The Role of Intrinsic Versus Extrinsic Cues during Zebrafish Retinal Development and Regeneration

Jeremy Ng Chi-Kei, B.Sc. (Hons)

A thesis submitted for the degree of *Doctor of Philosophy* at

Monash University in 2016

Enrolled through the Australian Regenerative Medicine Institute (ARMI)

&

Faculty of Medicine, Nursing and Health Sciences

Monash University, Australia

PhD Committee

Main Supervisor: Dr. Patricia Jusuf

Co-Supervisor: Prof. Peter Currie

External Supervisor: Dr. Una Greferath

Chair of the Committee: Assoc. Prof James Bourne

Copyright notice

Table of Content

Abstract

During retinal neurogenesis, the main regulators of cell fate specification are genes (intrinsic). Recently, studies have challenged this notion, and accumulating evidence suggests that environmental (extrinsic) cues also contribute to retinal development. However, how these extrinsic factors influence different aspects of retinal development remains largely unknown. In this thesis I show that intrinsic factors control the timing of cell cycle exit in an environment with reduced inhibitory neurons compared to a wild type environment. This first study was consistent with the status quo of cell cycle exit being primarily regulated intrinsically during development. However, I also show that fate specification of later born neurons is delayed in an environment missing a single neuron type. Thus, indicating that later born cells rely on feedback from the surrounding environment to determine fate choice. This observation expands our understanding of retinal development and may be able to progress research in therapeutic regeneration for developmental diseases through extrinsically regulating cell fate, such as in the area of cell transplantation therapy.

In response to injury in vertebrate retinas, different types of growth and cell signalling factors are produced. These factors have been tested *in vivo* and have been shown to improve regeneration in a number of different injury models (i.e. exposure to bright light, mechanical and chemical injury). However, the relative contribution of extrinsic factors and their influences on different aspects of regeneration such as on fate specification is largely unknown. In this thesis, my data show that an environment created from an injury that ablates only one neuron type will generate larger proportions of the missing neuron. This implies that there are environmental factors that are able to direct fate choice of progenitor cells after an injury. Thus, this provides

a platform to progress regenerative therapies for retinal diseases. For example, transplanting unspecified progenitors into the retina of a patient to selectively generate the missing neurons.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Publications during enrolment

1. Ng, Jeremy, Currie, P.D., Jusuf, P.R. 2013. The Regenerative Potential of the Vertebrate Retina – Lessons from the Zebrafish. Regenerative Biology of the Eye
2. Ng, Jeremy, Dudczig, S., Currie, P.D., Jusuf, P.R. 2016. Feedback from each retinal neuron population drives the expression of subsequent fate determinant genes without influencing the cell cycle exit timing. *Journal of Comparative Neurology*, In Press

Thesis including manuscripts submitted for publication general declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 journal article in press. The core theme of the thesis is understanding retinal development and regeneration. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Australian Regenerative Medicine Institute under the supervision of Doctor Patricia Yap & Professor Peter Currie.

In the case of the Chapter 3, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and extent of candidate's contribution
3	Feedback from each neuron population drives the expression of subsequent fate determinant genes without influencing the cell cycle exit timing	Article In Press	First Author, 80%

I have not renumbered sections of published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: **Date:**

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisor Doctor Patricia Yap for offering me the opportunity to conduct my PhD, to Professor Peter Currie for providing a place for a neural researcher in his muscle focused group, and to the both of them for leading me through diverse, exciting experiences through the projects. I would like to thank Patricia for the continuous support I received in my PhD, for her patience, kindness, joyfulness, and immense knowledge. Her guidance helped me through my research, troubleshooting, and during the write up of this thesis. I sincerely could not have asked for a better supervisor, mentor and friend, all in one, at this phase of my life.

I want to thank my fellow lab mates in the Currie Group, both past and present: to Stefanie Dudczig as a dear friend and brilliant technical support, to Liana Goodings, whom I cannot replace as a wonderful office neighbor, to Phong Nguyen for his brain and humor, to Ophelia Ehrlich for her bright smile of encouragement, to Zhenhua Li who is patience with me when I am his snoring roommate at science conferences, to Dr. Lee Miles for his astounding optimism, to Dr Wouter Masselink for all the awesome cakes and sometimes awkward, but sincerely appreciated jokes, to Dr Joe Berger for showing his soft side in caring for a confused student not of his own, to Dr Alasdair Wood whom I trade valued thoughts and conversations outside of scientific research, to Dr Ivana Mirkovic her almond fingers at lab meetings, to Carmen Sonntag and Silke Berger who takes up all the caring roles for getting cards and presents for the people in the lab (because I forget to do so), to Fruzsi Frenyes for all the excitement stored up within her, to Martin Day and Reinetta Tanujaya for surviving my first round of student supervision and being brilliant.

Not in the Currie group but of which my PhD experience would be in lack without: to Claude, Daniella, Natalie, Guizhi and Aude who adopted me into their lab family, providing advice and a home outside of the Currie lab, to Raquel and Mo who remain full of life even when experiments don't go to plan, to Ryan Debuque who has seen undergraduate, Honours and PhD life with me.

Finally, I would like to thank 3 important facets of my life, without which I would not have approached the finish line, still carrying my head up high, allowing for an enjoyable PhD experience. To my family back home in Malaysia, who has sacrificed having my physical presence back home for my freedom to chase after my own dreams overseas, to Megan, my beloved and best friend, who cheerleads from the benches and holds me up in the front, and to God, my Father and Friend, who has transformed me, and will continually do so from the inside out.

Published abstracts and conference presentations

The following abstracts have been accepted, published and presented at domestic or international conferences over the course of this degree

- Ng Jeremy, Currie, P.D., Jusuf, P.R. Neural fate specification biased towards cell specific retinal regeneration in zebrafish. June 2015, Comparative Regenerative Biology Course at MDI Biological Laboratories, Maine, USA

- Ng Jeremy, Currie, P.D., Jusuf, P.R. Environmental Cues direct cell fate specification during zebrafish retinal regeneration. Jan 2015, 16th Australia and New Zealand Zebrafish Meeting, Gold Coast, Australia (Oral Presentation)

- Ng Jeremy, Currie, P.D Jusuf, P.R. Environmental Cues direct cell fate specification during zebrafish retinal regeneration. Nov 2014, 7th Annual ANZSCDB Victorian Cell and Developmental Biology Symposium, Melbourne, Australia (Oral Presentation)

Awards

The author has been a recipient of the following awards during his degree:

- Goss Fellowship from MDI Biological Laboratories for the Comparative Regenerative Biology Course in Maine, USA (2015)

- Faculty of Medicine International Postgraduate Research Scholarship (MIPS) (2013 – 2016)

- Monash Graduate Scholarship (MGS) (2013 – 2016)

List of Figures and Tables

Figure X: Title (page)

*Figures and tables from manuscripts are not included.

Abbreviations

Abbreviations	Full Form
Ath5	See atoh7
Acta1	alpha 1a, skeletal muscle
Atoh7	Atonal homolog 7
ATP	Adenosine Triphosphate
bHLH	Basic helix loop helix
BMP	Bone morphogenic proteins
BrdU	Bromodeoxyuridine
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CT	Chamber Temperature
CMZ	Ciliary margin zone
DAPI	Diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMSO	Dimethyl sulfoxide
Dpf	Days post fertilization
Dpi	Days post injury
E3	Zebrafish media
E.coli	Escherichia coli
EGFR	Epidermal growth factor receptor
ELM	External limiting membrane
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factors

Abbreviations	Full Form
Mtz	metronidazole
Na^+	Sodium ion
NTR/nfsb	Nitroreductase
OCT	Optimal Cutting Temperature compound
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Pax6	Paired box protein 6
PBS	phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PFA	paraformaldehyde
Ptf1a	Pancreatic transcription factor 1 alpha
PTU	1-phenyl 2-thiourea
RAC1	Ras related C3 botulinum toxin substrate 1
RFP	Red fluorescent protein
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
RSC	Retinal stem cell
Rx	Retinal homeobox
SEM	Standard error of the mean
SHH	Sonic hedgehog
Std	standard
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

GAL4	Gal4 yeast transcription activator protein		UAS	Upstream Activation sequence
GCL	Ganglion Cell layer		Vsx1	Visual system homeobox 1
GFAP	Glial fibrillary acidic protein		Vsx2	Visual system homeobox 2
GFP	Green Fluorescent protein		WNT	Wnt signalling proteins
GTP	Guanosine-5'-triphosphate		WT	Wild Type
H_2b	H2B histone		Zn-5	Activated leukocyte cell adhesion molecule a
Hpf	Hours post fertilization			
IdU	Iododeoxyuridine			
IGF	Insulin growth factor			
IKNM	Interkinetic nuclear migration			
ILM	Internal limiting membrane			
INL	Inner nuclear layer			
IPL	Inner plexiform layer			
K^+	Potassium ion			
MO	Morpholino			
Lak	Lakritz			
MO2	Refer to MO			

Chapter 1. Introduction

1.1 Thesis Introduction

The human body is comprised of a multitude of cell types that work together to regulate the functional processes necessary for every day activity. One of these key processes that humans rely on daily is vision. Vision is initiated in the retina, which is a highly specialised neural sheet comprised of layers of nerve cells in the eye. The retina, which is part of the central nervous system (CNS), collects light and transforms it into electro-chemical signals to be processed by our brain that enables us to examine our surroundings. The retina has been a suitable model to study the CNS as it is easily accessible and is a highly organized part of the CNS (Masland 2012). Importantly, the main neuron types and many of the identified genes controlling their generation are similarly conserved as with other CNS regions. The accessibility of the retina is an advantage, for example live *in vivo* imaging of the retina can easily be performed.

The retina can be affected by a range of hereditary and acquired degenerative diseases that afflict different retinal neurons (D'Amico 1994). Retinal degeneration may result in visual impairment and if severe, complete blindness. Experimental studies such as injury or genetic ablation techniques have been conducted on a spectrum of animal models to understand the physiology of these diseases (Ng 2013). Identifying and characterizing potential sources of retinal progenitors and cues that drive regeneration provide opportunities to endogenously influence retinal regeneration.

In order to understand the full picture of how retinal diseases manifest, I first needed to comprehend the fundamentals of retinal cellular development and the regulatory networks that underlie the formation of a functional retina. Current research

suggests that many of the underlying mechanisms involved during retinal development are recapitulated during retinal regeneration of the organism (Mader and Cameron 2004, Martinez-De Luna, Kelly et al. 2011). Previous studies have demonstrated the similarities and differences between retinal development and regeneration to bring insights into ways to restore vision loss (Easter 2000, Lamba, Karl et al. 2008, Locker, El Yakoubi et al. 2010, Ramsden, Powner et al. 2013).

Of particular interest is the role of intrinsic and extrinsic cues that affect retinal development and regeneration and whether developmental and regenerative mechanisms are regulated entirely by either or a combination of intrinsic and extrinsic cues (Chacko, Das et al. 2003, Fischer and Reh 2003, Lenkowski, Qin et al. 2013, Ng 2013, Gorsuch and Hyde 2014). It would be ideal to study the function of these cues within a human system to be directly translatable. However, studying retinal disease and regeneration in a human model is highly challenging due to ethics and limited ability to regenerate after damage.

An alternative strategy is to study the function of intrinsic and extrinsic cues in lower vertebrates, such as amphibians, birds and fish. The advantage of lower vertebrates is that many of the models can readily regenerate damaged/loss organs and have allowed for tremendous progress in clinical regenerative field (Karl and Reh 2010, Barbosa-Sabanero, Hoffmann et al. 2012). Of the lower vertebrates, the teleosts, a group of bony fish, has been a relevant model in the discovery of mechanisms that regulate retinal development (Glass and Dahm 2004) and regeneration (Fleisch, Fraser et al. 2011). This is due to the high conservation of the retinal architecture, origin, order of neuron birth and gene expression with higher vertebrates such as rats and mice.

In this thesis, I will explain my experimental studies to address the regulatory process surrounding the development and regeneration of the vertebrate retina using the zebrafish model. In this introduction, I will cover the current knowledge surrounding retinal development in the vertebrate species including intrinsic gene expression. As highlighted earlier, understanding retinal development is beneficial to regenerative research and I will summarize some visual diseases, the current clinical approaches and the animal models used to study retinal regeneration, with particular attention to the zebrafish model. Lastly I will discuss the mechanisms of retinal regeneration, including the sources of vertebrate retinal progenitors, and current knowledge of the involvement of extrinsic factors during vertebrate retinal development and regeneration.

1.2 The Vertebrate Retina

1.2.1 Conservation of neuron types during development

A highlight of using the retina to study the CNS in vertebrates is the high level of morphological conservation among all vertebrate species, covering mammals, birds, amphibians and fish (Karl and Reh 2010, Barbosa-Sabanero, Hoffmann et al. 2012). This conservation can be observed in the architecture of the retina, represented by the six cell types found in all vertebrate species; three excitatory neurons types (cone and rod photoreceptors, bipolar and ganglion cells), two inhibitory neurons types (amacrine and horizontal cells) and Müller glia cells. Furthermore, these six cell types can be divided into various subclasses of neurons based on their morphology, location of processes and genetic markers in all vertebrates including the zebrafish that make up the diverse neuronal circuits of the retina (Raymond, Barthel et al. 1993, McMahon

1994, Mangrum, Dowling et al. 2002, Jusuf and Harris 2009, Wassle, Puller et al. 2009, Jusuf, Almeida et al. 2011).

These six conserved cell types show a conserved ordered retinal lamination in all vertebrate species studied. The retina is composed of three nuclear layers that houses the cell bodies and two plexiform layers in between that contains the synapses (Fig 1.1). Light that enters the eye passes through the retina and is processed by photoreceptors in the outer nuclear layer (ONL) and transmitted to the ganglion cell layer via the horizontal, amacrine and bipolar interneurons. Ganglion cells act as the output neurons of the retina, relaying visual information to the brain via their axonal bundle that leaves the eye as the optic nerve. The stereotypical laminated architecture of the retina allows neuron types to be distinguishable from each other without additional staining or labelling. Functionally, correct retinal lamination is necessary to allow for proper transmission of signals across the retina to the brain (Bibliowicz and Gross 2011, Chow, Almeida et al. 2015, Sustar, Perovsek et al. 2015). Thus, while relatively simple, the retina is composed of highly organised, precisely connected circuits that allow it to carry out its CNS role as the primary visual organ.

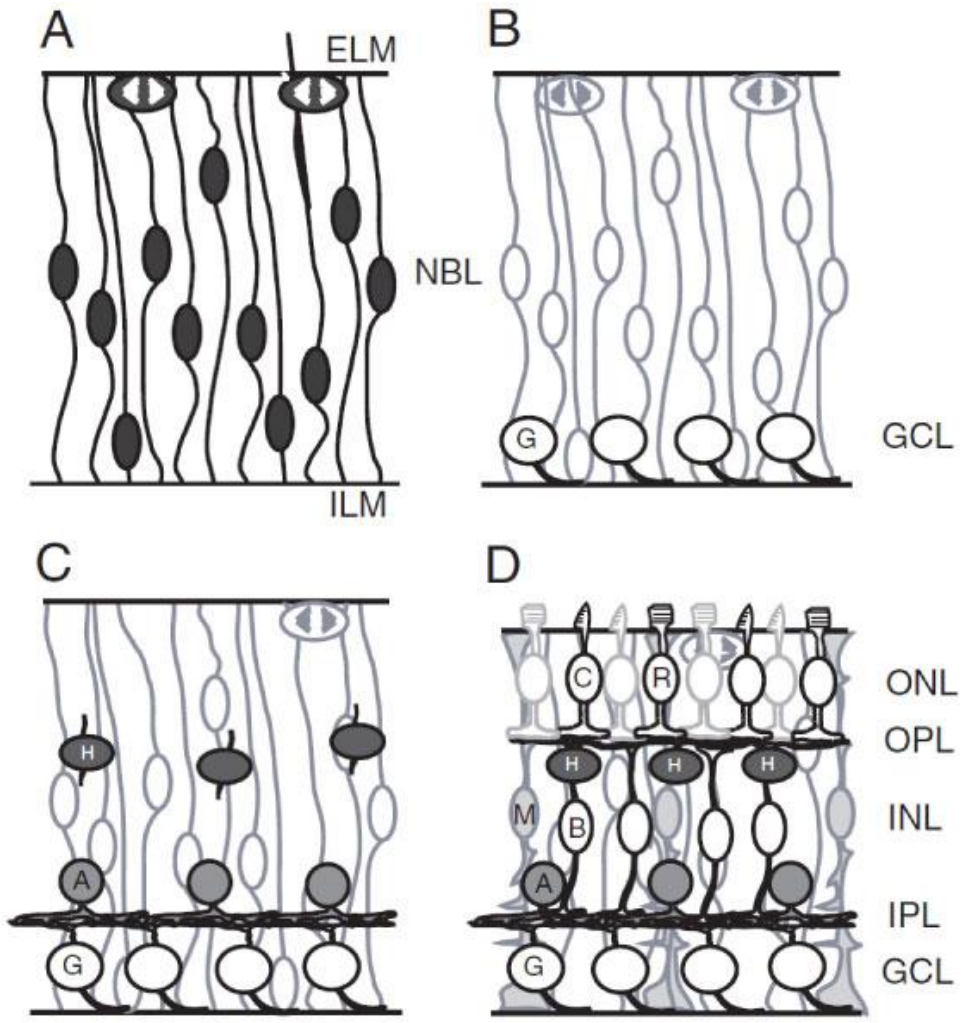
1.2.2 Conservation of retinal genesis

Early eye morphogenesis in zebrafish (Schmitt and Dowling 1994, Stenkamp 2015) is generally similar to that described in other vertebrate models (i.e. human, amphibians, birds and mammals), such as the process of evagination from the neural tube and the invagination process that follows to generate a concave shape (Mann 1964, Pei and Rhodin 1970, Grant 1980, Schook 1980a, Schook 1980b, Silver and Sapiro 1981, Schmitt and Dowling 1994). Some distinct differences do exist across species, for example evagination during zebrafish eye development occurs from a

solid neuroepithelial cell mass rather than in mammals where the hollow optic vesicle evaginates from the neural tube (Coulombre 1965, Walls 1967). Overall, the zebrafish remains as an excellent model of vertebrate retinal development.

As the vertebrate retina matures, progenitor cells undergo proliferation and begin to extend cytoplasmic processes from the external (ELM; apical) to the internal (ILM; basal) limiting membrane of the developing retina (Fig 1.1) (Young 1985, Wong and Godinho 2003, Stenkamp 2015). The nuclei of these progenitors undergo movement towards the apical end, where mitosis occurs, followed by movement towards the basal side of the developing retina. This process is termed interkinetic nuclear migration (IKNM) and occurs in both mammals (Noctor, Flint et al. 2001, Spear and Erickson 2012) and in zebrafish (Baye and Link 2007, Del Bene, Wehman et al. 2008, Norden, Young et al. 2009). Thus, progenitor nuclei undergo various stages of the cell cycle at various depths within the neuroepithelium, thus forming a pseudostratified layer of cells.

The final step in forming a stratified retina occurs at completion of the cell cycle. At this point, differentiation and migration takes place to form a correct cellular lamination and is followed by synapse formation & maturation into various retinal subtypes (Wong and Godinho 2003).



A= Amacrine cell
G= Ganglion cell
H= Horizontal cell
B= Bipolar cell
C=cone, R=rod
M=Müller cell

Figure 1.1 Schematic of a developing retina generating all 6 retinal cell types

(A) The retina arises from the neural ectoderm, which is initially a homogeneous population of multipotent progenitor cells arranged in a single-layered neuroepithelium. Cytoplasmic processes from these progenitors extend along the external (ELM) to internal limiting membrane (ILM). (B-D) Cells differentiate apically and complete differentiation in layers toward the ELM (basal). (B) Ganglion cells are the first cells to differentiate and these progenitors migrate towards the ILM to form the ganglion cell layer (GCL). (C) Amacrine and horizontal cells are the next to be born in the inner nuclear layer (INL), followed by the birth of cone photoreceptors (D) in the outer nuclear layer (ONL). Bipolar and Müller glia are the last cells to complete differentiation in the retina. Both the cone and rod photoreceptors form synaptic terminals with bipolar cells in the outer plexiform layer (OPL). The bipolar cells then form synaptic terminals with the ganglion cells in the ganglion layer in the inner plexiform layer (IPL). (Wong and Godinho 2003)

1.2.3 Conservation of Birth Order

In the CNS, neurons types are born in a stereotypical order at particular time points along development, termed as birthorder. During retinal development, the stereotypical order that gives rise to 6 types of retinal cell types is highly conserved across different species. Evidence of conserved developmental comes from studies that show similarities in vertebrate species; Xenopus (Stiemke and Hollyfield 1995), rat (Rapaport, Wong et al. 2004), mouse, monkey (La Vail, Rapaport et al. 1991), fish (Hollyfield 1972, Sharma and Ungar 1980, Nawrocki, BreMiller et al. 1985) and chick (Fujita and Horii 1963), though subtle species differences in the order of late born neuron types do exist.

It is widely accepted that the first post mitotic neurons to be born and to undergo differentiation are the ganglion cells in the GCL (Altshuler D 1991). All subsequent retinal subtypes are generated in a temporal distinct, yet overlapping manner in vertebrates (Young 1985, Rapaport, Fletcher et al. 1992, Rapaport, Wong et al. 2004). Amacrine and horizontal cells are generated subsequently, followed by cone and rod photoreceptors with the last retinal cells generated being bipolar and Müller glia.

In zebrafish, all retinal cell types initiate differentiation by 60 hours post fertilization (hpf) (Avanesov and Malicki 2010). Ganglion cell specific markers are first observed at 28 hpf at the anterior-ventral region of the developing retina (Kay, Finger-Baier et al. 2001), the region for earliest cell differentiation while bipolar cell specific markers are the last to be observed at 60 hpf. Müller glia, the only glia with a retinal progenitor origin, turn on their gene expression at 48 hpf (Bernardos and Raymond 2006). This stereotypical progression of cell fate specification is due to the highly choreographed spatio-temporal expression of specific genes within developing

multipotent progenitors, including those that control cell cycle progression as well as those that drive determination of specific neural fates.

1.3 Fate Determination during Vertebrate Retinal Development

The development of the vertebrate retina involves highly coordinated gene activity and signalling pathways, from neural plate induction up to cell specific fate specification to generate a functional, integrated neural system. Many of these genes and pathways involved during retinal development are also re-expressed during retinal regeneration in the adult vertebrate retina, as discussed below (Ng 2013).

Early developmental processes such as establishing the eye field are directed by coordinated genetic networks that function cell autonomously such as *Pax6, Rax, Lhx2* and *Six3* (Zuber, Gestri et al. 2003, Moore, Mood et al. 2004, Zaghloul, Yan et al. 2005, Lee, Bong et al. 2006). Additionally, developmental fate specification of new neurons from progenitors have also been shown to be influenced by stochastic activity of intrinsic gene activity in an independent gene expression model (Boije, Rulands et al. 2015) to produce clones of different combinations of neuronal cell types (Holt, Bertsch et al. 1988, He, Zhang et al. 2012).

Extrinsic feedback pathways are also involved in the early patterning of the eye structure. For example, neural plate induction from the anterior ectoderm is driven by the suppression or activity of secreted proteins and signalling networks such as the bone morphogenic proteins (BMP), Wnt signalling proteins (WNT) and fibroblast growth factors (FGF) (Sadler 2005, Barishak and Ofri 2007). This is followed by the formation of the presumptive eye field that is driven by a collection of signalling pathways that also includes WNT, FGF and Insulin like growth factors (Richard-

Parpaillon, Heligon et al. 2002, Eivers, McCarthy et al. 2004, Moore, Mood et al. 2004, Kim, Shin et al. 2007).

In contrast, later developmental processes such as the generation of progenitors are primarily controlled via intrinsic gene expression. A variety of homeobox gene activities have been identified to efficiency regulate progenitor formation. For example, paired box protein 6 (*pax6)* is the retinal master gene in both invertebrates and vertebrates (Halder, Callaerts et al. 1995, Gehring 2005). Interspecies misexpression of *pax6* is sufficient to induce the formation of ectopic eye structures (Onuma, Takahashi et al. 2002).

The conserved function of other gene families is limited to vertebrate progenitors. For example, the retinal homeobox (*rx*) gene is necessary for maintaining the retinal progenitor population in vertebrates. Loss of *rx* gene function leads to the absence of eye structures (Mathers, Grinberg et al. 1997, Andreazzoli, Gestri et al. 1999), while its misexpression results in excessive retinal tissue formation. Compared to invertebrate eye development, *rx* may not play a crucial role, as demonstrated in Drosophila. Drosophila *rx* null mutants did not show structural abnormalities in the eye, though it is expressed in the eye-disc in wild type larvae (Mathers, Grinberg et al. 1997, Davis, Tavsanli et al. 2003).

Another early progenitor transcription factor relevant to vertebrate development is the visual system homeobox 2 (*vsx2*). *Vsx2* is essential for normal vertebrate RPC proliferation as *vsx2* knockout mice develop small eyes (microphthalmia) (Burmeister, Novak et al. 1996). Vitorino et al 2009 showed that all multipotent retinal progenitor cells (RPCs) express *vsx2* before neuronal differentiation (24 hpf) during zebrafish development. These *vsx2*+ cells will either give rise to actively dividing daughter cells

or exit the cell cycle and differentiate into all the retinal neuron types and Müller glia cells. Furthermore, multipotent progenitors marked by *vsx2* have been shown to divide to generate a transient population of oligopotent progenitors (Vitorino, Jusuf et al. 2009). These transient progenitors turn on distinct fate specification genes at different developmental time points to generate different neuron subtypes (Fig 1.2).

A broad range of gene networks have been identified that can bias retinal progenitors towards specific fate changes throughout retinal development. Retinal cell fate specification genes often encode for basic helix loop helix (bHLH) and homeobox transcription factors (Hatakeyama and Kageyama 2004, reviewed in Agathocleous and Harris 2006, Harada, Harada et al. 2007, Ohsawa and Kageyama 2008). Once cell fate is determined, the cell differentiates and take on a specific neuronal phenotype (i.e. morphology and protein expression).

These two groups of transcription factors have been suggested to work together to specify neuronal fates, whereby homeobox genes provide layer specific identity, while bHLH regulates neural fate identity (Hatakeyama and Kageyama 2004). Transcription factors belonging to these two groups function within a gene regulatory network to influence progenitor cell fate switch into the six main retinal neuron types (Boije, MacDonald et al. 2014). Some of these well studied transcription factors that acts as retinal neuron master regulators include visual system homeobox 1 and 2 (vsx1, vsx2), atonal bHLH transcription factor 7 (atoh7) and pancreatic transcription factor 1 alpha (ptf1a). For example, atoh7 and ptf1a gene expression is necessary and sufficient for progenitors to become ganglion and inhibitory cell types respectively (Liu, Mo et al. 2001, Dullin, Locker et al. 2007, Hernandez, Matter-Sadzinski et al. 2007, Jusuf, Almeida et al. 2011), whereas vsx1 and vsx2 gene expression is necessary for progenitors to become bipolar neurons (Chow, Snow et al. 2001, Livne-Bar, Pacal et

al. 2006, Vitorino, Jusuf et al. 2009). Examining consequences of loss or gain of gene function for genes such as *vsx2, atoh7, ptf1a* and *vsx1* reveal a conserved role in mice (Burmeister, Novak et al. 1996, Brown, Patel et al. 2001, Hatakeyama, Tomita et al. 2001, Nakhai, Sel et al. 2007), zebrafish (Barabino, Spada et al. 1997, Kay, Finger-Baier et al. 2001, Dong, Provost et al. 2008, Vitorino, Jusuf et al. 2009), and humans (Reis, Khan et al. 2011, Prasov, Masud et al. 2012).

Differentiation of retinal neurons is divided into two stages; 1) generation of excitatory neuron lineage followed by 2) generation of inhibitory retinal neurons. In the first stage, cells that lose vsx2 expression will give rise to a variety of retinal progenitor pools: bipolar, Müller glia, ganglion and photoreceptors (rods and cones) (Vitorino, Jusuf et al. 2009). Differentiating progenitors turn off vsx2 expression to then upregulate the expression of either transcription factor *vsx1* or *atoh7* giving rise to bipolar and ganglion cells respectively (Vitorino, Jusuf et al. 2009).

Of these transcription factors, *vsx2* expression, which has a crucial role in regulating progenitor division, is also a key factor involved in driving fate specification of other cell types. Upregulation of *vsx2* expression occurs later in development in progenitors that maintained *vsx2* expression. These progenitors with upregulated *vsx2* expression give rise to Müller cells and a distinct subtype of bipolar cells compared to *vsx1*+ cells. These excitatory neuron fate specification genes are crucial in directing neuronal cell fate as altered gene expression during retinal development affects the proportions of each of these neuron types in fish (Ma, Yan et al. 2004, Vitorino, Jusuf et al. 2009, Jusuf, Almeida et al. 2011)

All excitatory neuron types (ganglion, bipolar and photoreceptors) and Müller glia cells are generated in the first stage from a pool of cells that maintain high

expression levels of *vsx1*, *vsx2* or *atoh7* (Jusuf, Almeida et al. 2011). The inhibitory cells are generated in the second stage, but these do not arise from a separate *vsx2*+ lineage but instead arise from downstream expression of excitatory neuron specific transcription factors. Upon the expression of the bHLH pancreas transcription factor 1a (*ptf1a*) in *vsx2*+ or *atoh7*+ cells, these cells turn down the expression of excitatory specifying transcription factors, and take on an inhibitory cell fate to become either amacrine or horizontal cells.

The transcription factor *ptf1a* is absolutely necessary for both amacrine and horizontal cell fates, as knockdown of *ptf1a* results in the respecification of “would be inhibitory cells” into excitatory cells (Jusuf, Almeida et al. 2011). Many more genes and factors that regulate cell fate bias act either upstream or downstream in a network have been identified in previous studies (Harada, Harada et al. 2007, Ohsawa and Kageyama 2008).

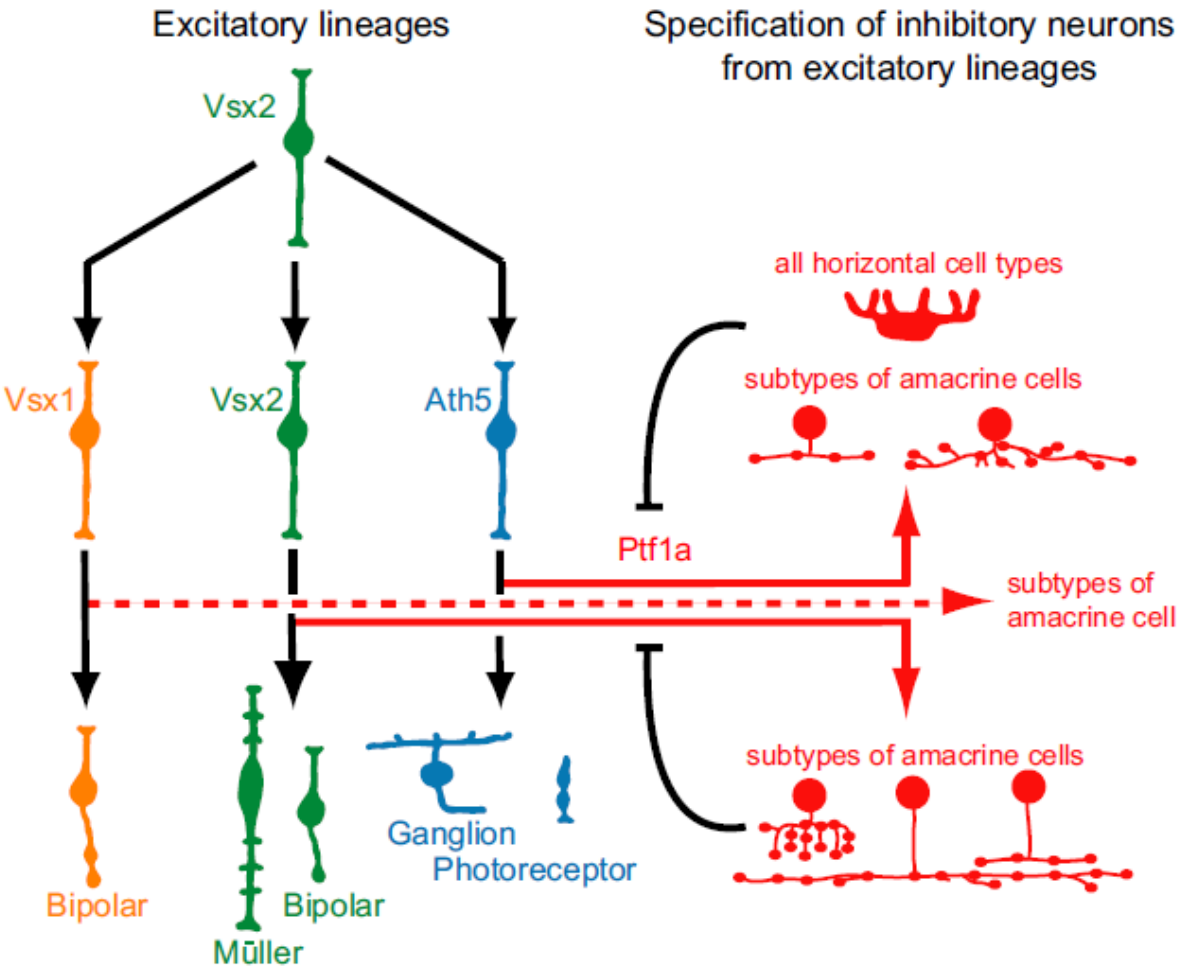

Excitatory lineages
Specification of inhibitory neurons from excitatory lineages
Vsx2
Vsx1
Vsx2
Ath5
all horizontal cell types
subtypes of amacrine cells
Ptf1a
subtypes of amacrine cell
subtypes of amacrine cells
Ganglion
Photoreceptor
Bipolar
Bipolar
Müller

Figure 1.2 Schematic summarizing current model of progenitor lineages for major retinal neuron subtypes. Fate determination occurs in 2 stages, which generates excitatory and inhibitory neurons respectively. Stage 1 involves the multipotent progenitor population (marked by visual system homeobox 2) which gives rise to separate transient oligopotent progenitors (marked by visual system homeobox 1 (*vsx1),* atonal homolog 5 (*ath5* – now known as *atoh7*) *and vsx2,* which generates all excitatory neurons at different time point throughout development. In stage 2, inhibitory neurons are generated from transient oligopotent progenitors (with *vsx1* progenitor population hypothesized) (Jusuf, Almeida et al. 2011)

1.4 Visual Diseases and Regeneration

Diseases that cause vision loss can occur to any age group, but mainly affect the aging population. In 2012, The World Health Organization estimated that individuals over the age of 50 comprise 65% of all those who are visually impaired on a global scale. In developed nations, the most common form of vision loss in individuals over 65 is caused by age-related macular degeneration (Banerjee 2006). Visual impairment can also be a secondary complication of other diseases, such as diabetic retinopathy, a condition that can develop in diabetic patients (Wilkinson-Berka and Miller 2008). With over 280 million people suffering from some form of visual impairment, there is an increase need to prevent, treat or even fully restore vision (Organization 2012). One way of potentially restoring vision may be found in cellular regeneration.

In response to any injury, cells either undergo regeneration and/or scar formation (Walmsley, Maan et al. 2015). Regeneration involves the re-growth of healthy cells to restore tissue function upon cell damage or death. Scar formation involves the deposition of extracellular matrix or fibrotic cells at the lesion site and results in tissues losing their innate function. Mammals are particularly limited in their regenerative potential and in response to injury, favour scar formation. In contrast, non-mammalian models such as teleosts, exhibit an indefinite ability to regenerate a multitude of cell types upon injury (Van Houcke, De Groef et al. 2015). This regenerative capacity is also present in the teleost retina.

1.5 Current Clinical Approach and Future Prospects

Clinical translation from bench to the bedside has made much progress in recent years, accumulating variations in visual prosthetics. Since its discovery in the

1960s (Brindley 1964), advancements in electronic technology and surgical technique have given rise to visual prosthesis devices that enable responses in the retina and higher brain centres to restore light perception, vision and acuity (O'Brien, Greferath et al. 2012). Clinically approved implants have been shown to provide low vision in retinitis pigmentosa (inherited degeneration) patients. One method is the implantation of a device in the subretinal space where photoreceptors have degenerated to stimulate retinal neurons (Chow, Chow et al. 2004, Chow, Bittner et al. 2010).

Other treatments for vision loss are gene and stem cell therapy (Jinturkar and Misra 2011, Phillips 2012). The theory of gene therapy involves correcting a defective allele using a wild type allele to restore the normal function of a gene. This is achieved by re-engineering viruses to remove all viral genes, replacing them with a transgene of interest and introducing this viral vector into the host to deliver the transgenetic material into the cell (Verma and Somia 1997). Gene therapy has had some success, including restoring sight to canine models, which modelled human X-linked retinitis pigmentosa (Beltran, Cideciyan et al. 2012). Recently, “Nighstar”, a gene therapy treatment targeting Leber congenital amaurosis type 2 (LCA2), has been clinically approved for to be used in patients (Schimmer and Breazzano 2015). This involves the use of an adeno-associated viral vector to deliver the RPE65 gene (mutated in the LCA2) to the eye via subretinal injections.

Stem cell therapy is another promising candidate, where the therapeutic aim of stem cells is the restoration of lost cell types into a host, which is able to perform the normal functions of that cell type after transplantation. This is not a new technique, with bone marrow stem cell therapy being the first stem cells used decades ago to treat leukaemia in mice before moving into humans (Santos 1971). Stem cell therapy has since come a long way, with many therapies currently undergoing clinical trials

(Faiella and Atoui 2016). The aim of current retinal stem cell therapies is to maintain surviving neurons and transplants of induced pluripotent stem cells (iPSc) to replace the retinal pigment epithelium (Baker and Brown 2009, Song and Bharti 2015).

However, it is evident that such methods are enormously challenging to make seamless in the area of safety, efficiency and ethics. Thus, alternate therapeutic models have been suggested, such as stimulating known stem cell sources in vertebrate animal models to regenerate. This may be done by delivering known extrinsic signals crucial for retinal development into different retinal injury environments. This method has been done experimentally with some signalling factors and the prospects of using this for regeneration studies appears promising (Fischer and Reh 2000, Del Debbio, Balasubramanian et al. 2010, Qin, Kidd et al. 2011). By understanding how extrinsic factors regulate retinal regeneration, further studies can include identifying secreted factors and signalling pathways that regulate the activation of the retinal regenerative process in humans to restore vision.

1.6 Vertebrate Models for Retinal Regeneration

1.6.1 Mammalian Retinal Regeneration

A capacity for tissues and organs to regenerate is not equivalent across the animal kingdom. Generally, the more basal the species in the phylogenetic tree, the more likely the animal is capable of robust and complete regeneration (reviewed in Tanaka and Reddien 2011, Knapp and Tanaka 2012). All vertebrate species do respond to some extent to retinal injury with regeneration-type responses, with amphibians and fish displaying the best regenerative capacity, followed by birds and to an even lesser extent in mammals (reviewed in Karl and Reh 2010).

The mouse model has been a key in representing mammalian studies through the establishment of elaborate knockout and recovery models, and culture and transplantation studies. In development, mouse retinal development completes by the early postnatal period in mammals (Young 1985) and continues to expand in size (Kuhrt, Gryga et al. 2012). Following this, no or few retinal cells are added to the retina in the lifetime of the mammal even after injury.

