, retinal cryosections were collected immediately following cessation of prodrug treatment at 8dpf and at 24-hour intervals thereafter until 11dpf (See cryosection preparation 2.2.8). Fluorescence from mCherry was visualized using ZEN 2010 software (version 6.0, Carl Zeiss AG, Oberkochen) with a Zeiss LSM 700 confocal microscope mounted on a Zeiss Axio Observer.Z1. The number of mCherry-positive photoreceptors was quantified per length of the outer plexiform layer then scaled to an average retinal size for comparison independent of plane of retinal section or differences in eye size using Image J (Wayne Rasband, National Institutes of Health, Bethesda MD; http://rsbweb.nib.gov/ij/index.html). The position of the mCherry-positive photoreceptors was

broadly categorized into two different regions: the central mature retina and the peripheral developing retina. We broadly categorized the central retina as 80% of the length of the outer plexiform layer while the peripheral retina was defined as two peripheral zones making up the final 20% of the outer plexiform layer.

2.1.6 Larval zebrafish optomotor response (OMR)

The optomotor response (OMR) is an innate visually mediated behaviour, wherein larval zebrafish swim in the direction of perceived motion. This behaviour is present as early as 6 dpf in larval zebrafish [122]. The optomotor apparatus consisted of a computer monitor (IBM 6734-HBO ThinkVision) laid flat and facing upwards to deliver a substrate-level visual stimulus. The visual stimulus was generated in PowerPoint (Microsoft, Redmond, Version 14.5.2) on a MacBook Pro (Apple, Cupertino, Version 10.11), and was connected to the monitor via a VGA cable with a Mini Display Port adapter. The parameters of the moving grating pattern stimulus were constant (velocity: 1.33cm/s, width of the bars: 1.35cm, and frequency 0.49Hz). The spectral outputs of the stimulus and computer monitor were recorded through an acrylic sheet to simulate the light conditions observed by the fish. Measurements were obtained using a spectrometer (USB4000-UV-VIS: Ocean Optics Inc., Dunedin FL) and an optic fiber cable with a cosine corrector (CC-3-UV-S: Ocean Optics Inc., Dunedin FL). The spectrometer was calibrated using a radiometric calibration source (HL-2000-CAL, Ocean Optics Inc.). Analysis of the spectral output was calculated using *SpectraSuite* software (Ocean Optics Inc., Dunedin FL). The experiment was preformed in a dark environment (verified using the spectrometer) to inhibit extraneous luminance motion cues. Optomotor behaviour was recorded from isolated larval zebrafish in a series of 8-10 long rectangular troughs (custom; 30cm x 1.4cm x 1.0cm, composed of 1.7mm thick transparent acrylic sheets bonded together). Troughs were filled with brackish

water (1250±50 μS) heated to 30°C. The positions of the larval zebrafish were recorded at the end of the trial using a digital camera (Cyber-shot DSC-HX50V, Sony Tokyo) mounted overhead of the OMR apparatus.

Larval zebrafish were selected randomly from a treatment population. Larval zebrafish had to display a robust avoidance response to a physical suction stimulus from a pipette to be selected for behavioural recording. This ensured that larval zebrafish were healthy and capable of a motor behavioural reflex. Larval zebrafish were acclimated to a reverse orientation stimulus for 1 minute prior to behavioural recording at which point larval zebrafish were positioned in a 2.5cm long starting zone prior to experimental recording. Larval zebrafish were exposed to the OMR stimulus for 1 minute upon which the screen shifted to a white background and displacement from the starting zone was captured. Each larval zebrafish was exposed to a series of optomotor stimuli in duplicate. The technical replicates were then averaged as a single biological replicate. The OMR data was collected from several separate trial dates and pooled. Recording of optomotor behaviour was limited to larval zebrafish aged 8-11 dpf because after 11 dpf zebrafish enter a fragile life stage following depletion of the yolk sac and transition to feeding.

2.1.7 Analyzing OMR results

To determine the OMR of larval zebrafish the photos recording larval displacement from the starting zone were analyzed using ImageJ. The mean percent of the trough the larval zebrafish swam in response to the optomotor stimulus was recorded as the strength of the optomotor behaviour. The strength of the optomotor behaviour was normalized to the wild type (non-transgenic sibling) treated with MTZ at each time point to emphasize treatment dependent effects rather than aging.

2.1.8 Larval zebrafish spontaneous activity

The spontaneous activity of a larval zebrafish is a useful control to test locomotor behaviour [99, 105]. Transgenic zebrafish were tested at 8dpf following 24 hour prodrug treatment. The behavioural apparatus was enclosed to minimize luminance motion cues. The apparatus consisted of a computer monitor (IBM 6734-HBO ThinkVision) laid horizontally projecting a white screen. A video camera (Colour CCTV Camera WV-CL930, Panasonic Tokyo) mounted overhead recorded movement. The behavioural arena was a petri dish (Fisher Scientific 100mm x 15 mm) filled with 30ml of brackish water (1250±50 μS) heated to 30°C. Each petri dish contained 10 zebrafish at a time. Zebrafish were selected randomly from a treatment population, though the presence of a robust escape response was required for selection. Zebrafish were acclimated for 20 minutes prior to behavioural recording, which lasted 10 minutes. Behavioural recordings were generated using *Ethovision* (Noldus Information Technologies Inc., Leesburg, VA, version XT 10.0). Frames were extracted from the video at a rate of 1 per second using *VLC media player* (VideoLan Organization, Paris, Version 2.2.2). The video frames were arranged using an ImageJ stack sorter plugin, each video frame was then compared to the next frame using a plugin to measure changes in pixel intensity (Delta F down plugin, Tony Collins, McMaster Biophotonics Facility, MBF-plugin collection). The plugin generates an image showing the location of pixels, which changed in intensity between frames. Thus only moving fish will appear, while stationary fish will be subtracted. The number of moving fish in each video frame was recorded using the particle analysis plugin. The rate of these "movement events" over 10 minutes were then compared to analyze for changes in locomotor behaviour. A Z-projection of the resultant frames created a movement profile to generate a visual depiction.

2.1.9 Preparing retinal cryosections

Retinal cryosections were prepared as previously described [123]. Larvae were fixed overnight at 4°C in 4% PFA, then cryopreserved step-wise in 0.1MPO_4 solutions with increasing concentrations of sucrose, and embedded in Tissue-Tek O.C.T embedding compound (VWR, Cat. No. 25608-930). Larvae were cryosectioned in 10 μm slices and thaw mounted onto SuperFrost Plus glass slides (Fisher, Pittsburgh, PA; Cat. No. 12-550-15). Tissue was air dried for 30 minutes prior to storage at -80°C until use.

2.1.10 Immunohistochemistry on retinal sections

Sections were removed from the -80°C and allowed to air thaw for 30 minutes at which point they were permeabilized using a PBS 3+ (0.1% Trition, 0.1% Tween, and 0.1% DMSO) solution for 20 minutes at room temperature. Slides were then treated with a reaction cocktail as described in the baseclick protocol (Baseclick GmbH, EdU Cell Proliferation Kit, Germany), which utilizes bioconjugation to attach a 6-FAM Azide fluorescent dye to 5-ethynyl-2′-deoxyuridine (EdU) via a "click reaction" [124]. Slides were incubated in a nuclear stain, TO-PRO-3 (Invitrogen, Cat. No. T3605; 1/5000 dilution), for 30 minutes after the "click reaction" had completed.

2.1.11 EdU application on larval zebrafish and staining retinal sections and retinal wholemounts

Larval Tg(*SWS1:KalTA4;UAS:nfsB-mCh-P2A-KalTA4*) and Tg(*SWS2:KalTA4;UAS:nfsB-mCh-P2A-KalTA4)* fish were treated with an E3 solution of 5mM EdU (a relatively high dose) with 0.3% DMSO for 24 hours at 6 dpf then incubated in 10mM MTZ for 24 hours then again exposed to an E3 solution of 5mM EdU with 0.3% DMSO for 48 hours. Larval zebrafish were euthanized and fixed at 3 different time points: 24 hours, 48 hours,

and 1-week post MTZ treatment. Following overnight in fixation in 4% PFA, one eye was enucleated and prepared for retinal wholemount, while the remaining eye was prepared for cryosection as previously described. Select larval Tg(*SWS1:KalTA4;UAS:nfsB-mCh-P2A-KalTA4*) and Tg(*SWS2:KalTA4;UAS:nfsB-mCh-P2A-KalTA4)* zebrafish were treated with an E3 solution of 0.3% DMSO as a drug vehicle control for the same time intervals as the EdU experimental treatment.

2.1.12 Identifying proliferating cells using EdU

The EdU cell proliferation assay detects DNA synthesis in proliferating cells using 5-ethynyl-2′-deoxyuridine (EdU) a synthetic nucleoside (thymidine) analogue that is incorporated into DNA during DNA synthesis in the S phase of the cell cycle [125]. EdU detection occurs through a "click chemistry" reaction with a florescent dye permitting identification of proliferating cells during the period of EdU exposure. Quantification of retinal sections examined the number of EdU positive cells as well as the number of EdU and mCherry positive cells.

2.1.13 Statistical analysis

Cone photoreceptor counts, retinal proliferation following ablation, and the spontaneous behaviour assay were evaluated using a Two-Way ANOVA with a Bonferroni post-hoc-test using Prism 6 (GraphPad Software Inc., San Diego CA). OMR behaviour was assessed via One-way ANOVA with post-hoc Tukey test run in SYSTAT 13 (Systat Software, San Jose CA). DF is degrees of freedom of the interacting factors and then residual degrees of freedom.

2.2 Electroretinogram (ERG) methods

2.2.1 Preparation of zebrafish for ERG

Adult zebrafish were maintained as described above. Prior to electrophysiological recording, zebrafish were fasted for one day. Fish were anesthetized by bathing in a solution of tricaine methanesulfonate (MS-222, 150mg/L), until they reached the stage IV of anesthesia [126]. Fish were then placed on a moist foam sponge and injected intraperitoneally as described [127]. The injection further anesthetized the fish using 5nL of Aquacalm^{TM} (Metomidate hydrochloride, Western Chemical Inc., Ferndale, WA, 0.003mg/g body weight) and pancuronium bromide (0.04mg/g body weight) an immobilizing agent. The zebrafish was then placed in a moist foam sponge cradle and intubated with aerated brackish water containing MS-222 and covered with a damp Kimwipe (Kimberly Clark, Cat. No. 34120, Roswell USA). The setup was located within a Faraday cage mounted onto a vibration isolation table (Technical Manufacturing Company).

Larval zebrafish were maintained and anesthetized using MS-222 (150mg/L), after which they were then pipetted onto a moist paper towel, which was placed onto a wet foam sponge within the faraday cage.

2.2.2 Attempted ERG recordings

Adult zebrafish ERG recording used a 3-electrode system: consisting of a recording, reference, and ground electrode. Various different types of electrodes were utilized from surgical steel needle electrode (ADInstruments, MAL1213, Sydney Australia), subdermal platinum needle electrode (Grass Technologies, Warwick, RI), and glass capillaries electrodes (filled with either E3 media, KCl (1M), or Ringer's solution) with either a sintered AgCl wire (World Precision Instruments, EP2, Sarasota, FL) or an AgCl half-cell pellet electrode (World Precision

Instruments, MEH6SF, Sarasota, FL). Various ground electrodes were used as well: Dri-Ref reference electrode (World Precision Instruments, DRIRED-5SH, Sarasota, FL), or an exposed AgCl disc electrode (Warner Instruments, E202, Hamden, CT). The recording electrode was typically positioned at the posterior dorsal surface of the eye using a micromanipulator in conjunction with an operating microscope. The reference electrode was positioned in the nares contralateral to the recording eye. The ground electrode was positioned either within the foam sponge or alongside the trunk of the zebrafish. The larval zebrafish setup utilized only two electrodes a recording electrode and a reference/ground electrode as previously described [109]. Once the electrodes were positioned the zebrafish was dark adapted for 5-10 minutes. Tear-gel was used to achieve good contact with the surface of the eye, and prevent desiccation.

We utilized two different ERG rigs; the first is a custom setup, which was used for adult and larval zebrafish recordings. The light stimulus was delivered to the zebrafish using a bifurcated optic fiber cable. The duration of stimulus exposure was controlled by a fast solenoid shutter (Melles Griot), while stimulus wavelength was controlled using a monochromator (Newport, Cornerstone monochromator, 74125, Irvine, CA). Light was collimated from the monochromator using a series of focusing lenses. Within this series a neutral density filter wheel can be placed to control light intensity. Light was produced from an ARC lamp 50-500W (Newport, 66901, Irvine, CA) with a digital arc lamp power supply (Newport, 69922, Irvine, CA). We tried amplifying the ERG response using different amplifiers: Animal BioAMP (ADInstruments, FE136, Colorado Springs, CO) or a differential amplifier (Warner Instruments, DP-301, Hamden, CT), which was able to differentially amplify 1000 times. ERG data was acquired from the amplifiers using a USB DAQ (ADInstruments, Powerlab 4/35, Colorado Springs, CO) and visualized using LabChart 7 (ADInstruments, Colorado Springs, CO). Filter settings were applied such that a 60Hz notch filter limited electrical noise, and various bandpass

filters, which were usually broad (between 0.3Hz and 300Hz) limited the risk of filtering out the signal while still limiting noise. ERG recording was preformed under dark conditions as verified using the previously mentioned spectrometer. Prior to ERG recording, the recording electrode, the reference electrode and the ground electrode were placed in a petri dish filled with E3 and the experimental preparation was optimized to ensure proper grounding and minimal noise in the system. We presented various stimuli as we screened through various wavelengths of light as well as various light frequencies, over durations times to generate potential ON and OFF responses. Alternatively, we utilized Diagnosys' ERG rig (Diagnosys, ColorDome, Lowell, MA) for larval zebrafish recordings. Following ERG recording zebrafish were euthanized using MS-222.