Hence, the dogma once considered the adult mammalian retina to be devoid of retinal stem cells (RSC) as reflected by the poor ability of mammals to regenerate multiple other cell types. However, there is evidence in the current literature suggesting that the mammalian eye may have multiple sources of adult RSCs (Jeon and Oh 2015, Stern and Temple 2015).

One source may be the mammalian ciliary body, as the teleost and Xenopus homolog (ciliary margin) is known to continually provide growth to the retina throughout the lifetime (Hollyfield 1968, Johns 1977). The ciliary body is located between the retina and iris. It comprises the ciliary muscle that alters the shape of the lens and the ciliary epithelium, which is responsible for the production of aqueous humor and controlling the levels of light that reaches the retina.

Primary cell culture of pigmented cells from rodent ciliary body generated clones that were able to proliferate, express differentiated neuron markers and differentiate into various retinal neurons such as photoreceptors (Ahmad, Tang et al. 2000, Tropepe, Coles et al. 2000). In addition, rat iris cell culture generated clones that express retinal neuron markers, suggesting that the iris may harbour retina stem cells (RSCs) in the adult eye (Haruta, Kosaka et al. 2001).

An alternative source of adult RSCs is from Müller glial cells (Lamba, Karl et al. 2008, Karl and Reh 2010, Locker, El Yakoubi et al. 2010). The Müller glia cells are radial glia cells that maintain retinal homeostasis by neurotransmitter recycling and retinoid metabolism in the retina (Reichenbach and Bringmann 2013). In fish and amphibians, Müller glia cells are a well characterized source of retinal progenitors during the regenerative period of amphibians and fish models (Karl and Reh 2010).

Species differences between the extent of regeneration, responses to various injury types and ability to regenerate some or all of the different retinal types may be in part due to intrinsic cellular / genetic differences of the cells acting as progenitors, or due to extrinsic signalling within the damaged tissue. Thus, understanding and comparing highly regenerative models with the more limited higher vertebrates will allow us to differentiate between intrinsic mechanisms we cannot easily change *in vivo,* and extrinsic signalling pathways that we can target to change and improve.

Major questions arise from the discovery of these potential mammalian RSCs. Why do mammals have such a limited capacity to regenerate after post-natal cell trauma though multiple sources have been suggested to harbour RSCs? And if retinal genesis, architecture, birth order and the fundamental genes for retina formation is conserved across multiple species, why are non-mammalian species much better at regenerating the retina than mammals? Through advanced understanding of retinal development and regeneration in both the mammalian and non-mammalian models, we may be able to address clinically relevant questions such as how are we able to restore vision.

1.6.2 Zebrafish as a Model for Retinal Regeneration

Understanding regeneration in the mammalian system such as in mice is necessary before translating research into human clinical studies. However, experimental studies addressing regeneration are difficult in higher organisms such as mammals. A major disadvantage of using the mammalian system is the scarce availability of real time *in vivo* imaging resulting in most current experiments to be conducted *in vitro* or *ex vivo*. Though informative, these experiments may not reflect the true nature of what is happening *in vivo* as the cells are removed from their natural microenvironment. In addition, the mammalian retina is quiescent during adulthood, limiting regeneration after any extent of retinal ablation.

In contrast, the retina of the teleost continues to actively grow throughout adulthood from a retinal progenitor population at the ciliary margin and is capable of regenerating itself after trauma throughout the animal's lifetime (Goldman 2014). In addition, a range of *in vivo* retina injury models has already been used in zebrafish that allows dynamic studies to be conducted (Ng 2013). This has encouraged the use of zebrafish as a prime animal model to study regeneration in the retina. Though therapeutic research and application in the retinal field needs to progress from a teleost into higher organisms, the teleost proves to be a powerful model to assist in understanding the fundamentals of retinal regeneration.

1.7 Retinal Progenitor Cell Sources

1.7.1 Adult vertebrate neurogenesis

Despite the conservation of morphology and gene regulation between vertebrate species, the regenerative capacity and source of regeneration among

vertebrates are not well conserved. In zebrafish, our understanding of adult neurogenesis in the central nervous system including cell sources and gene expression has grown significantly in recent years (reviewed by Grandel, Kaslin et al. 2006, Kaslin, Ganz et al. 2009, Schmidt, Strahle et al. 2013). The retina of non-mammalian species such as zebrafish, chicken and Xenopus are observed to have a much more extensive capacity of postnatal growth and regeneration after injury (Hitchcock and Raymond 2004, Stenkamp 2007, Sakami, Etter et al. 2008, Slack, Lin et al. 2008), however, potential sources of retinal stem cells have been suggested in mammals.

As previously described a source of retinal progenitor cells in the mammalian, avian and teleost retina are the Müller glia. However, not all Müller glia in the differentiated retina of these species are associated with adult retinal neurogenesis (Lamba, Karl et al. 2008, reviewed in Karl and Reh 2010, Locker, El Yakoubi et al. 2010). Müller cells are the major retinal glia cell type and the only one generated directly from retinal progenitors. Their cytoplasmic processes span the entire retina radially and surround the neuronal cell bodies, while their nuclei reside in the inner nuclear layer (INL). Like other glia cells of the nervous system, teleost Müller glia perform a range of functions, including recycling neurotransmitters, maintaining ion homeostasis and regulating neuronal survival and circuit formation in the retina (reviewed in Newman and Reichenbach 1996, Bringmann, Schopf et al. 2000, Mata, Radu et al. 2002, Bringmann, Pannicke et al. 2006, Jadhav, Roesch et al. 2009, Karl and Reh 2010).

Müller glia with slow, but ongoing proliferative activity, generate clusters of cells in the INL in the central differentiated retina. These migrate to the ONL to form rod precursors, which divide and differentiate into mature rod photoreceptors (Johns and

Fernald 1981, Raymond and Rivlin 1987, Julian, Ennis et al. 1998, Hitchcock and Raymond 2004, Bernardos, Barthel et al. 2007). These actively dividing Müller glia associated with adult neurogenesis are scattered throughout the retina, with greater density collecting at the CMZ (Julian, Ennis et al. 1998, Otteson, D'Costa et al. 2001).

1.7.2 Adult Vertebrate Regeneration

1.7.2.1 Ciliary margin zone (CMZ) and retinal pigment epithelium (RPE) regeneration

Amphibians in particular (salamanders, Xenopus, newts) have the capacity to regenerate the whole retina from the retinal pigment epithelium (RPE) if the retina is surgically removed or damaged (Reh, Jones et al. 1991, Araki 2007). These retinal pigmental cells dedifferentiate, re-enter the cell cycle, and their daughter cells are able to express retinal progenitor genes to regenerate a new retina (Stone and Steinitz 1957, Keefe 1973, Reh, Nagy et al. 1987). The chick also has the capacity to regenerate the neural retina from the RPE after being surgically removed, however this capacity is restricted to early embryonic development and is lost in later stages unlike amphibians (Coulombre and Coulombre 1965, Sakami, Etter et al. 2008).

The teleost is able to generate new retinal cells upon retinal injury from the CMZ and rod precursors (Maier and Wolburg 1979, Hitchcock and Raymond 1992, Braisted, Essman et al. 1994), though the majority of regenerating cells were recently identified to arise from resident Müller glia (Wu, Schneiderman et al. 2001, Fausett and Goldman 2006, Bernardos, Barthel et al. 2007). These glia are capable of directly replenishing all retinal neuron types.

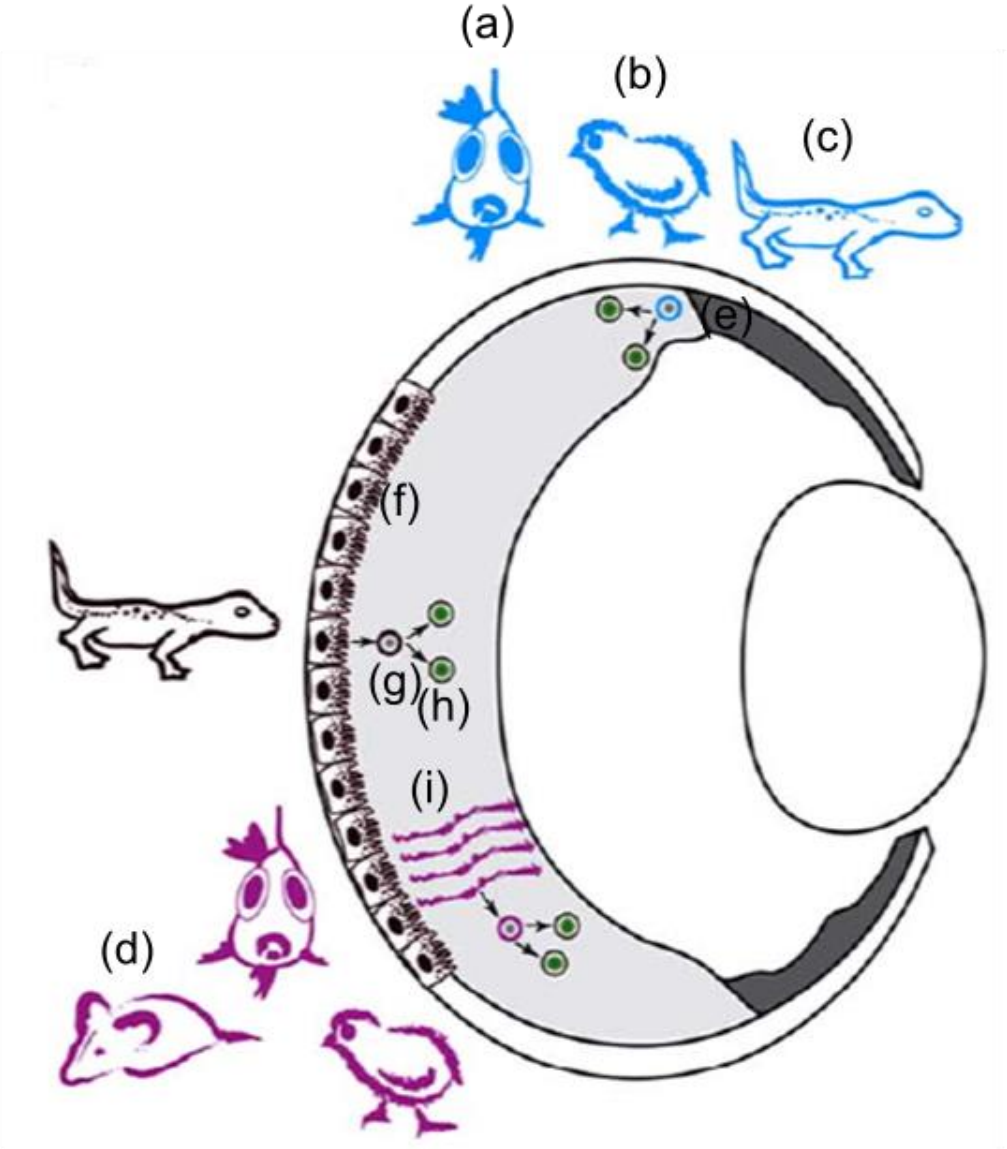
The pigment epithelium has also been identified as a source of retinal regeneration in other vertebrates, especially in amphibians (Karl and Reh 2010). The source of regenerative response depends on how quickly cell death is experienced and on the degree and type of damage sustained (Ng 2013). Different injury models have been shown to result in a regenerative response from either Müller glia or rod progenitors, arguably due to differences in the microenvironment after injury. The extent to which the CMZ responds to retinal injury has not been invested in great detail and might depend both on the location and physical extent of damage. The field has recently shifted towards generating a better understanding the Müller glia driven regenerative response (Fischer and Reh 2003).

1.7.2.2. Müller glia regeneration

In response to injury, resident Müller glia are activated, de-differentiate as they re-enter the cell cycle, and generate multipotent retinal progenitors, which subsequently differentiate into all retinal cells to result in cell replacement and functional recovery (Yurco and Cameron 2005, Fausett and Goldman 2006, Thummel, Kassen et al. 2008). As Müller glia are present in all vertebrate retinas, understanding and comparing any intrinsic limits in the regenerative potential in different species may allow us to develop strategies to potentially stimulate a regenerative response in humans.

Higher vertebrates including birds and rodents undergo a limited degree of retinal regeneration via activation of Müller glia (reviewed by Karl and Reh 2010). In birds, Müller glia, which express markers of embryonic retinal progenitors, re-enter into the cell cycle, but only for one division and only a small percentage of the Müller glia progeny successfully differentiate into new retinal neurons (Fischer and Reh 2001,

Fischer and Reh 2003, Hayes, Nelson et al. 2007). In rodents, also only a small percentage of Müller glia re-enters the cell cycle after injury (Bringmann, Iandiev et al. 2009), though a more extensive injury does increase the number of proliferating Müller glia (Wan, Zheng et al. 2008). Furthermore, even human Müller glia have a regenerative capacity to undergo indefinite proliferation and differentiation towards at least some retinal neuron fates at least *in vitro* (Lawrence, Singhal et al. 2007, Bhatia, Jayaram et al. 2011), suggesting the potential for glial driven mammalian regeneration and providing hope for an endogenous regenerative cell source.



a) Teleost
b) Birds
c) Salamanders
d) Rodents

e) CMZ
f) RPE
g) Progenitor cell
h) Mature cell
i) Müller glia

Figure 1.3 Schematic summarizing adult retinal stem cell sources in vertebrates. Teleost and salamanders are current model organisms that have more than one retinal stem cell source (CMZ and RPE/Müller glia) and are robust in regeneration after retinal injury. Other vertebrate model organisms are more limited in the effectiveness of regeneration (birds) and have a limited pool of progenitors (rodents) (Karl & Reh 2010). CMZ - Ciliary Margin Zone, RPE - Retinal Pigment Epithelium.

1.8 Injury Models in Teleost

Animal models can be used to study multiple facets of retinal diseases and regeneration. Research into regeneration in zebrafish has been conducted by inducing an injury (i.e. mechanical, light induced damage, chemical ablation, heat and transgenic knockout) or retinal disease models (Fleisch, Fraser et al. 2011). Moreover, there are techniques available that target non-cell specific populations or single neuron populations in specific retinal layers for ablation.

My research is focused on techniques that are best for generating different environments in a regenerative model using both mechanical and genetic ablation in order to assess whether the different extrinsic signals arising in these different environments influence aspects of regeneration. Summarized here are the established retinal injury models currently used in zebrafish.

1.8.1 Surgical lesion

Surgical lesioning of different regions of the CNS has been conducted for decades in both mammalian and non-mammalian animal models (rats, mouse, teleost, amphibians) (Cameron, Woolley et al. 1993, Gellrich, Schimming et al. 2002, Craig, Calinescu et al. 2008, Slack, Lin et al. 2008). Surgical removal of the majority of the adult retina (Hitchcock, Lindsey Myhr et al. 1992, Cameron and Easter 1995) and retinal needle stick injury have been conducted in teleosts to investigate retinal neuron regeneration. However, these techniques are not useful to study cell type specific regeneration as all retinal neuron subtypes are ablated and regenerate over time (Hitchcock, Lindsey Myhr et al. 1992).

1.8.2 Light and Laser ablation

Light induced lesioning is one of the most well characterised cell specific ablation methods in adults that targets photoreceptors. Bright light exposure induces photoreceptor cell death through apoptosis (Abler, Chang et al. 1996, Vihtelic and Hyde 2000) and leaves neighbouring cells unharmed. A benefit of light induced lesioning is a reduction in acute inflammatory response compared to surgical interventions (Shahinfar, Edward et al. 1991).

Studying retinal regeneration in adult teleosts using laser ablation is currently a limited field with published data only using an argon laser to target photoreceptor cells (Braisted, Essman et al. 1994, Wu, Schneiderman et al. 2001). However, as laser lesioning is seen to employ cell specific ablation, it would be an attractive technique to refine.

1.8.3 Chemical ablation

Retinal layer lesioning can be induced via toxic chemical exposure. An example is the use of Ouabain, a cardiac glycoside, which functions as a metabolic toxin that inhibits Na^+/K^+-ATPase normal function (Maier and Wolburg 1979), resulting in the depolarization of the plasma membrane that negatively affects normal signal transduction which ultimately leads to apoptosis (Valente, Capella et al. 2003). When intraocularly injected into the adult teleost, it is able to ablate retinal cells with low concentrations specifically damaging those retinal neurons closer to the injection site (Fimbel, Montgomery et al. 2007).

1.8.4 Transgenic conditional knock out

Transgenic zebrafish lines can be exposed to chemicals to cause specific cellular apoptosis for a restricted window of time, without affecting neighbouring cells. Of particular interest is the nitroreductase-metronidazole mediated conditional cell ablation paradigm in juvenile to adult fish (Pisharath 2007, Curado, Stainier et al. 2008). This inducible hybrid chemical-genetic cell ablation method using the bacterial *Escherichia coli* enzyme nitroreductase (NTR) and a non-toxic prodrug metrodinazole (Mtz) offers great spatial and temporal control. Upon Mtz addition, NTR, driven by a cell specific promoter, catalyzes the reduction of Mtz into a cytotoxic byproduct in NTR+ cells, inducing DNA fragmentation and ultimately resulting in endogenous cell death of only NTR+ cells, while leaving neighbouring cells unaffected.

Regeneration can be studied after the removal of Mtz allowing the organism to be devoid of any post treatment toxin effect. Bipolar and photoreceptor cell specific ablation studies in the zebrafish retina have been successfully conducted with this lesioning paradigm (Zhao, Ellingsen et al. 2009, Montgomery, Parsons et al. 2010).

Current nitroreductase – metronidazole conditional knockout models also utilizes in itself the Gal4-UAS transgenic system, allowing vast possibilities of transgenic combinations to study cell specific ablation (Scott, Mason et al. 2007, Scott and Baier 2009, Zhao, Ellingsen et al. 2009). In transgenic lines, a promoter of interest is upstream of the *gal4* gene, yeast transcription factor activator protein *gal4*. *gal4* specifically binds to an upstream activation sequence (*uas)* enhancer region to activate the expression of downstream genes. I will employ a combination of the Gal4-UAS and NTR-Mtz transgenic systems to manipulate the retinal environment and thus change the extrinsic signalling.

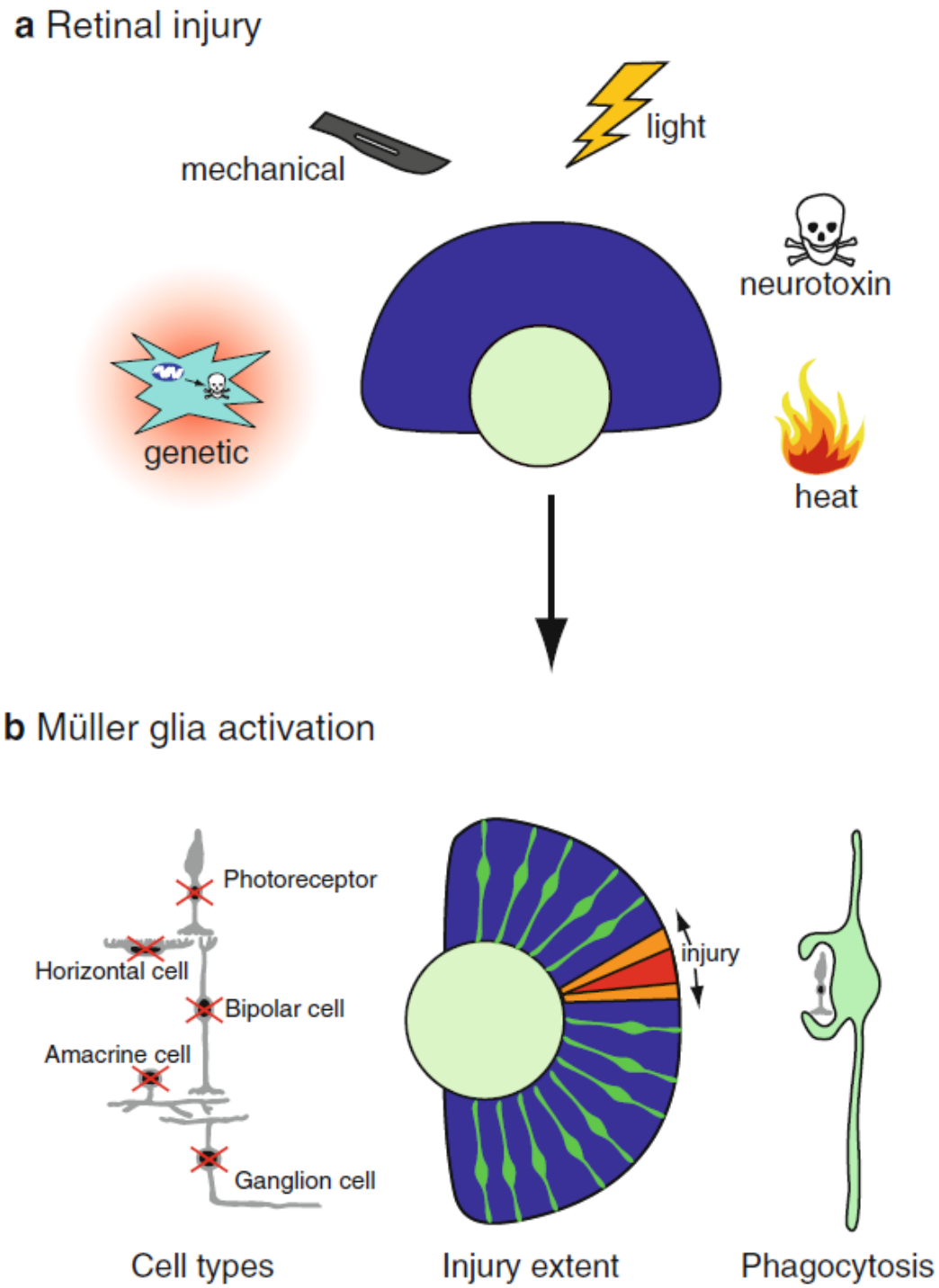

a Retinal injury
light
mechanical
neurotoxin
genetic
heat
b Müller glia activation
Photoreceptor
Horizontal cell
Bipolar cell
Amacrine cell
Ganglion cell
injury
Cell types
Injury extent
Phagocytosis

Figure 1.4 Müller glia driven regeneration. (a) Different injury models have been established in the zebrafish (i.e. genetic ablation, mechanical light & heat induced and various neurotoxins). All of these forms of injuries have been shown to activate a robust Müller glia regenerative response. (b) The type of retinal cell type that is injured, extent of injury and Müller glia phagocytotic activity have been shown to warrant different Müller glia driven regenerative responses (Ng 2013).

1.9 Extrinsic signalling during retinal regeneration

In response to injury, many different types of growth factors are produced to improve the different phases of regeneration, including FGF (Kostyk, D'Amore et al. 1994, Wen, Song et al. 1995, Valter, Maslim et al. 1998, Cao, Li et al. 2001, Walsh, Valter et al. 2001).

In zebrafish, FGF signalling is crucial for eye patterning and normal morphogenesis during development and regeneration of neurons after lesioning. FGF signalling is necessary for the maintenance of differentiated photoreceptors as inhibiting FGF signalling results in rapid photoreceptor degeneration and disorganization (Qin, Kidd et al. 2011, Hochmann, Kaslin et al. 2012). During retinal regeneration, inhibition of FGF signalling results in a vast reduction in proliferation after light lesioning (Hochmann, Kaslin et al. 2012) and intravitreal injections of FGF can contribute to an increase in rod progenitor proliferation (Qin, Kidd et al. 2011). The FGF pathway is similarly involved in amphibian and chick retinal regeneration (Yang, Wang et al. 2005, Spence, Aycinena et al. 2007).

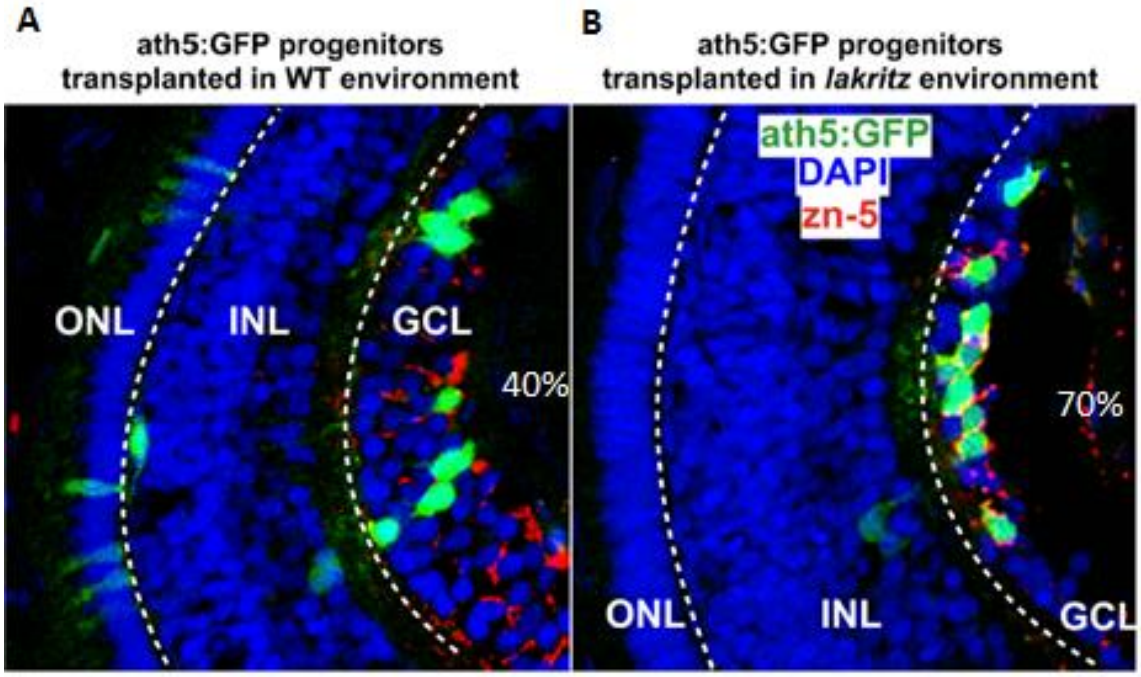
Other cell signalling pathways have been identified to contribute to vertebrate retinal regeneration, such as sonic hedgehog (SHH) (Spence, Madhavan et al. 2004, Spence, Aycinena et al. 2007, Wan, Zheng et al. 2007), *wnt* (Meyers, Hu et al. 2012), insulin growth factor (IGF) (Hansson, Holmgren et al. 1989, de la Rosa, Bondy et al. 1994), epidermal growth factor receptor (EGFR) (Wan, Ramachandran et al. 2012), notch (Del Debbio, Balasubramanian et al. 2010), BMP (Haynes, Gutierrez et al. 2007, Ueki and Reh 2013) Jak-Stat (Kassen, Ramanan et al. 2007), taurine (Lima, Drujan et al. 1990), retinoic acid (Hall, Else et al. 1990), Notch (Yurco and Cameron 2007), neurotrophin factor 3 (Santos, Monzon-Mayor et al. 2008) and ciliary neurotrophic factor (CNTF) (Kassen, Thummel et al. 2009, Wen, Tao et al. 2012).

Some of these extrinsic factors are now being tested in various animal models. The introduction of extrinsic factors in optic nerve crush injured mice results in improved survival and axonal regeneration and prevention of further damage to neurons (Lorenzetto, Ettorre et al. 2013). Intravitreal injection of constitutively active Ras-related C3 botulinum toxin substrate 1 (RAC1) Rho related small GTPase protects retinal ganglion cells from injury-induced death *in vivo*, permitted the elongation of axonal outgrowths to their target site and prevented axonal degradation. In addition, intraocular injections of CNTF and BMP4 into the chick retina was able to reduce the number of amacrine and bipolar cells dying after toxin induced injury when injected before the injury (Fischer, He et al. 2004). In the goldfish, computational modelling that constructs cellular pattern formation from different activated signalling pathways have shown how extrinsic factors can influence amacrine cell fate decisions in normal retinas (Tyler, Carney et al. 2005).

Cell fate progression during retina development and regeneration is governed primarily by intrinsic cues, however, as previously eluded to, extrinsic cues are able to influence neural fate specification in retinal progenitors across vertebrate species (Agathocleous and Harris 2009). Extrinsically secreted molecules from existing differentiated cell types in developing zebrafish retina are able to influence the proportion of other cell types generated from neighbouring retinal progenitors during embryonic retinal development (Poggi, Vitorino et al. 2005, Jusuf, Almeida et al. 2011).

Data from murine studies show that the integration of regenerated neurons is improved in diseased retina suggestive of the necessity of pro-regenerative injury induced factors (Chacko, Das et al. 2003). Similarly, embryonic Müller glia transplanted into adult chick retinas fail to differentiate into neurons, suggesting that the aging environment no longer supports this process (Fischer and Reh 2003).

Excitingly, bromodeoxyuridine (BrdU) incorporation in mouse retina can be increased by growth factors, showing that identification of external factors present a feasible avenue for increasing regeneration in mammals (Karl, Hayes et al. 2008, Takeda, Takamiya et al. 2008). Those these studies shown the importance of existing extrinsic feedback on retinal development and regeneration, the relative contribution of extrinsic factors on areas such as cell fate progression is largely unknown.


A
ath5:GFP progenitors
transplanted in WT environment
B
ath5:GFP progenitors
transplanted in lakritz environment
ath5:GFP
DAPI
zn-5
ONL
INL
GCL
40%
70%

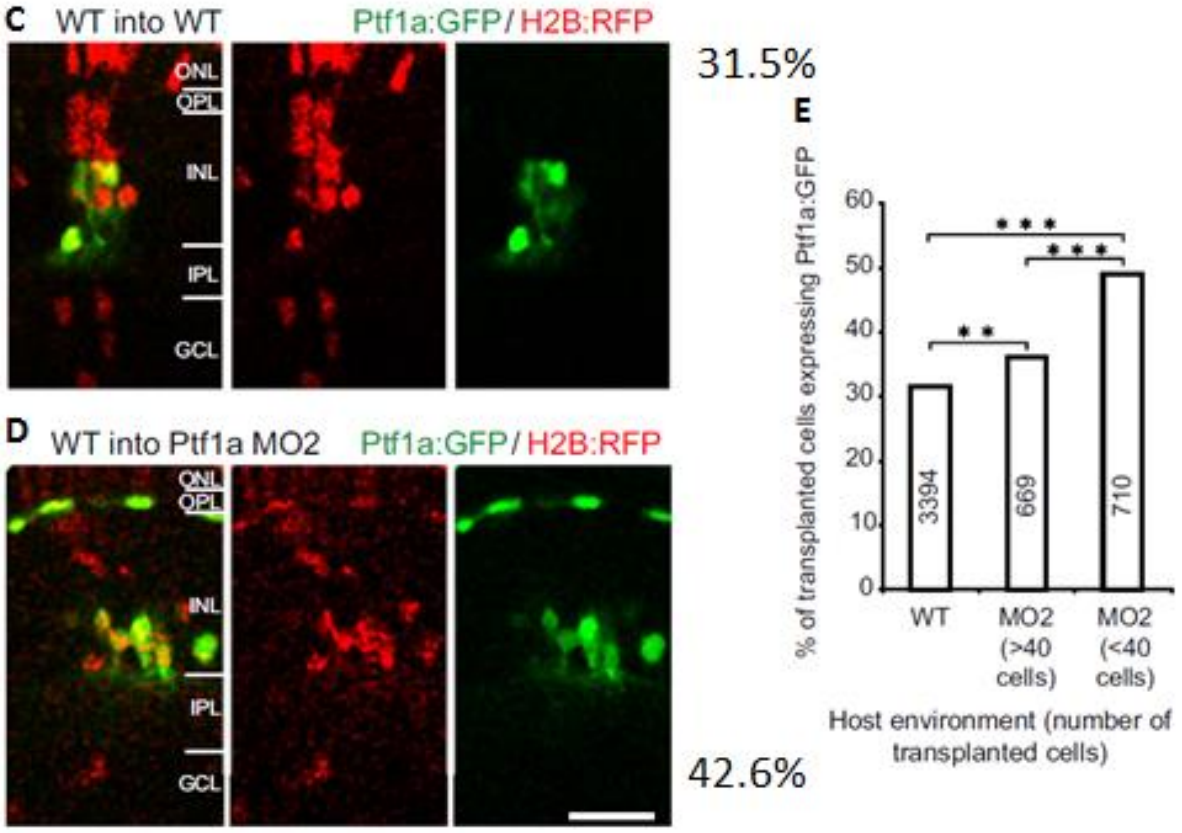

C WT into WT
Ptf1a:GFP / H2B:RFP
ONL
OPL
INL
IPL
GCL
31.5%
D WT into Ptf1a MO2
Ptf1a:GFP / H2B:RFP
42.6%
E
% of transplanted cells expressing Ptf1a:GFP
3394
669
710
WT
MO2 (>40 cells)
MO2 (<40 cells)
Host environment (number of transplanted cells)

Figure 1.5. Zebrafish retinal cross sections of wild type, morphants and mutants. (A, B), The retinal sections show 5 dpf Ath5:GFP (green cells) embryos, counterstained with DAPI (blue), and ganglion cells stained with Zn 5 (red). Extrinsic signals influence retina fate specification during embryonic development. Genetically wild type (WT) retinal progenitors marked by GFP that are transplanted into a WT environment generate fewer ganglion cells (A, 40%) marked by *zn-5* as when compared to transplantation of retinal progenitors into an environment that lacks ganglion cells (B, 70%). (C, D) The retinal sections show 5 dpf Ptf1a:GFP (green cells) embryos, and histones labelled with H2B:RFP (red) to observe for co-labelling. Photoreceptors are located apically and ganglion cells are located basally. WT progenitors are transplanted into a WT developing retina (C) and a retina that lacks inhibitory neurons (D). The transplanted cells generate more inhibitory neurons (42.6%) marked by GFP when compared to transplantation into a WT retina (C, 31.5%). This suggests that existing host retinal cells are signalling the transplanted donor progenitors to replenish at a greater capacity more of the missing cell type. ONL-Outer Nuclear Layer; INL-Inner Nuclear Layer; GCL-Ganglion Cell Layer; IPL-Inner Plexiform Layer; OPL-Outer Plexiform Layer; WT-Wild type; Ptf1a-pancreas transcription factor 1a; MO2-morpholino; GFP-Green Fluorescent Protein; RFP-Red Fluorescent Protein; H2B-Histone 2b protein; ath5/atoh7- atonal homolog 7; DAPI-diamidino-2-phenylindole (A, B - Jusuf et al 2011; C, D - Poggi et al 2005).

1.10 Final remarks

In this literature review, I have discussed that extrinsic cues are able to influence neural specification during retinal development in the zebrafish. However, it is not known, if extrinsic cues influence other phases of retinal development. As progenitors are generated in a predicted birthorder, it has been hypothesized that retinal progenitors receive signals in a feedback loop from neighbouring neurons to progress though differentiation. For example, retinal progenitors will firstly generate retinal ganglion cells until a sufficient number has been generated and will signal back to the progenitors to progress into generating the next cell type. This feedback is thought to be involved during the cell cycle progression and the onset of neuron gene expression during retinal development, which are the two areas to be assessed in my retinal development research aims.

Zebrafish are able to undergo robust regeneration after retinal injury. However, it is not known if the retinal progenitors are able to sense which cell types are missing and thus generate more of the missing cell types similar to the mechanisms involved during development. It is also unclear if this ability has been lost in adulthood and the progenitors are intrinsically programmed to generate a predicted proportion of neurons regardless of the cell types that are missing. I will assess, if the mechanisms involved during neural fate specification during retinal development are maintained in adult retinal regeneration.

1.11 Research Aims

My study will characterise the role of extrinsic cues on controlling (i) the cell cycle and (ii) cell competence progression during zebrafish retinal development and on influencing (iii) neuronal fate specification during zebrafish retinal regeneration with a focus on inhibitory neuron ablation.

The zebrafish has multiple advantages as a great developmental model; the optical clarity of the externally fertilized eggs, transgenic techniques, morpholino mediated knock down experiments and live *in vivo* timelapse imaging possibility. A healthy mating pair is able to produce over 200 eggs, with embryos hatching as early as 2 days post fertilization (dpf). The larvae feed off their yolk upon hatching and only require external feeding after 5 dpf and reach sexual maturity as early as 3 months. Such high offspring count and rapid development have placed the zebrafish at the forefront of functional experiments. As a model for neural regeneration, adult zebrafish are able to replenish all retinal neuron types primarily from the endogenous Müller glia population after extensive injury allowing the analysis of the mechanisms that follow retinal injury.

This thesis has 3 main result chapters:

In Chapter 3, the results obtained cumulated into a publication currently in press “Feedback from each neuron population drives the expression of subsequent fate determinant genes without influencing the cell cycle exit timing”. I assessed fate determination during retinal development, by observing if and how the absence of a specific neuron population/disrupted birth order influences progenitor cell cycle progression and the onset of fate gene expression during retinal development.

In Chapter 4, I assessed the differential fate specification during retinal regeneration after 2 distinct ablation models that I set up and characterised; a novel nitroreductase induced inhibitory neuron cell ablation regeneration model and a needle stick injury model that ablates all cell types.

In Chapter 5, I assessed the possibility of retinal regeneration in the absence of Müller glia. This was determined through the use of an established genetic ablation technique (previous chapter).

Chapter 2. Methods and Materials

2.1 List of suppliers and services

Adaptive Science Tools	Worcester, MA, USA
Adobe	San Jose, CA, USA
Ambion	Austin, TX, USA
Applied Biosystems	Grand Island, NY, USA
Argent Chemical Laboratories	Redmond, WA, USA
BD Bioscience	San Jose, CA, USA
Bio-Rad	Hercules, CA, USA
Biochemicals	Gymea, NSW, Australia
Bioline	Alexandria, NSW, Australia
Bitplane Scientific Software	South Windsor, CT, USA
Carl Zeiss Microimaging	Thornwood, NY, USA
Crown Scientific Pty Ltd	Moorebank, NSW, Australia
David Kopf Instruments	Tujunga, CA, USA
Developmental Studies Hybridoma Bank	Iowa City, IA, USA
DNAStar	Madison, WI, USA
Eppendorf	Hamburg, Germany
GraphPad Software	La Jolla, CA, USA
Harvard Apparatus	Holliston, MA, USA
Invitrogen	Grand Island, NY, USA
Merck	Kilsyth, Vic, Australia
Merck Millipore	Darmstadt, Germany
Narishige Scientific Instruments	Setagaya-ku, Tokyo, Japan
New England Biolabs	Beverly, MA, USA
Olympus Optical Co Ltd	Shinjuku-ku, Tokyo, Japan
Promega	Madison, WI, USA
Qiagen	Clifton Hill, Vic, Australia
Roche Molecular Biochemicals	Indianapolis, IN, USA
Sigma-Aldrich Pty Ltd	Sydney, NSW, Australia
Thermo Fisher Scientific	Scoresby, Vic, Australia
Worthington Biochemical Corporation	Lakewood, NJ, USA
ZIRC	Eugene, OR, USA

2.2 Zebrafish Techniques

2.2.1 Animal Husbandry

Zebrafish (*Danio rerio*) were housed, bred and raised at FishCore facility at Monash University in accordance with local animal guidelines. Embryos from mated zebrafish pairs were raised at 28.5^{0}C set incubators (Crown Scientific) and staged as previously described (Kimmel, Ballard et al. 1995). Depending on the experiments, development of zebrafish embryos was accelerated at 32^{0}C or slowed down at 25^{0}C set incubators after the 50% epiboly stage (5.25 hpf). Developmental stages from raised or lowered temperatures were calculated using standardized formula (Kimmel, Ballard et al. 1995). *Wild type* strains were obtained from ZIRC and included Tuebingen, Tupfel long fin, WIK, and AB. Transgenic fish strains were maintained by outcrossing fish to *wild type* strains every second generation.