2.3 Results

2.3.1 Characterizing the cell specific ablation of blue or ultraviolet sensitive cone photoreceptors

The transgenic fish denoted Tg(*SWS1:KalTA4;UAS:nfsB-mCh-P2A-KalTA4*) and Tg(*SWS2:KalTA4;UAS:nfsB-mCh-P2A-KalTA4*) (various alleles, see Table 1) were developed in the Allison laboratory to conditionally induce cell specific ablation of UV or blue sensitive cones, respectively, by the addition of the prodrug metronidazole (MTZ) to the embryo media (E3)(Fig. 3). Ablation is restricted to UV or blue cones based on cell specific transgene expression of nitroreductase. Nitroreductase (NTR) reduces the prodrug metronidazole converting it into a cell autonomous DNA crosslinking agent and thus initializing programmed cell death [112]. Induction of apoptosis in transgene expressing cells ceases upon removal of the prodrug, permitting regeneration of the ablated cells to occur. Recent work performed in our lab demonstrated that the ablation of targeted photoreceptor using this transgenic system does not

cause a toxic bystander effect on neighboring photoreceptors, as measured using a TUNEL stain to detect apoptotic cells [100].

Expression of the transgenic construct and subsequent ablation of transgene expressing cones was confirmed using mCherry, a fluorescent reporter, which was fused to NTR. KalTA4, a transcriptional activator, binds the UAS promoter initializing expression of the fusion protein. Expression of KalTA4 was driven in specific cells using either a UV cone specific promoter (*sws1*) or blue cone specific promoter (*sws2*)(Table 1). A second copy of KalTA4 was included alongside the fusion protein gene to engineer a positive feedback loop. This was included in an effort to maintain transgene expression into adulthood, because the UAS site is vulnerable to gene silencing due to methylation with age [128-130].

Expression of the NTR-mCherry fusion proteins in the appropriate cell types were identified following a 24-hour prodrug treatment at 7 days post fertilization (dpf) (Fig. 4A, 5A). MTZ exposure caused a significant 5-fold ($p<0.001$, $F_{1,48}= 99.67$) decrease in mCherry positive cells in the blue cone ablation model, and a 4-fold decrease ($p<0.001$, $F_{1,41}= 147.9$) in mCherry positive cells in the UV cone ablation model immediately following removal of the 24 hour prodrug treatment. Remnant mCherry fluorescence was observed immediately following ablation; it appeared improperly localized to the retinal pigment epithelium, and as dysmorphic blebs present in the outer nuclear layer. Healthy photoreceptor cells that were morphologically recognizable and mCherry positive were first observed at the in the peripheral developing retina at 48 hours following ablation (Fig. 4A, 5A). This can be attributed to the continuing growth of the larval retina from expansion of the ciliary marginal zones. Previous work performed in our lab supports the continued growth of the retina may experience modulation of the developmental pathway to bias cell fate to in order to regenerate the lost cone photoreceptor cell type [117]. While reappearance of mCherry positive cells occurred predominantly in the peripheral retina,

mCherry cells with healthy cone morphology were observed in the central retina at 72 hours following prodrug treatment, potentially derived from dedifferentiated Müller glia stem cells. Nonetheless, the observation of mCherry positive cells in the retina regardless of localization did not amount to a statistically significant increase.

2.3.2 Effects of cone photoreceptor ablation on retinal stem cell proliferation

In order to determine whether the targeted ablation of a specific cone subtype is sufficient to induce an injury response in the retina, I evaluated whether the number of actively dividing cells increases in the retina following prodrug treatment. Non-specific retinal damage is already known to induce a robust proliferative and regenerative response from retinal stem cell populations in zebrafish within 3 days [77]. The number of actively dividing cells was determined based on EdU (thymidine analogue) incorporation during DNA synthesis. This was compared relative to control fish (Tg DMSO), in which the EdU stain detected the number of actively dividing cells in an unaffected developing retina. Our findings showed that photoreceptor ablation induced an increase in cellular proliferation following photoreceptor ablation.

I first examined the induction of proliferation at 24 hours, 48 hours and 1 week after blue cone ablation (Fig. 6). MTZ-induced blue cone ablation caused an immediate increase in the number of proliferating cells in the ciliary marginal zone 24 hours after prodrug removal in the blue cone ablation model, however this two-fold increase in the number of proliferating cells was not statistically significant ($p=0.1884$, $F_{5,59}= 28.85$). No change in cell proliferation was detectable past 24 hours. A decrease in the number of EdU and mCherry positive cells at 1 week post-ablation was observed, which unexpectedly suggests the ratio of newly generated blue cones to other photoreceptor types may have shifted producing fewer blue cones in response to blue

cone ablation. Further visualization of the unidentified EdU positive cells is required to determine the photoreceptor ratios.

Analysis of retinal wholemounts demonstrated that 1 week after MTZ-mediated cone ablation mCherry-positive blue cones reappeared in the central retina, which suggests based on the position of the regenerated cones they may be derived from a Müller glial source (Fig. 7). MTZ mediated cone ablation was sufficient to induce cell proliferation in the central retina. Generation of EdU-and mCherry-positive blue cones was infrequent, but the regeneration of the blue cones was expected to be limited, because previous studies support that blue cones do not significantly regenerate without concurrent ablation of other cone photoreceptor cell types [88].

Additionally, I examined retinal sections for induction of cell proliferation following UV cone ablation. The results support that MTZ mediated UV cone ablation induces a significant increase in the number of EdU positive cells compared to the vehicle control treatment (DMSO) within 1 week following UV cone ablation (*$p<0.05$, $F_{5,89} = 28.85$)(Fig. 6). Additionally, an increase in the number of EdU- and mCherry-positive cells was observed relative to the vehicle control, supporting that biased regeneration of the ablated cone subtype is occurring in our model. Further analysis of the cone photoreceptor ratios following UV cone ablation is required to conclusively demonstrate whether regeneration is shifting the ratio of newly produced cone photoreceptor subtypes, specifically at the expense of rods as was previously observed using an older iteration of our UV cone ablation model [117].

The results from the retinal wholemounts of UV and blue cone ablation fish demonstrate that regeneration is occurring, as observed from the reappearance of transgene expressing cells (mCherry positive) in the central retina (Fig. 7). Cells that were both EdU and mCherry positive were putatively observed following blue cone ablation, supporting that blue cones have been generated in the central retina following prodrug treatment. In addition, some EdU positive cells

were not mCherry positive suggesting that the ablated subtype is not the sole photoreceptor being generated in response to cone ablation. Further analysis of the unidentified EdU positive cells is required to determine whether these are rod photoreceptors, which are generated periodically by Müller glia in adult zebrafish, a different cone subtype or whether the regenerated cone are the same subtype as was ablated, but have undergone UAS gene silencing [24]. In contrast to blue cone ablation, EdU staining in the central retina was limited following UV cone ablation and co-localization with mCherry was not observed (Fig. 7). However a larger sample size than n = 3 is required to assess the degree of regeneration in our model. I predict that with further characterization, our model of UV cone ablation will show an induction of a proliferative and regenerative response. Previous work done in our lab using a different iteration of the targeted UV cone ablation model demonstrated induction of a proliferative response following UV cone ablation, as well as biased UV cone regeneration at the expense of rod photoreceptors [117]. Furthermore, recent studies have shown that following ablation of UV cones, retinal stem cells will regenerate approximately 25% of the pre-ablation population of UV cones in the central retina [88].

2.3.3 Loss of visually mediated behaviour as measured by OMR

To investigate the functional impact of selective cone photoreceptor ablation on the vision of larval zebrafish, I induced UV or blue cone ablation at 7 dpf using a 24-hour treatment of MTZ prodrug. After prodrug treatment, larval zebrafish underwent visually mediated behaviour analysis to screen for defects in visual ability, which could potentially be recovered to investigate functional regeneration of vision. The optomotor response (OMR) is a robust visually mediated behaviour present as early as 6 dpf, which has been used previously to screen for visual system defects in larval zebrafish [131]. The goal of this behavioural test was to create an assay that

would best discriminate a difference in visual ability following cone ablation rather than to best stimulate an OMR. Various colours and intensities were screened in order to design an optical moving bar stimulus pattern for the behavioural assay (Fig. 8). I empirically determined that a relatively low contrast blue and dark red stimulus pattern was optimal (Table 2, and Fig. 8), as it demonstrated the most robust changes in visual ability between cone ablated and non-cone ablated fish. This phenotype could potentially be recovered to support functional regeneration of cone photoreceptors. Larval zebrafish were also sequentially exposed to a high contrast black and white OMR stimulus. We predicted that the ablation of a single cone type would result in an impaired OMR in a wavelength dependent manner, and that the OMR behaviour would remain intact when using a high contrast stimulus meant to evoke a robust OMR behaviour.

Transgenic zebrafish treated with the prodrug MTZ had a significantly reduced behavioural response immediately following removal of the prodrug ($p<0.001$), relative to the controls: transgenic fish treated with the DMSO vehicle, and non-transgenic siblings exposed to the prodrug metronidazole (Table 2, and Fig. 9). This deficit in visual mediated behaviour occurred in both UV and blue cone models of ablation, demonstrating that our model of cone ablation is sufficient to functionally affect visual ability. This decrease in visually mediated behaviour was observed using both the black and white (high contrast), and the blue and dark red (low contrast) stimulus, however the effect was more pronounced using the optimized low contrast stimulus (Table 2). The optomotor response is most attuned to input from the red cones [132], however our results demonstrate that input from short wavelength sensitive cones is necessary to evoke an optomotor response, supporting a potential inhibitory opponency by short wavelength cones in the OMR [132].

2.3.4 Functional recovery of visual behaviour following UV cone ablation

Morphological evidence using our novel transgenic model as well as previous studies have shown repopulation of the retina with mature UV cones within 1 week of targeted ablation of UV cones (Fig. 5,7)[117]. In order to determine whether this regeneration was sufficient to functionally restore vision we characterized the recovery of the visually mediated behaviour following UV cone ablation. As described above there was approximately a five-fold decrease ($p<0.0001$, $F_{2,938} = 117.2$) in visually mediated response immediately following removal of the prodrug (Fig. 9). Recovery of visually mediated behaviour occurred gradually over a course of 72 hours, by which point the behavioural response had significantly recovered ($p<0.01$, $F_{2,938}= 117.2$), and was comparable to control treatments. Behavioural analysis did not proceed past 72 hours post ablation (= 11dpf) because the health of the larval zebrafish became especially variable, as they decreasingly tolerate life in a dish culture.

The incremental recovery of visually mediated behaviour corresponded with the observed reappearance of mCherry positive cells within the outer nuclear layer. These mCherry positive cells displayed mature cone photoreceptor morphology and were first observed 48 hours post cessation of prodrug treatment (Fig. 5). These mCherry positive cells were localized solely to the developing periphery of the retina, which would be ideally situated to detect a substrate level stimulus such as the OMR. The first observation of mCherry positive cells in the central mature retina occurred at 72 hours post drug treatment. The correlation of the reappearance of newly generated UV cones to the gradual recovery of visually mediated behaviour strongly supports that our apparatus is able to measure functional regeneration of vision.

2.3.5 Functional recovery of visual behaviour is dependent on UV cone regeneration

In order to test the hypothesis that the generation of new UV cone photoreceptors following ablation is necessary for the gradual recovery of visually mediated behaviour (Fig. 9); larval zebrafish were exposed to a secondary 24 hour prodrug treatment. This secondary treatment 48 hours after cessation of the primary treatment should ablate any newly generated UV cones and prevent any associated recovery of visually mediated behaviour. Following the secondary treatment with MTZ, my results demonstrated that the visually mediated behaviour failed to recover, and was depressed relative to age-matched transgenic zebrafish (UV MTZ) that received only a single MTZ treatment at 7dpf (Fig. 10). Therefore newly generated UV cones were likely responsible for the gradual recovery of visually mediated behaviour.

2.3.6 Rapid recovery of visual behaviour following blue cone ablation

Given that the recovery of visually mediated behaviour occurred gradually following UV cone ablation and correlated to the reappearance of UV cones in the retina, I predicted that following ablation of blue cones, recovery of visually mediated behaviour would be gradual and depend on the generation of new blue cones. Ablation of blue cones resulted in a robust four-fold decrease in OMR behaviour immediately following removal of the prodrug ($p<0.05$, $F_{2,500} = 14.71$)(Fig. 9). However, recovery of visually mediated behaviour occurred within 24 hours ($p<0.05$, $F_{2,481} = 2.803$), which is more rapid than expected. This rapid recovery occurred when using either the black and white (high contrast) stimulus or the dark red and blue optimized stimulus (Table 2). The histological evidence demonstrated that blue cones were not present at this time point, as morphologically mature blue cones were first observed at the periphery of the retina 48 hours after the cessation of blue cone ablation (Fig. 4).

2.3.7 Functional recovery of visual behaviour is independent of blue cone regeneration

I tested the possibility that the quick recovery of visually mediated behaviour is due to undetected rapid generation of new blue cones. By extending the timeframe of MTZ exposure to encompass the 24-hour period in which the rapid recovery of visually mediated behaviour occurred I inhibited blue cone generation to prevent any associated recovery of vision. The OMR behaviour recovered independently from rapid blue cone generation (Fig. 11). This suggests that another mechanism may be involved in the quick recovery of visually mediated behaviour. One possible explanation is that blue cone ablation induces changes in the neural circuitry of vision to compensate for the loss of blue cone input (see Discussion for potential sources of plasticity and alternative hypotheses for compensation of lost cone input).

2.3.8 Blue cone ablation does not effect spontaneous movement of larval zebrafish

The OMR behaviour is a complex behaviour dependent on multiple systems such as vision to perceive the stimulus, and motor function to enact the swimming response. In order to determine if that the decreased OMR observed following photoreceptor ablation was due to a functional change in the visual ability and not the result of off-target effects impairing motor behaviour, I evaluated the spontaneous swimming behaviour of larval zebrafish immediately after prodrug treatment. No detectable changes in motor behaviour were observed following blue cone ablation in comparison to the vehicle treatment (Fig. 12). The lack of a motor defect due to blue cone ablation was further evidenced by the extended treatment of MTZ (Fig. 11). As stated above, this tested if the rapid recovery of OMR behaviour was due to the generation of new blue cones. The results demonstrated that OMR behaviour recovered independently of MTZ treatment. Therefore, the OMR behaviour was unaffected by the presence of MTZ, supporting that MTZ does not directly affect motor behaviour.

2.3.9 UV cone ablation decreases spontaneous movement of larval zebrafish

There was a significant 3-fold decrease in the spontaneous movement of UV cone ablation fish when treated with MTZ ($p<0.05$, $F_{1,17} = 7.946$)(Fig. 12). This suggests that potentially the gradual recovery of OMR behaviour following UV cone ablation could be independent of MTZ mediated changes in visual ability. Alternatively, it could suggest that the loss of UV cone input is affecting spontaneous movement, and therefore does not disprove that the UV cone ablation system is not experiencing defects to the visual system. Further testing is therefore required to determine whether vision is functionally affected by UV cone ablation, and to determine whether MTZ is directly affecting motor function or if UV cone input has an important role in vision mediating the spontaneous movement of larval zebrafish. This result may be restricted to the UV cone ablation because they are more numerous than blue cones in the larval zebrafish retina ratio (UV:blue = 1.15) [123]. Alternatively, the role of UV cones may be different than blue cones in the larval retina, and could be affecting swimming behaviour occurring during the spontaneous movement assay.

Recent work in our lab identified a second behavioural phenotype supporting that UV cone ablation does affect the visual ability of larval zebrafish. Larval zebrafish are known to disperse their pigment in response to UV light for UV light protection, and this response is mediated by short wavelength sensitive cones in the larvae [106]. Our model of cone ablation demonstrated a decreased ability to modulate their pigment distribution in response to UV light following UV cone ablation [100].

2.3.10 Electroretinogram (ERG) recordings

My goal was to use an ERG to identify a change in full field recordings of the eye after cone subtype specific ablation and then monitor the functional recovery of the response in an individual over time. I was not able to record an ERG from the surface of the eye of a larval or adult zebrafish. ERG recordings on the larval zebrafish were unable to filter out an artifact from the stimulus (xenon bulb flash) that obscured any potential electrical response from the eye during both the ON and OFF response. This artifact persisted when the larval zebrafish was euthanized as well as absent. Troubleshooting attempts to shield the electrodes from a potential photovoltaic effect were unsuccessful. The underlying reason the adult zebrafish ERG recordings were unsuccessful remains unknown. One significant problem was the signal to noise ratio during recordings was extremely low; whether this was from the stimulus failing to evoke a signal or from the magnitude of noise experienced during the recording, remains unknown. The rig was capable of recording electrical activity, as I was able to record the heartbeat of the zebrafish if I pierced the cornea and inserted a recording electrode into the eye, however the ERG response remained undetectable. This was an invasive procedure and the fish had to be euthanized following the recording, this limited the future potential for longitudinal studies of regeneration.

2.4 Discussion

My findings show that the two novel models of conditional cone photoreceptor ablation Tg(*SWS1:KalTA4;UAS:nfsB-mCh-P2A-KalTA4*) and Tg(*SWS2:KalTA4;UAS:nfsB-mCh-P2A-KalTA4*), successfully ablated the targeted cone subtype with specificity and temporal control through the addition of a prodrug. Using our two novel models in zebrafish, a teleost capable of functional regeneration of vision following injury to the retina, I compared cone subtype specific differences in degeneration as well as regeneration in an intact retina. Ablation using the NTR-MTZ system was previously demonstrated to be specific, lacking a detectable toxic bystander

effect on neighboring photoreceptors [100, 117]. The UV cone ablation model is an improvement on a previous iteration of the technology, which better extends transgene expression into adulthood. Blue cone ablation was chosen as a comparator to UV cone ablation because blue and UV cones utilize a single opsin gene, which are closely related paralogs attuned to short wavelength light, and they have a single cone morphology (as opposed to red/green double cones). This model is ideal to address the knowledge gap in cone neurogenesis, which must be addressed if stem cell replacement therapies are to successfully regenerate cones. Thus far, proof of concept has been shown for transplantation of photoreceptors in mice, with partial restoration of rod mediated visual acuity following rod transplantation in a degenerate mouse retina [16, 17]. Success with transplanting cone photoreceptors has been limited, since transplanted photoreceptors precursors preferentially assume a rod fate in the adult mouse retina [19]. The degree of vision recovery is dependent on the number of integrated photoreceptors [17]; for functional regeneration of vision to be optimally successful a significant number of cone photoreceptors must integrate into the retina. The zebrafish retina, which is a cone rich environment, has a robust regenerative response that can functionally regenerate vision following retinal lesion or targeted ablation of a specific type of cone photoreceptors [3, 4, 100]. Therefore my zebrafish model of cone specific ablation is expected to contribute to resolving the mechanisms involved in the integration cone photoreceptors and repair of visual circuits for the restoration of visual ability.

Our novel model of UV cone ablation induced a proliferative response in the central retina and a regenerative response was observed by the reappearance of the UV cones in the central and peripheral retina. Past work in our lab has demonstrated that specific ablation of UV cones induces a proliferative response associated with Müller glia, a source of retinal stem cells [117]. The regenerative response is biased to the generation of the ablated UV cones, but

because cell proliferation assays label both regenerating and developing cells in the larval retina it remains possible that UV cone ablation induces nonspecific generation of other cone types. The overrepresentation of UV cones came at the expense of rods, potentially signifying that rod precursors switched cell fate, adopting the UV cone fate instead. Proliferation in the non-ablated control retina can likely be attributed to the generation of rod photoreceptors to maintain visual acuity as the retina continuously expands [2, 49]. Our model of blue cone ablation also induced a proliferative response in the central retina; I observed the reappearance of blue cones in the central and peripheral retina potentially indicating a blue cone regenerative response. Recent evidence suggests that degeneration of different cone subtypes evokes a cone specific regenerative response, however blue cone ablation does not induce a significant regenerative response unless it is accompanied by ablation of another photoreceptor subtype such as red cones [88]. We have yet to assess the magnitude of the blue cone regenerative response evoked by blue cone ablation using our model, but I predict that our model will not produce a significant blue cone regenerative response as previously observed by Yoshimatsu et al. using their Tg(*sws2:nfsB-mCherry*) model of NTR-MTZ mediated blue cone ablation [88]. Nonetheless, it is possible that model specific variation in the expression of the transgene could result in different intensities of ablation and induction of regeneration. Initial quantification of the blue cone regenerative response suggests that proliferation is induced in the CMZ and central retina following blue cone ablation, but very few newly generated cells adopt the blue cone fate relative to non-ablated controls. Therefore, the regenerative response following blue cone ablation may not significantly regenerate the ablated cone subtype. Further characterization of the blue cone regenerative response is required to determine which cone photoreceptor subtypes are being generated to determine whether the proliferative response is tailored to replacing lost blue cones.

The blue or UV cone regenerative responses in the central retina indicate that photoreceptor regeneration can occur in the mature retina in the absence of developmental cues, however the regenerative response can vary with age. UV cone specific ablation does not elicit a regenerative response in the adult retina, and the gaps leftover in the photoreceptor mosaic are gradually filled with rods [133]. Nonetheless, it is possible to generate a substantial UV cone regenerative response if ablation is accompanied by ablation of other cone photoreceptor in the adult retina [133].

Given the robust regenerative potential of zebrafish, our models of cone subtype specific photoreceptor ablation are ideally suited to characterize the gene expression networks during regeneration to determine the factors involved in biasing retinal progenitor cell fate for specific photoreceptor subtypes, as well as the mechanisms of photoreceptor integration and the restoration of the visual circuitry in a cone subtype dependent manner.

I modified the optomotor response (OMR) assay, a visually mediated behaviour in zebrafish [99, 132], to test whether zebrafish are capable of recovering visual ability following cone subtype specific ablation, and determine whether regenerated photoreceptors that have integrated into the retina connecting to the visual circuitry are necessary to drive recovery of the visually guided behaviour. My findings demonstrated that the sudden loss of blue or UV cones resulted in a deficit in visually mediated behaviour of larval zebrafish. The deficit in visual ability was apparent when using a high contrast stimulus, designed to evoke a strong behavioural response, and when using a low contrast stimulus, optimized to be less perceptible to vision-compromised fish so to only evoke a behavioural response from zebrafish with intact vision. The optimized stimulus generated a more robust differential response between the cone-ablated versus non-ablated controls. The loss of the OMR using either high or low contrast stimuli suggests that

photoreceptors sensitive to short-wavelength light may be involved in motion perception and mediating the OMR response.

In our UV cone ablation model, visually mediated behaviour gradually recovered following removal of the prodrug. Recovery of visually mediated behaviour correlated to the reappearance of UV cones in the periphery of the retina, generated by the ciliary marginal zone (CMZ). The newly generated UV cones situated at the dorsal periphery of the retina were ideally positioned to detect substrate level visual cues such as the optomotor response. Recovery of visually mediated behaviour did not occur when newly generated UV cones were continually lost due to the presence of the prodrug. Supporting that newly generated UV cones from the CMZ integrate into the retina and functionally restore vision lost from UV cone ablation. Our findings support that following UV cone ablation the CMZ exhibits an increase in proliferation. These cells more frequently adopted the UV cone fate relative to non-ablated controls. Supporting that UV cone ablation induces biased regeneration as was previously observed [117], and that these regenerated UV cone photoreceptors can functionally integrate into the retina to restore the visual ability of the larval zebrafish.

In comparison, our blue cone ablation model demonstrated recovery of the visually mediated behavioural within 24 hours of prodrug removal. This was unexpectedly rapid given the histological evidence that blue cones had not yet been regenerated. This quick behavioural recovery occurred independently of blue cone generation, suggesting an unknown mechanism was responsible for recovery of visually mediated swimming behaviour. Because the OMR is dependent on movement for behavioural output, it is possible that a reduced OMR could be the result of a movement deficit caused by blue cone ablation. I validated the assumption that blue cone ablation does not induce a movement deficit using the spontaneous swim assay to asses movement behaviour [105]. Further analysis from our lab validated that a behavioural response

generated from the modified OMR assay was dependent on vision, and it reliably evoked directional movement [100]. This led me to speculate that the loss of blue cone input induces a yet unidentified mechanism to compensate for vision loss (for potential sources see section 3.1).

In addition, I examined whether UV cone ablation induced a motor deficit in larval zebrafish. I detected a decrease in movement behaviour following UV cone ablation using the spontaneous swim assay. This decrease in swimming behaviour suggests that UV cone input may have a role in guiding spontaneous swimming behaviour, or that UV cone ablation may have off-target effects resulting in a movement deficit. Our lab recently employed a second visually mediated behaviour that is dependent on UV cones, in order to assess if UV cone ablation functionally affected vision. Larval zebrafish normally respond to potentially harmful UV irradiation by dispersing the melanin within their pigment cells as a protective mechanism. This response is dependent on the short wavelength-sensitive cone photoreceptors, and is impaired when UV cones are ablated [100, 106]. This behaviour is independent of potential confounds such as movement, and gradually recovers following prodrug removal. This supports that UV cone ablation induces a vision deficit resulting in a decreased OMR, and that the regenerative response functionally restores visual ability.

Combination of transgenes	Purpose
Tg(sws1:KalTA4)ua3139; Tg(4X-uas:nfsB-mCherry-2A-KalTA4)ua3137	Ablate UV cone photoreceptors
Tg(sws2:KalTA4)ua3136; Tg(4X-UAS:nfsB-mCherry-2A-KalTA4)ua3135	Ablate Blue cone photoreceptors
Tg(sws2:KalTA4)ua3138; Tg(4X-UAS:nfsB-mCherry-2A-KalTA4)ua3137	Ablate Blue cone photoreceptors

Table 1. Transgenic fish engineered to ablate specific cone subtypes.