2.2.2 Microinjections

1 cell stage embryos collected from mating pair and are aligned on the side of a glass microscope slide on a petri dish. Injection needles were pulled from a 1.0mm O.D x 0.78mm I.D glass capillary (Harvard Apparatus) using a needle puller (Kopf needle/pipette puller, Model 730, David Kopf Instruments). Injections were performed using an IM 300 microinjector (Narishige), visualized under a Stemi SV6 dissecting microscope (Carl Zeiss Microimaging. Morpholinos (1mM) were resuspended in water containing 1% Fast Green FCF (Sigma-Aldrich) and backloaded into injection needles. 1 nl was injected into embryos.

2.2.3 3-amino benzoic acid ethyl ester (Tricaine)

The developmental age of the zebrafish affects the quantity of 0.4% tricaine (Argent Chemical Laboratories) required for anaesthesia. Tricaine was added

dropwise to the E3 media until the zebrafish no longer experience twitch reflex but with visible signs of a beating heart.

2.2.4 1-phenyl 2-thiourea (PTU)

Embryos that were set aside for live in vivo imaging were treated with 1-phenyl 2-thiourea (PTU, 100 µM, Sigma) from 24hpf onwards to inhibit pigment cell formation to maintain transparency.

2.3 Histology

2.3.1 Fixation

Embryos were fixed in 4% paraformaldehyde (PFA, ProSciTech) in phosphate buffered saline (PBS, pH 7.4) for 3 hours in room temperature, or overnight at 4^0C. Embryos were washed in PBS and incubated in a series of 15% sucrose and 7.5% gelatine / 15% sucrose in PBS solution. Embryos were mounted in Tissue-Tek Cryomold Molds (Sakura) and snap frozen in a cool bath of 100% ethanol and dry ice (-78^0C) in an insulated container and stored at -80^0C.

2.3.2 Cryosection

In snap frozen gelatine moulds, zebrafish embryonic stages <21 dpf were processed using a Leica CM3050S Cryostat. Sections were produced at -20ºC Objective Temperature (OT) and -25 ºC Chamber Temperature (CT) at 14 µm. Embryos were mounted on metal support structures held together with Tissue-Tek O.C.T Compound (Sakura) and sectioned with disposable blades, assisted with a 50mm anti-roll glass plate (Leica). Sections were collected onto SuperFrost® slides (Menzel-Gläser) and stored at -20^0C.

2.3.3 Immunohistochemistry

Day 1

Slides containing the sectioned embryos were fixed with 100 µl of 4% PFA at room temperature for 10 minutes, using a Parafilm M (Parafilm) cut out as a slide cover to spread the PFA out evenly. Slides were placed in a make shift incubation chamber, which was moisten tissue paper placed at the bottom of a slide box. The parafilm was then removed and given a 10 minute 1 x PBS wash. Depending on the nature of the antibody and tissue used, an antigen retrieval step was conducted (discussed in other Chapters). If an antigen retrieval step was conducted, slides were washed in 1 x PBS for 10 minutes. Slides were blocked in 100 µl of 5% foetal bovine serum/FBS and 0.5% Triton 100 in 1 x PBS using a parafilm cover, for 1 – 2 hours at room temperature. The parafilm was removed and the slides were dapped dry. Primary antibody solutions were made in the FBS blocking solution (antibodies used are discussed in following chapters), and slides were incubated overnight, in a parafilm cover in the incubation chamber, at room temperature

Day 2

Parafilm covers were removed and the slides were washed in a series of three, 1 x PBS washes at 10 minutes each. The slides were returned to the incubation chamber and incubated in a secondary antibody solution (discussed in following chapters), made up in 1 x PBS solution, for 2 – 3 hours at room temperature, covered in parafilm. The parafilm was removed and the slides were given a series of three, 1 x PBS washes at 10 minutes each, with 4',6-diamidino-2-phenylindole (DAPI) added to the final wash to counterstain the nuclei of the cells (1:10,000, Life Technology).

Sections were then mounted using 24 x 60 mm glass cover slips (Menzel Glaser) with Mowiol mounting agent (Sigma).

2.3.4 Imaging

Images of fixed sections were obtained on a Zeiss Z1 (20x, AxioCam HRm 13-megapixel, monochrome) with the Axiovision Release 4.8.2 (06-2010) software. Because differentiation in the retina proceeds in central to peripheral as well as ventral to dorsal waves, all analysis was conducted in the same retinal region. Sections surrounding the optic nerve were subdivided into four equal sectors drawn from the centre of the lens and the central two sectors, which contain the central most differentiated cells were quantified as done in similar studies (Jusuf, Almeida et al. 2011)

2.4 Live Imaging

2.4.1 Mounting

Embryos were anesthetized in tricaine and mounted laterally so that the whole eye is visible, with 1% low melt agarose (Sigma) in E3 medium on a petri dish.

2.4.2 Acquisition

Time-lapses were imaged on an inverted widefield fluorescent microscope (Olympus CellR Widefield Microscope) or Zeiss LSM710 confocal microscope. Using the 488 nm (GFP or Alexa 488 fluorophore) and 560 nm (RFP or Alexa 546 fluorophore) lasers images taken with a W Plan-Apochromat 20x/1.0 DIC M27 70mm objective at 1.5 zoom. Stacks of optical sections at 2 µm step intervals for a total of 170 µm stacks were taken every 30 mins at 28^0C for 24 hours.

2.5 Image Analysis

Image processing programs are listed: movie generation was conducted with Imaris 7.4 (Bitplane) and ImageJ (NIH), cell counting and analysis was conducted with Photoshop CS6 (Adobe). Programs further discussed in following chapters.

2.6 Statistics

All cell counting experiments were performed with a minimum of 3 independent replicates, across at least 2 separate experiments. Experiments involving movies were performed with a minimum of 20 independent replicates, across at least 3 separate experiments. Data is expressed as mean ± SEM. My analysis approach is covered in detail in the individual chapters, but are briefly covered here.

Chapter 3 - Quantification of cell cycle exit (non-parametric Mann-Whitney test) and quantification of time lapse analysis (non-parametric Mann-Whitney test).

Chapter 4 - Quantification of cell death (TUNEL), proliferation (BrdU, PCNA) and cell fate specification (BrdU, transgenic lines) (student t-test).

Chapter 5 - Quantification of Tg(*ptf1a:GFP / ptf1a:Gal4 / UAS:nfsb-mCherry*) cells in untreated and after metronidazole treatment (one-way ANOVA with post-hoc Tukey HSD and Bonferroni multiple correction).

Part B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following

Nature of contribution	Extent of contribution (%)
Concept, planning of experiments, morpholino and BrdU microinjections, transplantation, immunohistochemistry, imaging and data analysis, statistics, figure preparation, manuscript drafting	80%

The following co-authors contributed to the work.

Name	Nature of contribution
Patricia Jusuf	Concept, planning of experiments, manuscript drafting
Peter Currie	Concept, manuscript drafting
Stefanie Dudczig	Transplantation

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contribution to this work

Camdidate's Signature		Date
Main Supervisor's		Date

Feedback from each retinal neuron population drives expression of subsequent fate determinant genes without influencing the cell cycle exit timing

Jeremy Ng Chi Kei[1], Stefanie Dudczig[1], Peter D. Currie[1], Patricia R. Jusuf[1]

[1]Australian Regenerative Medicine Institute, Monash University, Clayton, VIC 3800, Australia

Abbreviated title: Neurons drive gene timing of subsequent fates

Associate Editor: Professor John L.R. Rubenstein

Keywords: histogenic birthorder, zebrafish, neurogenesis, competence progression, RRID: AB_233622, RRID:ZIRC_ZL84

Corresponding author: Patricia R Jusuf

Australian Regenerative Medicine Institute

Level 1, 15 Innovation Walk

Clayton Campus, Monash University

Clayton, VIC 3800

Australia

[redacted]

[redacted]

This work was supported by an ARC DECRA to P.R.J. (DE120101311). The Australian Regenerative Medicine Institute is supported by funds from the State Government of Victoria and the Australian Federal Government

Abstract

During neurogenesis progenitors balance proliferation and cell cycle exit together with expression of fate determinant genes to ensure that the correct number of each of these neuron types is generated. While intrinsic gene expression acting cell-autonomously within each progenitor drives these processes, the final number of neurons generated is also influenced by extrinsic cues, which represents a potential avenue to direct neurogenesis in developmental disorders or regenerative settings without the need to change intrinsic gene expression. Thus it is important to understand which of these stages of neurogenesis are amenable to such extrinsic influences. Additionally, all types of neurons are specified in a highly conserved histogenic order, though its significance is unknown. Here, we make use of conserved patterns of neurogenesis in the relatively simple, yet highly organised zebrafish retina model, in which such histogenic birthorder is well characterised. We directly visualised and quantified birthdates and cell fate determinant expression in wild type *vs* environments lacking different neuronal populations. Our work shows that extrinsic feedback from developing retinal neurons is important for the temporal expression of intrinsic fate determinants but not the timing of birthdates. We found no changes in cell cycle exit timing, but a significant delay in the expression of genes driving the generation only of later, but not earlier born cells, suggesting that the robustness of this process depends on continuous feedback from earlier formed cell types. Thus, extrinsic cues selectively influence cell fate determinant progression which may explain the function of the observed retinal histogenic order.

Introduction

During neurogenesis multipotent neural progenitors generate a fully functioning central nervous system (CNS) through distinct stages including proliferation, cell cycle exit, cell migration, differentiation and synaptogenesis. The genes acting within these progenitors to control these stages exhibit a high level of conservation both among CNS regions as well as among all vertebrate species. The neural retina has become an important CNS model to study the process of neurogenesis due to its relatively simple, yet highly organised neuronal architecture, and accessibility. In particular, the zebrafish retina model has for the first time allowed us to achieve the milestone of performing *in vivo* analysis of the entire development of a vertebrate CNS structure, which has opened up the possibility of directly assessing the timing and gene interactions.

The different stages of neurogenesis including proliferation vs. cell cycle exit and fate specification are regulated by highly co-ordinated intrinsic gene expression (Liu, Mo et al. 2001, Ma, Yan et al. 2004, Poggi, Vitorino et al. 2005, Fujitani, Fujitani et al. 2006, Vitorino, Jusuf et al. 2009, Jusuf, Almeida et al. 2011), as well as secreted signalling factors (Lillien and Cepko 1992, Van Raay and Vetter 2004, Hashimoto, Zhang et al. 2006, Wallace 2008, Jusuf, Almeida et al. 2011) and gradients (Del Bene, Wehman et al. 2008). How these signals interact to give rise to the final robustly organised neural tissue remains unclear, although common observations across species suggest that conserved developmental mechanisms evolved across all vertebrates.

A key developmental observation across all studied vertebrate species has been the distinct histogenic order in which the five main types of neurons of the retina

are generated (Karl and Reh 2010, Barbosa-Sabanero, Hoffmann et al. 2012). The high conservation of this histogenic order suggests an important developmental mechanism, though its significance and to what extent it might be important for the proper progression of neurogenesis and what signals control this progression remain unknown.

Since the different cell types are born at distinct developmental times, both the timing of cell cycle exit as well as timing of fate determinant gene expression together will influence the number of each neuron type generated. Whether or which of these key aspects is responsible for the observed difference in the proportion of different types generated in differing environments is unknown.

The switch between proliferative to differentiation phase within individual progenitors and the timing of this will determine both the final number of cells within the entire neural retina, but also the relative numbers of different cell types. Gene manipulations of intrinsic cell-autonomously acting factors such as $p27^{Kip1}$ and $p57^{Kip2}$ cyclin kinase inhibitors (Dyer and Cepko 2000, Dyer and Cepko 2001) and dynactin-1 (Del Bene, Wehman et al. 2008), as well as general extrinsic factors including Wnt/Frizzled, Hedgehog, vascular endothelial growth factor, transforming growth factorα, epidermal growth factor and fibroblast growth factor (FGF) signalling (Lillien and Cepko 1992, Van Raay and Vetter 2004, Moshiri, McGuire et al. 2005, Hashimoto, Zhang et al. 2006, Karl, Hayes et al. 2008, Takeda, Takamiya et al. 2008, Wallace 2008) can affect this proliferative to differentiation switch, which in turn affects cell fate specification, as early cell cycle exit will drive generation of early born cell types, such as ganglion cells (Ohnuma, Hopper et al. 2002).

Fate determination is directly controlled by basic helix loop helix (bHLH) and homeobox transcription factors are expressed within progenitors to bias them towards specific fates (Levine, Passini et al. 1997, Liu, Mo et al. 2001), for example, cone-rod homeobox, visual homeobox transcription factor 1 (Vsx1), and pancreas transcription factor 1a (Ptf1a) bHLH proteins are required for photoreceptor, bipolar, and horizontal / amacrine fates respectively (Hayashi, Huang et al. 2000, Poggi, Vitorino et al. 2005, Vitorino, Jusuf et al. 2009, Jusuf, Almeida et al. 2011). Other factors can also simultaneously affect multiple stages, such as visual homeobox transcription factor 2 (Vsx2), which maintains progenitors in the proliferative phase as well as driving bipolar fate determination (Vitorino, Jusuf et al. 2009). Similarly, the bHLH Atonal homolog 7 (Atoh7) drives cell cycle exit as well as specifies ganglion cell fate (Brown, Patel et al. 2001, Wang, Kim et al. 2001, Ohnuma, Hopper et al. 2002, Vitorino, Jusuf et al. 2009, He, Zhang et al. 2012). In the Atoh7 zebrafish mutant *lakritz* cell cycle exit is delayed resulting in larger clones with more of the remaining retinal neuron types generated instead of the first born ganglion cells (Kay, Finger-Baier et al. 2001, He, Zhang et al. 2012). Fate specification is also influenced by extrinsic cues including FGF signalling, which biases the precursor pool of photoreceptor progenitors towards amacrine cell fate (Frohns, Mager et al. 2009). Extrinsic cues directly from surrounding developing neurons also affect fate biases, as loss of specific retinal neurons during development can bias progenitors in the growing ciliary margin zone or transplanted wild type progenitors to generate a higher proportion of the missing cell type (Reh and Tully 1986, Poggi, Vitorino et al. 2005, Jusuf, Almeida et al. 2011).

At the same time, some aspects of neurogenesis are also driven stochastically. Thus, individual progenitors do not undergo the same predefined program, but rather

produce a whole variety of clone sizes composed of different combinations of neuronal cell types (Holt, Bertsch et al. 1988, He, Zhang et al. 2012).

Given the strong genetic control, yet some degree of stochasticity, we asked whether the function of the highly conserved histogenic birth order observed may lie in overseeing the process of cell cycle exit and gene expression timing to ensure the final accurate neural structure that is so robustly generated. Using the imaging, molecular and chimera advantages of the zebrafish vertebrate retina, we thus investigated whether the loss of a neuronal population within the developing retina affects cell cycle progression and timing of fate determinant gene expression to understand which developmental processes are either linked or independently controlled and the significance of the highly conserve birth order. Here we show that the timing of fate determination factors is directly influenced by the absence or presence of earlier born cell types in the environment, while the cell cycle progression and exit is timed independently.

.

Materials and Methods

Zebrafish husbandry

Zebrafish were housed, bred and raised at FishCore facility at Monash University in accordance with local animal guidelines. WIK (RRID:ZIRC_ZL84), Tg(*vsx1a*:GFP), Tg(*atoh7:GAP-RFP*), Tg(*ptf1a*:GFP) embryos of either gender were maintained according to standard protocol, staged as previously described (Kimmel, Ballard et al. 1995), and used exclusively before free feeding stages. Relevant numbers are described separately for each result.

Morpholino injections

Translation blocking and standard morpholino oligonucleotides (MO) obtained from Gene Tools were reconstituted as 1 mM stock solutions in water and injected into the yolk embryos during the 1-2 cell stage. A well established, previously characterised Ptf1a MO targeted against 44 bp upstream of the translational start site with sequences 5′-TTGCCCAGTAACAACAATCGCCTAC-3′ was used to inhibit generation of inhibitory horizontal and amacrine cells at 10-12 ng / embryo (Jusuf, Almeida et al. 2011). Standard MO with sequences 5'-CCTCTTACCTCAGTTACAATTTATA-3' injected up to 12 ng / embryo had no significant biological activity in zebrafish.

Transplantation

To follow transgene expression of wild type progenitor cells in different host environments, Tg(*atoh7:RFP*) or Tg(*vsx1:RFP*) all cells in donor embryos were labelled with *H2A-GFP* or *H2B-RFP* RNA injected into the yolk of single cell stage embryos. Hosts were wildtype embryos injected with either standard or Ptf1a MO as described above. At the blastula stage 10 – 50 cells were transplanted from donor

embryos into the animal poles of unlabelled host embryos (Jusuf, Harris et al. 2013). For this embryos were dechorionated using 0.5 µg/µl pronase and placed into 2% agarose moulds covered in E3. Embryos were subsequently transferred to agarose coated petri dishes in E3. N = 6 embryos (minimally) were examined for each transgenic line.

Bromodeoxyuridine (BrdU) injections

For injections, BrdU (B5002, Sigma) was diluted in 5 mM sodium citrate, pH 6.0 to a 10 mM stock solution and 3 nl was injected embryos starting at different developmental time points: 30, 36, 42, 48, 54, 60 and 66 hours postfertilisation (hpf) allowing for immediate availability and more accurate temporal control. Zebrafish were anaesthetised with 0.0006% tricaine in embryonic medium (E3) and placed onto petri dish coated with 2% agarose. The BrdU was injected into the third brain ventricle using a needle pulled from a 1.0 mm O.D x 0.78 mm I.D glass capillary (Harvard Apparatus) and a Narishige IM 300 microinjector. After the first injection zebrafish were maintained in 2.5 mM BrdU diluted in E3, and all embryos were fixed at 75 hpf when central retinal development is complete. All images were taken at 75 hpf. N = 20 embryos were used for each time point (30-66 hpf), in both standard and ptf1a MO group. The experiment was similarly replicated with n = 20 embryos per time point, per group.

Antibody Characterization

Detection of BrdU labelling was performed using immunohistochemistry with anti-BrdU antibody (Roche cat. No. 11170366001, BMC 9318 clone, RRID: AB_233622). This antibody was generated by immunizing BALB/c mice with a BrdU-bovine serum albumin conjugate. The BrdU-BSA conjugate recognizes and binds to bromodeoxyuridine and crossreacts with iodouridine, but not fluorodeoxy-uridine, nor

with any endogenous cellular components such as thymidine or uridine. The antibody raised against BrdU recognizes cells that have incorporated BrdU *in vivo* and *in vitro*, but does not show any nonspecific antibody binding in brain sections of mice that did not receive BrdU injections (Silvestroff, Bartucci et al. 2010) nor in zebrafish retinal sections that we did not expose to BrdU.

Table 1. Table of Primary Antibodies Used

Antigen	**Description of Immunogen**	**Source, Host Species, Cat. #, Clone or Lot#, RRID**	**Concentration Used**
BrdU-BSA conjugate	Labels proliferating cells. The epitope is inside the DNA helix. DNA has to be denatured to ssDNA before antibody efficiently binds to DNA-BrdU	Roche, mouse monoclonal, Cat# 11170366001 , RRID:AB_233622	0.1ug/ml

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) in phosphate buffered saline (PBS, pH 7.4), cryoprotected in 7.5% gelatine / 15% sucrose in PBS solution and cryostat sectioned at 14 µm thickness using a Leica CM3050S Cryostat. Antibody staining was performed using standard protocols with all steps performed at room temperature. Sections were blocked in 5% fetal bovine serum (FBS) / 0.5% Triton x-100 in PBS, incubated in mouse anti-BrdU primary antibody (1:500) diluted in the same block solution overnight at 4^{o}C. Secondary antibody used was anti-mouse Alexa Fluor-488 fluorophores (1:400, Life Technology diluted in the

same block). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:10,000, Life Technology). Sections were mounted with Mowiol (Sigma Aldrich).

Image acquisition

Images of fixed sections were obtained on a Zeiss Z1 (20x objective) using an AxioCam (HRm 13-megapixel, monochrome) and Axiovision software. Brightness and contrast were adjusted with Photoshop (Adobe).

Time-lapse imaging

Live *in vivo* time-lapse imaging was used to quantify the timing of transgene expression indicating cell fate determinant expression, specifically of transgenes such as the Tg(*vsx1:GFP*) which is weakly expressed in progenitors and then upregulated to drive differentiation of the last born bipolar populations. Imaging was conducted using previously described methods (Jusuf, Almeida et al. 2011). Briefly, embryos were anaesthetised in 0.0006% tricaine and mounted in 1% low melt agarose in E3 medium. Time-lapses were imaged on an inverted widefield fluorescent microscope (Olympus CellR Widefield Microscope) or Zeiss LSM710 confocal microscope. Using the 488 nm (GFP or Alexa 488 fluorophore) and 560 nm (RFP or Alexa 546 fluorophore) lasers, images taken with a W Plan-Apochromat 20x/1.0 DIC M27 70mm objective at 1.5 zoom. Stacks of optical sections at 2 µm step intervals for a total of 110 - 170 µm stacks were taken every 24 mins at 32^0C between 25 - 60 hpf. Montages were generated using Fiji software, and brightness and contrast were adjusted with Photoshop (Adobe).

Analysis

For quantification of cell cycle exit (250 – 300 cells / section from n = 20 embryos per time point), all analysis was conducted of the same retinal region because differentiation proceeds in central to peripheral as well as ventral to dorsal waves. Thus, sections surrounding the optic nerve were subdivided into four equal sectors drawn from the centre of the lens and the central two sectors, which contain the central most differentiated cells were quantified as done in similar studies (Jusuf, Almeida et al. 2011).

For quantification of timing of Tg(*ptf1a:GFP*) expression onset in *lakritz* mutants (*lak*), embryos (n = 93 mutants and 438 siblings) that showed very obvious start of expression in the eye were sorted every 0.5 hpf and kept in individual wells in embryo mix until 5 dpf, when homozygous mutants could be identified by the black body pigmentation. For similar phenotype identification of Tg(*vsx1:GFP*) embryos (n = 21 mutants and 61 siblings), individual embryos were released from the agarose after time-lapse imaging and similarly kept in individual wells in embryo mix until 5 dpf.

Statistical analyses were conducted using a non-parametric Mann-Whitney test (GraphPad) for pairwise comparison and Kruskal Wallis (GraphPad) for multiple groups.

Results

Timing of cell cycle exit occurs independently of earlier generated cell types

Different types of neurons are specified at distinct developmental times (Wong and Godinho 2003) following a histogenic order, which is conserved across all vertebrates (Fujita and Horii 1963, Nawrocki, BreMiller et al. 1985, La Vail, Rapaport et al. 1991, Stiemke and Hollyfield 1995, Rapaport, Wong et al. 2004). Thus, the balance between the timing of proliferation and differentiation within the progenitor populations plays an important role in determining the final number of different neuron types generated. Since feedback from existing cells in the developing tissue has been shown to influence the number of different neuron fates generated, we studied, whether such feedback might act through altering cell cycle progression, in particular the timing of cell cycle exit. In order to test this, we examined whether the absence of the two main types of neurons generated in the middle of the stereotypical birth order progression was sufficient to change the cell cycle exit timing of both earlier and later born neuron types, or whether progenitors had sufficient cues / gene expression programs to control this process completely independently.

The birth times of the main types of neurons were quantified using prolonged BrdU birthdating experiments established previously (Jusuf, Almeida et al. 2011). The birthdates of excitatory photoreceptors, bipolar cells and ganglion cells (excitatory neurons have glutamate receptors) were quantified in control retinas and compared to sibling retinas that lacked the two main inhibitory neuron types horizontal and amacrine cells (which release inhibitory neurotransmitters such as GABA and glycine). Inhibitory neurons are generated in the middle of the histogenic birthorder of neuron types in the retina. Their absence has been shown to positively influence transplanted

wild type progenitors to be biased towards generating more of the missing inhibitory neuron types (Jusuf, Almeida et al. 2011). If they also influence the cell cycle progression and cell cycle exit, then later born neurons (namely photoreceptors and bipolar cells) may have altered birthdates. Retinas without inhibitory neurons were generated as previously described using the Ptf1a morpholino (Jusuf, Almeida et al. 2011), which is cell-autonomously necessary and sufficient to specify inhibitory horizontal and amacrine cell fates in the retina. Unlike the Atoh7 transcription factor, which is expressed prior to the final mitosis and drives cell cycle exit and specifies ganglion cells (Kay, Finger-Baier et al. 2001, Poggi, Vitorino et al. 2005), Ptf1a expression is only switched on after the last progenitor division (Jusuf and Harris 2009) playing a role in progenitors that have already decided to exit the cell cycle. The normal developmental timing of morphant embryos was verified by comparing the timing of transgene expression in an unrelated tissue that does not express Ptf1a. For this, the timing of expression of reporter protein in Tg(*acta1:mCherry*) transgenic fish line was compared in uninjected, standard MO control and Ptf1a MO injected embryos. The mCherry reporter is driven by the alpha actin (acta1) promoter, which drives a major component of the contractile apparatus of skeletal muscle. Analysis of somite development timing at 31 hpf and 42 hpf showed comparable onset of transgene in uninjected, standard and Ptf1a MO injected embryos ($p > 0.05$, Kruskal-Wallis test, Figure 1, A-H).

To follow the timing of cell cycle exit (BrdU negative cells) for all of the different retinal cell fates, BrdU injections followed by BrdU immersion was conducted at distinct developmental time points in age matched control standard MO and Ptf1a MO injected siblings.

Comparison of the birthdates as quantified by the proportion of BrdU negative cells (i.e. those that had undergone terminal division prior to the time of BrdU injection) revealed robust developmental timing of ganglion (earlier born) as well as bipolar and photoreceptors (later born) cells. There is no significant difference between the timing when progenitors first exit the cell cycle and start as well as complete generating these neural populations, although the peak rate of differentiation can differ. Thus, the different types of excitatory retinal neurons are born and generated at the same developmental time independent of the presence or absence of inhibitory neurons (Figure 2, A-J). Temporal co-ordination of genes that control the timing of progenitor proliferation vs. cell cycle exit and differentiation are therefore independently controlled and are not driven by feedback from the previously born cell type as reflected by the comparable time at which half of each neuron populations is born (Table 2).

Table 2: Time point (extrapolate) whereby 50% of cells in the central retina is differentiated

Background	Ganglion Cells (hpf)	Bipolar Cells (hpf)	Photoreceptor Cells (hpf)
WT	42	50	56
Standard MO	42	50	56
No inhibitory (Ptf1a MO)	42	51	56

Fate determinant expression is timed by cues from previous neuron types

The second key process, which ultimately affects the final number of different neuron types generated by fate specification independent of cell cycle exit is the expression timing of cell-autonomous fate determinants within progenitors. In fact, the

conserved histogenic order and progression of such gene expression observed across all vertebrates may be the common mechanism to ensure sufficient numbers of each neuron type are generated, before progenitors change their competence to start generating the next neuron type. Whether the switch in gene expression from one to the next type of fate determinant is timed intrinsically within each progenitor, or whether this progression is driven by accumulating extrinsic feedback signals when sufficient earlier born cell types have been generated, remains unknown.

Thus, we compared the timing of expression of key fate determinant factors that specify ganglion cells (first born), horizontal and amacrine cells (intermediate born) or bipolar cells (last born) populations in the retina. Two distinct “environments” were generated to compare with the wild type control.

In the first set of experiments, standard MO injected embryos were compared to Ptf1a MO injected sibling embryos, in which the intermediate born inhibitory horizontal and amacrine neurons are lost in the retina (Figure 1 I, J) (Jusuf, Almeida et al. 2011). Here we quantified how the loss of such inhibitory neurons affected the timing of the earlier born Atoh7 expressing ganglion cells and later born Vsx1 expressing bipolar cells (Hayashi, Huang et al. 2000, Vitorino, Jusuf et al. 2009).

In the second set of experiments, homozygous *lakritz* mutants lacking functional Atoh7 (*lak*) (Kay, Finger-Baier et al. 2001) were compared with siblings with wild type phenotype. In *lak* mutants, the absence of Atoh7 causes the complete loss of the first born ganglion cells in the retina. Here, we quantified how the loss of ganglion cells affected the timing of gene expression of Ptf1a, which drives middle born horizontal and amacrine cell specification, or Vsx1, which drives late born bipolar specification.

As previously shown development and neurogenesis is first observed in the anterior ventral area and progresses around the retina as development progresses (Kay, Link et al. 2005). Thus the timing of first differentiation of ganglion, horizontal, amacrine and bipolar cells was analysed in this region by quantifying the onset of transgene expression in Tg(*atoh7:GAP-RFP*), Tg(*ptf1a:GFP*), Tg(*vsx1:GFP*) or appropriate double transgenic embryos. Ptf1a:GFP expression onset could clearly be visualised in living embryos, which were sorted for expression every 0.5 hpf. The Vsx1:GFP transgene is expressed at low levels in progenitors initially and upregulated strongly specifically as bipolar cells are specified and start differentiating. Thus, we quantified the onset of strong GFP expression in late born bipolar cells, which was visualised using time-lapse imaging at the confocal microscope. Atoh7:GAP-RFP was simultaneously imaged using double transgenics in these time-lapse movies.

In the Ptf1a morphants, we find no change in the timing of Atoh7:GAP-RFP transgene (around 28 hpf) and thus ganglion cell specification ($p = 0.0998$, Mann-Whitney test, Figure 3 A&C, Figure 4). Because ganglion cells are born prior to any inhibitory neurons, these results are consistent with timing of ganglion cell differentiation occurring independently of the presence of the later born inhibitory neurons.

The developmental timing of Vsx1 expression in the time-lapse imaging series showed that bipolar cells are generated at a significantly later time point (40 hpf ± 0.240 SEM) compared to the wild types (38 hpf ± 0.217 SEM) ($p < 0.0001$, Mann-Whitney test, Figure 3 B&D, Figure 4). While Vsx1 expression does turn on eventually and bipolar cells are indeed generated, the robust timing of this process is dependent on feedback from the intermediate born horizontal and amacrine cells.

Similarly, the loss of the firstborn ganglion cell population in the *lak* mutant also causes significant delays in the timing of expression of Ptf1a:GFP ($p < 0.0001$, Mann-Whitney test, Figure 4) and Vsx1:GFP ($p < 0.0346$, Mann-Whitney test, Figure 4) transgenes, showing again that usually signals from the earlier born cells generated do in fact control the switch and timing of relevant genes to generate the later born neuron types (Table 3).

Table 3: Summary of all timing of fate gene expression in all backgrounds used

Background	Ganglion fate Onset of Atoh7 (hpf)	Inhibitory cell fate Onset of Ptf1a (hpf)	Bipolar cell fate Onset of Vsx1 (hpf)
WT (lak sib)	-	33.48 ± 0.091	38.030 ± 0.357
No ganglion (lak)	-	34.63 ± 0.198	39.30 ± 0.529
WT (Standard MO)	28 ± 0.183	-	38 ± 0.217
WT (Standard MO-Chimera)	35 ± 2.96	-	38 ± 0.247
No inhibitory (Ptf1a MO)	28.5 ± 0.238	-	40.5 ± 0.240
No inhibitory (Ptf1a MO-Chimera)	40 ± 3.83	-	45 ± 1.70

Fate gene progression delayed in chimeras: Wildtype progenitors in different environments

The later born neuron types themselves do not usually express the genes that were knocked out to generate the different extrinsic environments and progenitors giving rise to these should not have been directly affected by the knock out conditions. We tested this directly by generating chimeras in which genetically wild type progenitors were transplanted into the different environment to assess, if the delay in

fate determinant gene expression was still observed. For these experiments, we used the Ptf1a vs Standard MO comparison, in which we can obtain sufficient embryos of each condition. Progenitors in wild type embryos were labelled either using *H2B-RFP* or *H2A-GFP* RNA injections into the yolk of one-cell stage embryos. At the blastula stage, labelled progenitors were transplanted into unlabelled host embryos at the same stage that had been injected either with standard (control) or Ptf1a MO to generate different environments for the wild type progenitors to develop in. Time-lapse imaging was performed as above to assess the onset of Vsx1:GFP and Atoh7:GAP-RFP transgenes in the transplanted wild type progenitors.

These chimeras confirmed that the onset of Atoh7:GAP-RFP in these wild type progenitors is unaffected by the loss of the later born inhibitory neurons. In the Ptf1a morphant hosts, we find no significant change in the timing of Atoh7:GAP-RFP transgene onset (n = 6 embryos, 35 hpf ± 2.96 SEM) compared to that in donors transplanted into control host background (n = 8 embryos, 40 hpf ± 3.83 SEM) ($p < 0.3623$ Mann-Whitney test, Figure 5). Thus the Atoh7 fate determinant transcription factor is expressed normally to generate ganglion cells, which are born prior to any inhibitory neurons. These results are consistent with timing of ganglion cell differentiation occurring independently of the presence of the later born inhibitory neurons. Additionally this result acts as a control to show that transplantation of cells into this host does not cause any delays in itself.

In these chimera experiments, we do however find a significant delay in the onset of Vsx1:GFP expression which signals bipolar differentiation. Thus, wildtype donor cells in a Ptf1a morphant host turned on Vsx1:GFP to generate bipolar cells at a significantly later time point (n = 6 embryos, 38 hpf ± 0.247 SEM) compared to control donor cells into a host background (n = 9 embryos, 45 hpf ± 1.70 SEM, $p < 0.0002$

Mann-Whitney test, Figure 5). Thus the robust timing of progression of fate determinant factor expression is directly dependent on the generation of and feedback from the previously born cell types (in this case horizontal and amacrine cells, Table 3).

The larger variation in the onset of transgenes in the chimeric experiments compared to that in the previous time-lapse experiments are likely due to the random integration of donor cells into different areas of the forming retina, including differing depth and differing circumferential location, which would increase variation due to the developmental waves in which retinogenesis occurs.

This confirms that our observed effect on fate determinant gene timing is due to the differences in cell composition in the environment, even when the progenitors we are following and analysing have completely wild type gene expression. Specifically, feedback from the relatively earlier born cell types is needed to control the precise switch of gene expression to start generating the later born cell types at the appropriate developmental time. Thus, signals from earlier born neurons drive the very robust timing of the onset of fate determinant expression within developing progenitors independently of cell cycle exit timing.

Discussion

It has long been described that different types of retinal neurons are born in a specific histogenic birthorder conserved across all vertebrates (Livesey, Young et al. 2004). In this context the generation of a fully functioning retina thus depends on appropriately timed cell cycle exit and fate determination. While it is known that progenitors can be biased towards different proportions of cell types by the absence or presence of neurons in the environment (Reh and Tully 1986, Poggi, Vitorino et al. 2005, Jusuf, Almeida et al. 2011), it remains unclear whether such feedback and the role of the histogenic order may in fact control the timing of these key stages during development.

The fate determination of retinal neural progenitors can be biased towards generating more of the missing cell type demonstrating feedback signalling from neurons as they are generated (Reh and Tully 1986, Poggi, Vitorino et al. 2005, Jusuf, Almeida et al. 2011). It has been shown that the number of cell types within the retina of mice can vary (Keeley, Whitney et al. 2014), suggesting that compensatory mechanisms exists to ensure that visual function is maintained with some variation. However, it is crucial to aim to make the correct proportion of cells during developing neuronal circuits to ensure necessary and sufficient connections are made. Since the different cell types are born at distinct developmental times, both the timing of cell cycle exit as well as timing of fate determinant gene expression together will influence the number of each neuron type generated. Whether or which of these key aspects is responsible for the observed difference in the proportion of different types generated in differing environments was unknown.

Here, we directly examined the timing of retinal progenitor cell cycle exit and fate specification during differentiation in a wild type environment, where all cell types are generated normally compared to one that is missing either the earliest born ganglion cells (*lak*) or the intermediate born inhibitory neuron types (Ptf1a morphant). In these changed environments we were able to quantify any changes in earlier born or later born cell populations. While the specificity of the Ptf1a MO has been well characterised (Lin, Biankin et al. 2004, Jusuf, Almeida et al. 2011), the use of the MO for this study is not to directly study gene function, but as a tool to generate a retina lacking inhibitory neurons (Jusuf, Almeida et al. 2011) regardless of its mode of action. In these changed environments we were able to quantify any changes in earlier born and later born cells populations.

In both experimental conditions, the lack of earliest born ganglion cells or intermediate born inhibitory cells did not affect the timing of cell cycle exit, suggesting this aspect is controlled robustly independent of such feedback. Thus, the neurogenic window for each cell type is determined independently of feedback cues from earlier born cell types (Figure 6).

However, in both changed environments (loss of first born ganglion cells in *lak* or loss of intermediate born inhibitory neurons in Ptf1a morphants), the timing of key fate determinants of later born, but not earlier born cell types was delayed. The onset of gene expression in different body organs (Acta1:mCherry expression in muscle) and gene expression of earlier born retinal neuron type (in this case Atoh7 expression in earlier born ganglion cells in the Ptf1a morphant) within the same embryo act as an internal experimental control. These showed no delay, revealing that the morpholino injection and embryo manipulation itself does not result in any developmental delay. As the same experimental procedure is performed, but the environment in which these

progenitors find themselves at this earlier time point is equivalent, while the later born neuron types are generated in an environment that is missing key feedback from neuron types that should have been generated previously (Figure 6). While there was only a few hours delay, the significant difference indicates that the timing is fine-tuned by such feedback. While the later born neurons do not express and do not require the genes that we knocked out to generate the different environments, we performed transplantation studies. In combination with *in vivo* imaging to look at the timing of transgene expression in truly “wild type” progenitors in these changed environments we found the same pattern showing no delay in the Atoh7 expression and a delay in Vsx1 expression. Again the Atoh7 expression acts as an internal control showing that expression of earlier genes are not affected by the presence or absence of later born neurons, and furthermore that the experimental procedure itself does not cause any gene expression delays.

The Vsx1 expression was also observed to be significantly delayed, though to a larger extent (7 hours). While this could be due in part to the actual setup (following wild type progenitors in a morphant host environment), transplantation itself has caveats that need to be carefully considered. We cannot accurately control the retinal position into which such transplanted cells integrate. Since gene expression starts in the anterior-ventral patch and spreads three dimensionally as a wave both towards posterior regions as well as deeper eye regions, transplanted cells even in the control condition will show vastly different timing of gene expression (> 12 hours for a single label, see Figure 2, (Poggi, Vitorino et al. 2005, Almeida, Boije et al. 2014). This is reflected by the increase in the standard deviation of this data even within the Atoh7 control expression. While we could track the circumferential location and show a

similar spread of control vs. morphant cells (Figure 5), the depth of cells could not be easily determined due to potential mounting angle differences.

Given that later born neurons are in fact generated (rather than arresting retinogenesis completely) suggests that intrinsic factors within progenitors are sufficient to progress retinogenesis with a recent study also showing that probabilistic gene expression can account largely for the resulting neural fate composition (Boije, Rulands et al. 2015). We believe that the feedback and changes we observe here may act to fine-tune this process. As the development of the zebrafish retina occurs rather rapidly and the difference between the first born (starting around 28 hpf) and last born (starting arouond 38 hpf) is only about 10 hpf at any given retinal location, these observed delays of a few hours can significantly influence the total number of each neuron type being generated.

Therefore, we believe that the highly conserved birthorder of neuron fates observed across vertebrate retinas acts specifically to ensure that the correct number of each neuron types are generated, before changing the gene expression of fate determinant genes to drive neurogenesis of the next cell type. The delay in gene expression of subsequent cell types that I observe may be a way to ensure that sufficient cells have been generated before neurogenesis progresses. While cell cycle progression and exit during zebrafish retinal development are independently controlled to ensure the correct overall number of neurons, the histogenic order and feedback from each generated cell type ensures that this overall number is correctly subdivided to specify the right proportion of each cell type.

Other acknowledgements

We thank Dr Lucia Poggi (Centre for Organismal Studies, Heidelberg, Germany), and Professors William A. Harris (Cambridge University, UK), Shin-ichi Higashijima (National Institute for Physiological Sciences, Okazaki, Japan) and Steven D. Leach (Johns Hopkins Medical Institute, Baltimore, MD) for the transgenic lines used in this study. We thank FishCore staff for taking care of our animal stocks and Monash Micro Imaging facility and Drs Ashley Siegel, Wouter Masselink and Phong Nguyen for technical assistance.

Conflict of interest statement

We have no conflict of interest.

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity for the data and the accuracy of the data analysis. JN carried out the experiments and wrote the first draft of the manuscript. SD carried out transplantations experiments. PC and PJ conceived the study, supervised and reviewed the manuscript.

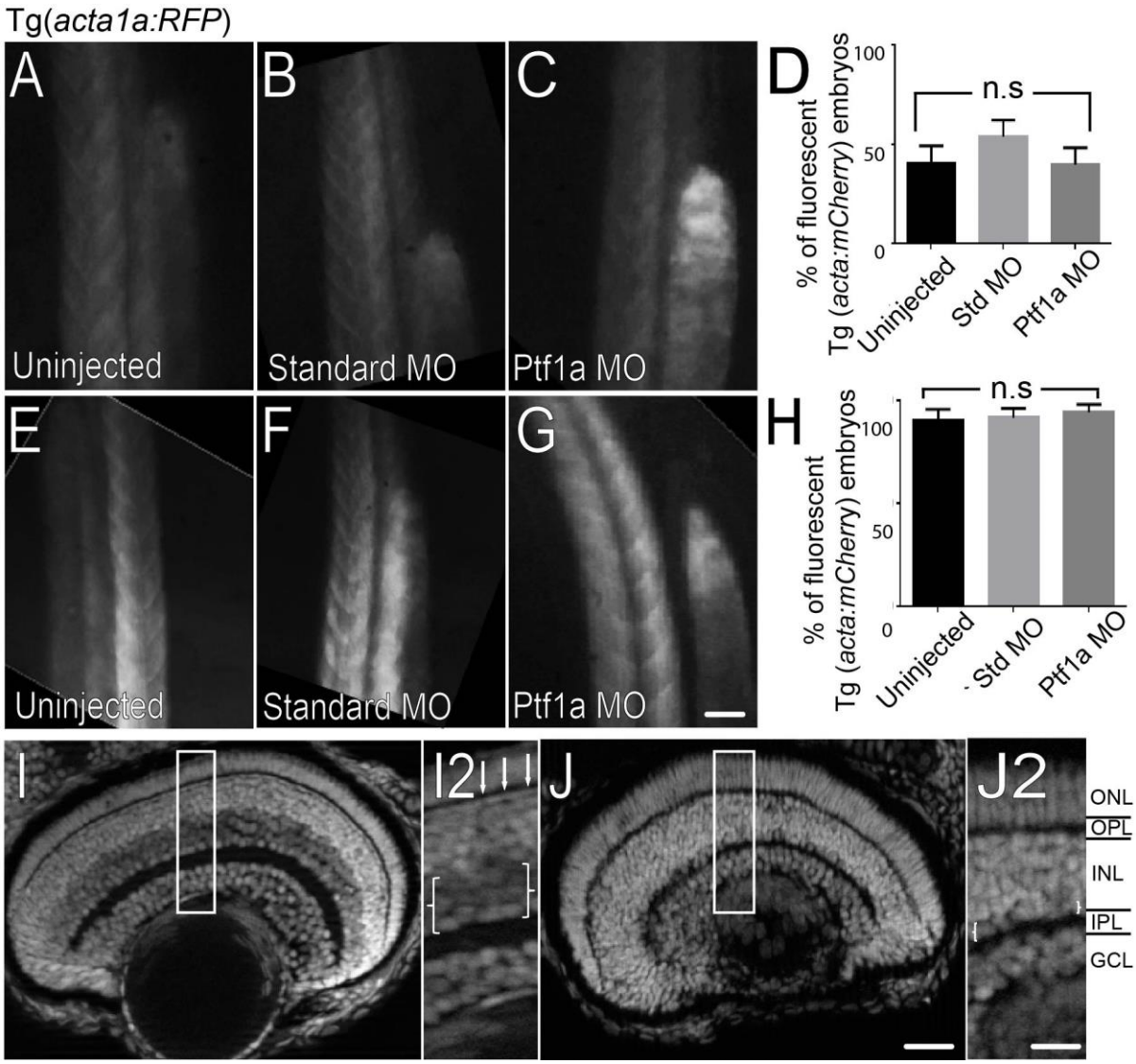

Tg(acta1a:RFP)
A
B
C
D
Uninjected
Standard MO
Ptf1a MO
% of fluorescent Tg (acta:mCherry) embryos
100
50
0
n.s
Uninjected
Std MO
Ptf1a MO
E
F
G
H
Uninjected
Standard MO
Ptf1a MO
% of fluorescent Tg (acta:mCherry) embryos
100
50
0
n.s
Uninjected
Std MO
Ptf1a MO
I
I2
J
J2
ONL
OPL
INL
IPL
GCL

Figure 1. Timing of embryonic development is unaltered when pancreas transcription factor 1a is knocked down

General developmental progression of embryos was assessed by comparing the developmental timing of embryonic structures that do not express pancreas transcription factor 1a (Ptf1a) and should not be affected by the knockdown. (A – H) Micrographs show the trunk region of Tg(*acta1*:*mCherry*) embryos, in which a fluorescent protein marker is expressed in developing muscles. Comparison at 31 hours postfertilisation (hpf) (A – D) and 42 hpf (E – H) showing micrographs of uninjected (A, E), standard morpholino (MO) injected (B, F) or Ptf1a MO injected (C, G) embryos. The number of embryos that had turned on the Acta1a:RFP transgene expression compared to the total number of embryos is indicated in each micrograph. Bar graphs (D, H) show proportion of larvae (n > 30 larvae per group) turning on Acta1:mCherry in the embryonic musculature of the trunk is comparable at 31 hpf (D, 40 – 50%) and 42 hpf (H, > 90%) in all groups ($p > 0.05$, Kruskal-Wallis test, error bars are SEM). (I-J) Micrographs of DAPI labelled cross sections through the central retina of 75 hpf standard (I) and Ptf1a MO injected embryos (J). Micrographs show loss of inhibitory neural retinal layers caused by efficient Ptf1a knockdown most evident in high power inserts (white boxes, I2 and J2). The size of the retina is comparable between the control and morphant, as described previously (Jusuf, Almeida et al. 2011). However, the composition of the INL in the morphant retina (J) is mainly made up of bipolar neurons, as compared to inhibitory neurons in the control retina (I). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; n.s.: not significant. Scale bar G (for A – G) = 50 µm, scale bar J (for I, J) = 20 µm, scale bar J2 (for I2, J2) = 10 µm.

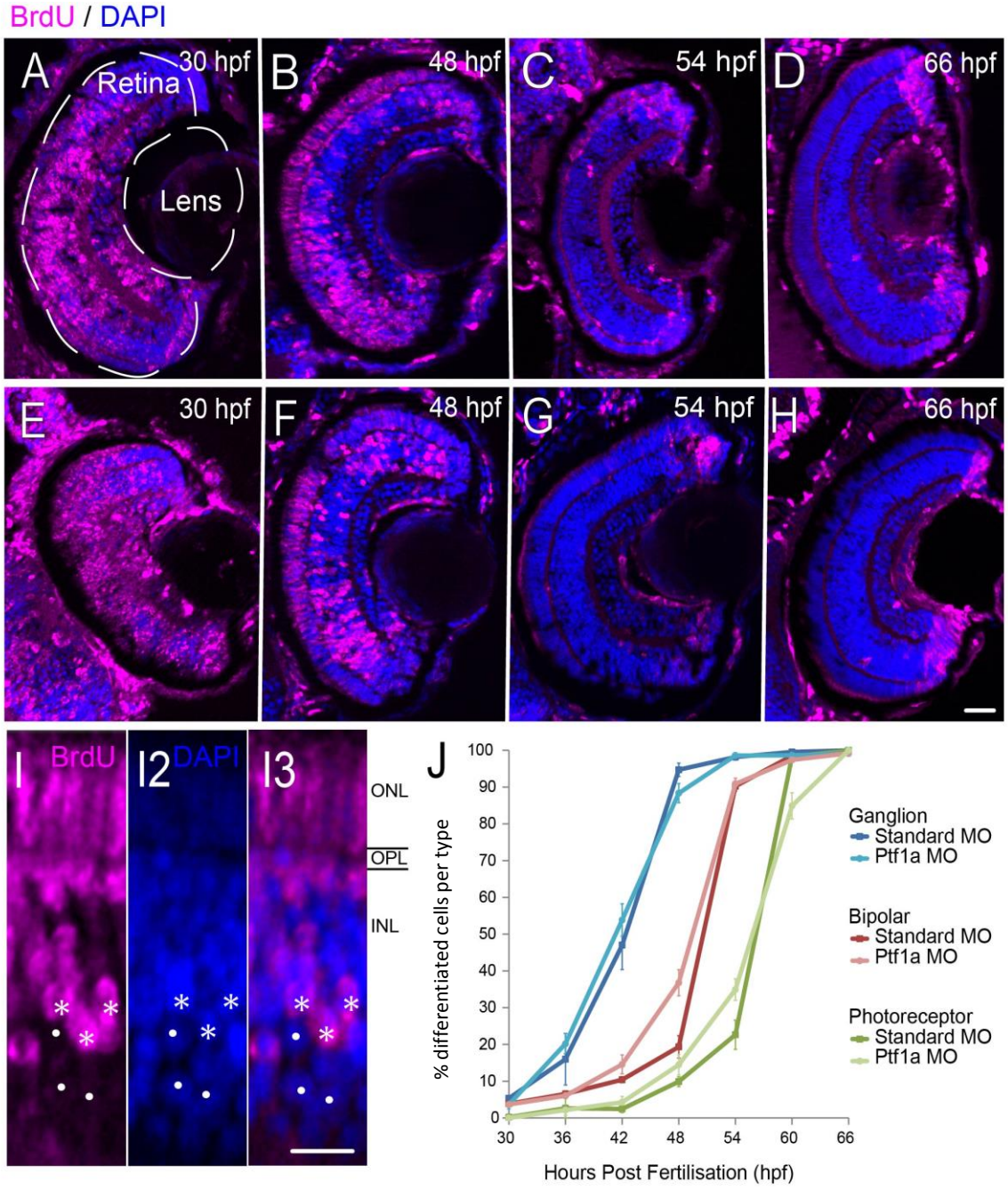

BrdU / DAPI
A
30 hpf
Retina
Lens
B
48 hpf
C
54 hpf
D
66 hpf
E
30 hpf
F
48 hpf
G
54 hpf
H
66 hpf
I
BrdU
I2
DAPI
I3
ONL
OPL
INL
J
% differentiated cells per type
Hours Post Fertilisation (hpf)
Ganglion
Standard MO
Ptf1a MO
Bipolar
Standard MO
Ptf1a MO
Photoreceptor
Standard MO
Ptf1a MO

Figure 2. Timing of neural birthdates is controlled independent from signalling from neighbouring neurons

(A - H) Micrographs of retinal sections of wild type embryos at 75 hpf (hours postfertilisation) injected either with standard (A - D) or Ptf1a (E - H) morpholino (MO) showing DAPI in blue and BrdU in magenta. Embryos were subsequently injected with BrdU followed by continuous BrdU incubation until 75 hpf starting at different ages (as indicated above each panel). (I) High power images of Ptf1a MO injected embryo with BrdU incubation starting at 30 hpf. Single channel BrdU (I, magenta), DAPI (I2, blue) and merged channels (I3) show examples of BrdU positive (asterisks) and BrdU negative (circles) nuclei (identified by DAPI labelling). (J) Graph shows comparison of the birthdates of ganglion cells (blue), bipolar cells (red) and photoreceptors (green) in standard MO (dark) vs. Ptf1a (lighter) MO injected embryos. Birthdates show no significant difference in the neurogenic window between standard and ptf1a MO injected embryos at all-time points for all cell types ($p > 0.05$), except for 48 hpf ganglion cells ($p < 0.05$), 48 hpf-bipolar cells ($p < 0.01$) and 54 hpf-photoreceptors ($p < 0.05$, Mann-Whitney test). However, though there is no change to the neurogenic window, the total number of excitatory cells increased over time, consistent with previous studies (Jusuf, Almeida et al. 2011). Error bars are SEM. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer. Scale bar A (for A – H) = 20 µm, scale bar I3 (for I – I3) = 10 µm.

Tg(*atoh7:RFP* / *vsx1:GFP*)

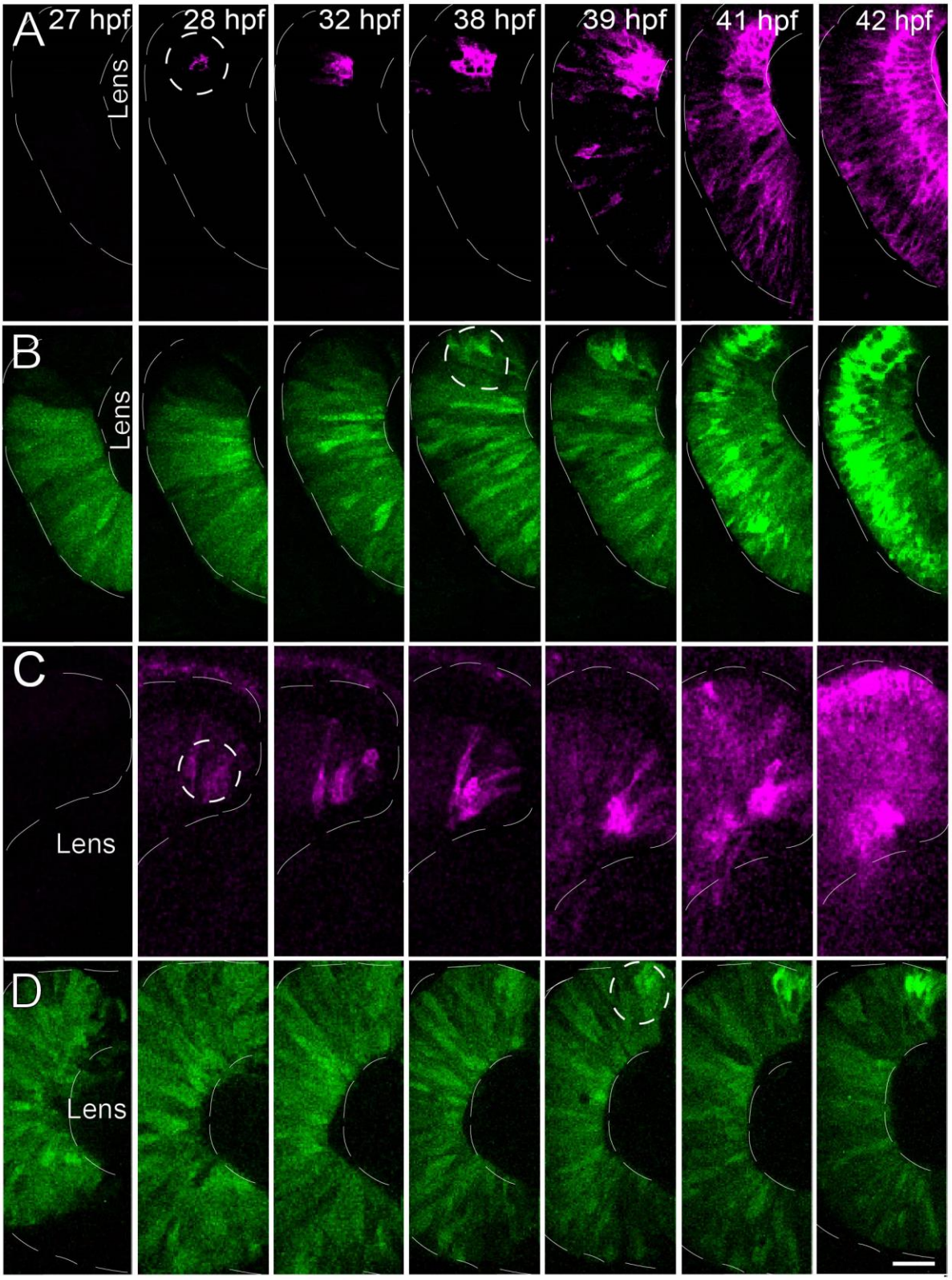


Figure 3. Timing of expression of later Vsx1, but not earlier Atoh7 factors is influenced by signals from inhibitory neurons

(A - D) Micrographs from time-lapse movies of developing retinas in Tg(*vsx1a*:*GFP* / *atoh7:GAP-RFP*) double transgenic embryos starting at 27 hpf (hours postfertilisation). (A, C) Atoh7*:*RFP (magenta) which marks the earliest born neurons (ganglion cells) is first expressed in the developing retina at 28 hpf, in both standard (A & B) and Ptf1a (C & D) morpholino injected embryos. The Ptf1a MO induced loss of the later born inhibitory neurons (horizontal and amacrine cells) does not affect the timing of the fate determinant Atoh7. (B, D) Vsx1:GFP (green) which marks progenitors is initially expressed in low levels and is upregulated in the later born bipolar cells is first expressed at 38 hpf in Ptf1a MO and at 40 hpf in standard MO injected embryos. Ptf1a MO induced loss of inhibitory neurons affects the robust timing of upregulation of the late born bipolar cell fate determinant Vsx1. Dashed circle indicates the first onset of Atoh7:RFP transgene or first upregulation of Vsx1:GFP transgene. Scale bar D (for A – D) = 20 µm.

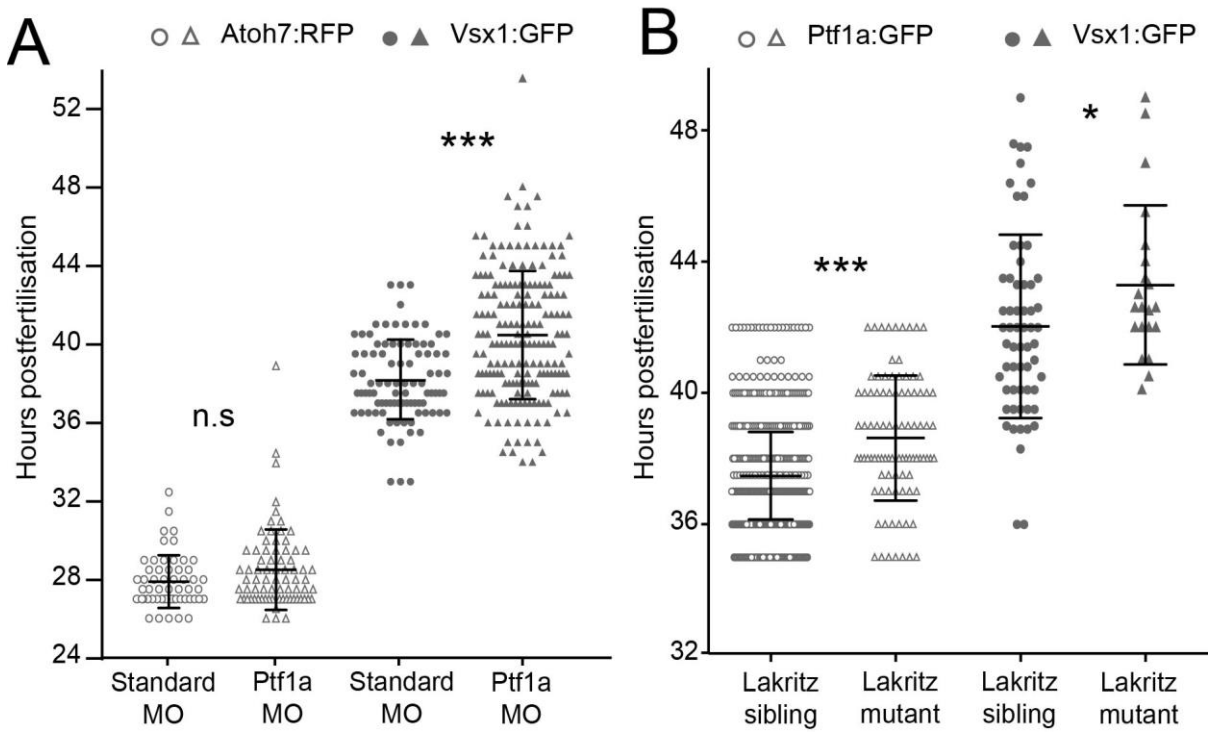
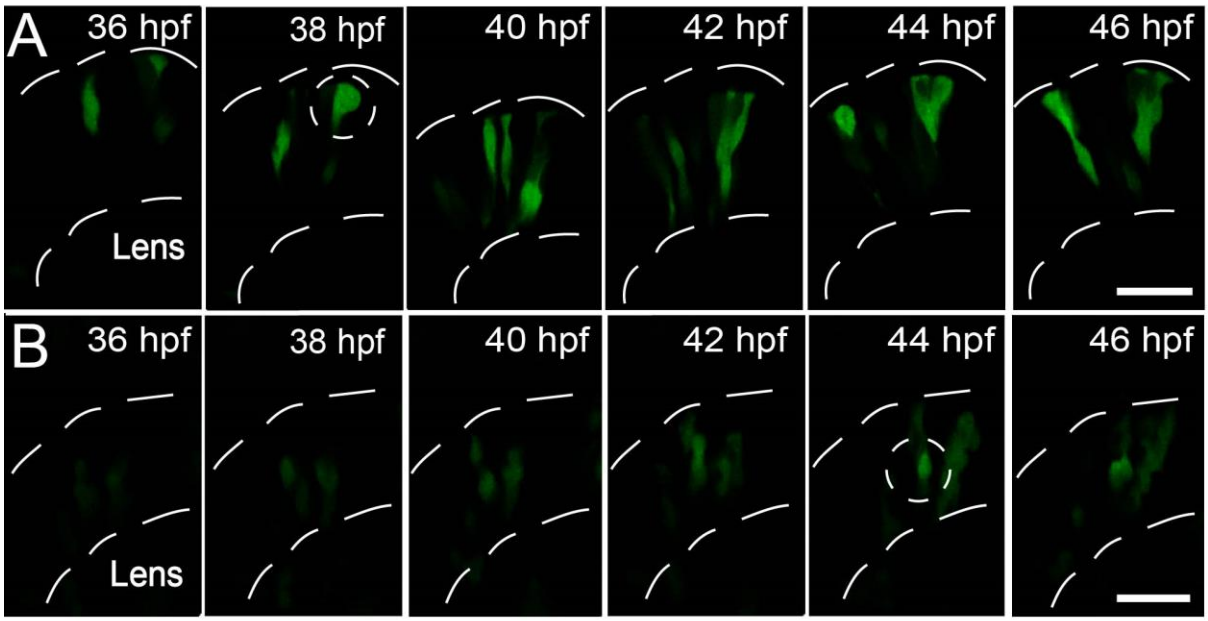

A
Atoh7:RFP
Vsx1:GFP
Hours postfertilisation
52
48
44
40
36
32
28
24
n.s
***
Standard MO
Ptf1a MO
Standard MO
Ptf1a MO
B
Ptf1a:GFP
Vsx1:GFP
Hours postfertilisation
48
44
40
36
32
***
*
Lakritz sibling
Lakritz mutant
Lakritz sibling
Lakritz mutant

Figure 4. Transgene onset for later cell fates is dependent on generation of earlier born cell types.

Box and Whisker plots shows the timing (hours postfertilisation – hpf) of Atoh7:RFP, Ptf1a:GFP and Vsx1:GFP transgenes onset for each embryo imaged. (A) Comparison of timing of transgene expression in standard or Ptf1a morpholino (MO) injected embryos. Atoh7:RFP expression (open symbols) onset in the first born ganglion cell neurons is comparable in standard vs Ptf1a injected MO (n.s. $p = 0.10$), whereas timing of Vsx1:GFP expression (closed symbols) onset in developing later born bipolar cell neurons in Ptf1a injected MO is significantly delayed compared to the standard injected MO. (B) Comparison of timing of transgene expression in *lak* (Atoh7 mutant) or sibling embryos. The timing of expression of Ptf1a:GFP (open symbols) and Vsx1:GFP (closed symbols), both driving generation of later born neuron types are significantly delayed in the *lak* mutants. * $p = 0.035$, *** $p < 0.0001$. Error bars are SEM.

Tg(*vsx1:GFP*)


A
36 hpf
38 hpf
40 hpf
42 hpf
44 hpf
46 hpf
Lens
B
36 hpf
38 hpf
40 hpf
42 hpf
44 hpf
46 hpf
Lens

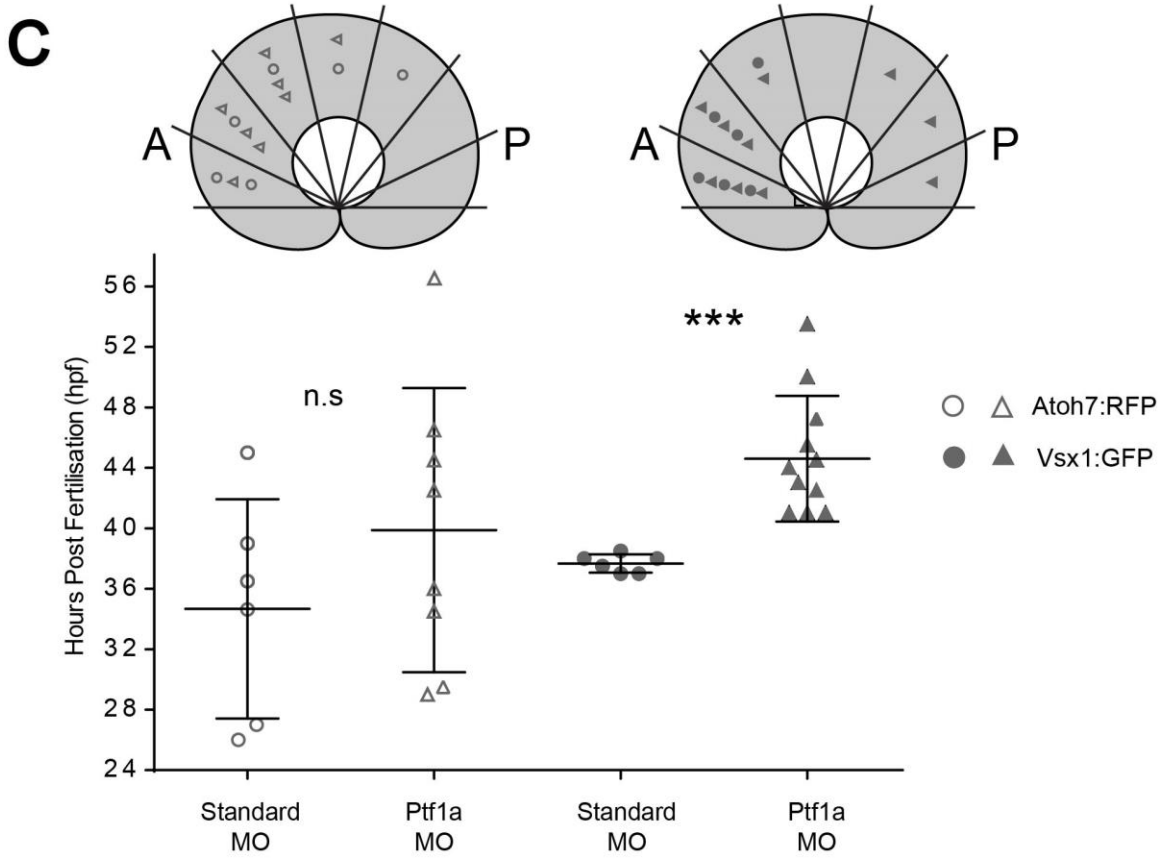

C
A
P
A
P
Hours Post Fertilisation (hpf)
56
52
48
44
40
36
32
28
24
n.s
***
Atoh7:RFP
Vsx1:GFP
Standard MO
Ptf1a MO
Standard MO
Ptf1a MO

Figure 5. Wild type donor cells transplanted into different environment show a delay in timing of expression of fate determinant genes of relatively later born neurons.

(A, B) Micrographs from time-lapse movies of developing wild type donor cells from Tg(*vsx1*:*GFP*) transgenic embryos in standard or Ptf1a morpholino (MO) injected host background starting at 36 hpf (hours postfertilisation). Vsx1:GFP marks progenitors and is upregulated in the latest born bipolar cells at 38 and 44 hpf in Ptf1a MO and standard MO injected embryos respectively. (C) Box and Whisker plot showing the timing of transgene (either Atoh7 – open symbols or Vsx1 – solid symbols) onset for each embryo imaged. Retinal schematics above each experimental data shows the spatial distribution of individual transplanted donor cells in standard MO (circles) and Ptf1a MO (triangles) injected host environments (A = Anterior, P = Posterior). Retinogenesis begins in the ventral anterior quadrant (near A) and progresses as a wave towards posterior regions (P). Timing of Atoh7:RFP expression onset in developing first born ganglion cell neurons is comparable in standard vs Ptf1a morphant hosts (n.s. $p = 0.3623$, Mann-Whitney test). Timing of Vsx1:GFP expression onset in developing first born bipolar cell neurons is significantly delayed in Ptf1a vs standard morphant hosts (*** $p < 0.0002$, Mann-Whitney test). Error bars are SEM. Scale bars A & B = 10 µm.

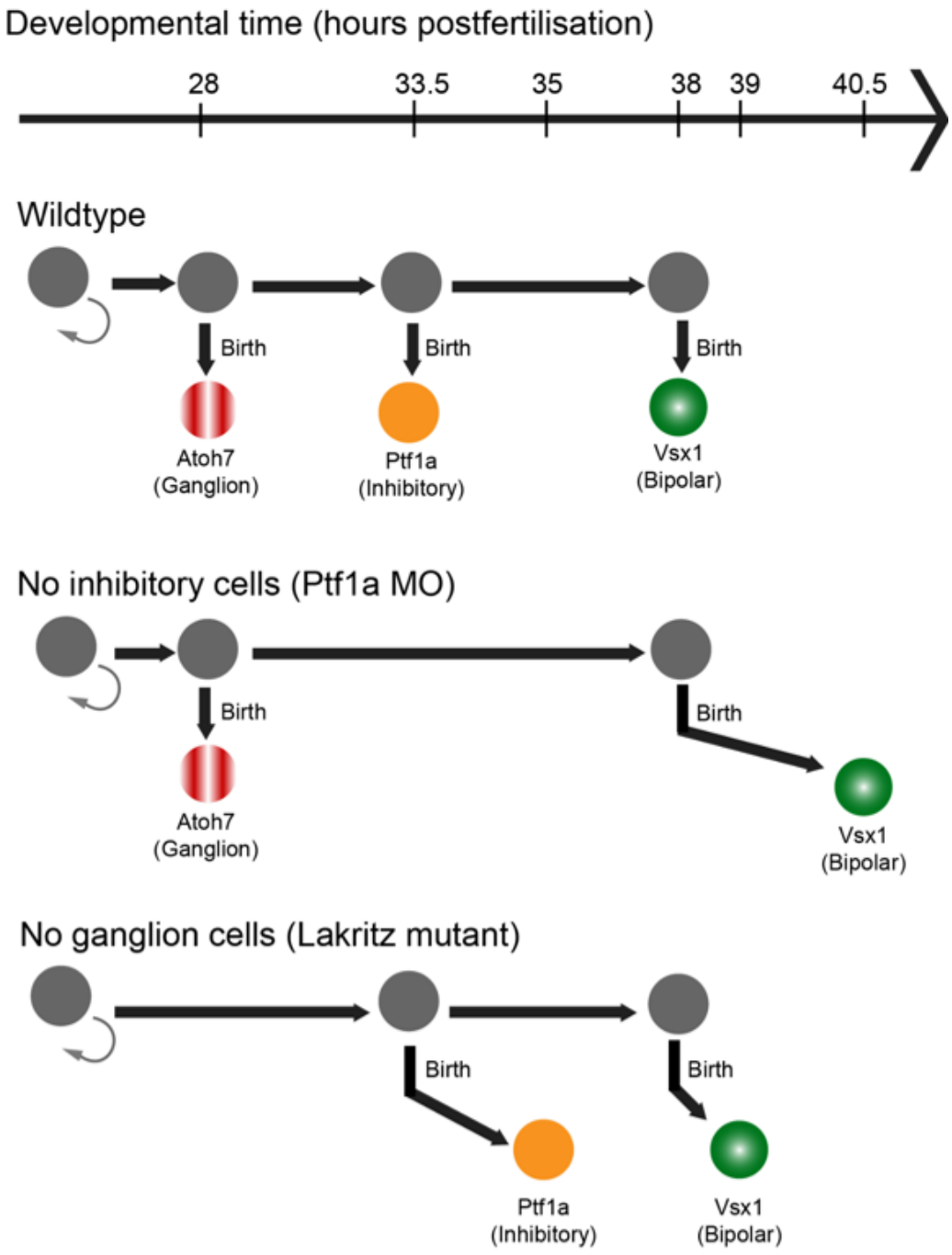

Developmental time (hours postfertilisation)
28
33.5
35
38
39
40.5
Wildtype
Birth
Birth
Birth
Atoh7
(Ganglion)
Ptf1a
(Inhibitory)
Vsx1
(Bipolar)
No inhibitory cells (Ptf1a MO)
Birth
Birth
Atoh7
(Ganglion)
Vsx1
(Bipolar)
No ganglion cells (Lakritz mutant)
Birth
Birth
Ptf1a
(Inhibitory)
Vsx1
(Bipolar)

Figure 6. Summary schematic of changes in cell birth and fate gene expression timing after loss of different neuronal population

During retinal development in a wild type environment, different types of neurons are born at distinct times and the expression of differential fate genes for each neuronal types similarly occurs in a stereotypical manner at distinct developmental times. In environments where a neuron population is missing during development, such as ganglion cells in the *lak* mutant or inhibitory cells (i.e. horizontal and amacrine cells) in the Ptf1a morphant, the birthdates are unchanged (vertical arrows still at the same developmental time), however, the timing of gene expression for later born neuron populations is delayed (such as Ptf1a and Vsx1), while the expression timing of earlier staged neuron fate genes (Atoh7) remains unaffected.

Chapter 4. Neural fate specification biased towards cell specific retinal regeneration in zebrafish

4.1 Introduction

All vertebrates studied to date have been shown to possess the potential for neural regeneration in the retina of the eye. However, retinal regenerative response is limited in higher vertebrate mammals, such as humans and rodents (Hamon, Roger et al. 2015). Mouse studies have shown that limited retinal regeneration can occur after an injury, however the process is impeded compared to other vertebrates and often results in scarring (Wan, Zheng et al. 2008, Bringmann, Iandiev et al. 2009). The mouse response to injury is in contrast to other vertebrate models such as the zebrafish, which can regenerate the correct number and cell types of neurons after different injuries fully restoring visual function (reviewed by Karl and Reh 2010; Fischer and Reh 2003). The extraordinary regenerative capacity of the zebrafish allows us to study how the different stages of neural regeneration are co-ordinated in vertebrates.

Retinal regeneration in zebrafish can involve activation of multiple endogenous cell sources, most notably the Müller glia, progenitors in the ciliary margin zone (CMZ) and rod precursors (Maier and Wolburg 1979, Hitchcock and Raymond 1992, Braisted, Essman et al. 1994, Wu, Schneiderman et al. 2001, Fausett and Goldman 2006, Bernardos, Barthel et al. 2007). Müller glia are present in all vertebrates and have been shown to be a cellular source of regeneration that can also be activated in mammals (Tropepe, Coles et al. 2000, Das, Mallya et al. 2006), such as in mouse (Wan, Zheng et al. 2008, Bringmann, Iandiev et al. 2009) and in human, as seen *in vitro* studies (Lawrence, Singhal et al. 2007, Bhatia, Jayaram et al. 2011).

Müller glia in mammals such as the mouse are able to divide and exhibit stem-like characteristics (Karl, Hayes et al. 2008, Takeda, Takamiya et al. 2008). The Müller glia of mice have limited self-renewing capabilities after injuries, such as photoreceptor degeneration due to retinal detachment (Kaneko, Nishiguchi et al. 2008) or failure of Müller glia to produce mature photoreceptors *in vivo* in mice after light lesion (Geiger, Barben et al. 2015). Isolated Müller glia, however, can be cultured to mature retinal neurons that integrate into the retina (Johnson and Martin 2008, Lorenc, Jaldin-Fincati et al. 2015). Hence, these studies demonstrate that mammalian Müller glia have the potential to regenerate new neurons.

In lower vertebrates such as chicken, zebrafish and amphibians the Müller glia are often the source of retinal regeneration and more responsive to injury (Karl and Reh 2010). For example in chicken, toxin induced injuries result in Müller glia dedifferentiation and re-entry of cells into the cell cycle (Fischer and Reh 2003), and take on stem cell characteristics (Hayes, Nelson et al. 2007). Though possessing regenerative capacity, Müller glia in chick undergo differential responses depending on age of the glia and the environment, as observed in older Müller glia not dedifferentiating after an injury, as well as embryonic Müller glia unable to differentiate into new neurons after transplanted into an injured, older host environment (Fischer and Reh 2003).

In contrast, amphibians exhibit highly robust retinal regeneration from the retinal pigment epithelium (RPE) (Araki 2007), and little regenerative activity from Müller glia cells after retinal neuron chemical ablation or complete retinal removal (Reh 1987, Grigorian and Poplinskaia 1999, Novikova Iu, Poplinskaia et al. 2008, Karl and Reh 2010).

In zebrafish however, mechanical, light and chemical damage to the retina are able to stimulate a robust regenerative response from Müller glia *in vivo* (Vihtelic and Hyde 2000, Senut, Gulati-Leekha et al. 2004, Curado, Stainier et al. 2008, Sherpa, Fimbel et al. 2008, Scott and Baier 2009, Zhao, Ellingsen et al. 2009, Montgomery, Parsons et al. 2010).

Though Müller glia regeneration has been observed in various vertebrates using different injury paradigms, there remain unanswered questions surrounding the process. Species and injury type differences appear to contribute to both the self-renewing capability and the extent of robust regenerative activity from Müller glia, suggesting that there are variations between species and little is still known on what activates the regenerative responses in these models. Molecular differences have been observed between species, for example in the chick and zebrafish Müller glia after injury, however whether this difference may result in a differential regenerative response is unknown (Boije, Ring et al. 2010, Ghai, Zelinka et al. 2010, Nelson and Hyde 2012). Different lesioning paradigms can also give rise to different regenerative responses in the Müller glia. For example in the zebrafish, glutamine synthetase expression is turned off after light ablation (Bringmann, Pannicke et al. 2006, Thummel, Kassen et al. 2008) but remains unaltered in an optic crush model (Chen and Weber 2002).

Furthermore, the extent of injury has been observed to influence the activation of a Müller glia regenerative response. Low insult via shorter and lower intensity light lesions to the photoreceptors in zebrafish only activates regeneration from rod precursors and not Müller glia. However, a large lesion from longer and higher light intensities activates a Müller glia response to produce new photoreceptor cells

(Vihtelic, Soverly et al. 2006, Morris, Scholz et al. 2008, Montgomery, Parsons et al. 2010).