Blue cone Ablation		Hours since end of drug treatment															
		0				24				48				72			
Stimulus colour pattern		BK & WT	B.RD & BL	RD & BL	D. RD & BL	BK & WT	B.RD & BL	RD & BL	D. RD & BL	BK & WT	B.RD & BL	RD & BL	D. RD & BL	BK & WT	B.RD & BL	RD & BL	D. RD & BL
Treatment group	WT in MTZ	35 ± 8 (33)	13 ± 7 (23)	16 ± 6 (30)	41 ± 7 (32)	27 ± 5 (50)	16 ± 4 (41)	26 ± 5 (28)	35 ± 7 (29)	47 ± 6 (46)	33 ± 6 (34)	35 ± 7 (33)	53 ± 7 (35)	61 ± 9 (24)	40 ± 9 (18)	66 ± 8 (19)	60 ± 11 (14)
	Tg (Blue) in DMSO	20 ± 5 (47)	19 ± 5 (45)	20 ± 5 (44)	28 ± 4 (29)	35 ± 6 (47)	28 ± 6 (43)	32 ± 6 (44)	37 ± 7 (35)	45 ± 6 (55)	38 ± 6 (46)	44 ± 6 (46)	47 ± 7 (43)	68 ± 8 (25)	34 ± 9 (19)	39 ± 8 (17)	43 ± 11 (14)
	Tg (Blue) in MTZ	15 ± 3 (54)	4 ± 2 (58)	6 ± 3 (55)	*7 ± 3 (54)	25 ± 6 (50)	17 ± 5 (42)	22 ± 5 (45)	31 ± 6 (39)	37 ± 6 (39)	29 ± 5 (47)	42 ± 6 (48)	53 ± 6 (46)	61 ± 9 (23)	48 ± 11 (16)	49 ± 11 (15)	60 ± 11 (18)

UV cone Ablation		Hours since end of drug treatment							
		0		24		48		72	
Stimulus colour pattern		BL & WT	D. RD & BL	BL & WT	D. RD & BL	BL & WT	D. RD & BL	BL & WT	D. RD & BL
Treatment group	WT in MTZ	47 ± 18 (31)	40 ± 17 (31)	50 ± 18 (59)	47 ± 18 (59)	52 ± 17 (45)	52 ± 17 (46)	33 ± 13 (29)	35 ± 13 (30)
	Tg(UV) in DMSO	55 ± 15 (31)	50 ± 16 (19)	60 ± 16 (65)	59 ± 16 (52)	57 ± 16 (60)	54 ± 15 (58)	44 ± 16 (17)	56 ± 17 (17)
	Tg(UV) in MTZ	** 5 ± 6 (29)	** 1 ± 1 (30)	** 12 ± 7 (60)	** 8 ± 7 (54)	** 23 ± 14 (44)	** 12 ± 8 (40)	37 ± 18 (32)	30 ± 16 (33)

Table 2. Visually mediated behaviour is lost following ablation of blue or UV cone; recovery time is dependent on the type of cone photoreceptor ablated. Mean of fish movement (% of trough travelled) ± standard error and sample size (number of fish) are reported. WT in MTZ are non-transgenic siblings treated with the prodrug MTZ. Tg(Blue) are transgenic zebrafish expressing nitroreductase in their Blue cones. Tg(UV) are transgenic zebrafish expressing nitroreductase in their UV cones. DMSO is the prodrug vehicle control. Stimulus colour of moving bars: BL & WT = black and white, B.RD & BL = bright red and blue, RD & BL = red and blue, and D.RD & BL = dark red and blue (see Figure S1. for example and spectral output). * Statistically significant difference (*$p<0.05$; **$p<0.0001$) respective to the control fish at the same timepoint (Two-Way ANOVA and post-hoc Bonferroni test). Normalized data plotted in Figure 3 and 4, raw data plotted in Figure S1.

	Stimulus colour pattern	BL & WT	D. RD & BL
Treatment group	WT in MTZ	51 ± 11 (10)	54 ± 12 (9)
	Tg(Blue) in DMSO	49 ± 13 (10)	44 ± 13 (10)
	Tg(Blue) in MTZ	49 ± 14 (10)	42 ± 13 (10)

Table 3. Rapid functional recovery of visually evoked behavioural response is not dependent on blue cone generation or regeneration.

No significant differences were found between these treatments. Fish were treated for 48 hours with MTZ at 7 dpf to prevent the quick recovery of visually mediated behaviour as observed in Table 2 and Figure 4 after zebrafish were treated with MTZ at 7 dpf for 24 hours. Mean of fish movement (% of trough travelled) ± standard error and sample size (number of fish) are reported. Stimulus colour of moving bars: BL & WT = black and white, and D. RD & BL = dark red and blue. Normalized data presented in Figure 5.

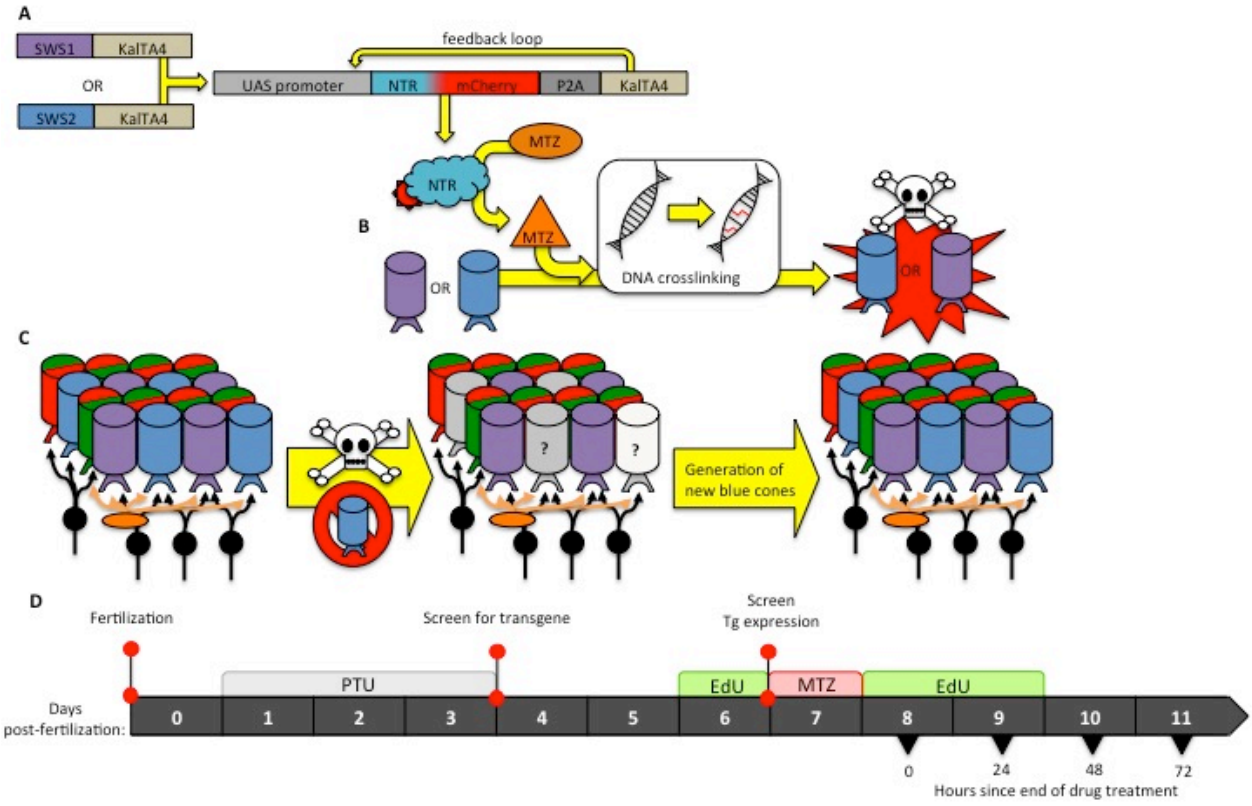


Figure 3. Novel chemical-transgenic models of conditional targeted cone photoreceptor ablation.

Transgenic zebrafish were engineered to express nitroreductase in either UV or blue cone photoreceptors, which includes conditional cell ablation when in the presence of an innocuous prodrug. **(A)** UAS/KalTA4 system of cell specific expression utilizes either the UV or blue cones specific opsin gene (sws1 or sws2 promoter respectively) to drive expression of KalTA4 (an optimized variant of Gal4) in separate transgenic lines of zebrafish. KalTA4 binds and initiates expression of the UAS promoter, and subsequently a bacterial enzyme nitroreductase (NTR), which is fused to a fluorescent reporter mCherry, as well as a second copy of KalTA4 creating a positive feedback loop "Kaloop" to maintain expression of the transgene into adulthood. **(B)** Nitroreductase (NTR) converts the prodrug metronidazole (MTZ) into a cell autonomous DNA cross-linking agent, initiating apoptosis in transgene expressing cone photoreceptors. MTZ exposure occurs as a bath treatment thought the addition of the prodrug to the tank water. **(C)** Conditional ablation depends on the presence of the prodrug, when the prodrug has been removed ablation ceases and regeneration may begin. Teleosts are able to regenerate any type of retinal cell following injury. For regeneration to be successful newly generated photoreceptors must synapse with the pre-existing circuitry to properly integrate and functionally restore lost vision. **(D)** Experimental timeline. Larvae were treated with 1-phenol-2-thiourea (PTU) to block synthesis of the pigment melanin prior to screening for the transgene and robust expression. Larval zebrafish were treated with MTZ for 24 hours at 7 days post fertilization (dpf). Behavioural recording and histological samples were taken following prodrug treatment (8 dpf) and each day afterwards. Select larval zebrafish were treated with EdU as part of a cell proliferation assay at 6 dpf for 24 hours and again following prodrug treatment for 48 hours.

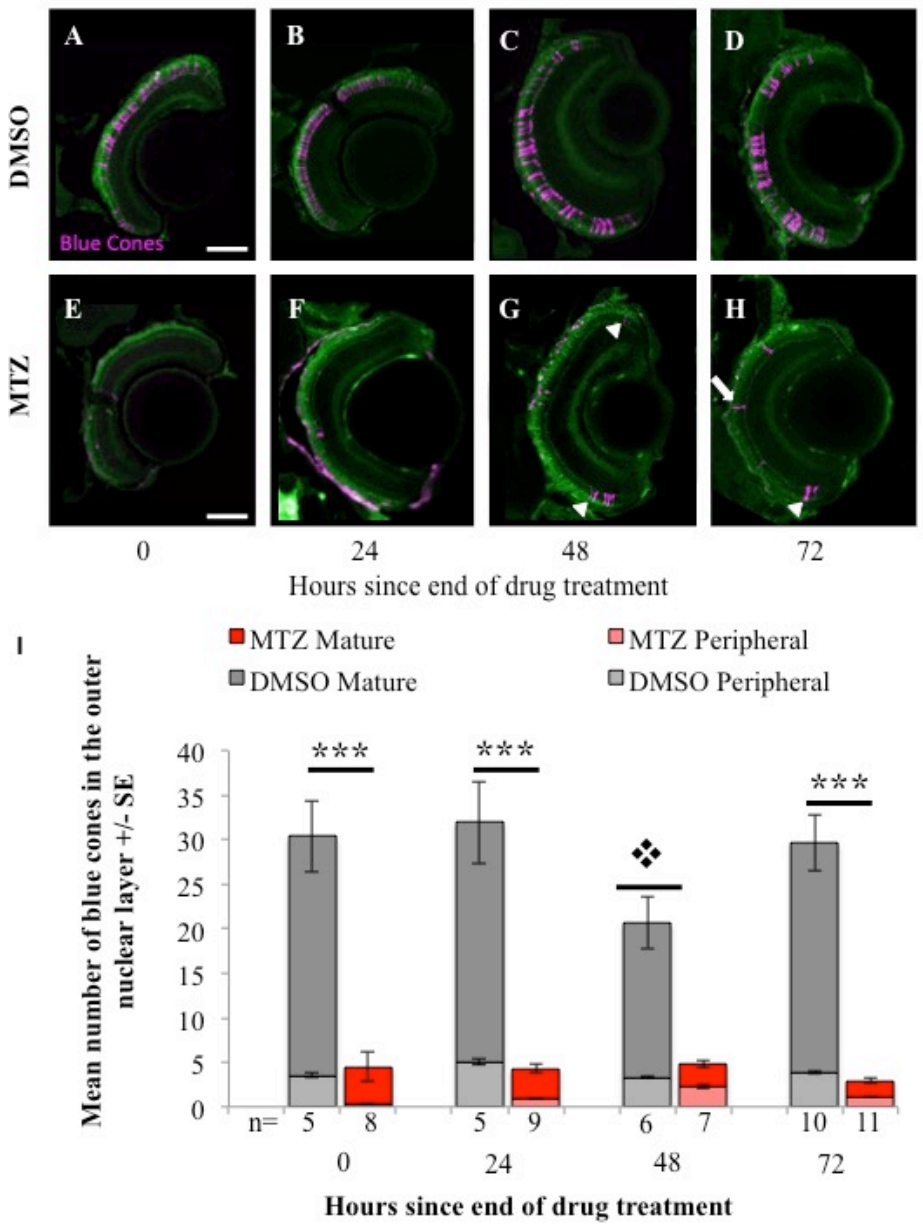


Figure 4. Blue cone photoreceptors are efficiently ablated upon addition of the prodrug.

Larval zebrafish expressed the transgenes Tg(sws2:KalTA4; UAS:nfsB-mCherry-KalTA4) in the blue cones. Larva treated with the prodrug metronidazole (MTZ) for 24 hours beginning at 7 dpf had a statistically significant decrease in the number of mCherry positive blue cones (magenta), compared to the vehicle control (0.2% DMSO). Autofluorescence (Green). MTZ treatment yielded a significant decrease in the number of mCherry positive cells in both the central and peripheral regions of the outer nuclear layer (*** = total retina, central retina, and peripheral retina $p<0.001$, ❖ = total retina, and central retina $p<0.001$ Two-Way ANOVA) compared to vehicle control (DMSO). Newly generated blue cone were first observed 48 hours after prodrug treatment in the peripheral retina (Arrowhead). Blue cones reappeared in the central retina 72 hours after prodrug treatment (Arrow). Sample size (n = number of larvae) reported on each bar. Scale bars are 50 μm.