Lastly, the role of the retinal environment is important, as it can bias the development of retinal neurons in vertebrates regeneration such as chick, fish and amphibians (Ng 2013). In development, it is established that progenitors bias fate choices by sensing which cell types are underrepresented in the local microenvironment and favour the production of these missing cell types (Poggi, Vitorino et al. 2005, Jusuf and Harris 2009). In regeneration, a similar process to development could be occurring where Müller glia are able to sense environmental signals and respond by altering progenitor fate choice to produce the missing cell types. If this theory is correct in regeneration, the ability of Müller glia to sense and adjust gene expression would be a powerful regenerative tool that can be exploited after cell specific visual disorders including retinitis pigmentosa (photoreceptor cell loss) or glaucoma (ganglion cell loss).

As compared to other vertebrate models, the zebrafish is able to show a remarkable ability to restore the architecture of the retina after injury, including functional recovery (Saszik, Bilotta et al. 1999, Mensinger and Powers 2007, Sherpa, Fimbel et al. 2008). The system has been a useful system to understand the activation and the proliferative potential of Müller glia and to characterise the phases of regeneration.

Such characterization of the temporal timing of cell death (Vihtelic, Soverly et al. 2006, Montgomery, Parsons et al. 2010), proliferation (Fimbel, Montgomery et al. 2007, Thummel, Kassen et al. 2008, Thomas, Nelson et al. 2012), gene expression changes (Livesey, Young et al. 2004, Roesch, Jadhav et al. 2008, Ramachandran,

Fausett et al. 2010), and timing & signalling that are activated during neural differentiation (Fischer and Reh 2003, Karl, Hayes et al. 2008, Fraser, DuVal et al. 2013), has revealed many similarities to developmental neurogenesis.

Similar signalling pathways activated during neurogenesis are also involved in different regenerative stages, especially during Müller glia dedifferentiation to turn on progenitor markers. Due to the similarities observed retinal regeneration is believed to recapitulate developmental neurogenesis, though to what extent or which developmental events are recapitulated has not been investigated thoroughly.

In order to further understand the role of the environment on Müller glia driven regeneration, I generated models that result in the degeneration of specific retinal neurons at a defined acute time point in large cohorts. As previously discussed, variable regenerative responses as observed from available injury models were not able to satisfy these aspects, thus I established two distinct but comparable injury models; (1) a mechanical, non-specific cell ablation model to ablate all cell types within the retina and (2) a chemical, specific cell ablation model that specifically targets only inhibitory neurons. By comparing these models, it will be possible to observe the endogenous regenerative process and determine whether Müller glia are able to adjust fate determination of newly regenerated neurons to replace only the missing cell populations (ie replace inhibitory neurons) or the uninjured population (ie. replace all neurons).

Complete characterisation was carried out to compare the regenerative process in these two injury models by assessing the time period of cell death, types of cells affected, origin of regenerative stem cells, proliferation of progenitor and extent, timing and fate specification of regenerated neurons.

4.2 Methods and Materials

4.2.1 Zebrafish husbandry

Zebrafish were housed, bred and raised at FishCore facility at Monash University in accordance with local animal guidelines. Fishlines used include TU, Tg(*ptf1a:Gal4*) kindly provided by Prof. Leach, Tg*(UAS:nfsb-mCherry*) (Davison, Akitake et al. 2007) a gift from Prof. Lieschke, Tg(*gfap:GFP*) generated by Dr Bernardos and Prof. Raymond (Bernardos and Raymond 2006), Tg(*vsx1:GFP*) provided by Prof. Higashijima (Kimura, Okamura et al. 2006), Tg(*atoh7:GFP*) generated by Drs Zolessi and Poggi (Zolessi, Poggi et al. 2006). These single lines were crossed to generate relevant double and triple transgenic lines such as Tg(*atoh7:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*) and Tg(*vsx1:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*). Juveniles of either gender were maintained according to standard protocol, staged as previously described (Kimmel, Ballard et al. 1995) and used both before and after free feeding stages.

4.2.2 Mechanical Ablation (Needle Stick injury)

1 week old TU zebrafish were anaesthetised in 0.0006% tricaine and were placed on 2% low melt agarose coated petri dishes. Retinal injury was performed using glass needles, pulled from a 1.0mm O.D x 0.78mm I.D glass capillary (Harvard Apparatus), at 6 different points on the eye. Zebrafish were then recovered in fresh E3 solution and were monitored as they were returned to the flow system.

4.2.3 Genetic Ablation (Metronidazole treated Nitroreductase injury)

1 week old Tg(*ptf1a:Gal4* / *UAS:nfsb-mCherry*) zebrafish were incubated in a solution of 10mM metronidazole / 0.2% DMSO in E3 (NaCl, KCl, $CaCl_2.2H_2O$,

$MgCl_2.6H_20$, methylene blue) solution for 8 hours at 28^oC. Zebrafish were washed 3 times in fresh E3 media, and monitored as they were returned to the flow system.

4.2.4 5-bromo-2'-deoxyuridine (BrdU) exposure

For swim exposure, BrdU was diluted in embryonic medium (E3) and adjusted to pH 7.0 to a 2 mM solution. Embryos that completed mechanical or genetic ablation injury were treated in the BrdU solution through swim exposure. Embryos were swim exposed for 24 hours to BrdU at stages 0 to 7 days post injury. For prolonged BrdU pulse experiments, embryos were swum overnight for 16 hours every day from 3 dpi to 7 dpi, and washed in fresh E3 media during the day (2 washes).

4.2.5 Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4), cryoprotected in 7.5% gelatine / 15% sucrose in PBS solution and cryostat sectioned at 14 µm thickness using a Leica CM3050S Cryostat. Antibody staining was performed using standard protocols with all steps performed at room temperature. Sections were blocked in 5% fetal bovine serum (FBS) / 0.5% Triton x-100 in PBS, incubated in mouse anti-BrdU (1:500, Life Technology), in situ cell death detection kit, fluorescein (Sigma Aldrich) & sheep anti-fluorescein Fab fragment antibody (1:500, Roche), rabbit anti-PCNA antibody (1:500, Abcam) diluted in the same block solution overnight at 4^oC. Secondary antibody used was anti-mouse, anti-rabbit and anti-sheep Alexa Fluor-488 fluorophores (1:400, Life Technology diluted in the same block). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (1:10,000, Life Technology). Sections were mounted with Mowiol (Sigma-Aldrich).

4.2.6 Image acquisition

Images of fixed sections were obtained on a Zeiss Z1 (20x objective) using an AxioCam (HRm 13-megapixel, monochrome) and Axiovision software. Brightness and contrast were adjusted with Photoshop (Adobe).

4.2.7 Analysis

For quantification of cell death, cell cycle exit and cell fate specification (standardized to retinal width of 1000 µm from n = 5 embryos per time point), all terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), proliferating cell nuclear antigen (PCNA), Atoh7:GFP and Vsx1:GFP co-labelled cells were analysed across the entire length of the retina, excluding the ciliary margin zone (CMZ).

For quantification of Tg(*ptf1a:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*) cells after genetic ablation injury (n = 4 mutants), cells were counted from the central retina in a fixed retinal width of 50 µm by 100 µm height. Statistical analyses were conducted using a student T test (GraphPad).

4.3 Results

4.3.1 Cell death in distinct neural populations can be efficiently targeted by specificity of injury

Different injury ablations can lead to different regenerative capacities within vertebrates (Ng 2013). Here I established a nitroreductase-metronidazole induced (genetic) ablation injury model targeting specifically the inhibitory neuron populations in the retina, namely horizontal and amacrine cells. First the efficacy of this injury model was assessed, by characterising and comparing the time course, extent and cell type specificity of this genetic ablation injury with a mechanical ablation injury targeted to cause damage across all retinal layers, hence affecting all of the different retinal neuron types.

Disruption to the retinal architecture shortly after injury was clearly seen in both the mechanical and genetic ablation injury model as revealed by DAPI nuclear staining (Figure 4.1 A-D). The injury track of the needle can be easily observed immediately after mechanical ablation injury, with obvious disruption to all cell layers at this site (Figure 4.1 B). By performing the injury at 6 evenly spaced points, we would be able to observe a regenerative response across the retina, but within localised regions. In the genetic ablation injury model, inhibitory neurons were targeted for ablation injury using a *ptf1a* promoter to drive expression of nitroreductase enzyme, which in turn converts the pro-drug metronidazole into a cytotoxin within these inhibitory neuron populations. In contrast to the 6-point needle injury, the genetic ablation resulted in a pan retinal injury. In this model, striking neuronal loss can be observed at 3 days post-injury (dpi, Figure 4.1 D).

The total number of horizontal inhibitory cells identified as a single layer of flattened nuclei in the outermost row of the inner nuclear layer and amacrine inhibitory cells identified as the weaker DAPI labelled neurons in the inner half of the inner nuclear layer is greatly reduced in the genetic ablated model by 51% for amacrine cells (41 ± 2 SEM untreated vs. 21 ± 1.5 SEM post-injury, n = 6 or 7 embryos) and 67% for horizontal cells (9 ± 0.775 SEM untreated vs. 3 ± 0.842 SEM post-injury, n = 6 or 7 embryos), while all other cell layers appear to be unaffected by this ablation injury.

In order to analyse the extent and timing of cell death post-injury, as well as the specificity of affected neuron types post injury. TUNEL staining was used at 0, 1, 2, 3, 4, 5, 7 and 10 dpi to label and quantify DNA fragmentation occurring in cells in different layers (Figure 4.1 E - J). After mechanical ablation injury the cell density of TUNEL labelling is already observed in 129 ± 63.3 SEM cells / 1000 µm retinal width, n = 7 embryos at 0 dpi (Figure 4.1 E,) (The number of cells are calculated over a given retinal width for consistency in all measurements). The number of TUNEL labelled cells peaks at 1 dpi (139 ± 36.9 SEM cells / 1000 µm retinal width, n = 6 embryos) and was almost completely gone by 3 dpi (6 ± 2.22 SEM cells / 1000 µm retinal width, n = 5 embryos) (Figure 4.1 K).

After genetic ablation injury, TUNEL positive cells are also immediately seen with the peak number of TUNEL cells similarly observed at 1 dpi (203 ± 34.6 SEM cells / 1000 µm retinal width, n = 5 embryos) and drastically reduced by 3 dpi (16 ± 1.33 SEM / 1000 µm retinal width, n = 3 embryos), though unlike the mechanical ablation injury, the number of TUNEL positive cells is greatly reduced by 4 dpi (Figure 4.1 H - J, L). Overall, the genetic ablation resulted in a more extensive cell death (450 TUNEL

cells per 1000 µm retinal width) as compared to the mechanical injury (400 TUNEL cells per 1000 µm retinal width), possibly due to the pan retinal genetic ablation.

Analyses of retinal layers in which TUNEL positive cells were observed showed the expected cellular distribution. After mechanical ablation injury, in which the needle penetrated through all retinal layers, TUNEL positive cells were observed in all retinal cell layers (Figure 4.1 K). In contrast after genetic ablation injury, which uses nitroreductase expression only in inhibitory neurons to convert harmless metronidazole into a cytotoxin, TUNEL labelled cells can be seen primarily in the inhibitory cell layers from 0 dpi in the genetic ablation injury (81% of TUNEL positive cells, with 50% in the amacrine and 31% in the horizontal layer) as compared to the mechanical ablation injury at 0 dpi (34% of total TUNEL positive cells, with 30% in the amacrine and 4% in the horizontal layer).

In addition, genetic ablation injury resulted in a sharp drop in the number nitroreductase (nfsb) positive cells as time progresses (Figure 4.1 L), reinforcing the inhibitory neuron specific cell death. In both ablation injury models, the timing of TUNEL labelled cell death is comparable as the temporal progression, peak in cell death and fall in cell numbers occurs within a similar time range (Figure 4.1 E-J). Thus, differential cell type specific injury resulting in comparable cell death progression can be achieved using these two distinct ablation injury models.

4.3.2 Progenitor proliferation is comparable in mechanical vs genetic ablation models

The temporal stages of progenitor activation and proliferation in the retina were compared following ablation injury of neurons in both the mechanical and genetic ablation injury models to assess whether the regenerative response after such

different injuries differs. Cell proliferation was assessed using immunohistochemical labelling for proliferating cell nuclear antigen (PCNA), a factor necessary for DNA polymerase (Paunesku, Mittal et al. 2001) or daily 24 hour 5-bromo-2'-deoxyuridine (BrdU) pulses.

PCNA positive clusters of cells can be seen to span across retinal layers starting at 4 dpi in the mechanical ablation injury (Figure 4.2 B) and at 5 dpi in the genetic ablation injury injury model (Figure 4.2 F), suggestive of individual proliferative clones arising from individual progenitors. The number of PCNA positive cells is largest between 4 and 6 dpi in mechanical ablation injury (Figure 4.2 D, 4 dpi: 82 ± 20.82 SEM cells / 1000µm retinal width; 5 dpi: 42 ± 9.56 SEM cells / 1000µm retinal width; 6 dpi: 32 ± 8.77 SEM cells / 1000µm retinal width) and between 5 and 7 dpi in genetic ablation injury (Figure 4.2 H; 5 dpi: 49 ± 10.86 SEM cells / 1000µm retinal width; 6 dpi: 41 ± 4.19 SEM cells / 1000µm retinal width; 7 dpi: 47± 9.68 SEM cells / 1000µm retinal width). The total number of PCNA positive cells after mechanical (Figure 4.2 D) and genetic (Figure 4.2 H) ablation injury indicate that most of the proliferation occurred within a 3-day period with proliferation in the mechanical ablation injury possible occurring slightly earlier.

Additionally, to label proliferating cells each day in my novel genetic ablation injury, consecutive 24 hour BrdU pulse labelling was performed each day from 0 to 7 dpi (Figure 4.3 A-G). The highest BrdU incorporation within the observed time frame in the genetic ablation injury model was observed at 4 dpi (51 ± 17.72 SEM cells / 1000 µm retinal width). By 7 dpi, the number of BrdU positive cells was dramatically reduced (5 ± 2.67 SEM cells / 1000 µm retinal width) and overall retinal architecture was recovered in both ablation injury models by this stage (Figure 4.3 I, J). The period

of peak progenitor proliferation in both the mechanical and genetic ablation injury model is broadly similar.

4.3.3 Regenerating proliferative cells arise from Müller glia

Different ablation injury models can result in differences in origin of the endogenous cellular source of regenerating cells. In zebrafish for instance, light exposure induced photoreceptor ablation injury (Penn 1985, Abler, Chang et al. 1996, Vihtelic and Hyde 2000, Bernardos, Barthel et al. 2007) or ouabain induced ganglion cell ablation injury (Maier and Wolburg 1979, Fimbel, Montgomery et al. 2007, Sherpa, Fimbel et al. 2008) results in differential progenitor activation from the ciliary margin zone (CMZ) and rod precursors or Müller glia respectively (Maier and Wolburg 1979, Hitchcock and Raymond 1992, Braisted, Essman et al. 1994).

Müller glia are commonly activated across vertebrates and remain the predominant endogenous regenerative cell source after large injuries (Wan, Zheng et al. 2008) including after mechanical lesions in zebrafish (Yurco and Cameron 2005). Understanding potential environmental influences on progenitor behaviour, I first assessed the endogenous progenitor source activated in response to my two distinct ablation injury models. Using the transgenic fish Tg(*gfap:GFP*), in which the promoter marks mature Müller glia with GFP reporter protein, I assessed co-labelling with the proliferation markers after ablation injury.

As described above, genetic ablation injury was conducted at 7 dpf and sections of embryos fixed at 3, 4, 5, 6 & 7 dpi were stained with PCNA (Figure 4.4 B). Consistent with previous papers that described Müller glia as the regenerative source of origin after mechanical injury, I observed PCNA co-labelling with GFAP positive

cells. GFAP positive cells that co-label with PCNA first formed clusters that spans across retinal layers at 3 dpi (Figure 4.4 C-G).

At 5 dpi, 97% of PCNA cells were co-labelled with Gfap:GFP, though even at this stage, most of the co-labelled glia showed a reduction of GFP level as compared to neighbouring non-proliferative non-activated Müller glia. At subsequent days the proportion of co-localised cells decreased and by 6 dpi only 57% of PCNA positive cells were GFAP positive. This is consistent with the reduction in GFP levels at earlier stages and with the interpretation that activated glia de-differentiate (and thus down regulate glial markers such as GFAP) to enter a progenitor like state (Jadhav, Roesch et al. 2009, Meyers, Hu et al. 2012). At 7 dpi, only 29% of PCNA positive cells were GFAP positive, and were weaker for GFP when compared to non PCNA co-labelled Müller glia cells. All of the observations are in line with my interpretation that the primary cell source of progenitor proliferation comes from Müller glia in both ablation injury models.

4.3.4 Environment signals cell type specific regeneration at early stages

In order to determine progenitor fate specification during regeneration, I performed prolonged BrdU labelling. Based on the time course of proliferation assessed both from the PCNA labelling and 24 hour BrdU pulse exposures, I treated zebrafish juveniles injured at 7 dpf with either ablation injury models with a prolonged BrdU pulse, by incubation in BrdU overnight (16 hours) from 3 to 7 dpi (with recovery in embryo medium during the day), to encompass the peak proliferative stage (Figure 4.3 K, M).

Leaving embryos in BrdU for the entire period unexpectedly resulted in less BrdU labelled cells and started to show detrimental health effects on embryos, suggesting that such extensive exposure may have toxic side-effects both for the animal and the cells (data not shown). Samples immediately processed after this prolonged pulse at 7 dpi resulted in labelling of 117 cells ± 37.2 SEM / 1000 µm retinal width in the mechanical ablation injury model and 169 cells ± 29.15 SEM / 1000 µm retinal width after genetic ablation injury (Figure 4.3 K-N). In the mechanical ablation injury model (Figure 4.5 A) at 7 dpi, BrdU positive cells appear to be distributed into all layers regenerating all of the five main neuron types. This population of BrdU positive cells are still actively proliferating to generate new clones even after exposure to BrdU, as the total number of BrdU cells increased by 70% (70 BrdU positive cells per 1000 µm retinal width) between 7 to 10 dpi. By 10 dpi and subsequent analysed time points, the total number of BrdU cells in each cell layer is roughly proportionate to the overall size of the layer and remains so in subsequent stages.

In the genetic ablation injury model (Figure 4.5 B) at 7 dpi, BrdU positive cells appear to be distributed mainly in the amacrine neuron layer (65% cells) and other BrdU cells are distributed into the other 4 layers. Similarly to the mechanical ablation injury model, this population of BrdU positive cells are still actively proliferating to generate new clones in the absence of BrdU exposure, as the total number of BrdU cells increased by 400% (150 BrdU positive cells per 1000 µm retinal width) between 7 to 10 dpi. By 10 dpi onwards, when retinal architecture seemed to have recovered, most of the proliferating cells were distributed mainly to the other neural layers, while only a small proportion of BrdU cells was added on to the amacrine neuron layer. This suggests that there may be an overshoot of activated regenerating neurons that

subsequently gives rise to all retinal neurons in an environment that no longer shows a cell specific ablation bias.

In both injury models, I observed a reduction in the number of BrdU positive cells from 10 dpi onwards, which could be either due to subsequent cell division or some cell death occurring. The proportion of cells in each layer remains stable during regeneration, suggesting that the observed reduction in BrdU cells affects all forms of neuron types in equal proportions.

My results show that in the environment that ablated inhibitory neurons, BrdU positive cells were mainly located in the amacrine inhibitory neuron layer early on (7 dpi) during regeneration, whereas BrdU positive cells were distributed in all cell layers proportionally in the early stages of the mechanical injury, which ablates all neuron types. At the later stages of regeneration (10 dpi onwards), the newly generated BrdU positive cells were distributed to other layers in the cell specific genetic ablation model, potentially contributing to all cell types similarly to that observed in the mechanical ablation model.

4.3.5 Gene expression of BrdU positive cells

Since newly generated BrdU positive cells were found in all the layers in both ablation injury models, I asked whether any of the BrdU positive cells at these stages analysed were expressing differentiation markers appropriate for cells in these layers. Thus, the expression of key fate determinant factors were in BrdU positive cells by using Tg(*atoh7:GFP*) and Tg(*vsx1:GFP*). Tg(atoh7:GFP) transgene is expressed during neurogenesis to direct progenitor cells into ganglion cell fate within the ganglion cell layer (Liu, Mo et al. 2001, Kimura, Okamura et al. 2006). Tg(vsx1:GFP) transgene is expressed during neurogenesis to direct progenitors into bipolar cell fate within the

inner nuclear layer of the retina (Chow, Snow et al. 2001, Zolessi, Poggi et al. 2006). Both genetic and mechanical ablation injuries were conducted and the time course of gene expression vs relative layer distribution of BrdU positive cells was assessed at 7, 10, 14, 17 and 21 dpi.

In the Tg(*atoh7:GFP*) mechanically injured model, 23% (37 ± 20.392 SEM cells / 1000 µm retinal width, n = 3 embryos) of all BrdU positive cells were located in the ganglion cell layer by 14 dpi, and 75% (28 ± 14.22 SEM cells / 1000 µm retinal width, n = 3 embryos) of these co-labelled with Atoh7:GFP (Figure 4.6, B, M), with similar results at 17 dpi (Figure 4.6, C, M). Thus, at these times, the cells within the ganglion cell layer have appropriately migrated and started differentiating.

Genetic ablation in the Tg(*atoh7:GFP*) transgenic line at 17 dpi, 24% of all BrdU positive cells were located in the ganglion cell layer, but none of these cells were expressing Atoh7:GFP at any stage of my analysis, although Atoh7 expression was turned on at comparable stages in BrdU positive cells in other layers.

Additionally, in both injuries at 17 dpi Atoh7:GFP expression was also observed in a comparable small proportion of BrdU positive cells in the amacrine layer (20% in mechanical injury, 15% in genetic ablation injury, Figure 4.6 N, Q). These could represent amacrine cells arising from the Atoh7 lineage as observed during development (Jusuf and Harris 2009) or possibly displaced ganglion cells which are common in other vertebrate species, but are not observed during zebrafish retinal development.

In development, Vsx1 is first expressed at low levels in progenitor cells and upregulated in cells that are differentiating as bipolar cells. In my mechanical ablation injury model 78% of BrdU cells in the bipolar layer (43 ± 21.121 SEM cells / 1000 µm

retinal width out of the total BrdU positive population of 55 ± 23.524 SEM cells / 1000 µm retinal width) were co-labelled with Vsx1:GFP at 10 dpi (Figure 4.6 D, O). In the genetic ablation injury model 73% of BrdU cells in the bipolar layer (17 ± 2.397 SEM BrdU positive cells / 1000 µm retinal out of the total BrdU positive population of 24 ± 8.549 SEM cells / 1000 µm retinal width) were co-labelled with Vsx1:GFP at 14 dpi (Figure 4.6 E, O). Vsx1:GFP expression was strongly maintained in BrdU positive cells in the appropriate retinal bipolar layer at later stages in mechanical (95%, 14 &17 dpi) and genetic (100%, 17 dpi) ablation injury. Thus, cells proliferating in the first week after injury do migrate to appropriate layers to differentiate into the expected cell types as revealed by fate specific transgene expression at similar times in both injury models.

Strong Vsx1:GFP expression in the bipolar cell layer is expressed earlier than ganglion cell markers in the mechanical ablation injury, suggesting that regeneration may not recapitulate developmental cell order, though a thorough investigation of this needs to be performed.

4.4 Discussion

The recent identification that all vertebrates, including humans, have the potential to regenerate neurons in the CNS has opened up intense research in the field of regenerative neuroscience. Processes involved in neural regeneration are believed to recapitulate developmental processes, which are much better characterized to date.

Due to the highly conserved neuron types as well as genetic control during development across all vertebrates studied, the retina has become a great model system for investigating developmental processes and has yielded a wealth of knowledge in regards to neurogenesis of the CNS.

For instance, developmental fate specification of new neurons generated from equipotential progenitors are shown to be primarily driven intrinsically by the stochastic expression of specific transcription factors driving fate specification within the progenitors that express these factors (Boije, Rulands et al. 2015). Many of these factors are re-expressed in the regenerating progenitor cells following retinal injuries such as paired box 6 (Pax6), oligodendrocyte transcription factor 2 (Olig2) and Notch1 as shown through microarray expression studies (Raymond, Barthel et al. 2006, Kassen, Ramanan et al. 2007, Thummel, Kassen et al. 2008).

However, other important cellular events involved during development have not been investigated thoroughly during regeneration to concretely imply that the events are similarly conserved. Two developmental events play important roles in regulating regeneration and are highly conserved across vertebrates. These are the occurrence of a developmental like histogenic birth order and the contribution of extrinsic factors in determining fate choice of progenitors.

In development, the importance of extrinsic factor contribution was exemplified in studies in the zebrafish (Poggi, Vitorino et al. 2005, Jusuf and Harris 2009) and Xenopus (Reh and Tully 1986), which showed that extrinsic factors can bias the generation of particular neuron types to restore the missing proportion of cells. In addition, the conserved order of neuronal birth across all vertebrates studied suggests an importance role during normal retina development (Wong and Godinho 2003, Barbosa-Sabanero, Hoffmann et al. 2012). In chapter 3, I discovered two key concepts; different neuron types are born in a distinct histogenic order and that the final number of each neuron type, while determined intrinsically, can be biased by extrinsic factors through controlling the timing of important fate determinant gene expression.

In the current chapter, I now show that in line with the dogma that regeneration mimics development, progenitors that are activated in my regenerative models are also able to use extrinsic feedback information from the environment to specifically direct fate specification towards the missing cell type. However, closer analysis at the gene expression order in regeneration unexpectedly revealed that the timing of fate gene expression, and thus the histogenic order of neurogenesis, does not adhere to that observed in normal development, suggesting that regenerating progenitors are not entirely equivalent to their developmental counterpart.

4.4.1. Comparison of adult injury models ablating different cell populations

I established two injury models for ablating different neuronal populations within the zebrafish retina to assess the cells types generated when different cell populations are injured. These included a needle stick injury, recapitulating a physical trauma by inserting a needle through the zebrafish retina. The second injury involves a genetic-

chemical ablation using bacterial nitroreductase to cause cytotoxic conversion of the drug metronidazole (Lindmark and Muller 1976) specifically in the inhibitory horizontal and amacrine cells in the retina. While trauma after accidents can result in general neuronal death of multiple types found in the affected area, the genetic-chemical ablation model is more reminiscent of human visual degenerative disorders and other disorders of the CNS, in which genetic mutations cause degeneration primarily to one of the neuronal subtypes (Nash, Wright et al. 2015).

Both of my injuries result in a proliferative regeneration response of Müller glia, which drives retinal regeneration. This was expected, as Müller glia have been shown to drive regeneration across different injury models in the fish (Fischer and Reh 2003, Boije, Ring et al. 2010, Nelson, Gorsuch et al. 2012, Pollak, Wilken et al. 2013). The activation process is likely to change across different injury models, however once activated, the ability for Müller glia to regenerate neurons and the general phases Müller glia progress through appear to be comparable across injury models (Bernardos, Barthel et al. 2007, Fimbel, Montgomery et al. 2007, Curado, Stainier et al. 2008).

I show that cell death occurs in all cell types in all layers in the needle stick injury model, while the nitroreductase model results in inhibitory neurons specific cell death, both as expected by my experimental design. The subsequent regenerative response was comparable in timing and extent of proliferation. My established injury models thus exhibit cell type specific ablation and the activation of the classical Müller glia regenerative response in line with previously established injury models, thus making both injuries suitable to compare Müller glia response in two different injury environments.

Surprisingly, in both injury models the total number of cells that appeared to have recovered was more than the expected numbers observed from tracing proliferating cells through bromodeoxyuridine (BrdU) labelling, which marks actively dividing cells. The number of BrdU positive cells (Figure 4.5) was 250 cells per 1000 µm section, approximately half of the total number of cells (400 cells per 1000 µm section) that underwent cell death in genetic ablation injury over 2 days (Figure 4.1). As TUNEL marks cells undergoing apoptosis for only 1 – 2 hours (Perlman, Maillard et al. 1997), it is likely the 400 TUNEL cell count is an underestimate on how many neurons undergoes apoptosis. This further emphasize that recovery to the architecture of the retina is not solely driven by BrdU proliferating cells. This will be discussed further in Chapter 5, however, this does indicate the presence of BrdU unlabelled regenerated cells, suggesting that an alternate source of regeneration may exist to regenerate the retina. Therefore, the ability for Müller glia derived progenitors to respond to environmental cues should be similar in other models and other species.

4.4.2. Regenerating progenitors mimics the fate bias of developmental progenitors

In both injury models, I followed dividing cells by tracking BrdU labelled cells through the proliferative phase. I quantified the cells after the cells differentiate and settle in their final cell layer, as the proportions were stable and are unchanged. The needle stick injury resulted in the regeneration of all cell types in similar proportions to each other. On the other hand, the chemical ablation resulted in the specific regeneration of the missing cell types, which can be observed most pronounced in the early time stages of regeneration. Thus I showed that neurogenesis was primarily biased towards regeneration of the affected cell type, possibly to regenerate the right proportion of cells that are missing.

Other injury studies that used nitroreductase to target other neuron types such as photoreceptors (Fleisch, Fraser et al. 2011) and bipolar cells (Zhao, Ellingsen et al. 2009), progenitors were also observed to successfully regenerate the missing neurons and restore visual function. However, whether regeneration of other cell types occurred in parallel was not characterised in detail in these studies. Based on my results, it is likely that in these published studies, the progenitors would equally be able to respond to extrinsic cues and initially regenerate primarily the affected neuron types followed by potentially regeneration of other neuron types in parallel.

This ability of regenerating progenitors to adjust the type of neuron generated to restore the correct proportion of neuron types can be crucial to the final outcome of functional recovery. The correct proportion of neurons guides the proper development of functional circuits, a network of interconnected neurons, formed by the occurrence of well-timed regulatory events (Purves D 2001). In this thesis, I reviewed the contribution of intrinsic gene regulation, the role of a histogenic order of birth and extrinsic feedback on adjusting progenitor fate bias during development.

The importance of the correct proportions of neuron types is illustrated by the many different processes in which this process is controlled developmentally to result in a surprisingly robust anatomical and thus functional bauplan. Other events necessary to generate the proper neuronal circuits during development include for example the initial overproduction of CNS neuron types, which is reduced later in development after synapse formation through programmed cell death (Burek and Oppenheim 1996). The importance of cell death can also be seen in other organs, for example, during the generation of fingers of limbs, whereby the cells between digits undergo apoptosis in development (Ganan, Macias et al. 1996). Once generated, homotypic interaction occurs between cell bodies and processes of the same neuron

subtype to ensure that the remaining neurons are distributed evenly to form semi-regular mosaics (Lee, Cowgill et al. 2011). These mosaic arrangements allow for an even distribution of all the cells types across the retina, giving all regions of the visual field access to the necessary processes and elements.

In the retina, mosaics of different neural subtypes are formed independently, but within each subtype, the neurites form distinct mosaic with processes from the same number of cells responsible for analysing visual information that enters the eye from any given area of the visual field (Stevens 1998). Individual neurites can also be pruned after synapse formation and as a result of neural activity, which is important for the release of neurotropic support from postsynaptic partners (Okawa, Hoon et al. 2014, Kostadinov and Sanes 2015). In addition, forming proper circuits involve neuron pathfinding, whereby mature neurons send out axons to reach their right targets by following specific path, and this event involves dynamic changes in the neuron receptors composition (Harris, Holt et al. 1987) to respond to different chemoattractants or chemorepellents (Bashaw and Goodman 1999, Sun, Bahri et al. 2000).

All of these complex events that lead to the development of a neural circuit imply that if a subtype was generated during retinal regeneration was uncoordinated, progenitors may either generate too many non-affected or too few of the affected neuron subtypes compared to an uninjured state. As dendritic plasticity can play a compensatory role to counteract variations of cell numbers in the outer plexiform layer (OPL) (Reese, Keeley et al. 2011), making additional, non-ablated neurons may not disrupt functional coverage of the neurons. However, the extent of compensation is not known, whereby large variations of cell numbers after regeneration may affect functional circuits.

Although I did not test the functional circuits (i.e. behavioural testing), I did demonstrate an important mechanism, in which reactivated progenitor cells can sense the neural composition of the surrounding environment and adjust its fate choice the same way that developmental progenitors can. An interesting experiment would be to genetically drive the over- or under production of specific cell types followed by probing the visual function. For instance, if regenerating progenitors in our nitroreductase experiment only regenerated a few of the missing inhibitory neurons, in addition to many of the unaffected types, would all cells still integrate in functional circuits. We would also observe if the regenerated cells will visually evoked behaviours relying on inhibitory circuits, such as for example motion detection recover. This could be tested using a optokinetic or optomotor test established in zebrafish, which uses a repeated stimuli to assess visual function during swimming (Maaswinkel and Li 2003, Zou, Yin et al. 2010).

4.4.3. Environmental contribution to general regeneration

My results show that regenerating progenitors are able to sense the cells types that are missing in the environment and selectively bias fate specification to generate more of the missing cell type. The possible influence of environments on regenerative processes has been described previously in different models.

In mice, successful regeneration of the sciatic nerve after it was cut was shown to be dependent on the presence and orientation of the proximal and distal stumps cut out, whereas if one of the stumps was omitted, the sciatic nerve does not regenerate (Scaravilli 1984). Further, in mouse culture, neural progenitors can be influenced by extrinsic factors and directed to differentiate either into neuron, muscle or glia cells when cultured in different media (Brannvall, Corell et al. 2008).

In birds after an injury to hair cells through drug treatment, the fate choice at which progenitors decide to either divide into two hair cells, two non-hair cells or one of each, appears to be dependent on the time course of regeneration after damage, suggesting that there is an extrinsic regulation on time and quantity to determine the fate choice of progenitors (Stone and Rubel 2000).

Reptilian tail regeneration is another example of a process dependent on extrinsic factors, whereby the wound epithelium generated from the injury is characterized by a unique profile of proteins and factors generated such as fibroblast growth factors and matrix metalloproteinases that helped direct the cells within the blastemal cap towards different cell fate choices (Gilbert, Payne et al. 2013). In summary, studies have shown that an environment that influences regeneration is a highly conserved process.

I specifically show that the environment results in a fate choice bias. However, caveats need to be drawn when extrapolating the data for broader application. Further investigation is required to identify if other CNS progenitors can similarly sense cell types that are missing and specifically regenerate these cells. This would identify if environmentally directing regeneration is a viable option for therapeutic approaches. For example, using progenitors of the brain for cell replacement therapies in Parkinson's or Alzheimer's disease models, which specifically affect dopaminergic neuron types in the substantia nigra or results in a build-up of amyloid aggregations in the brain, respectively (Mohamed, Shakeri et al. 2016, Zhu, Li et al. 2016).

An additional challenge that is more relevant in the brain or spinal cord than the retina includes the relevant environmental chemotactic cues to control pathfinding of neurites in particularly long ranged axon projections. Within the retinal neuron

populations connections are made relatively proximal, though the output ganglion cells will face the same challenges. If such necessary signals to direct pathfinding are no longer present or regenerating progenitors are not able to reexpress the correct sequence of the relevant receptors to respond to chemoattraction or repulsion, then proper neuronal pathfinding and subsequent correct circuit formation and circuit function will fail, even if regenerating stem cells know which cell types to replace.

Nonetheless, this study opens up the field to identifying these unknown environmental factors that are involved in directing cell specific regeneration and thus contribute to the research efforts of activating cell specific regeneration in the human retina, which currently appears promising as based on *in vitro* human studies (Bhatia, Jayaram et al. 2011, Giannelli, Demontis et al. 2011).

4.4.4. Gene Expression order and histogenic fate specification not recapitulated in regeneration

By using transgenic lines, I confirmed that progenitors are generating the appropriate mature neuron types in the right retinal layers. Thus, neurons produced within the mature retina are able to use the existing environment, which is quite different to the neuroepithelium during development, to complete late stage differentiation events including migration. This again suggests that progenitors or precursors (ie. grown *in vitro* and reintroduced at this stage rather than as mature neurons) are highly involved in feedback signalling between the tissue environment to contribute towards appropriate regeneration, which represents the best way to ensure an accurate reconstituted retina.

I used prolonged BrdU exposure to fate track newly formed regenerating cells. While it does not influence the result, this method does have a few experimental

caveats. Firstly, the BrdU pulse consists of 16 hours on and 8 hours off exposure for the benefit of the animal health. Thus, while dividing cells may undergo S phase within the off period without BrdU exposure, the cells division can still be observed using immunohistochemistry for residual BrdU and it is unlikely that many dividing cells will be unlabelled.

Secondly, labelled cells at early time point may have yet to reach their final destination or may be undergoing interkinetic nuclear migration (IKNM) (Lahne, Li et al. 2015), similar to what is observed in progenitors during development (Baye and Link 2007, Del Bene, Wehman et al. 2008, Norden, Young et al. 2009). However, my transgenes for fate differentiation markers show that layer location correlates well with the expected neuron fate, at least by 7 dpi, suggesting that these neurons reach their future layer relatively quickly and start terminal differentiation in the appropriate retinal location. In addition, I observed a decrease in the number of BrdU labelled cells after 10 dpi. This may be due in part by dilution of BrdU in subsequent cell division, or a lack of integration and loss of some of these cells through for example apoptosis. This is however, unlikely as both PCNA and TUNEL staining are minimal at relevant time points.

These experiments did show unexpected differences compared to retinal development in the timing of expression such as fate specification genes. As highlighted in Chapter 3, during development, gene expression for fate determinant genes occurs in temporally distinct, though slightly overlapping events, such that neurons exiting the cell cycle tend to give rise to a specific cell type at any stage of development. I showed that the order of gene expression still occurs regardless of extrinsic environment, however, it does specifically affect the timing of the gene expression switch (Chapter 3). Thus, I similarly expected the same order of gene

expression to occur in regeneration, if it does indeed recapitulate developmental processes. In contrast, I found that the fate determining genes were not expressed in the same order as in development and some of the later expressed genes were active in parallel with the earlier expressed genes. This thus supports my alternate hypothesis that not all processes in regeneration recapitulate development. This is in line with a few differences observed in the transcriptome of progenitors during regeneration versus development, such as differential expression of GAP-43, a growth associated protein, which is turned on in progenitors during development but not during regeneration (Udvadia, Koster et al. 2001). This difference in birth order timing may also be a result of different cohorts of Müller glia that were activated to regenerate at different time point. Thus, further research is required to analyse the timing of clonal formation from different activated glia population to observe if this developmental birth order is recapitulated during regeneration. Given the observations and caveats present, this highlights that we cannot simply presume that all events during development are immediately applicable and is driven in the same way during regeneration.

It may also be important to note that mechanisms observed during retinal development, such as all the events involved in establishing circuit formation, may not be as applicable to the entire CNS as it is likely restricted to cells and CNS regions and cannot be simply extrapolated. This caveat is also applied across species, as for full mammalian regeneration, the various events leading up to the development of the right proportion of neurons to generate the right neural circuit needs to be similarly assessed to the zebrafish. This includes a more efficient activation of available stem cells and increasing their proliferative function, fate determination and the final differentiation stage such as pathfinding. If any of these do not occur efficiently, the

final function will be jeopardized and thus influence the therapeutic relevance of experimental studies.

4.4.5 Implication of studies towards human regeneration

This study reveals important implications in the design for future regenerative therapies. For example, current approaches include directing stem cells *in vitro* towards photoreceptor fate for transplantation therapies. Photoreceptors are of particular interest as they are the most prevalent cell type affected in degenerative visual disorders, affecting more than half of all cases of blindness in the aged population due to diseases such as age related macular degeneration and retinitis pigmentosa (Pearson 2014).