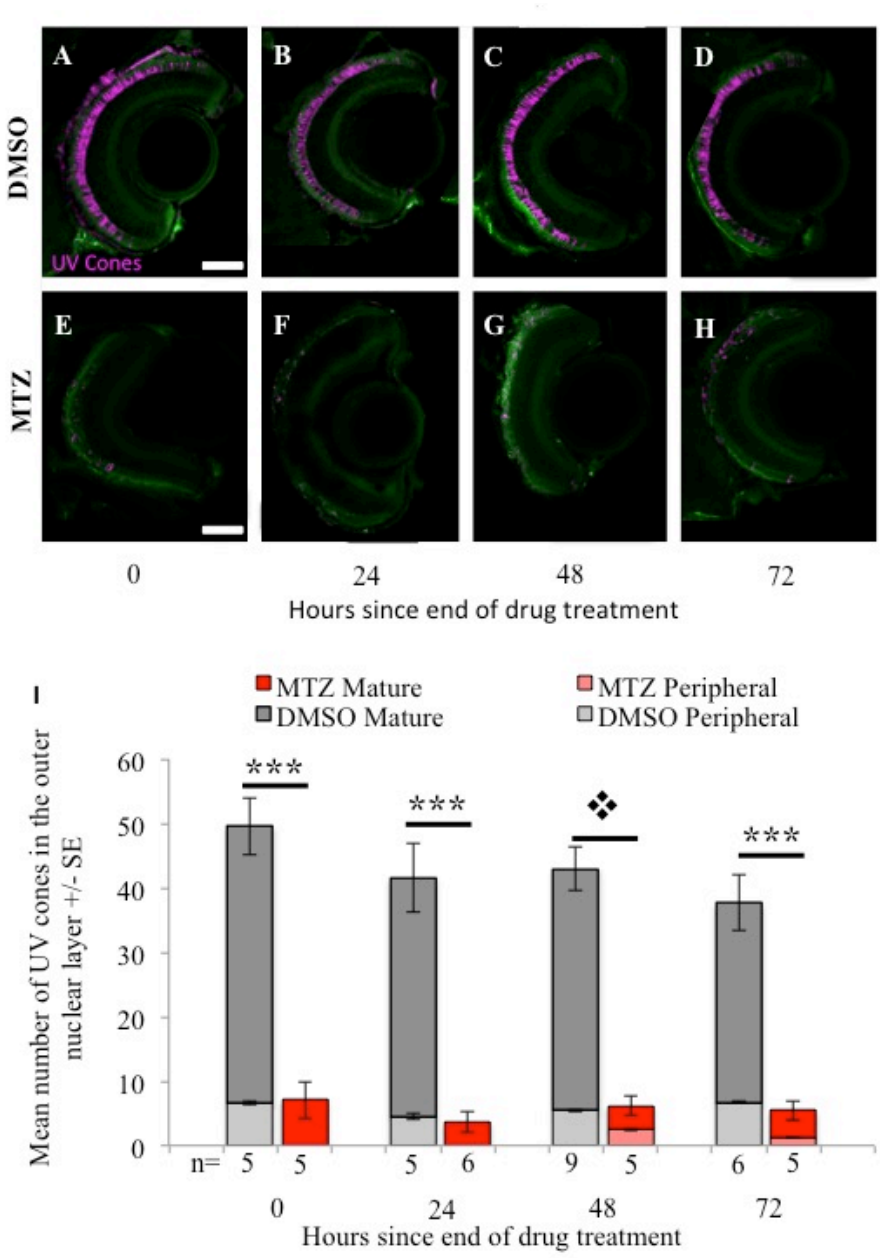
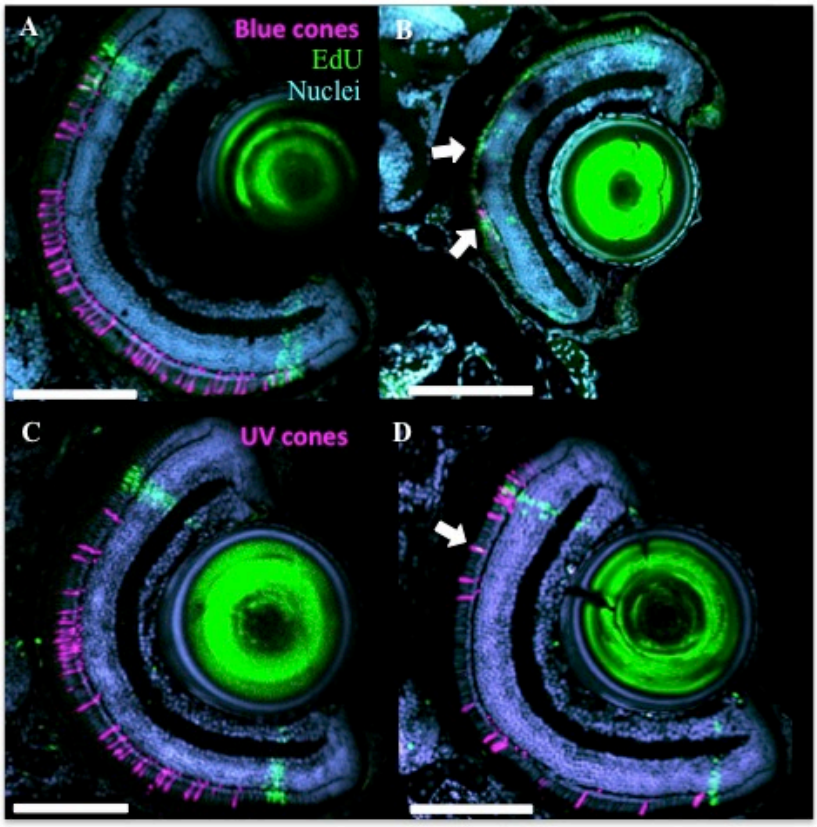
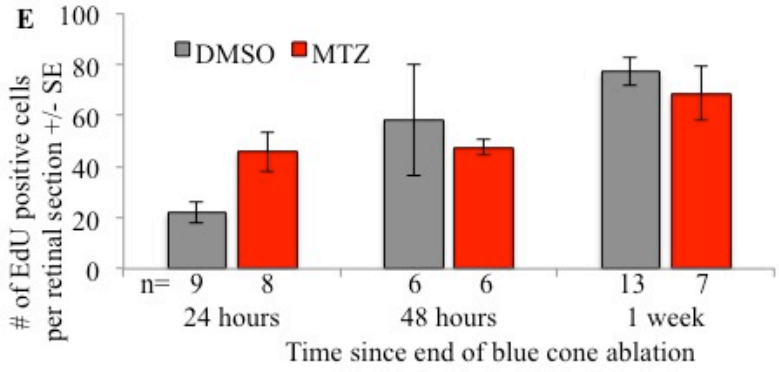
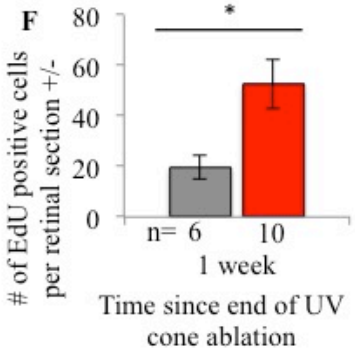


Figure 5. UV cone photoreceptors are efficiently ablated upon the addition of the prodrug.

Larval zebrafish expressed the Tg(sws1:KalTA4; UAS:nfsB-mCherry-KalTA4) in the UV cones. Larva treated with the prodrug MTZ for 24 hours at 7 dpf had a decrease in the number of mCherry positive UV cones (magenta) compared to the vehicle control (DMSO). Autofluorescence (Green). MTZ treatment yielded a significant decrease in the number of mCherry positive cells in both the central and peripheral regions of the outer nuclear layer (*** = total retina, central retina $p<0.001$ and peripheral retina $p<0.05$, ❖ = total retina, and central retina $p<0.001$ Two-Way ANOVA) compared to vehicle control (DMSO). UV cones reappeared were first observed reappearing in the peripheral retina 48 hours after prodrug treatment (Arrowhead). Sample size (n = number of larvae) reported on each bar. Scale bars are 50 μm.


A
Blue cones
EdU
Nuclei
B
C
UV cones
D


E
100
80
60
40
20
0
DMSO
MTZ
# of EdU positive cells per retinal section +/- SE
n= 9 8 6 6 13 7
24 hours
48 hours
1 week
Time since end of blue cone ablation


F
80
60
40
20
0
*
# of EdU positive cells per retinal section +/-
n= 6 10
1 week
Time since end of UV cone ablation

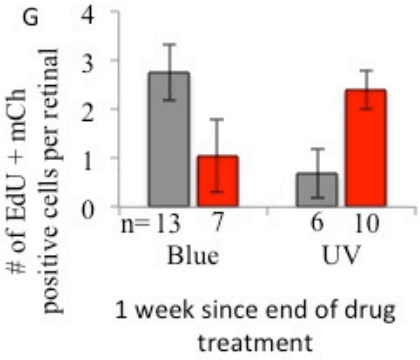

G
4
3
2
1
0
# of EdU + mCh positive cells per retinal
n= 13 7 6 10
Blue
UV
1 week since end of drug treatment

Figure 6. Ablation of UV and blue cones induced increased cell proliferation at different time points.

Larval zebrafish were treated with EdU, which incorporates into the DNA of proliferating cells. Larval zebrafish were treated with EdU at 6dpf for 24 hours and again following prodrug treatment for 48 hours. EdU positive cells in the vehicle treated fish demonstrate the normal expansion of the retina due to proliferating retinal stem cells at the ciliary marginal zone **(A, C)**. Prodrug treatment induced cell proliferation in the central retina (Arrows)(B, D). Ablation of blue cones induced increased cell proliferation in the retina immediately following prodrug treatment **(E)**. A significant increase in cell proliferation was detected 1 week following UV cone ablation **(F)**. The number of mCherry positive cells that colocalized with EdU decreased with MTZ exposure for blue cone ablation whereas it increased following UV cone ablation **(G)**. *$p<0.05$ experimental relative to vehicle control (Two-Way ANOVA). Sample size (n = number of larvae tested) reported on each bar.

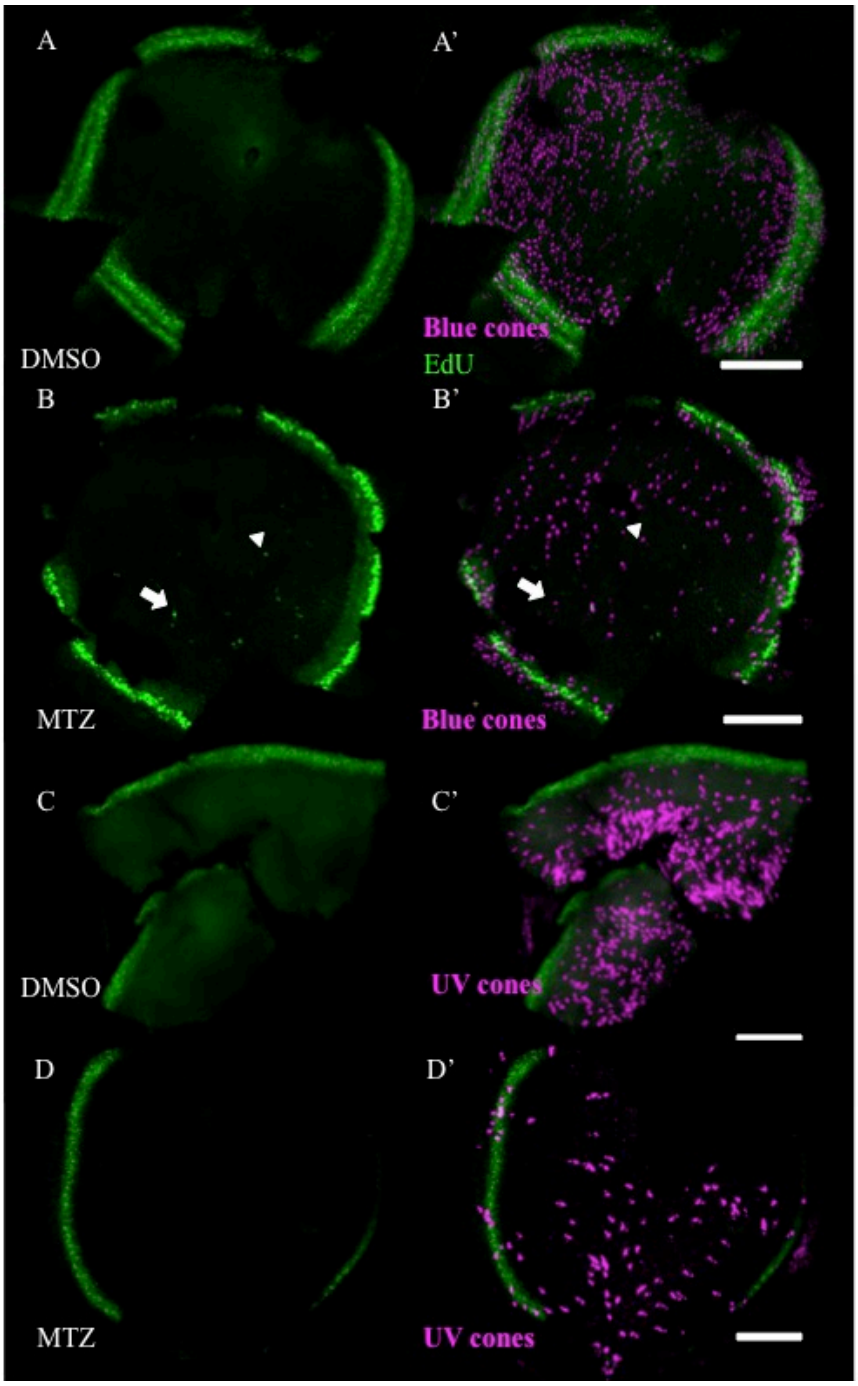

A
A'
DMSO
Blue cones
EdU
B
B'
MTZ
Blue cones
C
C'
DMSO
UV cones
D
D'
MTZ
UV cones

Figure 7. Following cone photoreceptor ablation of either blue or UV cones, mCherry positive cells reappear in the central retina.

Larval zebrafish were treated with EdU at 6 dpf for 24 hours. Larval zebrafish were then treated with the prodrug for 24 hours, and then were exposed again to EdU for 48 hours (see Figure 3 timeline). Following ablation of blue or UV cone ablation, larval zebrafish recovered for 1 week at which point they were fixed (A-B' and C-D' respectively). EdU stains proliferating cells (green), mCherry positive cells co-express nitroreductase (magenta). In the central retina, EdU and mCherry positive cell show a newly generated blue cone potentially from a Müller glial source (arrowhead). EdU positive mCherry negative (arrow).