By generating mature photoreceptors *in vitro* and transplanting them, factors and events usually supplied from the surrounding tissue that may be necessary in establishing proper circuits may be missing. Late differentiation processes including migration, terminal morphological changes including neurite outgrowth and synapse formation depend on other cell types such as postsynaptic partner neurons. For example, during retinal development, cells are able to use the existing scaffold of neurites in the inner plexiform layer later to generate proper stratification (Randlett, MacDonald et al. 2013, Chow, Almeida et al. 2015). Therefore, by generating photoreceptors outside of this context, these final processes may not occur with the necessary feedback and may lead to low efficiency of integration into existing neuronal network.

My results suggest that a better approach would be introducing retinal progenitors at a relatively early stage into the deficient host environment, thus by using the scaffold and associated signalling in the existing environment, generate the right

cell types in the right proportions and assist in directing proper migration and neurite & synaptic formation. Thus, the closer therapeutic approaches mimic the events at the right time in an *in vivo* setting, the more likely regeneration be successful.

4.6 Conclusion

My results make seminal contributions to the field of regeneration, showing the involvement of the extrinsic environment after an injury to efficiently and accurately drive neurogenesis, a field that has been previously dominated by the contributions of intrinsic gene control. In addition, I show that the processes described during development form an important starting point for our understanding, but further studies are needed to be applicable in a human clinical setting (Bhatia, Jayaram et al. 2011, Giannelli, Demontis et al. 2011).

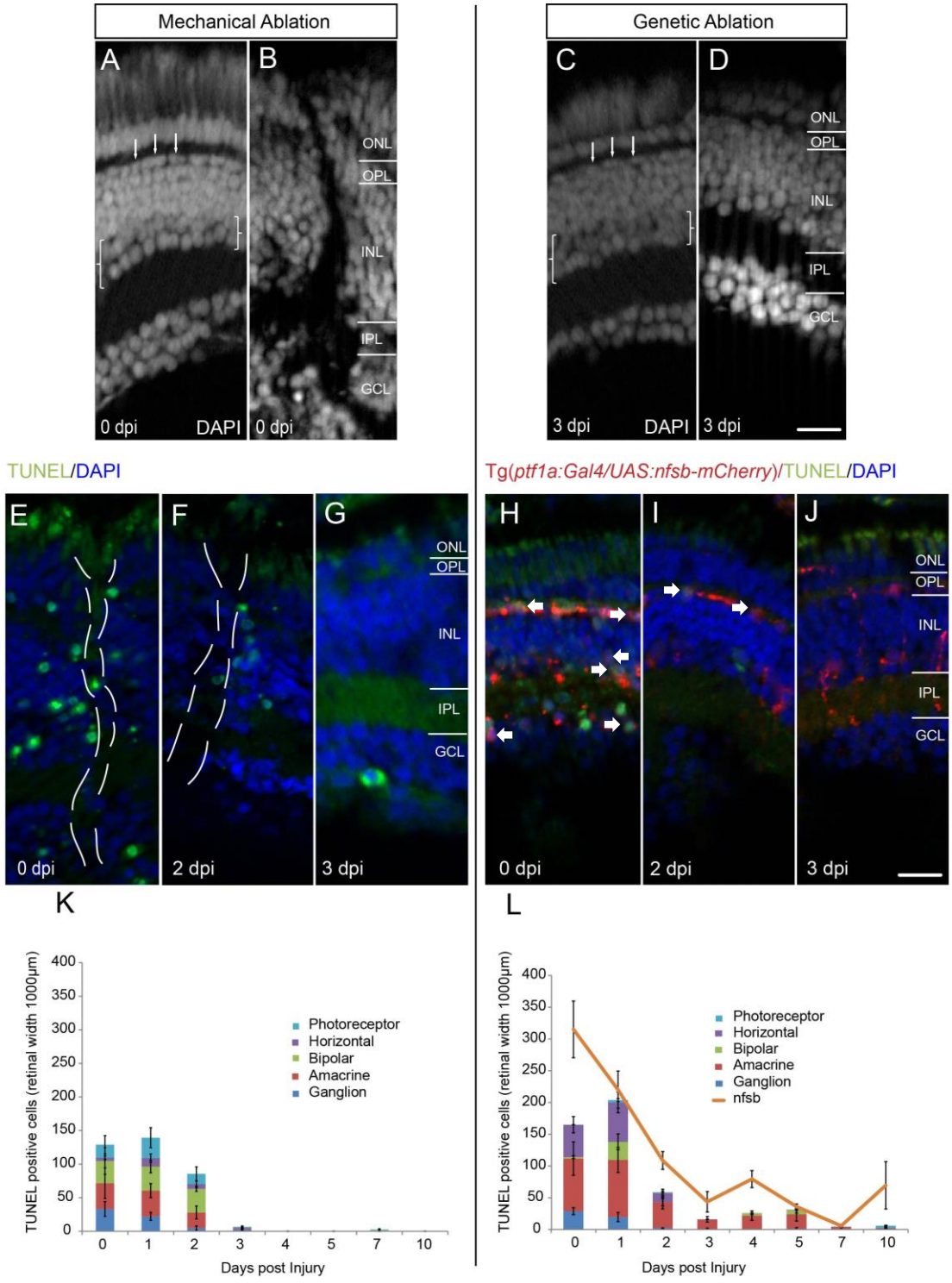

Mechanical Ablation
Genetic Ablation
A
B
C
D
ONL
OPL
INL
IPL
GCL
0 dpi
DAPI
0 dpi
3 dpi
DAPI
3 dpi
TUNEL/DAPI
Tg(ptf1a:Gal4/UAS:nfsb-mCherry)/TUNEL/DAPI
E
F
G
H
I
J
0 dpi
2 dpi
3 dpi
0 dpi
2 dpi
3 dpi
K
L
TUNEL positive cells (retinal width 1000µm)
Photoreceptor
Horizontal
Bipolar
Amacrine
Ganglion
nfsb
Days post Injury

Figure 4.1. Neuron type specific cell death shows comparable timecourse in two distinct injury models. (A - J) Micrographs of retinal sections after mechanical injury (A, B, E – G) or metronidazole induced genetic ablation in Tg(*ptf1a:Gal4 / UAS:nfsb-mCherry*) (C, D, H - J). (A, C) Retinal architecture of uninjured retina. Brackets indicate the amacrine neuron layer (weaker DAPI staining in the inner half of the INL) and arrows indicate the horizontal neuron layer (first row of flattened nuclei in the inner nuclear layer – INL). (B, D) Retinal architecture of injured retina revealed by DAPI staining shows disruption caused by the needle track immediately after ablation injury, affecting neuron types in each retinal layer (B) and reduction in the horizontal cell layer and amacrine cells (D). (E - J) TUNEL labelling at different days post-injury (dpi) in both injury models. TUNEL staining is observed in all retinal layers early after mechanical ablation (E – G) and specifically in horizontal and amacrine layers, where nitroreductase labelled neurons are also located in (H - J). Quantification of TUNEL positive cells in the different retinal layers across days post injury reveals a peak in cell death in the first two days distributed across all retinal layers in the mechanical ablation (K) and primarily confined to inhibitory neurons after genetic ablation (L). (L) Loss of nitroreductase-mCherry positive cells follows the cell death observed in genetic ablation (orange line). ONL: outer nuclear layer; OPL: outer plexiform layer; IPL: inner plexiform layer; GCL: ganglion cell layer; nfsb: Nitroreductase. Scale bar in D (for A – D) = 50 µm, scale bar in J (for E – J) = 50 µm. Error bars are SEM.

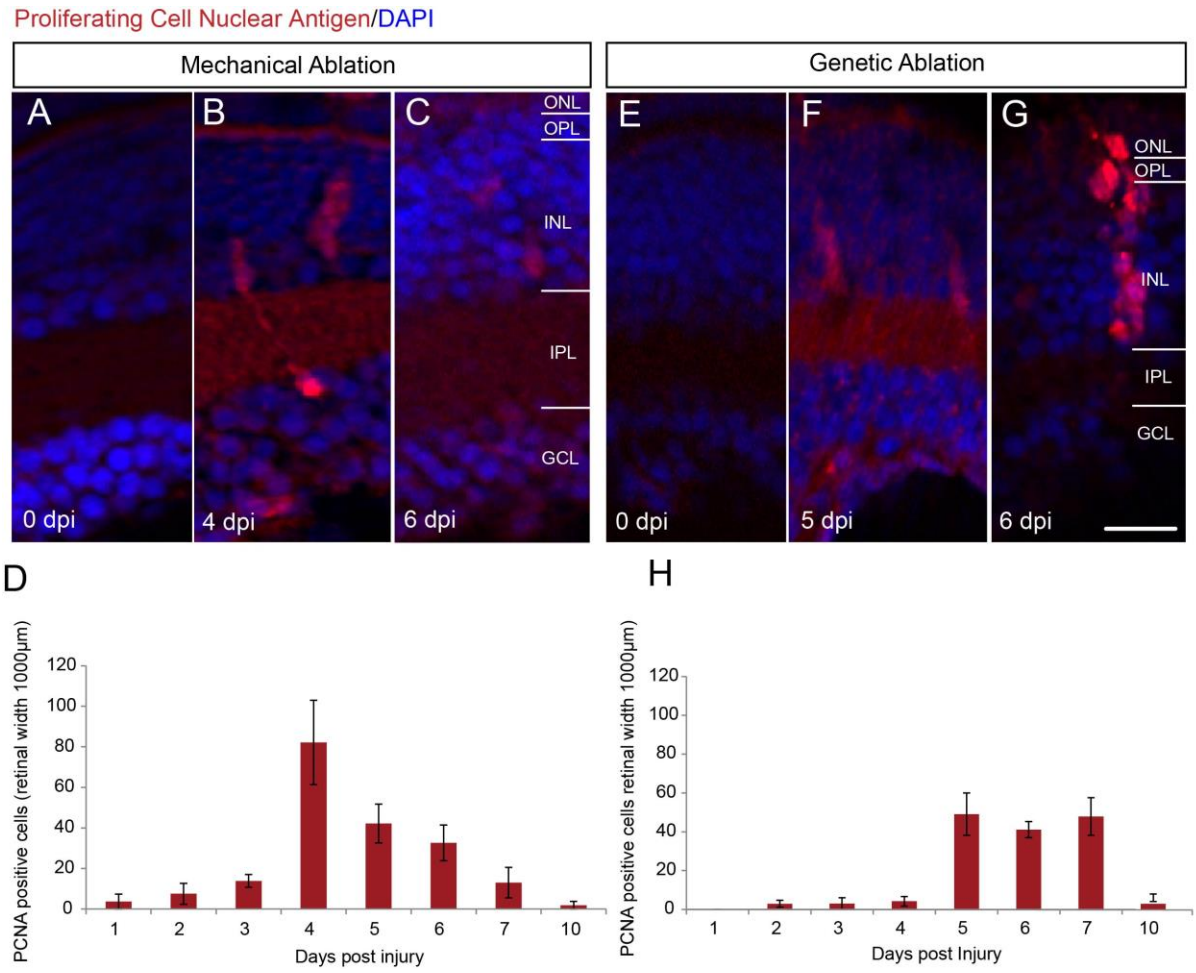

Proliferating Cell Nuclear Antigen/DAPI
Mechanical Ablation
Genetic Ablation
A
B
C
E
F
G
ONL
OPL
INL
IPL
GCL
0 dpi
4 dpi
6 dpi
0 dpi
5 dpi
6 dpi
D
H
PCNA positive cells (retinal width 1000µm)
Days post injury
PCNA positive cells retinal width 1000µm)
Days post Injury

Figure 4.2 Timing of PCNA labelled proliferation is comparable between injury models (A - C, E - G) Micrographs of retinal sections after mechanical injury (A - C) and genetic ablation injury (E - G). Retinal sections were stained for PCNA (red) (proliferating cell nuclear antigen) and showed cell clusters that spans across retinal layers in both injury models (B, F, G). (D, H) The graph shows the total number of PCNA cells after mechanical (D) and genetic ablation injury (H) model, suggesting that broadly, proliferation does not begin until 3 - 4 dpi and is active for at least three days. Each time point (1-10 dpi) for both mechanical and genetic ablation represents the total average of n=5 embryos. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar in G (for A – C, E - G) = 50 µm. Error bars are SEM.

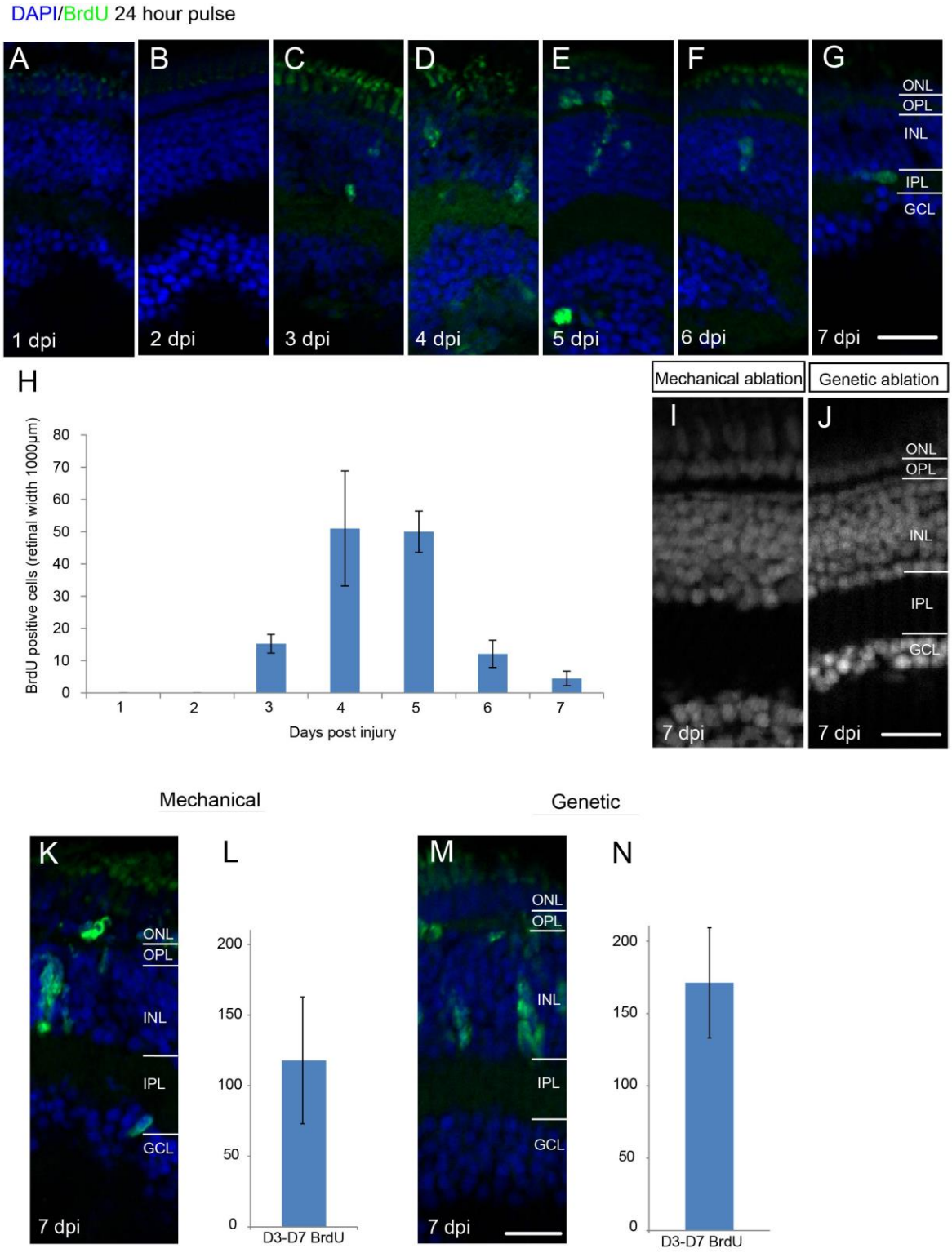

DAPI/BrdU 24 hour pulse
A
1 dpi
B
2 dpi
C
3 dpi
D
4 dpi
E
5 dpi
F
6 dpi
G
ONL
OPL
INL
IPL
GCL
7 dpi
H
BrdU positive cells (retinal width 1000µm)
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
Days post injury
Mechanical ablation
Genetic ablation
I
7 dpi
J
ONL
OPL
INL
IPL
GCL
7 dpi
Mechanical
Genetic
K
ONL
OPL
INL
IPL
GCL
7 dpi
L
0
50
100
150
200
D3-D7 BrdU
M
ONL
OPL
INL
IPL
GCL
7 dpi
N
0
50
100
150
200
D3-D7 BrdU

Figure 4.3. Peak BrdU incorporation is comparable between both injury models. (A - G) Micrographs of retinal sections after mechanical injury stained with DAPI (blue) and for BrdU (green). (A - G) BrdU positive cell clusters were observed between 3 to 7 days post-injury (dpi) that span across retinal layers . (H) The graph shows that most BrdU positive cells were most abundant within a 2 – 3 day time period. (I - J) Micrographs of retinal sections of mechanical (I) and genetic (J) ablated retina at 7 dpi, showing that the retinal architecture for both ablation injuries are similar to an uninjured retina (Figure 1 A, C). (K, M) Micrographs of mechanical and genetic ablated juveniles in overnight BrdU exposure between 3 - 7 dpi and the total number of cells are depicted in the graph (L, N). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar G (for A – G) = 50 µm, scale bar in J (for I – J) = 50 µm, scale bar in M (for K, M) = 50 µm. Error bars are SEM.

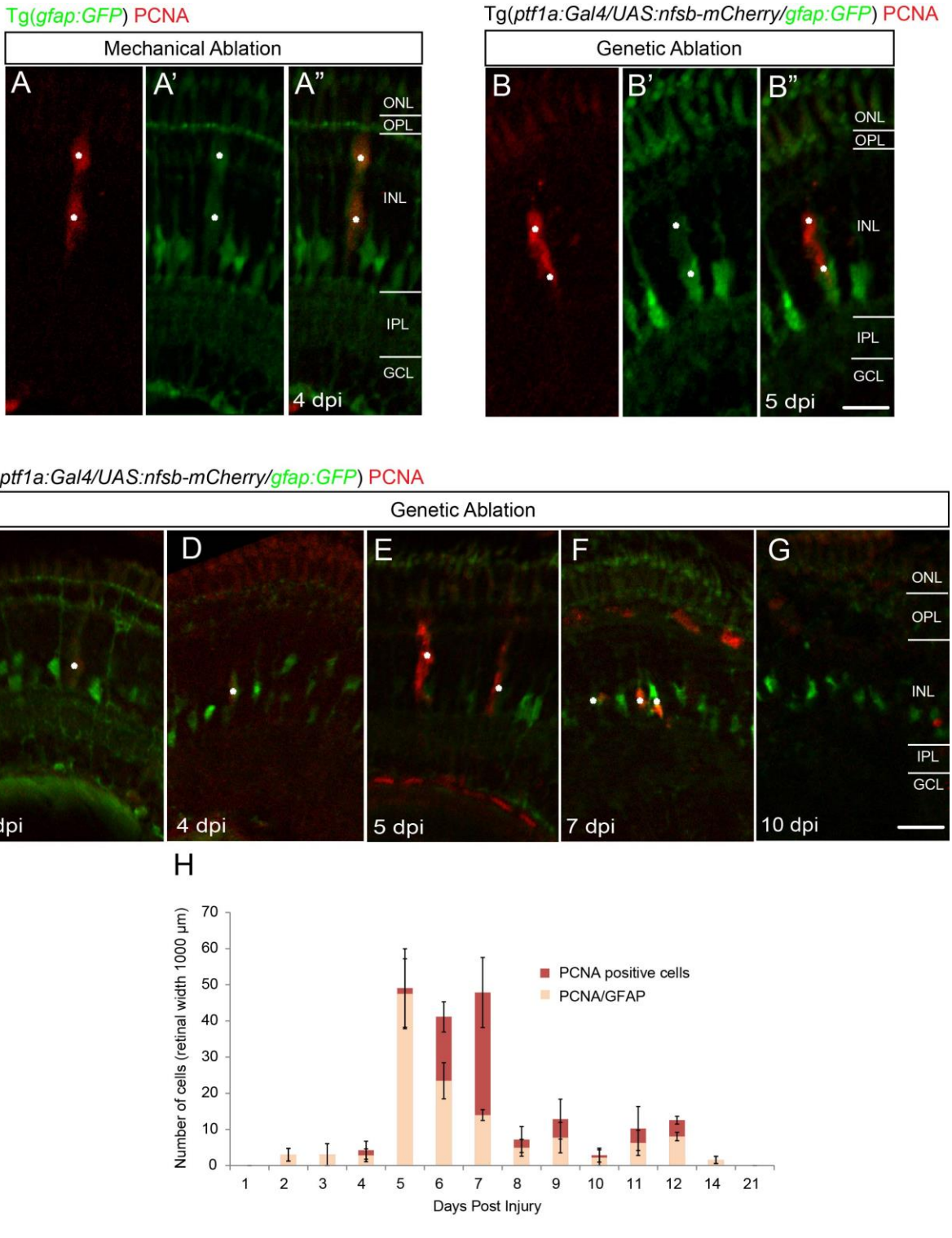
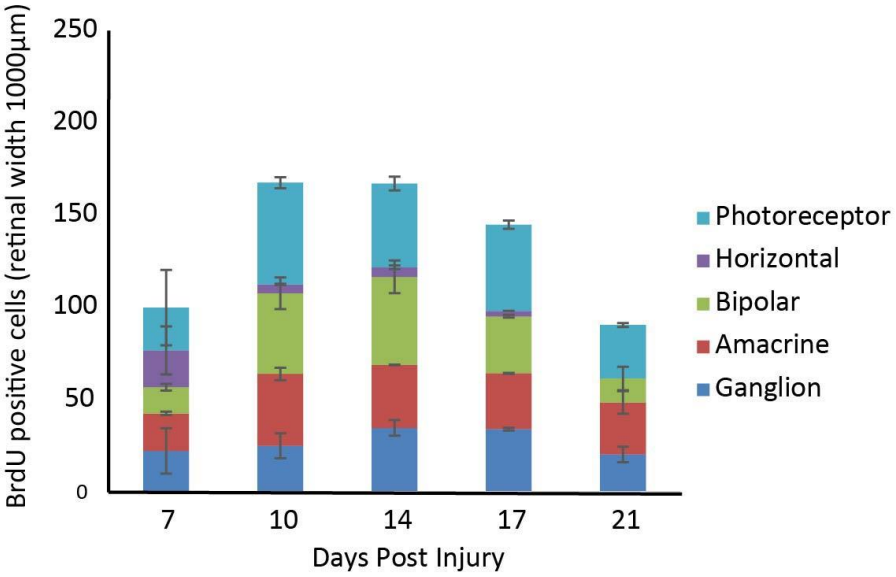

Tg(gfap:GFP) PCNA
Mechanical Ablation
A
A'
A"
ONL
OPL
INL
IPL
GCL
4 dpi
Tg(ptf1a:Gal4/UAS:nfsb-mCherry/gfap:GFP) PCNA
Genetic Ablation
B
B'
B"
ONL
OPL
INL
IPL
GCL
5 dpi
Tg(ptf1a:Gal4/UAS:nfsb-mCherry/gfap:GFP) PCNA
Genetic Ablation
C
D
E
F
G
ONL
OPL
INL
IPL
GCL
3 dpi
4 dpi
5 dpi
7 dpi
10 dpi
H
Number of cells (retinal width 1000 µm)
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
14
21
Days Post Injury
PCNA positive cells
PCNA/GFAP

Figure 4.4. Progenitors and clones arise from Müller glia. (A - B) Micrographs of retinal sections of mechanical (A) and genetic (B) ablated Tg(gfap:GFP) lines, with Müller glia cells (A', B') stained for PCNA (A, B), represented as a time series (C - G). (H) Graph showing the total number of Gfap:GFP Müller glia co-labelled with PCNA across 21 days after injury. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar B (for A – B) = 50 µm, scale bar in G (for C – G) = 50 µm. Error bars are SEM.

A


BrdU positive cells (retinal width 1000µm)
250
200
150
100
50
0
7
10
14
17
21
Days Post Injury
Photoreceptor
Horizontal
Bipolar
Amacrine
Ganglion

B

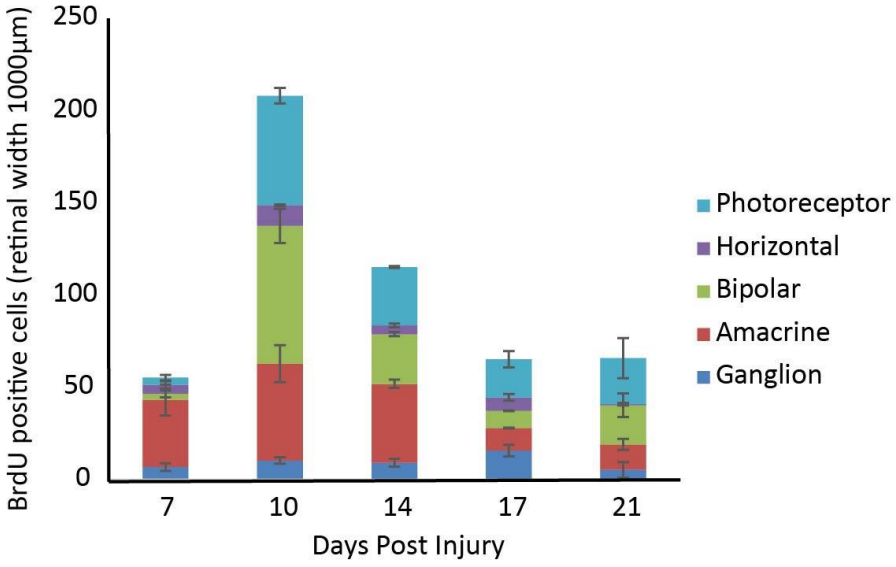

BrdU positive cells (retinal width 1000µm)
250
200
150
100
50
0
7
10
14
17
21
Days Post Injury
Photoreceptor
Horizontal
Bipolar
Amacrine
Ganglion

Figure 4.5. Cell type specific replacement. The graphs indicate the total number of BrdU cells in each retinal layer across 5 time points observed in the mechanical (A) and genetic (B) ablation injury models. More BrdU positive cells are found at 7 dpi in the genetic ablation model (B) than at 7 dpi in the mechanical ablation injury. (A - B) In both injuries, the total number of cells per layer increases after 7 dpi and decreases by 14 dpi (genetic) and 17 dpi (mechanical).

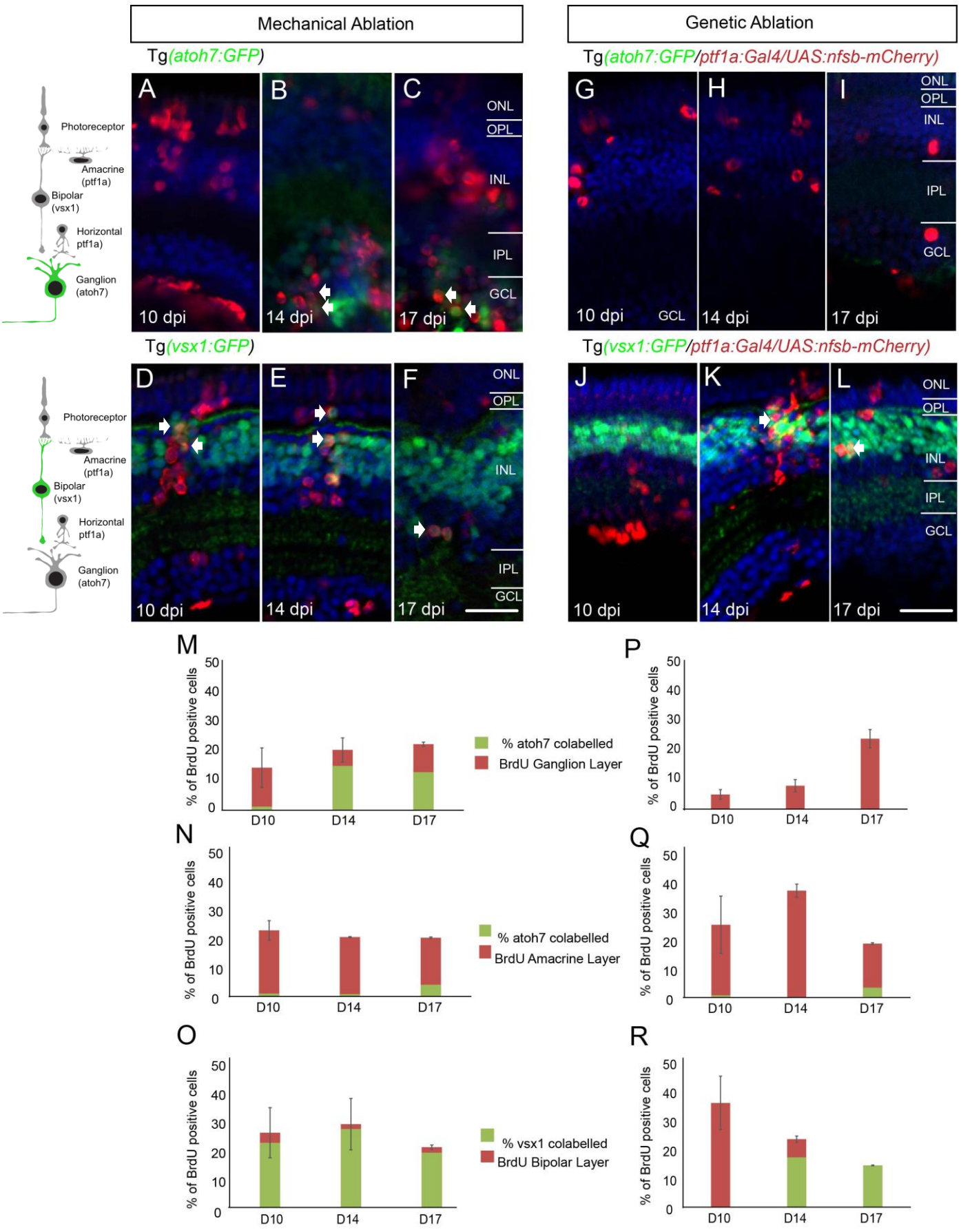

Mechanical Ablation
Genetic Ablation
Tg(atoh7:GFP)
Tg(atoh7:GFP/ptf1a:Gal4/UAS:nfsb-mCherry)
Tg(vsx1:GFP)
Tg(vsx1:GFP/ptf1a:Gal4/UAS:nfsb-mCherry)
Photoreceptor
Amacrine (ptf1a)
Bipolar (vsx1)
Horizontal ptf1a)
Ganglion (atoh7)
A
B
C
D
E
F
G
H
I
J
K
L
ONL
OPL
INL
IPL
GCL
10 dpi
14 dpi
17 dpi
M
N
O
P
Q
R
% of BrdU positive cells
D10
D14
D17
% atoh7 colabelled
BrdU Ganglion Layer
BrdU Amacrine Layer
% vsx1 colabelled
BrdU Bipolar Layer

Figure 4.6. Timing and layer distribution of fate determinant gene expression within regenerating proliferative neurons. (A - L) Micrographs of retinal sections of mechanically (A - F) and genetic (G - L) ablated Tg(*atoh7:GFP*) and Tg(*vsx1:GFP*) stained for BrdU (red). A schematic is located on the left of the panels to show the gene expression profile in the relevant cell types. Cells labelled by GFP (vsx1 or atoh7) are indicated in the schematic. (A - F) In the mechanical injured juveniles, BrdU co-labelled cells with atoh7 can be observed as early as 14 days post-injury (dpi) in the GCL layer and co-labelled cells with vsx1 can be observed as early as 10 dpi in the bipolar cell layer. No BrdU co-labelled cells with atoh7 is observed in the GCL of genetic ablated retina but can be observed by 17 dpi in the amacrine cell layer. BrdU co-labelled vsx1 positive cells can be observed as early as 14 dpi in the bipolar cell layer. (M - R) Graphs of percentage of BrdU positive cells distributed in either (M, P) ganglion, (N, Q) amacrine and (O, R) bipolar cell layers. Red bars indicate % of BrdU positive cells in each layer, green bars indicate % of BrdU positive cells that are also atoh7 or vsx1 co-labelled cells. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar F (for A – F) = 50 µm, scale bar in L (for G – L) = 50 µm. Error bars are SEM.

Chapter 5. Regenerative cell sources in the CNS

5.1 Introduction

The regenerative capacity for different body organs exists across different animal phylum groups, including in the central nervous system (CNS) of vertebrates and invertebrates (Lie, Song et al. 2004, Chen and Chisholm 2011, Fleisch, Fraser et al. 2011, Nawabi, Zukor et al. 2012) which exhibits varied degrees of regeneration across different organisms, despite the conservation of morphology and gene regulation between species (Nawrocki, BreMiller et al. 1985, La Vail, Rapaport et al. 1991, Stiemke and Hollyfield 1995, Vitorino, Jusuf et al. 2009, Reis, Khan et al. 2011).

Salamanders, birds and fish (Karl and Reh 2010) can efficiently regenerate different parts of the CNS including brain, spinal cord, retina, whereas mammals exhibit regeneration to a much lesser extent. Adult neurogenesis occurs at low levels at homeostasis in these lower vertebrates (Kaslin, Ganz et al. 2008).

In mammals, most of neurons of the CNS are generated embryonically, however sites of restricted adult neurogenesis still occur (Rakic 2002) for example in structures such as the dentate gyrus and subventricular zone (SVZ) in the mammalian brain (Ming and Song 2011). In contrast, neurogenesis continues on more extensively in non-mammalian species throughout adulthood and add to different brain regions (Doetsch and Scharff 2001, Garcia-Verdugo, Ferron et al. 2002, Zupanc and Sirbulescu 2011), such as in the lateral ventricle of birds (Alvarez-Buylla, Theelen et al. 1990), medial cortex of reptiles (Marchioro, Nunes et al. 2005) and even up to 16 distinct regions along the anterior-posterior axis of the adult zebrafish (Grandel, Kaslin et al. 2006).

In response to injury or disease to the CNS, both mammalian and non-mammalian species are able to undergo neurogenesis to generate new neurons. Patients who suffer from CNS related diseases exhibit limited, proliferative neurogenesis in restricted regions of the adult brain (Curtis, Penney et al. 2003, Jin, Peel et al. 2004, Goldman 2005). Brain ischemia in rodents also causes increased proliferation in the SVC and recruitment of neural precursors to the site of injury (Zhang, Zhang et al. 2004). In non-mammalian species, dynamic and functional integration has been observed following neuron proliferation and replacement after injury; regeneration of neurons and function after selective ablation of neurons in birds (Scharff, Kirn et al. 2000), robust, structural recovery after mechanical lesion to the adult reptile cortex (Romero-Aleman, Monzon-Mayor et al. 2004) and widespread adult neurogenesis and brain plasticity post-injury in teleost (Kishimoto, Shimizu et al. 2012).

An ideal model to study CNS regeneration is the retina, which is well conserved across most vertebrates. The retina is easy to access and can be visualised non-invasively. The major difference in vertebrates is that non-mammalian species such as the zebrafish, chick and Xenopus, the retina is able to undergo extensive post-natal growth and regeneration after injury (Stenkamp 2007, Sakami, Etter et al. 2008, Slack, Lin et al. 2008). This is in contrast to the mammalian eye where cell proliferation ceases after birth, though the eye and retina undergoes passive growth by stretching (Kuhrt, Gryga et al. 2012).

A range of retinal diseases exist whereby different neural to glia types can be affected, resulting in partial or total blindness (D'Amico 1994), such as retinitis pigmentosa or glaucoma. Many different approaches are being taken to improve current forms of therapy. These include for example gene therapy using microRNAs

(Sundermeier and Palczewski 2015) and stem cell replacement therapy such as using embryonic or induced pluripotent stem cell derived precursor cells (Garcia, Mendonca et al. 2015, Jayakody, Gonzalez-Cordero et al. 2015). Clinical trials within this space are active. However, there are ongoing concerns for the safety and efficacy of these therapies in humans (Collins and Thrasher 2015, Deleidi and Yu 2016).

A drawback of transplanting mature neurons to overcome blindness is that the neurons do not re-establish normal axonal topographic relationships with their targets. This leads to the regenerated neurons to compete with the existing neurons and a reduction in innervation efficiency (Tan and Harvey 1997, Koeberle and Bahr 2004). Given the drawbacks in using such technology in the current state, a complimenting measure to eradicating visual diseases may involve learning from other vertebrates that are efficient at regenerating the retina. For example, it would be beneficial to identify if a mechanism exists in the zebrafish to perform complete retinal regeneration in the absence of progenitor cell source, as this may indicate additional sources of regeneration.

Various endogenous cell sources contributing to regenerating the retina have been identified across different species. In salamanders the retinal pigment epithelium (RPE) duplicates and the new layer can remarkably replace the entire retina if it is surgically removed or damaged (Reh, Nagy et al. 1987, Reh, Jones et al. 1991, Araki 2007). In contrast, the RPE of birds directly generates retinal progenitor cells and subsequently retinal neurons, which accounts for a more restrictive form of regeneration (Coulombre and Coulombre 1965, Pittack, Jones et al. 1991, Sakami, Etter et al. 2008). In the zebrafish, sources of regeneration are from the ciliary margin zone (CMZ), rod precursors and Müller glia, where the variety of cell sources may result from different injury paradigms such as through mechanical, light, or chemical

injury (Maier and Wolburg 1979, Braisted, Essman et al. 1994, Vihtelic and Hyde 2000, Fausett and Goldman 2006, Bernardos, Barthel et al. 2007, Stenkamp 2007). This suggests that regeneration may be driven by more than one mechanism depending on the stimulus.

As previously described in Chapter 4, Müller glia contribute to regeneration across most injury paradigms in vertebrates studied. Müller glia activation, proliferation and the integration of newly generated neurons into the existing system exists across all vertebrate systems observed such as zebrafish, mouse (Wan, Zheng et al. 2008) and human (Lawrence, Singhal et al. 2007, Bhatia, Jayaram et al. 2011) does differs across species in the extent of regeneration.

In adult mice, BrdU incorporation was observed specifically in the Müller glia after neurotoxic injury (Karl, Hayes et al. 2008, Takeda, Takamiya et al. 2008). These Müller glia were able to dedifferentiate, migrate, re-enter the cell cycle and induce neurogenesis in both *in vivo* and in cell culture conditions. After toxin induced injury in the chick retina, Müller glia cells may lose the mature GFAP marker and re-enter the cell cycle, as suggested through double staining for PCNA and BrdU (Fischer and Reh 2003).

In adult zebrafish for example, Müller glia have been suggested to be the primary source of regeneration and act as an endogenous stem cell source after injury or disease as it directly interact with other cell types (Peterson, Fadool et al. 2001, Nagashima, Barthel et al. 2013). Evidence for zebrafish Müller glia post injury activation to the generation of progenitors that undergo neurogenesis has been intensely studied and is explored in detail below.

After injury in the zebrafish, Müller glia cells are activated and re-enter the cell cycle by expressing markers such as proliferating cell nuclear antigen (PCNA) and activating signalling pathways that are involved in cell proliferation (Thummel, Kassen et al. 2008, Meyers, Hu et al. 2012). These Müller glia cells then dedifferentiate (Fimbel, Montgomery et al. 2007) and begin expressing genes involved in DNA synthesis and proliferation such as PCNA, and can be stained for BrdU upon exposure (Cameron, Gentile et al. 2005, Fausett and Goldman 2006, Bernardos, Barthel et al. 2007, Kassen, Ramanan et al. 2007, Kassen, Thummel et al. 2008, Meyers, Hu et al. 2012, Wan, Ramachandran et al. 2012) as well as progenitor markers (Ramachandran, Fausett et al. 2010, Thummel, Enright et al. 2010).

Microarray data reveal that stimulation of regenerative events after injury involves the regulation of over 900 transcripts in the zebrafish Müller glia, a number of which regulate developmental signalling pathways such as TGF-β, Hedgehog and Notch. (Qin, Barthel et al. 2009). Müller glia and proliferative cells from dedifferentiated glia share also certain stem cell markers found during normal development and progenitors in the CMZ, including visual homeobox transcription factors 1 and 2 (Vsx1, Vsx2), Notch1, Notch3, N-cadherin, Paired box 6 (Pax6), Atonal7 (Atoh7), Oligodendrocyte transcription factor (Olig2), Ascl1 and Neurogenin1 (Ngn1) (Levine, Hitchcock et al. 1994, Hitchcock, Macdonald et al. 1996, Sullivan, Barthel et al. 1997, Marquardt, Ashery-Padan et al. 2001, Wu, Schneiderman et al. 2001, Liu, Londraville et al. 2002, Senut, Gulati-Leekha et al. 2004, Yurco and Cameron 2005, Raymond, Barthel et al. 2006, Fimbel, Montgomery et al. 2007, Kassen, Ramanan et al. 2007, Thummel, Kassen et al. 2008).

These cells then express differentiation markers (Vihtelic and Hyde 2000, Inoue, Hojo et al. 2002, Vihtelic, Soverly et al. 2006) to regenerate a functional

zebrafish retina (Yurco and Cameron 2005, Fausett and Goldman 2006, Thummel, Kassen et al. 2008). Evidence of Müller glia contribution to neurogenesis after injury has recently been observed using fate mapping to show that Müller glia are able to give rise to all retinal cell types except rod photoreceptors after light and heat lesion (Wilson, Wen et al. 2015), which is similar to progenitors during developmental neurogenesis giving rise to all neuronal cell types. Thus as the activated gene networks appear comparable, this led to the retaining dogma that adult retinal regeneration fully recapitulates what occurs during retinal development.

However, molecular differences do appear to exist (Raymond, Barthel et al. 2006) as newly generated neurons do not fully reconstitute all developmental structural patterns (Vihtelic and Hyde 2000), suggesting that regeneration does not completely recapitulate developmental neurogenesis. Evidence for zebrafish Müller glia post injury activation for the generation of progenitors that undergo neurogenesis has been intensely studied and is explored in detail below.

Much retinal regenerative research has gone into understanding how cells are activated to undergo a proliferative form of regeneration, particularly through Müller glia, and the extent of repair by proliferative clones to the retina. In chapter 4, I briefly mentioned that the total number of cells recovered in the two different injury models was more than expected from the number of proliferating cells labelled with bromodeoxyuridine (BrdU). Thus, indicating either non-proliferative Müller glia regeneration or alternative regeneration cell sources.

A non-proliferative form of retinal regeneration in vertebrates that can efficiently regenerate the retina has not been identified. Observing if such a form of regeneration exists after injury or disease can thus open novel avenues for retinal regeneration

research. As proliferative regeneration only occurs in low levels in humans (Lawrence, Singhal et al. 2007, Bhatia, Jayaram et al. 2011), studying if regeneration can occur in the absence of a proliferative, Müller glia driven response, may lead to the possibility of activating such a non-proliferative regenerative response in humans in the future.

In this study, I thus investigated if the classical Müller glia mode of retinal regeneration is the only stem cell source after injury. I also examined if there are any additional cell sources that can contribute and/or revert to progenitor like to participate in regeneration. Finally, I reviewed if proliferative regeneration is the only form of regeneration in the zebrafish retina. This form of regeneration is described in most injury models, however data from my previous studies indicate that proliferative regeneration may not be the only contributor (Chapter 4).

Using the zebrafish retina I identified for the first time an early response that involves novel cellular regeneration unlike any described previously. I characterise the time course of regeneration and identified that this response occurs prior to the peak of proliferative regeneration. Additionally, I show that this non-proliferative regenerative response is not initiated in Müller glia cells and does in fact occur even when the development of Müller glia is inhibited. This early non-glia non-proliferative response can partially, but not completely rescue the loss of inhibitory neurons in my cell type specific ablation model.

5.2 Methods and Materials

5.2.1 Zebrafish husbandry

Fish lines used include Tg(*ptf1a:Gal4*) and Tg(*ptf1a:GFP*), kindly provided by Prof. Leach (Godinho, Mumm et al. 2005) and Tg*(UAS:nfsb-mCherry*) (Davison,

Akitake et al. 2007) a gift from Prof. Lieschke. These single lines were crossed to generate the relevant triple transgenic line Tg(*ptf1a:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*), Juveniles of either gender were maintained according to standard protocol, staged as previously described (Kimmel, Ballard et al. 1995). Zebrafish were housed, bred and raised at FishCore facility at Monash University in accordance with local animal guidelines and used both before and after free feeding stages.

5.2.2 Metronidazole treatment

One week old Tg(*ptf1a:Gal4* / *UAS:nfsb-mCherry*) zebrafish were incubated in a solution of 10 mM metronidazole / 0.2 % dimethyl sulfoxide (DMSO) in E3 solution for 8 hours at 28^{o}C. Zebrafish were then washed 3 times in fresh E3 media, and were monitored as they were returned to the flow system.

5.2.3 Inhibition of Müller glia development using N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) exposure

Embryos were exposed to 25 µM DAPT (γ-secretase inhibitor) / 0.1 % DMSO in E3 media from 48 hpf to 6 dpf (MacDonald, Randlett et al. 2015). Embryos were washed in E3 solution and left to recover overnight before further treatment.

5.2.4 Histological retinal tissue processing

Embryos were fixed in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4), cryoprotected in 7.5 % gelatine / 15 % sucrose in PBS solution and cryostat sectioned at 14 µm thickness using a Leica CM3050S Cryostat. Sections were counterstained for nuclei with 4',6-diamidino-2-phenylindole (DAPI) (1;10,000, Life Technology). Sections were mounted with Mowiol (Sigma-Aldrich).

5.2.5 Image acquisition

Images of fixed sections were obtained on a Zeiss Z1 (20x objective) using an AxioCam (HRm 13-megapixel, monochrome) and Axiovision software. Brightness and contrast were adjusted with Photoshop (Adobe).

5.2.6 Analysis

For quantification of Tg(*ptf1a:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*) cells in untreated and after metronidazole treatment were counted from the central retina in a fixed 50 µm width window for consistency (n = 20 embryos for both untreated and metronidazole treatment). Statistical analyses were conducted using F-statistic of one-way ANOVA with post-hoc Tukey HSD and Bonferroni multiple correction (GraphPad).

5.3 Results

5.3.1 Recovery of retinal architecture after genetic ablation injury within 5 days

I used a genetic ablation injury paradigm I previously established (Chapter 4) in which Tg(*ptf1a:Gal4 / UAS:nfsb-mCherry*) zebrafish were used to drive expression of the nitroreductase (nfsb) enzyme specifically in pancreas transcription factor 1 alpha (Ptf1a) expressing retinal neurons. Ptf1a is necessary and sufficient for the generation of inhibitory neurons during retina development (Jusuf and Harris 2009), so these specifically express nitroreductase in this setup.

At 7 days postfertilisation (dpf) zebrafish were treated with metronidazole by placing them in fish water containing this pro-drug. This is taken up by all cells, but only within nitroreductase expressing cells is metronidazole converted into a cytotoxin, targets specifically only in these inhibitory neurons. This very efficiently results in TUNEL labelled cell death specifically in these inhibitory nfsb-mCherry expressing neurons horizontal and amacrine retinal cell populations (Chapter 4).

Using this genetic ablation method, the degree of regeneration after neuronal cell loss was assessed and compared to uninjured retinas. The retinal cell population prior to and after genetic ablation injury was compared using nuclear labelling with DAPI to reveal the anatomical retinal architecture.

Layer organisation and other morphological hallmarks can be used to identify each of the main neural populations in such DAPI labelled retinas. The horizontal inhibitory neurons can be identified as a single layer of flattened nuclei in the outermost row of the inner nuclear layer and amacrine inhibitory cells identified as the weaker DAPI labelled neurons in the inner half of the inner nuclear layer (Figure 5.1 A – F).

The study was conducted using the Tg(*ptf1a:Gal4 / UAS:nfsb-mCherry*) fish and the total number of amacrine (Figure 5.1 A - F, brackets) and horizontal cells (Figure 5.1 A - F, arrowheads) was compared between injured and uninjured fish. All cells were quantified within a fixed 50 µm width window across all retina sections and compared between control pre-injury (0 dpi) and treated embryos at two stages after injury at 3 and 7 dpi (Figure 5.1 A - F).

In treated juveniles, the number of inhibitory amacrine (p value <0.0001) and horizontal (p value < 0.0001) cells is significantly reduced at 3 dpi compared to an uninjured retina (0 dpi) (Figure 5.1 H) (Table 5.1). This cell loss follows the peak of TUNEL labelling that occurs between 0 – 2 dpi after such ablation (Chapter 4). No significant changes were observed at 3 dpi in the other retinal neuron types when compared to the uninjured retina (Table 5.1). By 7 dpi, retinal layers appear to be fully recovered (Figure 5.1 A - F). At this stage both amacrine cell numbers (p value = 0.8404) and horizontal cell numbers (p value = 0.7911) are comparable to an uninjured retina (0 dpi) (Table 5.1). Thus at 7 dpi, the total number of inhibitory neurons is restored. Untreated retinas do not show any significant changes to the number of cells at any of the time points we observed. (Figure 5.1 G).

After such genetic ablation injury the proliferative peak of regenerating progenitors occurs between 3 – 7 dpi as quantified using either proliferating cell nuclear antigen (PCNA) or consecutive 24 hour bromodeoxyuridine (BrdU) pulse labelling techniques (Chapter 4). This proliferation wave was initiated from cells co-expressing mature glial markers at 5 dpi (Chapter 4), with less co-labelling observed in subsequent days, consistent with the timing of Müller glia dedifferentiation but not ruling out other proliferation initiated events from other cell sources.

Given my DAPI results showed that the retinal architecture was recovered by 7 dpi (Figure 5.1 H), this suggests that the recovery of lost inhibitory neurons may occur prior to glia mediated proliferative regeneration. This interpretation is consistent with my previous observation that most BrdU positive cells were localize to cell layers other than inhibitory neuron layers by 10 dpi and very few newly generated neurons were added to the inhibitory layer at 10 dpi (Chapter 4 Figure 5).

5.3.2 Rapid recovery of missing inhibitory neurons

As the architecture of the retina appeared to be recovered by 7 dpi, I wanted to verify the identity of the cells in the regenerated horizontal and amacrine cell layers. Here, I asked if the cells observed after the recovery of the architecture as visualized using DAPI after genetic ablation in the inhibitory neuron layers are Ptf1a:GFP positive cells, as this transcription factor is absolutely necessary for the generation of these inhibitory neurons during development. For this, I used the Tg(*ptf1a:GFP / ptf1a:Gal4 / UAS:nfsb-mCherry*) line, whereby all newly generated inhibitory neurons are GFP positive. The total number of Ptf1a:GFP positive cells was quantified within a 50 µm retinal width every day for 14 days in both injured (metronidazole treated) and uninjured (untreated) retinas.

Untreated retinas at equivalent dpi do not show any significant changes to any cell populations at any time points, including 3 dpi equivalent (p value = 0.8712) and 4 dpi equivalent (p value = 0.9923) (Figure 5.2, Table 5.2). In contrast in treated juveniles, the total number of Ptf1a:GFP positive inhibitory neurons (amacrine and horizontal) cells at 3 dpi (p value <0.0001) and 4 dpi (p value < 0.0001) was significantly reduced compared to pre-injured retinas at 0 dpi (Figure 5.2, Table 5.2). The number of Ptf1a:GFP positive cells recovers to levels comparable to 0 dpi already

by 5 dpi (p value = 0.5186). In contrast, this entire period of inhibitory cell loss and recovery back to pre-injury levels occurs prior to the differentiation of inhibitory neurons from BrdU labelled progenitors, which occurs between 7 to 10 dpi (Chapter 4). This indicates the presence of a novel early mechanism of regeneration that does not rely on glia activation and proliferation as the source of neurogenesis.

5.3.3 Partial non-proliferative regeneration after inhibition of Müller glia development

Most vertebrate models which undergo efficient retinal regeneration rely on a Müller glia mediated, proliferative response (Karl and Reh 2010). In this genetic ablation injury model, such classical regeneration events reveal a similar response (Chapter 4). However, the early non-proliferative form of regeneration described here may or may not involve these same glial cells. Thus, I investigated, whether the loss of Müller glia will result in the loss of this early cellular regeneration described here.

Proper development of the vertebrate retina requires Notch signalling pathway to regulate processes such as progenitor proliferation, inhibition of retinal neuron differentiation and promotion of Müller glia differentiation in species such as mammals (Livesey and Cepko 2001, Ahmad, Das et al. 2004), amphibians (Schneider, Turner et al. 2001) and fish (Perron and Harris 2000). In the zebrafish, while complete loss of Notch signalling (i.e. mindbomb mutant) leads to patterning defects (Bernardos, Lentz et al. 2005), delaying Notch inhibition to after 30-33 hpf selectively blocks Müller glia development (48 hpf) without affecting neurogenesis in the retina (Randlett, MacDonald et al. 2013, MacDonald, Randlett et al. 2015). DAPT treatment was conducted in the Tg(gfap:GFP) line, in which Müller glia are labelled in green starting at 36 hpf just prior to Müller glia generation (MacDonald, Randlett et al. 2015). Juveniles were analysed immediately after injury (0 days post injury after DAPT

treatment), 3, 5, 7 and 10 dpi to observe, if glia development was successfully blocked, and if Müller glia were generated after withdrawal from DAPT.

Untreated juveniles at 7 dpf show GFP positive Müller glia cells (46 ± 2.5 SEM cells) in the central retina and at the CMZ (12 ± 1 SEM cells) (Figure 5.3 A) as usual (glia+ fish), but juveniles that were treated with DAPT efficiently inhibit Müller glia development causing a significant reduction in the number of glia present at 7 dpf (0 dpt) (2 ± 0.577 SEM cells at the central retina and 2 ± 0.333 SEM cells at the CMZ, $p < 0.001$) (Figure 5.3 B), resulting in what I will refer to glia- fish. Müller glia return in low numbers by 3-days post treatment (dpt) (1 ± 0.4 SEM cells at the central retina and 6 ± 1.281 SEM cells at the CMZ) (Figure 5.3 C) and are maintained at low levels at 5 dpt (1 ± 0.5 SEM cells at the central retina and 8 ± 1 SEM cells at the CMZ) (Figure 5.3 D) and 7 dpt (0 cells at the central retina and 9.5 ± 0.5 SEM cells at the CMZ) (Figure 5.3 E). By 10 dpt, more GFP positive cells were observed in the retina (18 cells at the central retina and 16 cells at the CMZ) (Figure 5.3 F).

Given that I can achieve efficient inhibition of Müller glia development up to the stage of the genetic ablation injury, I used this experimental paradigm to assess the regenerative response of inhibitory neurons with or without the presence of Müller glia. For this, I conducted genetic ablation in Tg(*ptf1a:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*) juveniles to compare the differences in the number of Ptf1a:GFP cells (Figure 5.4). The total number of Ptf1a:GFP positive cells was quantified within a 50 µm retinal width for 10 days every day in DAPT treated (glia-) metronidazole treated fish and compared to no DAPT treated (glia+) metronidazole treated fish.

Consistent with previous data where the genetic ablation shows specific loss of inhibitory neurons, similar reduction was also observed in glia- fish. However, unlike

the glia+ treated fish, the loss of inhibitory neurons can already be first observed two days after retinal injury in glia- fish juveniles as compared to 3 dpi in glia+ fish (glia+ fish n = 30 ± 1 SEM cells vs glia+ fish n = 38 cells, p = 0.0073). By 3 dpi, the degenerative extent in both injuries was comparable with maximal loss of both amacrine and horizontal cells (glia- fish n = 22 ± 1.5 SEM cells vs glia+ fish n = 22 ± 1.5 SEM cells, p = 0.0812) (Figure 5.4 G). Following inhibitory cell loss, the onset of cellular recovery was similar post injuries with or without Müller glia at 4 dpi (glia- fish n = 28 cells ± 2.2 SEM vs glia+ fish n = 28 ± 0.7 SEM cells, p = 0.8809). However by 5 dpi, the extent of maximal cell recovery was significantly different between glia- and glia+ fish (glia- fish n = 32 ± 1 SEM cells vs glia+ fish n = 38 cells ± 4 SEM, p = 0.0259) (Figure 5.4 G).

By 10 dpi, no significant difference was observed between the glia- fish vs glia+ fish genetic ablation model groups (glia- fish n= 30 ± 1.6 SEM cells vs glia+ fish n = 34 ± 1.2 SEM cells, p = 0.1124) (Figure 5.4 G). This may be due to Müller glia being more broadly generated in the glia- fish at 10 dpi (Figure 5.3 F). Thus, this recovery of Müller glia within the retina could be contributing to the glia driven, progenitor derived, and proliferative regeneration that usually occurs by this stage (Chapter 4). This form of glia dependent recovery may be able to complete regeneration of the retina after a genetic ablation, after a partial regeneration was observed via early non-glia regeneration.

This study shows that the extent of cell degeneration and subsequent onset of recovery after a genetic injury is comparable regardless of whether Müller glia are present. However, the extent of maximal recovery differs significantly. In the glia+ fish, the numbers of inhibitory neurons are recovered to a state where the architecture is similar to an uninjured retina. In contrast, complete regeneration of the inhibitory

neurons is not achieved in the glia- fish, possibly due to the limitations set by not having the primary proliferative cell source available. .

5.4 Discussion

Although the mammalian CNS has been shown to be capable of limited regeneration, complete and functional regeneration of any CNS structure such as the brain and spinal cord currently remains unattainable (Shimazaki 2016). The current dogma follows that a neuron in an *in vivo* environment is the final endpoint of the cell fate. However, history has shown that research causes paradigm shifts in dogmas, such as the relatively recent emergence of induced pluripotent stem cells (Takahashi and Yamanaka 2006), which allow mature cell types *in vitro* to be induced to dedifferentiate and take on other cell fates, showing mature cells are capable of plasticity. Hence, though once thought impossible, the mammalian system has progressively shown higher capacity to undergo regeneration, primarily driven by understanding the regenerative mechanisms in lower organisms such as the zebrafish. Within the scope of CNS regeneration, much of the mechanisms driving regeneration have been discovered by studying the retina as both its cell composition and genetic makeup are well conserved across vertebrate species.

In the retina, Müller glia derived progenitors can be activated to regenerate neurons after being inflicted with a wide range of retinal injuries, including light damage, mechanical injury, chemical and genetic ablation (Vihtelic and Hyde 2000, Bernardos, Barthel et al. 2007, Ng 2013). This also occurs across all vertebrate models studied including after chemical and light injuries in the mouse (Karl, Hayes et al. 2008, Ueki, Wilken et al. 2015). Additionally, inhibition of glial proliferation results in a failure of retinal neurons to regenerate after light damage (Thummel, Kassen et al. 2008), showing that Müller glia are the primary regenerative source. This is consistent with the glial response I observed in both of the injury models I used for studying retinal regeneration. However, in the work described in this chapter I made

the exciting discovery of a two phased regenerative response, with the second phase representing the classical described activation of glia, their dedifferentiation into progenitors, cell cycle re-entry and subsequent neurogenesis as described in Chapter 4. However, I identified a completely novel, faster occurring first phase of regeneration in addition to this described regenerative model. This response was characterised by being able to rapidly contribute towards replacing lost neurons without contributions of proliferation and without Müller glia.

5.4.1. Non-proliferative regenerative source

After the nitroreductase injury described in Chapter 4, which leads to efficient loss of inhibitory neurons at 0 dpi, a recovery of 80% of these neurons can already be found at 5 dpi, prior to Müller glia proliferation that peaks between 4 – 7 dpi. Interestingly, my 24 hour pulse BrdU experiments in the first 7 days showed very minimal proliferation throughout that time period, though massive amount of cell death and TUNEL labelling were observed the between 0 – 2 dpi. Additionally, using PCNA or repeated BrdU pulses every 12 hours from 4 to 7 dpi, these newly generated cells were not labelled. While caveats need to be noted when using these labelling techniques, including BrdU only labelling cells between the end of G1 and during the S-phase (Ng Chi Kei, Dudczig et al. 2016), the complete lack of BrdU labelling in the first 5 days when this regenerative response occurs suggests that only very few, if any of these newly generated cells arise from dividing cells. Thus, it may be possible that Müller glia may be able to contribute to the newly generated neurons without division. However, any regeneration without proliferation will presumably result in the regeneration of one cell at the simultaneous loss of whatever the original cell was. Thus, if this was the only regenerative mechanism at a given time, this would have potential limitations if it may result in further cell loss of another cell type. While this

may contribute towards the restoration of neurons important for forming functional circuits, the actual total number of cells will not change. Thus, it will be interesting to observe in future retinal studies if the loss of cells can be compensated by allowing remaining cells to increase their neurite arbor to cover the same area, though this in turn may come at a cost for spatial resolution (Montague and Friedlander 1991, Sagasti, Guido et al. 2005).

Similar disadvantages would occur in other CNS regions, if the total number of neurons were reduced significantly. However, this form of regeneration potentially has limitations as sacrificing one cell type to replace another will result in the retina to lose part or most of its function. Nonetheless, the potential of regenerating neurons from local cell sources without the delay of re-entering the cell cycle to proliferate may represents a necessary immediate but incomplete form of healing to prevent further degeneration and cell loss, while maintaining the structure of the retina before a later form of functional recovery.

5.4.2. Non Müller glia regeneration

This first phase early regenerative response occurs prior to Müller glia dedifferentiation (the loss of the mature Müller glia marker GFAP) (Bernardos and Raymond 2006) and progenitor proliferation. Additionally, the numbers of neurons replaced in this early face outnumbered the locally present glia found in the tissue. Thus, I tested if regeneration could occur in the partial absence of glia.

For this, I pharmacologically inhibited the Notch signalling pathway with DAPT at 36 hpf to specifically inhibit Müller glia differentiation during development. While Notch signalling also plays a crucial role during normal development of the retinal neurons, delaying Notch inhibition until 36 hpf does not affect the development of other

neurons but only inhibits Müller glia differentiation, which is the last cell types generated in the retina (MacDonald, Randlett et al. 2015). By specifically blocking the development of Müller glia during development until 6 dpf, mature Müller glia are completely absent at 7 dpf (equivalent to 0 dpi when the injury was performed). I remove DAPT treatment at the time of injury to allow regenerative processes to occur in its normal signalling environment including Notch signalling. I observed that surprisingly, early regeneration does occur in the absence of Müller glia.

In retinas lacking Müller glia, the glia are generated past this stage, with about 4 mature gfap expressing Müller glia observable on the day DAPT treatment was stopped and significant recovery observed only by 10 days after injury, when I can observe about 40 newly generated gfap:GFP cells / retinal section. This suggests that when analysing the results, I need to take into account the generated gfap:GFP that can contribute to the regenerative process at least after 10 dpi. I found that regeneration does occur in the absence of Müller glia. Between 3 and 4 dpi, a comparable recovery was observed even in the absence of Müller glia, corresponding to 3 and 4 dpi nitroreductase injury in the presence of Müller glia.

Two additional major observations can be observed in this study. Firstly, after genetic ablation, a retina devoid of mature Müller glia exhibits a steady decline of the number of inhibitory neurons at 2 dpi, as compared to a similar rate of decline observed at 3 dpi in injured retinas with Müller glia at the same stage. Müller glia may provide a form of neuroprotection to delay cell death and hence no rapid cell loss is seen in the environment with Müller glia (Bringmann, Iandiev et al. 2009). However, when the peak neural loss occurs at 1 dpi, the number of ablated neurons are comparable, suggesting that such neuroprotection is not sufficient to prevent eventual cell death.

Neuroprotection originating from Müller glia is also observed in the mouse model, whereby upon injury Müller glia regulate neurodegeneration by secretion of neuroprotective factors that are pro-survival such as IGF, FGF and LIF, which prevents rapid and further neuronal cell death (Arroba, Alvarez-Lindo et al. 2014, Hauck, von Toerne et al. 2014). In addition, the fast decline in inhibitory neuron numbers in the absence of Müller glia may be due to a lack of tensile strength throughout the retina as the Müller glia provides mechanical support (MacDonald, Randlett et al. 2015). Hence, in my model of ablating 2 major populations, the integrity of the retina may be compromised, resulting in an exacerbation of cell loss.

An intriguing second observation from these data is that cellular recovery without Müller glia is incomplete at 5 dpi. This incomplete regeneration is observed up to 10 dpi, where at 10 dpi the number of neurons recovered was similar to a wild type retina after injury at the same time point. This recovery period at 10 dpi corresponds to the time point when large numbers of gfap:GFP cells are generated (due to removal of DAPT and removal of Notch inhibition) which may participate in complete regeneration. It is interesting to speculate that this incomplete form of regeneration may be regulated by an active mechanism to control not only the extent of neurons regenerated, but also control and minimize the loss of cells that are contributing to this non-proliferative regeneration. If too many cells are actively participating in this non-proliferative form of recovery, the pool of cells contributing to regeneration will become exhausted. Thus, while inhibitory neurons are regenerated at this stage in the absence of Müller glia, the total number of horizontal and amacrine neurons are not fully recovered to a state such as an injured wild type retina.

The incomplete recovery may be sufficient for restoring adequate retinal function, and a complete recovery of all the missing cell types may be done in the

slower second proliferative regeneration stage involving Müller glia. A slower regenerative phase may not lead to the exhaustion of other cell populations due to the self-renewing capacity of the progenitor cells. By studying signals involved in Müller glia driven regeneration via methods such as comparative transcriptomic studies in the zebrafish, we may be able to recreate the second phased regeneration in a clinical setting.

5.4.3 Alternative Cell Sources of Regeneration

From this study, I show that the total number of cell types may be generated to recover the retina even in the absence of Müller glia. Other studies have shown that though Müller glia have been described as the primary source of regeneration, other cell sources have been shown to contribute to regeneration primarily in response to very specific injuries, such as rod precursors or ciliary margin zone (CMZ) cells in light and chemical injury models (Maier and Wolburg 1979, Hitchcock and Raymond 1992, Braisted, Essman et al. 1994) particular in vertebrates such as zebrafish. These cell sources represent potential cell populations that may contribute to recovery in an environment lacking Müller glia.

The CMZ is responsible for adult neurogenesis through the lifetime of the zebrafish, whose eyes continue growing by addition of neurons in contrast to mammalian retinas. The CMZ is mainly composed of slowly dividing stem cells and transient rapidly amplifying dividing cells. Currently, the degree of contribution the CMZ makes during regeneration is unclear.

Since transiently amplifying cells of the CMZ are limited in the number of divisions (Centanin, Ander et al. 2014), reactivation of stem cells to increase the pool of transient stem cells may viably contribute to regeneration. This would presumably

include asymmetric division to make one stem daughter cell and one amplifying daughter cell to maintain the stem cell pool (Horvitz and Herskowitz 1992, Rhyu, Jan et al. 1994). Some lateral migration of neurons such as horizontal and amacrine cells does occur in the retina (Reese, Necessary et al. 1999, Godinho, Williams et al. 2007). However, as we only viewed regeneration in a short time window (7 dpi – 21 dpi), it is likely that we did not observe the migration of cells from the CMZ towards the central, regenerating retina. Thus, as for short term regeneration (3 dpi – 7 dpi), there has been no evidence to date that new cells generated from the CMZ will migrate laterally during regeneration, as the CMZ is located at the very boundary of the retina and usually only contributes cells to this region. In fact, long range lateral migration, at least during development, is a rare occurrence in the retina, whereby clones arising from individual progenitor cells form radial columns (Holt, Bertsch et al. 1988, Wong and Rapaport 2009). This fits with my PCNA staining observation, whereby proliferative cells form associated clusters span radially, but not laterally in the retina.

With evidence from the current literature, it is unlikely that the regeneration I observed in the absence of Müller glia is solely a CMZ mediated form of regeneration, though proper investigation is required to rule the CMZ out as a cell source. However, given that non-proliferative regeneration results in generating one cell type at the cost of losing others, the total cell number after cell loss caused by ablation is actually not recovered in this process. Given that the CMZ is a continuous growth zone in the zebrafish, the cells generated by the CMZ might be recruited to the regenerative process by simply adding newly generated neurons of particular subtypes to the periphery of the retina at an increased pace to replace the total number of cells lost and to ensure that the size of the eye remains stable. This could be tested with a combination of BrdU and EdU labelling and a comparative analysis between stem cell

versus the amplifying stem cell compartments in the fish between pre- and post-injury conditions, to identify if, in post injury conditions, an increase in the rate of proliferation can be observed.

The other described source of adult neurogenesis is rod precursors, cells that arise from a Müller glia origin migrate to the photoreceptor layer and continue to slowly divide cells to give rise to new rods specifically within the photoreceptor layer. Rod precursors are generally believed to be restricted to a photoreceptor fate as determined through distinct antibody staining from multipotent progenitors (Knight and Raymond 1990, Morrow, Belliveau et al. 1998, Ng 2013). Hence, rod precursors (ie. having a specified fate but is not matured yet) are not considered able to give rise to other neuronal lineages. However, due to the physical proximity of the photoreceptor layer with the horizontal layer that is lost in my injury model, it is intriguing to speculate that rod precursors may contribute towards horizontal cell replacement. This will depend on whether they are able to respond to appropriate signalling from dying horizontal cells as well as how far along the photoreceptor lineage they have progressed and whether they are, in essence, able to switch towards a horizontal fate.

Recently, similar precursors to other neuron types have been identified during development, such as bipolar precursor cells (Pilz, Shitamukai et al. 2013, Weber, Ramos et al. 2014) and amacrine precursor cells (Godinho, Williams et al. 2007). A shared similarity between these precursor cells is the limited division that they can take and that they have a very restricted fate. Thus, though precursor cells may represent another potential source of adult neurogenesis, precursor cells are by their nature and definition so far believed not to be able to explain the extensive regeneration I observe in the absence of Müller glia.

Future studies to identify if additional cell sources are contributing to this first phase of regeneration may involve isolating regenerating cell populations from a Müller glia depleted versus wild type retina. Sequencing can be conducted to identify differences between the two populations, generate knockout lines based on the sequencing data and conduct injury modelling in parallel with the Müller glia ablated retina. Thus by doing so, I may be able to identify the regulatory factors involved in functional regeneration such as formation of the previously described neural circuits.

5.4.4. Neural Transdifferentiation

In light of a possible non-proliferative form of regeneration, it would be particularly interesting to investigate the possibility of transdifferentiation of mature neurons into the missing cell type during retinal regeneration in the absence of Müller glia. Transdifferentiation of the retinal pigment epithelium (RPE) to regenerate missing neurons has been described *in vivo* in amphibians and in chick upon supplication of factors such as fibroblast growth factors (Park and Hollenberg 1989, Reh, Jones et al. 1991, Araki 2007). In the zebrafish, studies have shown that the RPE is unable to regenerate the retina (Karl and Reh 2010) and thus, transdifferentiation has not been described *in vivo* in CNS structures of the zebrafish. However, transdifferentiation *in vivo* has been observed in the zebrafish heart, whereby atrial cardiomyocytes transdifferentiate into ventricular cardiomyocytes after injury (Zhang, Han et al. 2013, Kikuchi 2015), suggesting that transdifferentiation may still be a possibility in the retina.

Mature neurons are believed to be the final stage in the final fate in the neuronal cell line. However, the process of transdifferentiation is defined as when one cell type directly transforms into another (Eguchi, Abe et al. 1974, Kragl, Knapp et al. 2009). In development, fate switching from one cell type to another has been described in

inhibitory neurons (Jusuf and Harris 2009). But this process involves the redirection of progenitor cells into another fate path, not transdifferentiation of mature neurons.

Since Müller glia respond via dedifferentiation into multipotent progenitor cells, this is not direct transdifferentiation but instead goes through a stem cell like state. This is also different from quiescent stem cells like those described in the bone marrow (Stamm, Westphal et al. 2003) and satellite muscle stem cells (Collins, Olsen et al. 2005), as these cells have only one function that is to divide upon activation, unlike Müller glia, which are heavily involved in maintaining the functional activity of the retina (Bringmann, Schopf et al. 2000, Bringmann, Pannicke et al. 2006, Kassen, Thummel et al. 2009).

Possible experiments to observe transdifferentiation in the retina may be to combine my nitroreductase retinal ablation model with a transgenic line that labels all retinal neurons through different fluorescent proteins (Almeida, Boije et al. 2014). By conducting *live* imaging on this transgenic line upon ablation, I may be able to observe if one neuron type migrates into another neuronal layer, and if transdifferentiation occurs to observe if cells of different colors are generated within a radial column. If transdifferentiation within the retina is shown to be true, similar methods can then be applied to other organ systems if the arrangement of cell types is organized, and possibly even to other species. As transdifferentiation does not increase the number of cells, long term labelling of proliferative cells in regenerating zebrafish may be necessary, such as using the Tg*(ccnb1*:EGFP) to label proliferating cell (Kassen, Thummel et al. 2008), if more cells are eventually generated to replace the initial transdifferentiated cell types. Additionally, similar BrdU labelling and tracking techniques can be utilized to observe, if long term (3 – 6 months) lateral migration of

cells from the CMZ occurs in regenerating retinas, and how the extent of cells regenerated compares to uninjured retinas.

By understanding whether transdifferentiation can occur *in vivo* and if so, how transdifferentiation is initiated in different cell types across different animal species, it may be possible to direct transdifferentiation *in vitro* and *in vivo* into cell types of interest for therapeutic purposes. The field of induced pluripotent stem cells, though being artificially induced in a dish, does show us the potential that mature cell types have to be reprogrammed. For example, mesenchymal stem cells, adipose derived stem cells and hepatic stellate cells must undergo global alteration of their epigenetic state in order to undergo transdifferentiation reprogramming (Alexanian 2015, Forcales 2015, Page, Paoli et al. 2016). Thus, by looking towards epigenetics as a method to activate cells to regenerate cells in a case they would not otherwise, therein available is the generation of large numbers of regenerating cells, trandifferentiated from a particular cell type into a completely different cell type.

Having explored transdifferentiation, while it does occur in other species, fate changing from one mature cell type into another is a complex process. Thus, other forms of potential regenerative source like different neuron precursors may be more likely. Hence, further investigation is required into all the potential regenerative cell sources as discussed.

5.5 Conclusion

From my results, at least in the zebrafish, there appear to be regenerative mechanisms other than Müller glia that recover the retina. This mechanism has not been well documented *in vivo* and thus whether it is a transdifferential process or

another undescribed event, the cell type involved in regeneration requires identification. Moreover, as this non-Müller glia driven form of regeneration is observed in the zebrafish retina, for applicable purposes into the clinical field, similar studies will need to be conducted within mammalian models, whereby the introduction of these factors is conducted upon injury to observe if a similar mode of regeneration occurs.

Currently, the activation of regeneration via externally introduced factors is an alternative therapeutic approach as the cell types remain in their natural retinal environment, as compared to cell therapy approaches whereby genetic changes are directly made into the cells *in vitro* before reintroducing them back into the patient environment after all the external changes. In addition, further insights into the possibility of other forms of regeneration would be ideal, especially if the knowledge is applied to other CNS structures or regions without a known stem cell source, such as the brain whereby the only stem cell niches are in the subventricular and subgranular zones (Doetsch, Caille et al. 1999, Jin, Minami et al. 2001).

In essence, this study opens up the field to a possibility of an unexplored regenerative cell source other than Müller glia driven proliferative regeneration with significant impact in understanding vertebrate neural regeneration.

Table 5.1 Total number of retinal cell types in untreated fish and after genetic ablation.