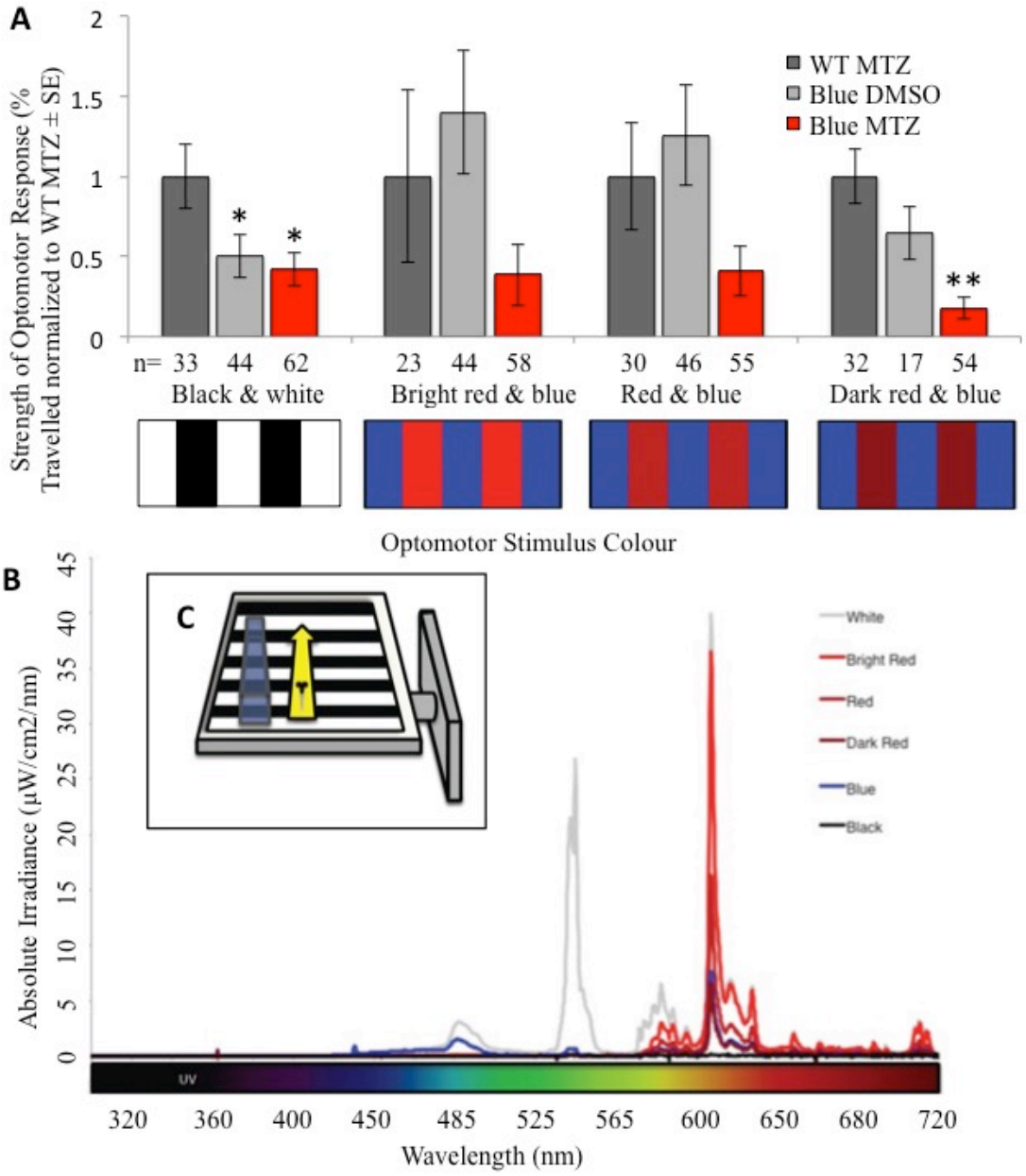

A
Strength of Optomotor Response (% Travelled normalized to WT MTZ ± SE)
2
1.5
1
0.5
0
WT MTZ
Blue DMSO
Blue MTZ
*
*
**
n= 33 44 62
Black & white
23 44 58
Bright red & blue
30 46 55
Red & blue
32 17 54
Dark red & blue
Optomotor Stimulus Colour
B
C
Absolute Irradiance (µW/cm2/nm)
0 5 10 15 20 25 30 35 40 45
White
Bright Red
Red
Dark Red
Blue
Black
UV
320 360 400 450 485 525 565 600 650 680 720
Wavelength (nm)

Figure 8. Characterization of the stimuli in the optomotor response (OMR) assay.

The OMR behaviour of larval zebrafish measured visual function using various colour patterns (shown below the respective OMR) (A). Behaviour was measured immediately following the removal of the prodrug to identify changes in visually mediated behaviour. Responses of transgenic zebrafish larvae treated with MTZ (red bars) were measured relative to control fish (grey bars). Spectral irradiance of each colour used in the optomotor response assay (B). Optomotor apparatus, which consisted of an LCD computer monitor laid horizontally and a series of narrow troughs containing one larval zebrafish each (C). In this schematic larvae were positioned starting at one end of the trough and were then exposed a series of the moving bars for 1 minute. The moving bars travelled in the direction of the arrow, perpendicular to the long axis of the trough. Optomotor response was recorded from a camera positioned overhead, which measured OMR as percent of trough travelled from to the starting zone. Larval zebrafish were acclimated to a stimulus in the reverse direction for 1 minute prior to behavioural recording. **$p<0.05$ experimental relative to controls, *$p<0.05$ experimental compared to the wild-type control (Two-Way ANOVA). Sample size (n = number of larvae tested) reported on each bar.

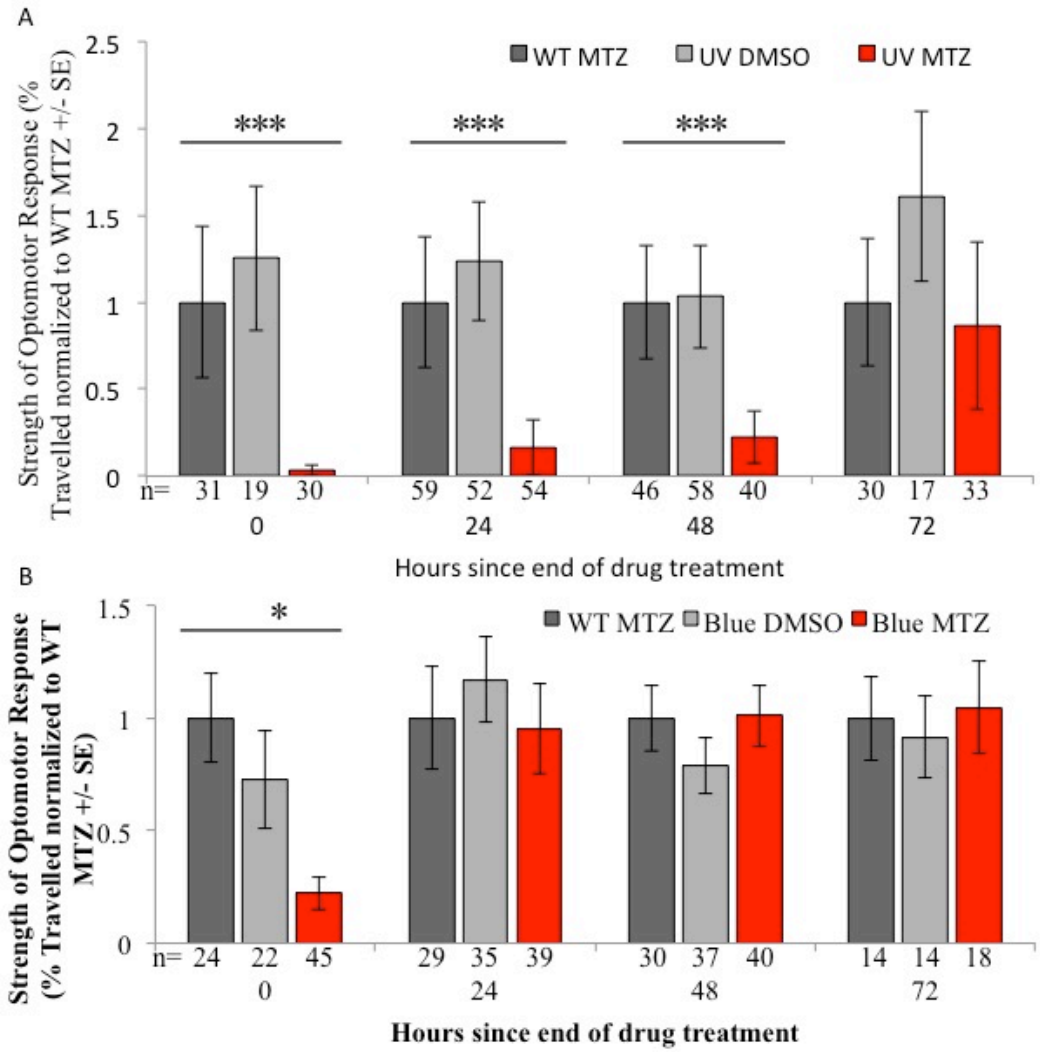


Figure 9. Visually mediated behaviour is reduced following ablation of UV or blue cones, however the time period for the recovery visually mediated behaviour depends on the type of cone photoreceptor ablated.

The visually evoked optomotor response (OMR) stimulates directional movement in larval zebrafish. Ablation of UV or blue cone immediately impairs the behavioural response (A and B, respectively). Following UV cone ablation OMR behaviour recovered gradually and reached control levels (Grey bars) 72 hours after prodrug treatment (A). Following the ablation of blue cones, recovery of the OMR to control levels occurred within 24 hours of prodrug removal, which is more rapid than expected. “WT MTZ” are the non transgenic siblings treated with MTZ (dark grey), “UV DMSO” and “Blue DMSO” are the transgenic fish that received the drug vehicle treatment (DMSO)(light grey bars). “UV MTZ” and “Blue MTZ” are transgenic fish that have had their respective UV or Blue cones ablated by prodrug treatment prior to behavioural testing (red bars). Sample size (n = number of larvae tested) reported on each bar. Data is normalized to “WT MTZ” for each respective time point. ***$p<0.001$ experimental relative to controls, *$p<0.05$ experimental compared to the wild-type control (Two-Way ANOVA).

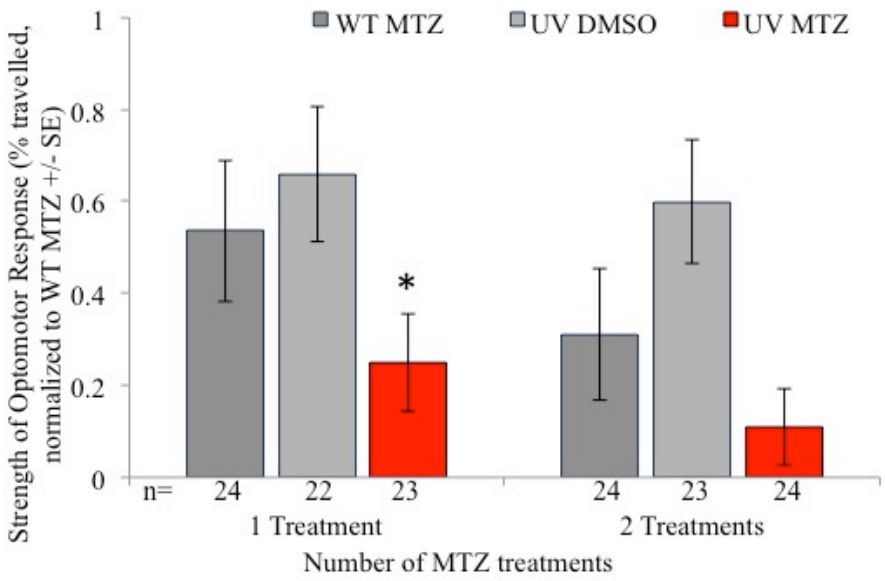


Figure 10. Functional recovery of visually evoked behaviour did not occur when UV cone generation was inhibited.

Larval zebrafish were first treated with the prodrug at 7 days post fertilization for 24 hours. Select larval zebrafish were then treated a second time for 24 hours, following a 48 hours recovery period from the first treatment. This was to ablate any newly generated UV cones to determine test whether they were responsible for the gradual increase in visually mediated behaviour, which reached control levels 72 hours after prodrug treatment (see Figure 9). OMR failed to recover to control levels (grey bars) when treated twice with the prodrug. Additionally, UV cone ablated fish treated only at 7 dpf failed to regenerate the visually mediated behaviour. *$p<0.01$ experimental compared to the wild-type control (One-Way ANOVA). Sample size (n = number of larvae tested) reported on each bar.

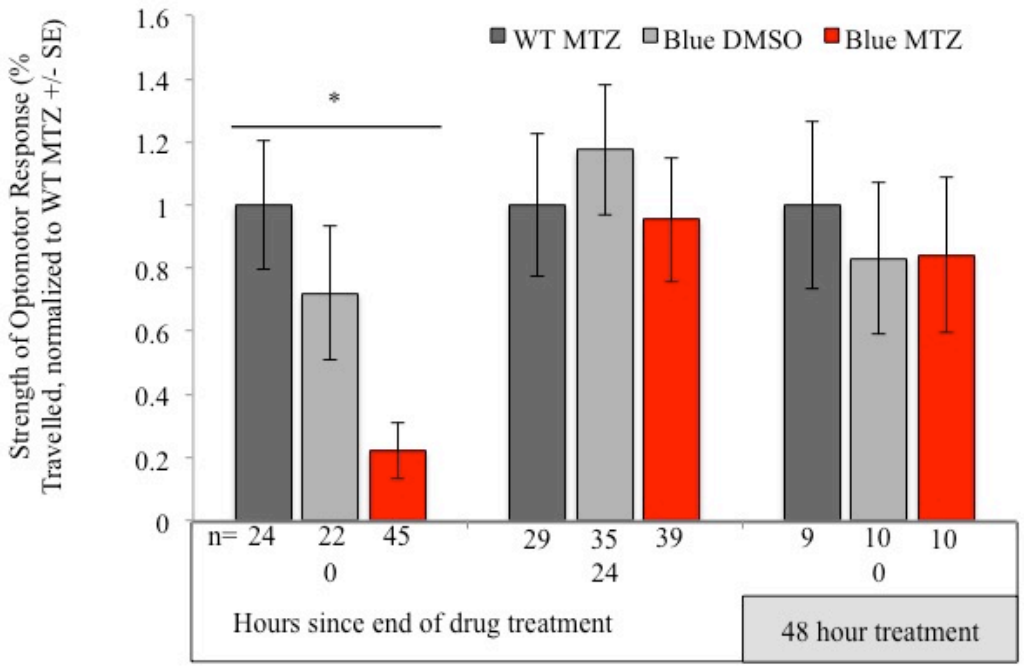


Figure 11. Functional recovery of visually evoked behavioural response is not dependent on the generation of new blue cones.

Larvae were treated with the prodrug MTZ for 24 hours beginning at 7 days post fertilization (dpf). Select larvae received a prolonged 48-hour MTZ treatment at 7 dpf to inhibit the generation of new blue cones, which did not prevent rapid recovery of visually mediated behaviour. Sample size (n = number of larvae tested) reported on each bar. *$p<0.05$ experimental compared to the wild-type control (Two-Way ANOVA).

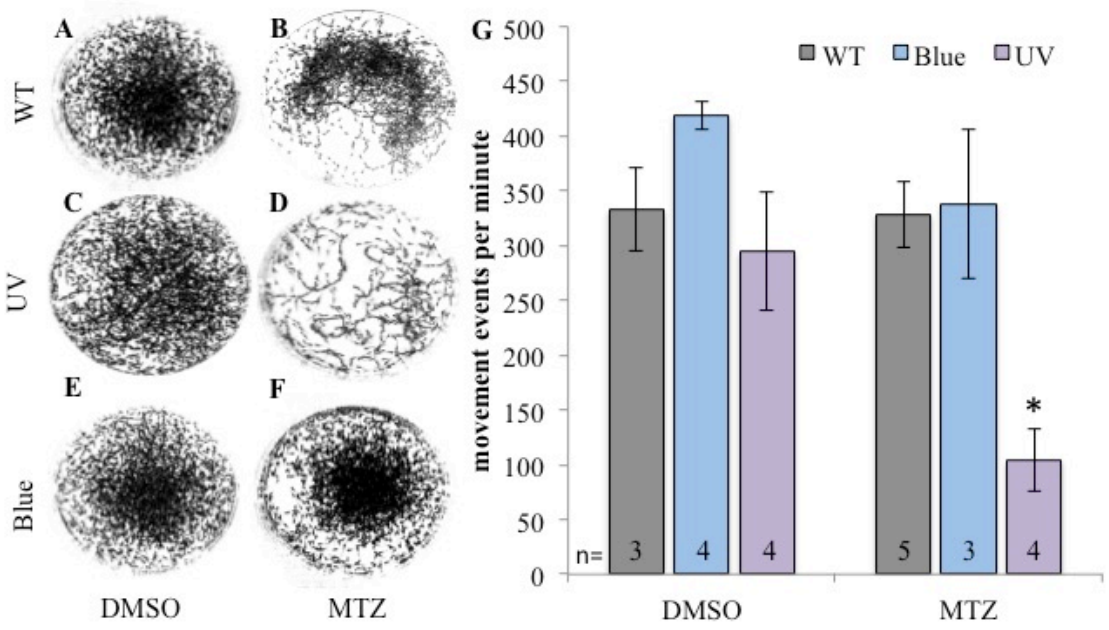


Figure 12. The effect of blue or UV cone photoreceptor ablation on the spontaneous motor activity of larval zebrafish is dependent on the subtype of cone photoreceptor ablated.

Spontaneous movement of 10 zebrafish in a petri dish was recorded over a period of 10 minutes using an overhead camera. The movement profile shows an overlay of all movement **(A-F)**. The number of movement events were recorded every second **(G)**. Ablation of blue cones did not affect spontaneous behaviour relative to the non-transgenic (WT) control. Ablation of UV cones significantly decreased spontaneous behaviour. *$p<0.05$ experimental relative to controls (Two-Way ANOVA). Sample size (n = number of larvae tested) reported on each bar.

Chapter 3. Interpretation and Future Directions

Having successfully demonstrated targeted ablation of blue or UV cone photoreceptors using our novel model of conditional transgenic-chemical ablation, in conjunction with our tractable visually mediated behavioural assay, our system is ideal for interrogating the mechanisms involved in functional regeneration of cone-mediated vision. By inducing photoreceptor specific cell death we will be able to characterize how the retinal circuitry reacts to the loss of a specific cone photoreceptor subtype and whether the visual circuitry is restored during regeneration of the photoreceptors. Furthermore, observing the retina remodel in the context of rapid recovery of visual ability following blue cone ablation will be important in determining the functional significance of retinal plasticity. Using our model, we will be able to induce regeneration of the ablated cone photoreceptor subtype in a biased manner, which is ideal to determine the mechanisms involved in photoreceptor fate determination. Additionally, using our most recent iteration of blue or UV cone ablation we can expand our characterization of functional regeneration into the adult zebrafish.

3.1 Speculation on the mechanism of rapid recovery of vision following blue cone ablation

Recovery of visually mediated behaviour occurred faster than predicted and was independent of blue cone generation suggesting that another mechanism may be involved. I speculate that quick recovery may be due to compensation by retinal remodelling. Retinal remodelling is known to occur due to the loss of cone photoreceptors or loss of cone input [7, 134, 135], and initiates the stereotypical remodelling events at the molecular, synaptic, cell, and circuit and levels [136]. The functional implications of retinal remodelling on vision are poorly understood, especially for interneurons such as horizontal cell, bipolar cells, and amacrine cells which undergo morphological and physiological remodelling, sprouting neurites and changing

their firing patterns [137-139]. Restoration of visual ability due solely to retinal remodelling has not been previously documented.

A 24 hour timeframe for rapid remodelling is not unfeasible, rapid remodelling is a normal process observed during development. For example, chick retinal ganglion cells exhibit a high degree of dendritic motility specifically at the terminal filopodia-like processes. These dendritic processes can move several micrometers per second. These movements are associated with initial synaptogenesis and are regulated by glutamatergic (bipolar cell input) and cholinergic (amacrine cell) transmission [140, 141]. However, these dendritic movements slow as the retina matures and synaptogenesis advances. Retinal plasticity during development is important to understand in the context of vision circuit assembly as some mechanisms can be recapitulated in regeneration of the mature retina. Rapid retinal remodelling can also be observed in the context of light adaptation, and the circadian remodelling of neuronal circuits, resulting in the modification in the number of ribbon synapses in teleost fish, and structural remodelling of rod bipolar resulting in the loss of bipolar cell terminal spinules and irregular axon terminal shape cells as observed in the rat retina [81, 142].

The rapid recovery of visually mediated behaviour following blue cone ablation could potentially be due to rapid morphological remodelling of interneurons to form novel visual circuits using new synaptic partners. When photoreceptors are dysfunctional or absent, synaptogenesis with non-preferred binding partners occurs; ON cone bipolar cells form ectopic synapses with rods when cones are dysfunctional in CNGA3$^{-/-}$ mice or absent in coneless mice [143, 144]. The localization of these synapses can also be abnormal; rod bipolar cells and horizontal cells extend ectopic contacts to the outer nuclear layer, contacting rod cell bodies and retracted rod spherules in nob2 mice [145]. Whether these aberrant connections with novel synaptic partners are able to compensate for loss of input and restore visual acuity is unknown.

A recent study examined whether the ablation of a specific cone photoreceptor subtype would affect the cone connectivity pattern and whether regeneration could rewire the original circuit. H3 horizontal cells prefer specific synaptic partners such as blue and UV cones, while avoiding red and blue cones. After blue cone ablation or UV cone ablation, H3 horizontal cells did not form novel contacts with non–preferred targets (red or green cones) or alter H3 horizontal cell connectivity [88]. Yet, UV cone ablation did trigger transient remodelling of H3 horizontal cells. H3 horizontal cells extend transient neurites that subsequently retract. Prolonged inhibition of UV cone regeneration alters the connectivity pattern as H3 horizontal cells synapse with non-preferred targets, suggesting there may be a critical period in which H3 cells maintain postsynaptic connectivity preferences prior to remodelling to non-preferred synaptic partners. The timeframe of this transient period is unknown, but abnormal rewiring begins within four days post ablation [88]. Future studies should determine whether prodrug treatment for 24 hours is sufficient to delay regeneration past this theoretical transient period to determine whether H3 cells are altering their connectivity patterns. My research suggests that blue cone ablation may not be sufficient to trigger a transient period that maintains synaptic connectivity of H3 cells for the same duration, because we witnessed a rapid recovery of visual ability potentially as a result of synapsing with non-preferred synaptic partners. Further examination of the regenerative signals that inhibit remodelling of the retinal circuitry is significant as it may explain why the timeframe for functional regeneration is different based on the cone subtype ablated. Therefore, I speculate that because blue cone ablation does not induce a significant regenerative response, functional recovery of visually mediated behaviour occurs rapidly due to uninhibited remodelling. By comparison, UV cone ablation induces a robust regenerative response, which inhibits remodelling, so functional recovery occurs on a gradual timeline as the UV cones regenerate and integrate into the photoreceptor layer to restore the visual circuit. Alternatively,

the retinal circuitry may be rewiring to surviving UV or blue cones photoreceptors, which avoided ablation to restore visual acuity. Approximately 80% of UV cones expressed the nfsB-mCherry transgene [100], leaving a subset of UV cones which would have avoided ablation.

Alternatively, I speculate that rapid recovery of visually mediated behaviour could be the result of a physiological change in the signaling of the circuitry. For example, in retinitis pigmentosa ON-cone bipolar cell (ON-CBC) and rod bipolar cells (RBC) undergo reprogramming; the mGluR6-mediated response diminishes and begins to aberrantly express iGluR [146]. ON-cone bipolar cells normally express mGluR6, while OFF-cone bipolar cells express ionotropic AMPA/kainate GluRs [147]. Altering the synaptic potential of ON-cone bipolar cells in the context of blue or UV cone ablation could alter the visual circuit and functionally affect visual ability. Spectral responses cannot be explained solely by the spectral sensitivity of the various cone photoreceptors, colour opponency is an important process for colour vision. Light stimuli of various wavelengths can stimulate different types of potentials. For example certain horizontal cells are either depolarized or hyperpolarized based on the stimulus wavelength [148]. Alterations to presynaptic receptors may compensate for the loss of cone input to maintain opponency. From a spectral sensitivity standpoint, the retina has the capacity to compensate for the loss of blue cones, but not UV cones, because they are uniquely sensitive to UV light. Therefore, following blue cone ablation the retina could become trichromatic without complete loss of spectral sensitivity. This change in cone photoreceptor input could also evoke a temporary disruption to the processing of colour input, which is recovered through adaptation by the visual centers.

Quick recovery of visually mediated behaviour could also be due to a non-specific effects of NTR-MTZ mediated ablation such as leftover apoptotic bodies, hypertrophy of Müller glia cells, or toxic effects of MTZ. However, this is unlikely to produce a differential response

between UV and blue cone ablated fish as clearance of the remnant bodies was not observed to be any different between the models. Additionally the recovery of visually mediated behaviour occurred in the presence of MTZ during the prolonged treatment. Any toxic effects due to prodrug exposure were controlled during behavioural recording by comparing the results of a non-transgenic fish treated with MTZ and a transgenic fish treated with a vehicle control. Behavioural responses were relatively similar between the two controls.

3.2 Assessing the regenerative response following blue cone ablation

Recent evidence demonstrated that ablation of blue cones does not evoke a significant regenerative response without concurrent ablation of an additional cone subtype [88]. Our findings demonstrate that blue cones ablation elicits a proliferative response and blue cones reappear in the central retina. The magnitude of this regenerative response may be comparatively lower than UV ablation, however it is important to determine whether the regenerative response is tailored to regeneration of the ablated blue cones. Our lab has been successful in the past using *in situ* hybridization of the various opsins to determine the ratio of newly generated cone subtypes in conjunction with a cell proliferation assay [117]. It is important to determine whether blue cone regeneration is favored as potential genetic expression profiling could identify important upregulated genes specific to biased blue cone fate determination. The short wavelength sensitive1 (sws1) or blue cone was lost during vertebrate evolution, and a transcriptional regulator of blue cone fate remains elusive. Therein, zebrafish are ideally suited for studying the regulation of cone photoreceptor fate.

3.2.1 Characterizing retinal regeneration by manipulating fate determination and retinal remodelling

Driving cone photoreceptor fate from retinal stem cells is a major hurdle in cone photoreceptor replacement therapies in the mature retina [19]. Observing regeneration of the zebrafish retina is a valuable complement to mammalian studies, aiming because the zebrafish retina is cone dense environment capable of robust functional regeneration of cone mediated vision in which to assess the necessity of various genetic factors for cone photoreceptor cell fate. I propose using our model of cone subtype specific ablation to induce production of retinal progenitors. Regeneration following UV or blue cone ablation does not specifically regenerate the lost photoreceptor type, demonstrating that retinal progenitors are competent to form various neuron types. Thyroid hormone (TH) has been previously implicated as having an effect on photoreceptor cell fate during both development and maturation in teleosts [93, 149]. Generation of red cones during development requires thyroid hormone receptor β gene (trβ2) activity, but it is unknown whether trβ2 is required for red cone generation by the Müller glia during regeneration. We are proposing to alter thyroid hormone levels during regeneration to determine whether exogenous TH treatment can influence the fate of retinal progenitor cells favoring the red cone fate. Exogenous treatment with TH does not induce an opsin switch in zebrafish unlike juvenile rainbow trout [149, 150]. Alternatively, trβ2 morphants have a decreased number of red cones with an increased number of UV cones [54]. Work performed in mice has shown this inhibition of UV opsin expression occurs through heterodimerzation of trβ2with retinoid X receptor. Our lab has been developing an inducible dominant negative trβ2, which is resistant to thyroid hormone. We propose using this transgenic construct or methimazole, a common drug used to inhibit T3 and T4 synthesis, to test if UV cone generation is favored at the expense of red cones generation during regeneration following UV cone or blue cone ablation.

3.3 Identifying the source of retinal plasticity following cone ablation

My findings suggest that rapid recovery of visually mediated behaviour observed following blue cone ablation could be the first documented instance of retinal remodelling functionally compensating for loss of photoreceptor input to maintain vision. In order to verify this claim, we must first identify the source of plasticity and establish that retinal remodelling is responsible for recovery of vision.

Cone photoreceptor specific ablation offers an unprecedented opportunity to assess how newly regenerated cone photoreceptors integrate into the retina and form synapses with pre-existing partners to restore the visual circuit and vision. Determining how the retina adapts, the factors that guide synapses, and formation of circuits could have important ramifications in developing novel strategies for therapeutic treatment of photoreceptor loss.

3.3.1 Identifying changes in the neural activity of the visual system using CaMPARI

Retinal remodelling due to the loss of specific subtypes of cone photoreceptor could be occurring at various levels in the retina resulting in physiological or morphological changes in the visual circuitry. Identifying the source of functional regeneration will be difficult, however our lab is employing a promising new technique called CaMPARI (calcium-modulated photoactivatable ratiometric integrator) in zebrafish. CaMPARI is a genetically encoded calcium indicator (GECIs) that utilizes a fluorescent protein that can change wavelength emission from green to red irreversibly in the presence of high intracellular calcium and UV light [151]. This creates a snapshot of active nerve cells during behaviour. Our lab aims to express CaMPARI transgenically in all retinal neurons using the pan neuronal marker *elavl3*. This method will be utilized in conjunction with our blue and UV models of conditional cone photoreceptor ablation to identify how photoreceptor ablation impacts the neural activity of the visual circuits. The UV

light used for photoconversion of the chromophore will also function as a visual stimulus, which is both advantageous and a limitation as it reduces some of the flexibility of the assay to respond to a specific wavelength or intensity of light. Simultaneous illumination with an additional light source may be effective method to overcome a single UV light response and measure wavelength specific changes in neuron activity.

The ability to compare between our different models of cone ablation is invaluable considering that following UV cone ablation, visual recovery is dependent on the generation of new UV cones, in contrast visual recovery following blue cone ablation is independent of blue cone generation. Therein the ablation of these two cone subtypes may evoke different reactions from the retina that can be characterized by their activity profile. Therein, the CaMPARI method could potentially demonstrate functional regeneration as newly generated UV cones integrate in the retina to gradually restore the visual circuit. Whereas, the quick recovery of visually mediated behaviour following blue cone ablation suggests the retina can remodel to quickly visual ability, potentially suggesting altered signaling of the retinal circuit.

Alterations in neuron activity due to photoreceptor ablation will demonstrate how the activity of the visual circuitry changes functionally. We will then be able to answer whether functional regeneration requires restoration of the original activity pattern or if regenerated photoreceptors alter the existing visual circuit to functionally restore vision. Additionally, we aim to determine how the activity of the retina changes when regeneration of the ablated cell is interrupted by continuous ablation due to prodrug treatment. This could be used to assess whether ectopic synapses with non-preferred targets has functional implication for the spectral sensitivity of the neuron activity. For example, mature H3 horizontal cells do not normally synapse with green or red cones, however when UV cones are ablated and regeneration is delayed the H3 cell synapses with green and red cones[88]. By profiling the activity of these neurons in response to

various wavelengths of light we will be able to determine how the visual circuitry changes in response to loss of photoreceptor input. Identifying the cells that experience alterations in activity will be important for further studies aiming to characterize the mechanism of retinal remodelling due to photoreceptor ablation.

Preliminary attempts to drive CaMPARI in a specific retinal cells types has already begun. Cell specific expression of CaMPARI in retinal ganglion cells (isl2b:CaMPARI), and bipolar cells (nyx:CaMPARI) will permit our lab to assess how neuron activity is altered in response to cone subtype specific ablation. By assessing the retina's activity when exposed to UV light at various time points following ablation of UV or blue cones, our model will be ideal for addressing how the signaling of the retinal circuit changes during degeneration as well as during regeneration. However, because photoconversion is irreversible we will have to compare between individuals.

3.3.2 Assessing re-wiring to other photoreceptor subtypes following blue cone ablation

Our lab has already begun preliminary experiments to determine whether blue cone ablation induces re-wiring of the blue cone's synaptic partners to UV cones to compensate for the loss of short-wavelength input to rapidly recover visual ability. In crossing our two models of cone photoreceptor ablation we will test whether UV cones are necessary for the rapid recovery of visually mediated behaviour following blue cone ablation. We have considered re-wiring to other cone types, however there is currently no model for ablation of green cones, and Yoshimatsu et al.'s red cone ablation model Tg(thrb:Gal4;clmc:GFP;UAS:nfsB-mCherry) lack's characterization of off-target effects, which would be expected to influence the green cone due to the double cone morphology [88]. Nonetheless, I predict that rapid recovery of visually mediated behaviour would not occur following ablation of red or green cones, because red and green cones

are the dominant input types and their chromatic input is necessary to evoke an optomotor response [99]. Therefore, we predict that the recovery of visually mediated response would not occur until red or green photoreceptors had been functionally regenerated in sufficient numbers. Because green and red cones are predicted to be necessary for the OMR, we would not be able to test if blue cone ablation triggers re-wiring to red or green cones through simultaneous ablation of both blue cones and red or green cones. Nonetheless this prediction merits further examination to determine if blue cone ablation is triggering re-wiring to red or green cones. Alternatively, a behavioural assay wherein blue cone input is the predominant input type would be required could resolve whether blue cone ablation triggers re-wiring to red or green cone, as well as imaging the neuron activity using CaMPARI.

3.3.3 Characterization of interneurons morphology during remodelling

One of the most commonly identifiable signs of retinal remodelling occurs as interneurons extend neurites into the outer nuclear layer or retract their dendrites following photoreceptor dysfunction or loss. These abnormal neurite sprouts can form invaginating or flat connections both of which have ribbon synapses [85, 86]. Nonetheless, the functionality of these synapses and impact on visual ability is unknown. These sprouts typically originate from bipolar cells or horizontal cells. In order to identify if such remodelling is occurring in our model of UV or blue cone ablation we propose injecting a transgenic construct expressing either a bipolar cell specific promoter such as *nyx* or horizontal cell specific connexins such as *cx.55.5* driving expression of a fluorescent reporter gene for visualization of cell morphology. Using transient transfection to achieve mosaic expression in a subset of neurons will facilitate monitoring individual cells and their respective contacts. Larval zebrafish are amenable to live imagining,

previous studies have detected retinal remodelling in real time using larvae immobilized in low melting point agarose [88, 152].

3.4 Functional regeneration following cell-specific ablation of cone photoreceptors in the adult zebrafish retina

The regenerative response generally decreases with age as neurogenesis decreases and becomes restricted to certain regions in many vertebrate species [153]. Even in zebrafish, the robust regenerative response to injury attenuates with age [154]. It is important to identify the cause of age-related decline of neurogenesis/regenerative ability in vertebrates, in order to maximize the efficiency of regenerative therapies. Transplanted photoreceptor precursors can successfully integrate into the adult retina and restore visual acuity in mice [17]. Rod photoreceptor precursors can integrate into the mature and immature retina with similar efficiencies demonstrating that optimal integration depends on the developmental stage of the donor cell [17]. However, whether this is true for cone photoreceptor precursors remains unknown.

Adult zebrafish are an ideal model to study the mechanisms involved in photoreceptor precursor integration using their robust endogenous regenerative response. Using our transgenic model of UV or blue cone ablation, which has been optimized to maintain transgene expression into adulthood, we propose inducing photoreceptor regeneration in a mature retina to determine whether the regenerative response is sufficient to functionally restore visual ability. Recent work preformed by our lab has shown that MTZ bath works to induce NTR-MTZ ablation in adult zebrafish (unpublished). Monitoring the regeneration using the fluorescent markers present in our models of cone ablation is simple using our custom fundus lens to monitor the disappearance and reappearance of regenerated neurons *in vivo* [155]. Additionally, cone photoreceptor loss in the

adult zebrafish retina is obvious due to the cone photoreceptor mosaic. This mosaic provides a potentially powerful tool in determining whether positional effects and cell interactions have an important role in driving cone fate in the regenerating retina. Previous studies have demonstrated that following injury to the retina the photoreceptor mosaic fails to reform in the adult retina [77], suggesting that regeneration utilizes a different mechanism to determine cell fate. Failure to reform the photoreceptor mosaic would require remodelling of the interneurons to accommodate the new pattern of photoreceptors.

Because the cone photoreceptor mosaic has a strict organization, the ratio of UV to blue cones is expected to be similar. However, there is an increased number of blue cones relative to UV cones in the adult retina (UV:blue = 0.83) [123]. This could potentially change the magnitude of the cone specific regenerative response if it is based on the relative number of photoreceptors ablated; the larval retina had a higher proportion of UV cones and exhibited a significant regenerative response (ratio UV:blue = 1.15). The differential regenerative response in larval zebrafish could have also been the result of the magnitude of photoreceptor ablation, which would have increased with age as the retina expanded and cone photoreceptors were continually added to the periphery.

In order to determine whether regenerated photoreceptors can integrate into the retina and restore the visual circuit I propose testing functional regeneration of vision using either the optomotor behaviour, or the optokinetic response, two tractable visually mediated behaviours present in adult zebrafish, or alternatively an electroretinogram to assess recovery of the photoreceptor and interneuron response. Assessing the functional regeneration in the adult retina will further validate that recovery of visually mediated behaviour occurs in the absence of rapid larval development, and is due to regeneration of cone photoreceptors restoring the visual circuit to recover visual ability.

3.5 Cone photoreceptor replacement therapy in adult zebrafish

Transplanting cone photoreceptor precursors into the adult zebrafish retina would be an excellent complement to previous murine studies [19]. One of the major hurdles to overcome in cone photoreceptor replacement therapies is that the rod-dominated retinal environment of mice may not be conducive for photoreceptor precursors to assume the cone photoreceptor fate. Therefore, the cone dense retina of the zebrafish is an attractive environment to determine which extrinsic factors are necessary for transplanted cone precursors to assume the cone fate. I predict that retinal precursors may still not assume the cone photoreceptor fate in an intact zebrafish retina. This is because a full contingent of cones photoreceptors is already present, and their presence may cause feedback inhibition, preventing any further generation of cone photoreceptors. The removal of feedback inhibition by ablating UV cone photoreceptors may explain why our lab subsequently observed a bias towards the regeneration of UV cones following UV cone ablation. If feedback inhibition prevents transplanted cone precursors from adopting the cone fate, I expect the precursors will predominantly assume the rod photoreceptor fate, because rod inhibition is most likely absent as the mature retina normally adds rods periodically from Müller glia sources. Alternatively, they may differentiate in a cone to rod ratio similar to that of the host retina as previously observed in mice [19].

Pairing cone photoreceptor transplantation with our models of conditional photoreceptor ablation as well as our behavioural assays would ensure a cone photoreceptor deficient environment as well as a cone specific vision deficit that can be recovered. This would allow us to determine whether transplanted cone photoreceptor precursors can assume a cone photoreceptor fate and integrate with high enough efficiency and in great enough numbers to functionally regenerate vision. In utilizing our model of cone photoreceptor ablation we will be able to compare our previous results characterizing the regenerative potential of endogenous

regeneration to cone photoreceptor replacement therapy to determine whether they have the same characteristics and capacity for functional regeneration of vision. Prolonged prodrug treatment, utilizing the NTR-MTZ method of cell specific ablation, will prevent regeneration of transgene expressing blue or UV cones. I expect that the regenerative signals present following cone photoreceptor specific ablation will have a positive effect on the integration efficiency of transplanted cone photoreceptor cells, which would not normally be present in the degenerate mammalian retina.

3.6 Functional integration via electrophysiological response

The electroretinogram (ERG) is an invaluable non-invasive technique, used to assess retinal function by measuring the light-induced electrical activity of the eye [156, 157]. Because the technique is non-invasive, the ERG can be utilized to monitor regeneration of the visual circuit in an individual zebrafish over time. Therein we can measure recovery of the photoreceptor response (a-wave), the integration of photoreceptors with ON-bipolar cells (b-wave), and integration with OFF-bipolar cells (d-wave) [3, 4, 93]. Our rig when working can potentially record cone specific responses by measuring the ERG response generated by specific wavelengths using a monochromator. With limited modifications this rig could be used to measure both larval and adult zebrafish ERGs. While I was unsuccessful in operating/assembling the ERG rig, future studies would benefit greatly using electrophysiological data to support functional regeneration.

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