The table compares the total number of DAPI labelled cell nuclei within a 50 µm width retinal window for 5 neuron cell types. Data includes the SEM for each layer, p value corresponding to the F-statistic of one-way ANOVA with post-hoc Tukey HSD and Bonferroni multiple correction (indicated in the table as ******* for significant differences, non-significance is not indicated in the table). The first section indicates the total number in a genetic ablated retina, and the second section indicates the total numbers in an untreated retina (dpie – days post injury equivalent to dpi in treated retina)

dpi	Photoreceptor	Horizontal	Bipolar	Amacrine	Ganglion
	Cell number (in 50 µm retinal width) ± SEM, number of embryos				
0	17.5 ± 1.4, n=6	6.7 ± 1.4, n=6	43.8 ± 2.9, n=6	27.5 ± 2.3, n=6	31.2 ± 1.8, n=6
3	16.4 ± 1.0, n=9	3.6 ± 0.5, n=9, *******	42.2 ± 1.3, n=9	8.9 ± 1.1, n=9, *******	34.1 ± 1.7, n=9
7	17 ± 1.8, n=5	7.2 ± 0.9, n=5	46.4 ± 2.8, n=5	26.6 ± 1.2, n=5	29.2 ± 2.2, n=5
dpie	Photoreceptor	Horizontal	Bipolar	Amacrine	Ganglion
	Cell number (in 50 µm retinal width) ± SEM, number of embryos				
0	17.5 ± 1.7, n=6	6.8 ± 0.6, n=6	41.7 ± 2.9, n=6	27.3 ± 2.0, n=6	34 ± 2.5, n=6
3	16 ± 1.4, n=9	7.5 ± 0.6, n=9	42.4 ± 2.5, n=9	31.6 ± 1.5, n=9	38.7 ± 1.8, n=9
7	22.8 ± 2.3, n=5	7 ± 0.7, n=5	47 ± 2.9, n=5	28.6 ± 2.3, n=5	33.8 ± 1.9, n=5

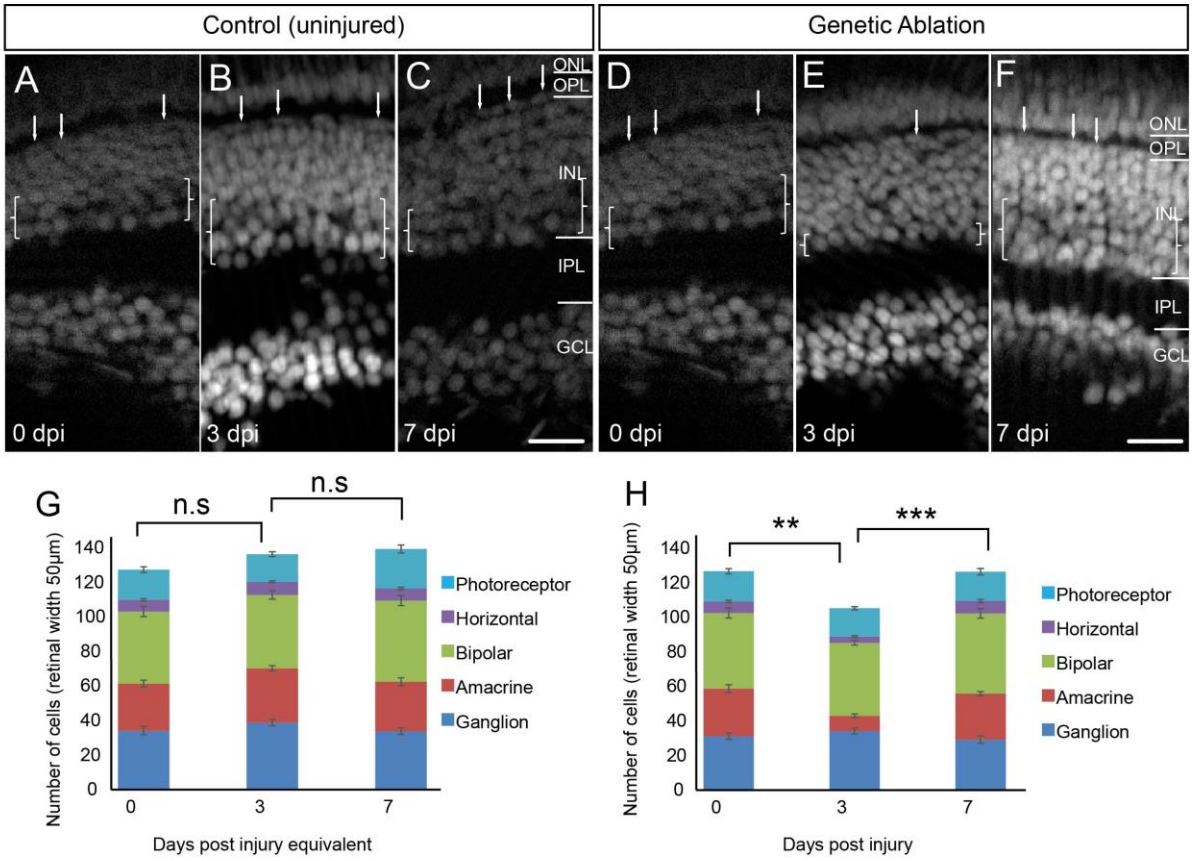

Control (uninjured)
Genetic Ablation
A
B
C
D
E
F
ONL
OPL
INL
IPL
GCL
0 dpi
3 dpi
7 dpi
0 dpi
3 dpi
7 dpi
G
n.s
n.s
Number of cells (retinal width 50µm)
140
120
100
80
60
40
20
0
0
3
7
Days post injury equivalent
Photoreceptor
Horizontal
Bipolar
Amacrine
Ganglion
H
**
***
Number of cells (retinal width 50µm)
140
120
100
80
60
40
20
0
0
3
7
Days post injury
Photoreceptor
Horizontal
Bipolar
Amacrine
Ganglion

Figure 5.1. Regeneration of retinal architecture after genetic ablation injury. (A - F) Micrographs of retinal sections in uninjured control (A - C) and genetic ablated juveniles (D - F) in Tg(*ptf1a:Gal4 / UAS:nfsb-mCherry*) juveniles stained with 4',6-diamidino-2-phenylindole (DAPI) at 0, 3 and 7 days post injury (dpi). Amacrine cells can be distinguished from difference in DAPI contrast (in brackets) to other layers. Horizontal cells are morphologically flattened (indicated by arrows). In the uninjured controls, the number of cells within a fixed area is unchanged (G), however in the genetic ablation juveniles, the number of amacrine cells is reduced to 32 % and similarly the horizontal cell number is reduced to 49 % compared to the total number of these cells in an uninjured retina at the same age, i.e. 3 dpi (H). The architecture of the retina recovers following 3 dpi in each subsequent day. By 7 dpi in the genetic ablation model, there is no significant difference between the total numbers of cells with the uninjured retina, showing quick recovery. The p-values of horizontal and amacrine layers at 3 dpi in the genetic ablated fish, corresponding to the F-statistic of one-way ANOVA with post-hoc Tukey HSD and Bonferroni multiple correction, were lower than 0.01 when compared to all the other paired samples (H). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar A (for A – G) = 50 µm. Error bars are SEM.

Table 5.2 Total number of inhibitory neurons after genetic ablation without DAPT (glia+) and DAPT treated (glia-) retinas.

The table compares the total number of Ptf1a:GFP positive cells within a 50 µm width retinal window. Data includes the SEM for each layer, p value corresponding to the F-statistic of one-way ANOVA with post-hoc Tukey HSD and Bonferroni multiple correction (indicated in the table as ******* for significant differences, non-significance is not indicated in the table). The first section indicates the total number of inhibitory neurons in a retina lacking Müller glia that is genetic ablated (glia-), and the second section indicates the total numbers of inhibitory neurons in a retina with Müller glia that is normal (dpie – days post injury equivalent to dpi in treated retina).

dpi (glia-)	0	1	2	3	4	5	6	7	10	14
Average	41.1	37.6	42.5	21.5	28.1	38.3	37.5	36.6	33.7	34.6
SEM	2.6	2.1	0.5	1.5 ***	0.7 ***	1.8	4.0	1.7	1.2	1.6
dpi (glia+)	0	1	2	3	4	5	6	7	10	14
Average	41	45.17	41.3	40.8	39.7	44.8	38.7	39.3	34	41.7
SEM	2.0	3.6	2.2	2.3	2.5	3.1	1.9	3.8	2.8	2.8

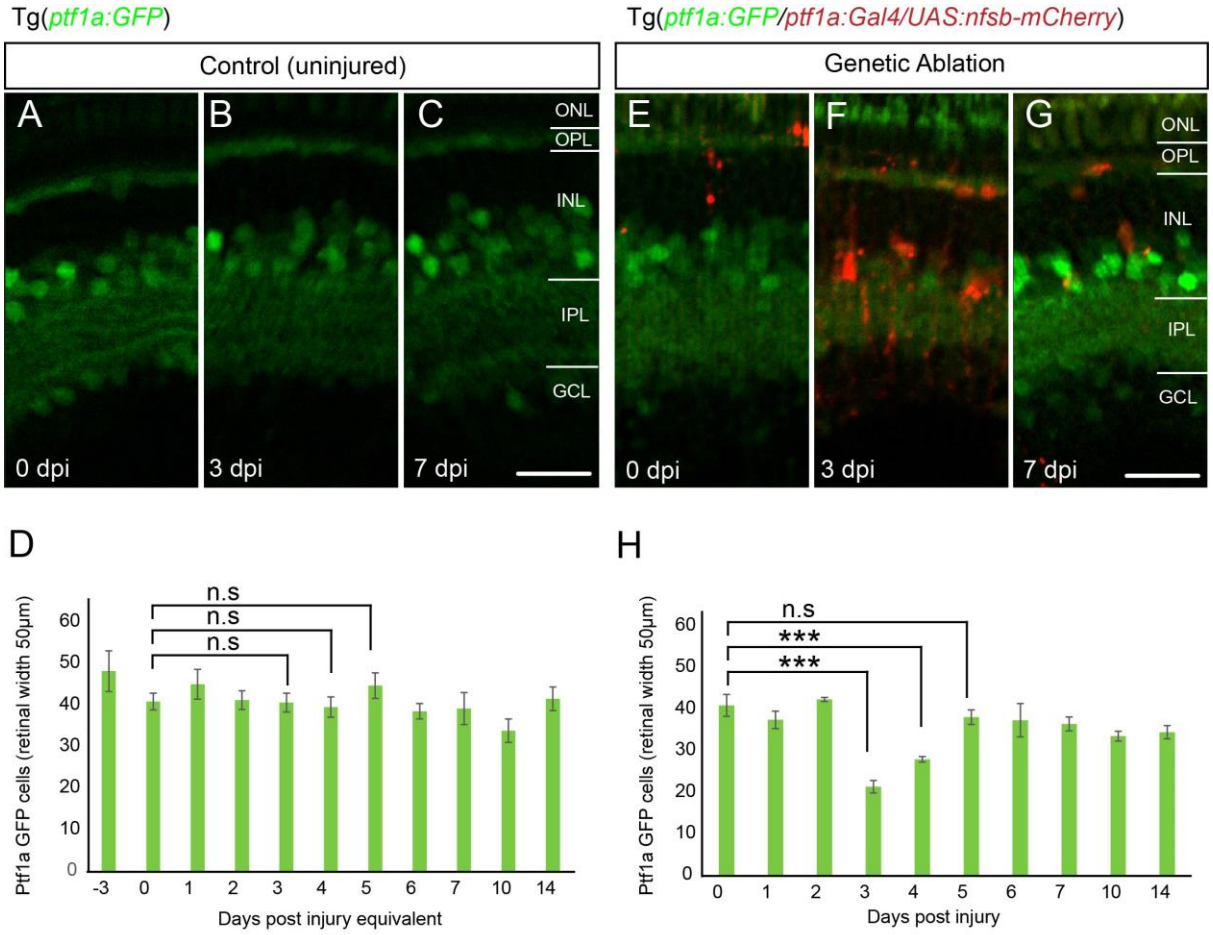

Tg(ptf1a:GFP)
Tg(ptf1a:GFP/ptf1a:Gal4/UAS:nfsb-mCherry)
Control (uninjured)
Genetic Ablation
A
B
C
E
F
G
ONL
OPL
INL
IPL
GCL
0 dpi
3 dpi
7 dpi
0 dpi
3 dpi
7 dpi
D
H
Ptf1a GFP cells (retinal width 50µm)
n.s
n.s
n.s
***
***
Days post injury equivalent
Days post injury

Figure 5.2 Rapid recovery of missing neuron population after cell loss. (A - C, E - G) Micrographs of retinal sections in uninjured control (A - C), and genetic ablated juveniles (E - G) in Tg(*ptf1a:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*) juveniles at 0, 3 and 7 days post injury (dpi). (A - C) In the uninjured control, the total number of Ptf1a:GFP cells remains constant throughout the length of time studied (D). At 3 dpi in the genetic ablated retina, the number of Ptf1a:GFP labelled inhibitory neurons (amacrine & horizontal cells) drops to 50 % compared to the total number of cells in the retinal sections in uninjured controls. At 4 dpi, the number of Ptf1a1:GFP positive cells increased in the genetic ablated retina as compared to 3 dpi. By 5 dpi, the total number of Ptf1a:GFP positive cells in the inhibitory neuron layer was not significantly different to an uninjured retina, indicating full recovery of the architecture was reached (H). The p-values of 3 and 4 dpi in the glia- fish, corresponding to the F-statistic of one-way ANOVA with post-hoc Tukey HSD and Bonferroni multiple correction, were less than 0.01 when compared to all the other paired samples (H). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar A (for A – G) = 50 µm. Error bars are SEM.

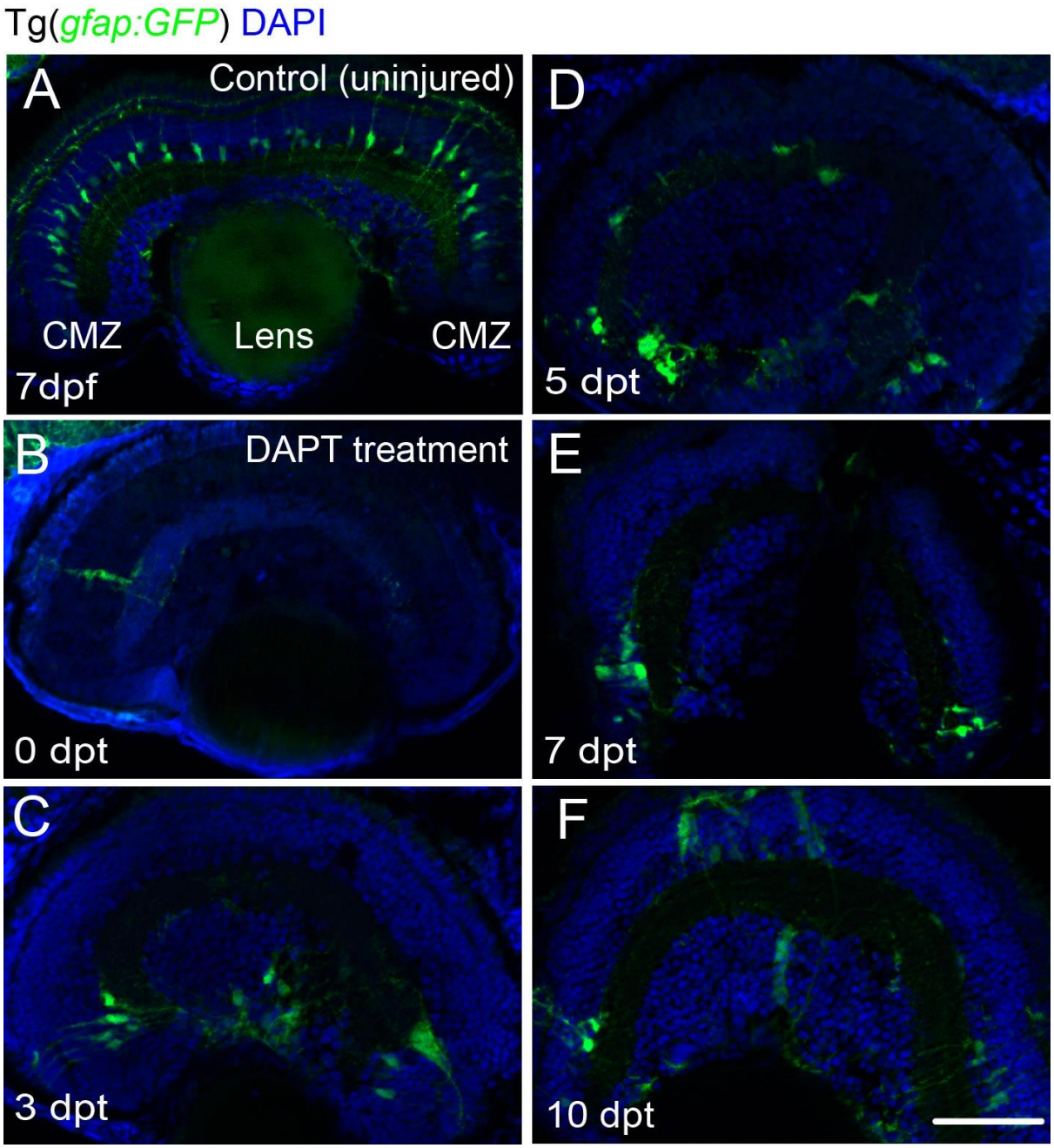
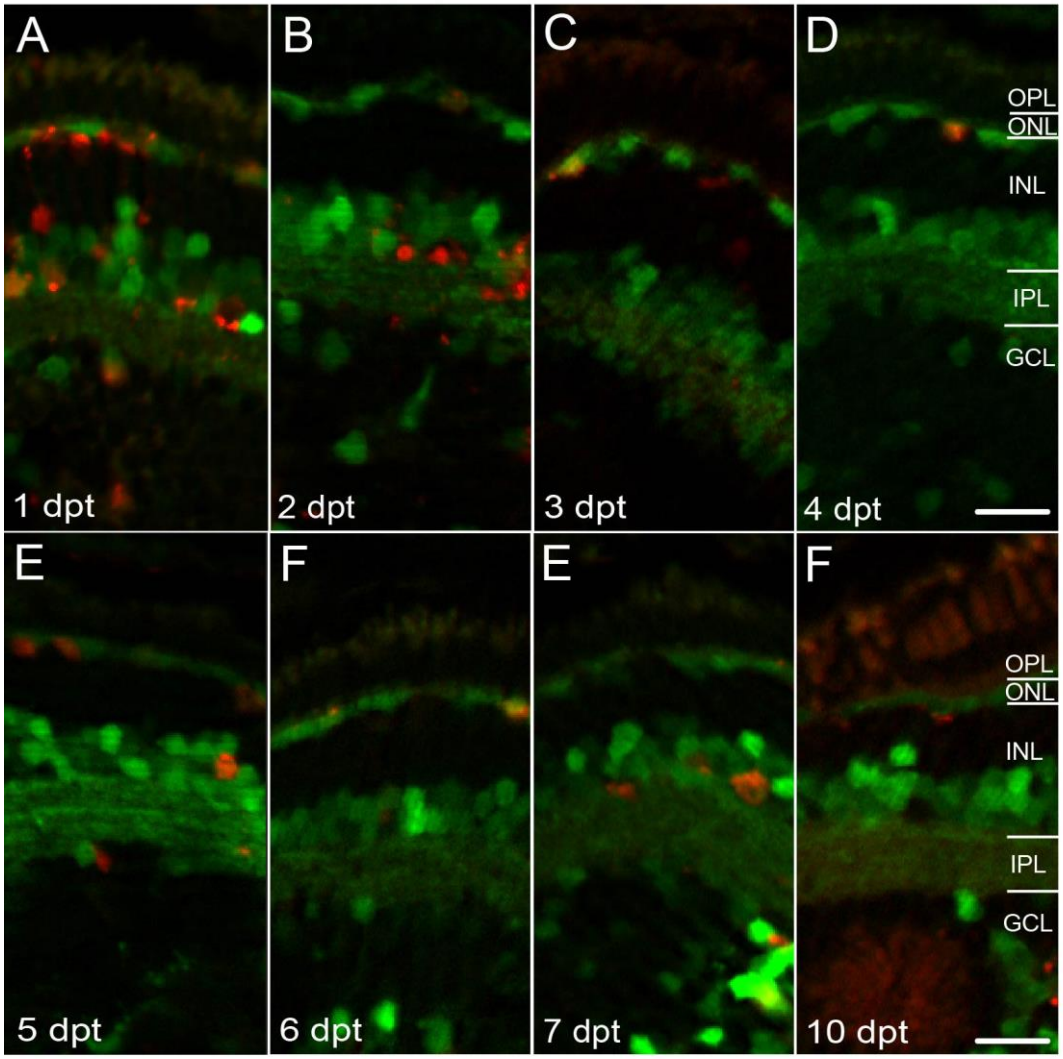

Tg(gfap:GFP) DAPI
A
Control (uninjured)
CMZ
Lens
CMZ
7dpf
D
5 dpt
B
DAPT treatment
0 dpt
E
7 dpt
C
3 dpt
F
10 dpt

Figure 5.3 Inhibition of Müller glia development (A - F) Micrographs of retinal sections of Tg(*gfap:GFP*) juveniles treated with N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) at 48 hpf until 6 days post fertilization (dpf). Juveniles were stained with DAPI and analysed each day between 0 – 7, as well as at 10 days post DAPT treatment (dpt). (A) Control untreated juveniles show Gfap:GFP positive Müller glia spanning the entire retina. In contrast, juveniles that were treated with DAPT do not develop Müller glia during the time of exposure as revealed by the lack of Gfap:GFP positive cells. (B - F) Few Müller glia are present immediately (B) after treatment. Some Müller glia are appearing at the retinal periphery by 3 dpt, which could represent glia developing from the growing ciliary margin zone (CMZ). By 10 dpt Gfap:GFP positive cells are also observed in the central region of the retina. Scale bar A (for A – F) = 20 µm.

Tg(*ptf1a:GFP/ptf1a:Gal4/UAS:nfsb-mCherry*)

DAPT and Genetic ablation



G

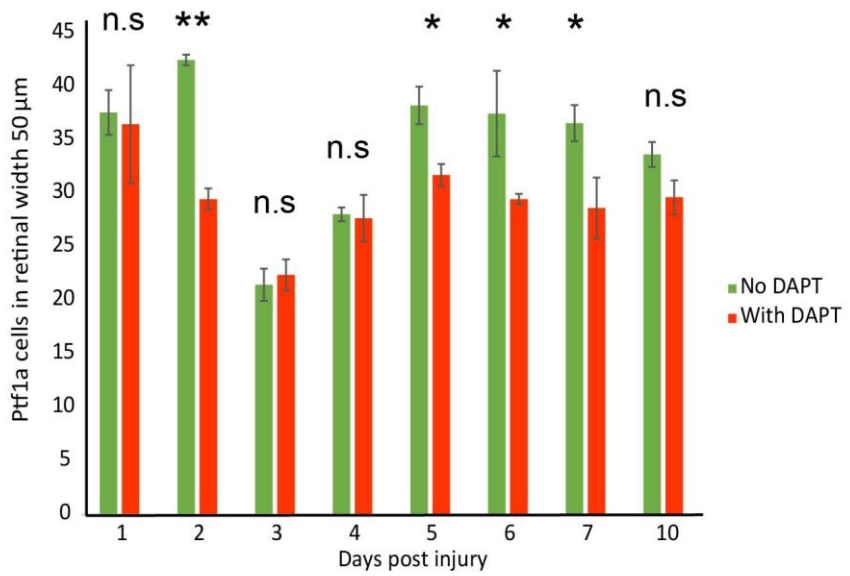


Figure 5.4 Non-Müller glia driven proliferation. (A - F) Micrographs of retinal sections following genetic ablation of inhibitory neurons in the absence of Müller glia (glia- fish). Tg(*ptf1a:GFP* / *ptf1a:Gal4* / *UAS:nfsb-mCherry*) embryos were treated with DAPT from 48 hpf to 6 dpf to inhibit the development of Müller glia. Following this, juveniles were treated with metronidazole to genetically ablate all retinal Ptf1a positive inhibitory neurons in an environment without Müller glia. Loss of inhibitory neurons can be observed at 3 dpi and recovery to the inhibitory neuron layer can be observed to start by 4 dpi. (G) The graph indicates the total number of inhibitory neurons in the retina at subsequent time points after genetic ablation of inhibitory neurons in the glia- fish (DAPT treatment) versus in the glia+ fish (no DAPT treatment). At 4 dpi, similar numbers of Ptf1a:GFP positive inhibitory neurons was observed in both environments with or without Müller glia. Following this in the glia+ fish, the total number Ptf1a:GFP positive inhibitory neurons recovered to that in an uninjured state by 5 dpt. However in the glia- fish up to 7 dpt, the total number Ptf1a:GFP positive inhibitory neurons do not fully recover to the total number of inhibitory neurons as in an uninjured state. No significant differences can be observed at 10 dpi between treatment groups ($p = 0.1124$). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar A (for A – F) = 50 µm. Error bars are SEM.

Chapter 6: Concluding Remarks

At the start of this project, not much was known about how retinal developmental events are regulated by environmental signals (Reh and Tully 1986, Poggi, Vitorino et al. 2005, Brzezinski 2010, Jusuf, Almeida et al. 2011) and only recently was it suggested to be done through stochastic gene expression (Boije, Rulands et al. 2015). The literature showed that environmental cues could influence the developmental fate choice of retinal progenitors. However, how and when these cues accurately and consistently generate a retina with the appropriate number of cells at the right size for optimal function is unknown. In addition, many cellular signalling events during retinal regeneration in adult zebrafish are similar to retinal development events, however regulators such as environmental factors have not been assessed during regeneration. In this study, I provided evidence on the involvement of environmental factors in both the development and regeneration of the retina, which can be used for future research work, which involves supplementing signalling factors for developmental or regenerative therapeutic studies.

During development, I showed that the perturbation of the generated cells in the retinal environment delays the expression of subsequent fate determinant genes. The sequence of when retinal neurons are born is very well conserved across all vertebrate models. It has thus been suggested that this progression has a role in regulating key developmental events during development. Here I show that this conserved birth order may function to ensure the generation of the right number of retinal neurons, by regulating the timing switch of fate genes of neurons during neurogenesis. Information from this study may provide some insights into the histogenic birth order of other CNS structures such as different regions of the brain

(Kohwi and Doe 2013) and by understanding regulation of the timing of cell birth, how to effectively generate specific neurons for therapeutic repair.

Much of the knowledge surrounding cellular events that occurs during retinal development has been applied to regeneration. This is due to the observed similarities during regeneration such as the sequence of photoreceptor differentiation and the formation of ciliary margin zone (CMZ) like structures. Thus regeneration has been said to recapitulate retinal development (Mader and Cameron 2004, Martinez-De Luna, Kelly et al. 2011). This suggests that other events such as fate specification of newly generated neurons are also primarily directed by intrinsic signalling, as this is observed during developmental neurogenesis. However, the involvement of environmental cues on fate specification of progenitors during regeneration is largely unknown.

In order to assess fate determination of progenitors during retinal regeneration, I established two ablation models affecting different neuronal populations. Early cellular events after injury such as cell death, origin & activation of stem cell source and proliferation events are broadly comparable. The main difference between regeneration and development is the role of environmental signals to direct cell specific regeneration to replace the ablated population. These different injuries may result in damages to different tissue and cell structures, hence activating regeneration via different pathways. However, as cellular events post-injury were comparable, it is less likely to influence the overall regeneration of the retinal structure, such as the final size of the regenerated retina. This suggests that progenitors during regeneration share similar fate specification to the developing retina, whereby progenitors during regeneration are not solely reliant on intrinsic factors, but retain an ability to sense their surroundings for fate determination such as what is observed in development.

The histogenic order of cell birth in the retina during development is not recapitulated in regeneration. This important difference allows for the regeneration of missing cell types, rather than regenerating cell types strictly according to the proportions observed during normal development and a linear birth order of cells.

The study also indicates the potential use of externally introducing therapeutic factors directly into the retina to allow for cell specific recovery using endogenous stem cells, if present in humans. It would be interesting to identify if there are any differences between a homeostatic state compared to a post-genetic ablated model for inhibitory neurons using an electroretinogram (ERG), to observe for any functional similarities or differences in the newly generated neurons (Chrispell, Rebrik et al. 2015).

Finally, I made a novel observation of a non-Müller glia, non-proliferative regeneration that has not been previously described in the zebrafish. These regenerative cells contributed to partial regeneration of the total cell number within the retina, and the presence of Müller glia appears to complete the regenerative process to restore the cell number to that of an uninjured retina. This unexpected data suggests the possibility of other regenerating stem cell sources that have yet to be characterized and could represent novel endogenous cells to target for regenerative approaches. Thus, future work should assess if this non-proliferative, non-glia form of regeneration occurs in other vertebrate species including mammals such as the mouse.

As previously discussed, further investigations need to be taken to look if the ciliary margin zone (CMZ), neuron precursors and the process of transdifferentiation may contribute to regeneration, in parallel with Müller glia regeneration. Understanding if this occurs would open possibilities of identifying additional cell sources of

regeneration, which may develop into a potential therapeutic cell source whether cultured *in vitro* or activated *in vivo* in humans.

My thesis thus implicates the influence of the environment during both developmental and regenerative neurogenesis in the vertebrate retina. The retinal environment appears to assist in determining the fate bias of progenitors. It remains unclear, if similar mechanisms are also observed across other CNS structures, if this is observed only in zebrafish, if is conserved within the teleost class, or even to a larger extent in mammals. If proven correct, these studies provide a platform to understand the therapeutic potential in a setting with large numbers of neurons as seen in humans where it is estimated that there are 80 billion neurons in the human brain compared to 200 million in the mouse (Herculano-Houzel and Lent 2005).

Lastly, by understanding the mechanisms which regulate progenitor proliferation and fate choice we can learn why animal models such as the zebrafish have sustained regenerative capacities in the CNS and other systems throughout adulthood, whereas it is strictly limited in the mammalian models. Thus, by studying regeneration in vertebrate species it will be possible to improve mammalian regeneration and open up the avenue for future regenerative medicine in human patients.

Reference

Abler, A. S., C. J. Chang, J. Ful, M. O. Tso and T. T. Lam (1996). "Photic injury triggers apoptosis of photoreceptor cells." Res Commun Mol Pathol Pharmacol **92**(2): 177-189.

Agathocleous, M. and W. A. Harris (2006). Cell determination. Retinal Development. E. Sernagor, S. Eglen, W. A. Harris and R. O. Wong. New York, Cambridge University Press**:** 75-98.

Agathocleous, M. and W. A. Harris (2009). "From progenitors to differentiated cells in the vertebrate retina." Annu Rev Cell Dev Biol **25**: 45-69.

Ahmad, I., A. V. Das, J. James, S. Bhattacharya and X. Zhao (2004). "Neural stem cells in the mammalian eye: types and regulation." Semin Cell Dev Biol **15**(1): 53-62.

Ahmad, I., L. Tang and H. Pham (2000). "Identification of neural progenitors in the adult mammalian eye." Biochem Biophys Res Commun **270**(2): 517-521.

Alexanian, A. R. (2015). "Epigenetic modulators promote mesenchymal stem cell phenotype switches." Int J Biochem Cell Biol **64**: 190-194.

Almeida, A. D., H. Boije, R. W. Chow, J. He, J. Tham, S. C. Suzuki and W. A. Harris (2014). "Spectrum of Fates: a new approach to the study of the developing zebrafish retina." Development **141**(9): 1971-1980.

Altshuler D, T. D., Cepko C (1991). "Specification of cell types in the vertebrate retina. In Development of the Visual System." Boston, MIT Press,: pp37-58.

Alvarez-Buylla, A., M. Theelen and F. Nottebohm (1990). "Proliferation "hot spots" in adult avian ventricular zone reveal radial cell division." Neuron **5**(1): 101-109.

Andreazzoli, M., G. Gestri, D. Angeloni, E. Menna and G. Barsacchi (1999). "Role of Xrx1 in Xenopus eye and anterior brain development." Development **126**(11): 2451-2460.

Araki, M. (2007). "Regeneration of the amphibian retina: role of tissue interaction and related signaling molecules on RPE transdifferentiation." Dev Growth Differ **49**(2): 109-120.

Arroba, A. I., N. Alvarez-Lindo, N. van Rooijen and E. J. de la Rosa (2014). "Microglia-Muller glia crosstalk in the rd10 mouse model of retinitis pigmentosa." Adv Exp Med Biol **801**: 373-379.

Avanesov, A. and J. Malicki (2010). "Analysis of the retina in the zebrafish model." Methods Cell Biol **100**: 153-204.

Baker, P. S. and G. C. Brown (2009). "Stem-cell therapy in retinal disease." Curr Opin Ophthalmol **20**(3): 175-181.

Banerjee, S. (2006). "A review of developments in the management of retinal diseases." J R Soc Med **99**(3): 125-127.

Barabino, S. M., F. Spada, F. Cotelli and E. Boncinelli (1997). "Inactivation of the zebrafish homologue of Chx10 by antisense oligonucleotides causes eye malformations similar to the ocular retardation phenotype." Mech Dev **63**(2): 133-143.

Barbosa-Sabanero, K., A. Hoffmann, C. Judge, N. Lightcap, P. A. Tsonis and K. Del Rio-Tsonis (2012). "Lens and retina regeneration: new perspectives from model organisms." Biochem J **447**(3): 321-334.

Barishak, R. Y. and R. Ofri (2007). "Embryogenetics: gene control of the embryogenesis of the eye." Vet Ophthalmol **10**(3): 133-136.

Bashaw, G. J. and C. S. Goodman (1999). "Chimeric axon guidance receptors: the cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion." Cell **97**(7): 917-926.

Baye, L. M. and B. A. Link (2007). "Interkinetic nuclear migration and the selection of neurogenic cell divisions during vertebrate retinogenesis." J Neurosci **27**(38): 10143-10152.

Beltran, W. A., A. V. Cideciyan, A. S. Lewin, S. Iwabe, H. Khanna, A. Sumaroka, V. A. Chiodo, D. S. Fajardo, A. J. Roman, W. T. Deng, M. Swider, T. S. Aleman, S. L. Boye, S. Genini, A. Swaroop, W. W. Hauswirth, S. G. Jacobson and G. D. Aguirre (2012). "Gene therapy rescues photoreceptor blindness in dogs and paves the way for treating human X-linked retinitis pigmentosa." Proc Natl Acad Sci U S A **109**(6): 2132-2137.

Bernardos, R. L., L. K. Barthel, J. R. Meyers and P. A. Raymond (2007). "Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells." J Neurosci **27**(26): 7028-7040.
Bernardos, R. L., S. I. Lentz, M. S. Wolfe and P. A. Raymond (2005). "Notch-Delta signaling is required for spatial patterning and Muller glia differentiation in the zebrafish retina." Dev Biol **278**(2): 381-395.
Bernardos, R. L. and P. A. Raymond (2006). "GFAP transgenic zebrafish." Gene Expression Patterns **6**(8): 1007-1013.
Bernardos, R. L. and P. A. Raymond (2006). "GFAP transgenic zebrafish." Gene Expr Patterns **6**(8): 1007-1013.
Bhatia, B., H. Jayaram, S. Singhal, M. F. Jones and G. A. Limb (2011). "Differences between the neurogenic and proliferative abilities of Muller glia with stem cell characteristics and the ciliary epithelium from the adult human eye." Exp Eye Res **93**(6): 852-861.
Bibliowicz, J. and J. M. Gross (2011). "Ectopic proliferation contributes to retinal dysplasia in the juvenile zebrafish patched2 mutant eye." Invest Ophthalmol Vis Sci **52**(12): 8868-8877.
Boije, H., R. B. MacDonald and W. A. Harris (2014). "Reconciling competence and transcriptional hierarchies with stochasticity in retinal lineages." Curr Opin Neurobiol **27**: 68-74.
Boije, H., H. Ring, M. Lopez-Gallardo, C. Prada and F. Hallbook (2010). "Pax2 is expressed in a subpopulation of Muller cells in the central chick retina." Dev Dyn **239**(6): 1858-1866.
Boije, H., S. Rulands, S. Dudczig, B. D. Simons and W. A. Harris (2015). "The Independent Probabilistic Firing of Transcription Factors: A Paradigm for Clonal Variability in the Zebrafish Retina." Dev Cell **34**(5): 532-543.
Braisted, J. E., T. F. Essman and P. A. Raymond (1994). "Selective regeneration of photoreceptors in goldfish retina." Development **120**(9): 2409-2419.
Brannvall, K., M. Corell, K. Forsberg-Nilsson and A. Fex Svenningsen (2008). "Environmental cues from CNS, PNS, and ENS cells regulate CNS progenitor differentiation." Neuroreport **19**(13): 1283-1289.
Brindley, G. S. (1964). "A NEW INTERACTION OF LIGHT AND ELECTRICITY IN STIMULATING THE HUMAN RETINA." J Physiol **171**: 514-520.
Bringmann, A., I. Iandiev, T. Pannicke, A. Wurm, M. Hollborn, P. Wiedemann, N. N. Osborne and A. Reichenbach (2009). "Cellular signaling and factors involved in Muller cell gliosis: neuroprotective and detrimental effects." Prog Retin Eye Res **28**(6): 423-451.
Bringmann, A., T. Pannicke, J. Grosche, M. Francke, P. Wiedemann, S. N. Skatchkov, N. N. Osborne and A. Reichenbach (2006). "Muller cells in the healthy and diseased retina." Prog Retin Eye Res **25**(4): 397-424.
Bringmann, A., S. Schopf and A. Reichenbach (2000). "Developmental regulation of calcium channel-mediated currents in retinal glial (Muller) cells." J Neurophysiol **84**(6): 2975-2983.
Brown, N. L., S. Patel, J. Brzezinski and T. Glaser (2001). "Math5 is required for retinal ganglion cell and optic nerve formation." Development **128**(13): 2497-2508.
Brzezinski, J. R., TA. (2010). "Retinal Histogenesis." The Retina and Its Disorders.
Burek, M. J. and R. W. Oppenheim (1996). "Programmed cell death in the developing nervous system." Brain Pathol **6**(4): 427-446.
Burmeister, M., J. Novak, M. Y. Liang, S. Basu, L. Ploder, N. L. Hawes, D. Vidgen, F. Hoover, D. Goldman, V. I. Kalnins, T. H. Roderick, B. A. Taylor, M. H. Hankin and R. R. McInnes (1996). "Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation." Nat Genet **12**(4): 376-384.
Cameron, D. A. and S. S. Easter, Jr. (1995). "Cone photoreceptor regeneration in adult fish retina: phenotypic determination and mosaic pattern formation." J Neurosci **15**(3 Pt 2): 2255-2271.
Cameron, D. A., K. L. Gentile, F. A. Middleton and P. Yurco (2005). "Gene expression profiles of intact and regenerating zebrafish retina." Mol Vis **11**: 775-791.

Cameron, H. A., C. S. Woolley, B. S. McEwen and E. Gould (1993). "Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat." Neuroscience **56**(2): 337-344.
Cao, W., F. Li, R. H. Steinberg and M. M. Lavail (2001). "Development of normal and injury-induced gene expression of aFGF, bFGF, CNTF, BDNF, GFAP and IGF-I in the rat retina." Exp Eye Res **72**(5): 591-604.
Centanin, L., J. J. Ander, B. Hoeckendorf, K. Lust, T. Kellner, I. Kraemer, C. Urbany, E. Hasel, W. A. Harris, B. D. Simons and J. Wittbrodt (2014). "Exclusive multipotency and preferential asymmetric divisions in post-embryonic neural stem cells of the fish retina." Development **141**(18): 3472-3482.
Chacko, D. M., A. V. Das, X. Zhao, J. James, S. Bhattacharya and I. Ahmad (2003). "Transplantation of ocular stem cells: the role of injury in incorporation and differentiation of grafted cells in the retina." Vision Res **43**(8): 937-946.
Chen, H. and A. J. Weber (2002). "Expression of glial fibrillary acidic protein and glutamine synthetase by Müller cells after optic nerve damage and intravitreal application of brain-derived neurotrophic factor." GLIA **38**(2): 115-125.
Chen, L. and A. D. Chisholm (2011). "Axon regeneration mechanisms: insights from C. elegans." Trends Cell Biol **21**(10): 577-584.
Chow, A. Y., A. K. Bittner and M. T. Pardue (2010). "The artificial silicon retina in retinitis pigmentosa patients (an American Ophthalmological Association thesis)." Trans Am Ophthalmol Soc **108**: 120-154.
Chow, A. Y., V. Y. Chow, K. H. Packo, J. S. Pollack, G. A. Peyman and R. Schuchard (2004). "The artificial silicon retina microchip for the treatment of vision loss from retinitis pigmentosa." Arch Ophthalmol **122**(4): 460-469.
Chow, R. L., B. Snow, J. Novak, J. Looser, C. Freund, D. Vidgen, L. Ploder and R. R. McInnes (2001). "Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells." Mech Dev **109**(2): 315-322.
Chow, R. W., A. D. Almeida, O. Randlett, C. Norden and W. A. Harris (2015). "Inhibitory neuron migration and IPL formation in the developing zebrafish retina." Development **142**(15): 2665-2677.
Chrispell, J. D., T. I. Rebrik and E. R. Weiss (2015). "Electroretinogram analysis of the visual response in zebrafish larvae." J Vis Exp(97).
Collins, C. A., I. Olsen, P. S. Zammit, L. Heslop, A. Petrie, T. A. Partridge and J. E. Morgan (2005). "Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche." Cell **122**(2): 289-301.
Collins, M. and A. Thrasher (2015). "Gene therapy: progress and predictions." Proc Biol Sci **282**(1821).
Coulombre, A. J. (1965). "The Eye." In R.I. DeHaan and H. Ursprung (eds): Organogenesis. New York: Holt, Reinehart and Winston: 219-251.
Coulombre, J. L. and A. J. Coulombre (1965). "Regeneration of neural retina from the pigmented epithelium in the chick embryo." Dev Biol **12**(1): 79-92.
Craig, S. E., A. A. Calinescu and P. F. Hitchcock (2008). "Identification of the molecular signatures integral to regenerating photoreceptors in the retina of the zebra fish." J Ocul Biol Dis Infor **1**(2-4): 73-84.
Curado, S., D. Y. Stainier and R. M. Anderson (2008). "Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies." Nat Protoc **3**(6): 948-954.
Curtis, M. A., E. B. Penney, A. G. Pearson, W. M. van Roon-Mom, N. J. Butterworth, M. Dragunow, B. Connor and R. L. Faull (2003). "Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain." Proc Natl Acad Sci U S A **100**(15): 9023-9027.
D'Amico, D. J. (1994). "Diseases of the Retina." New England Journal of Medicine **331**(2): 95-106.
Das, A. V., K. B. Mallya, X. Zhao, F. Ahmad, S. Bhattacharya, W. B. Thoreson, G. V. Hegde and I. Ahmad (2006). "Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling." Dev Biol **299**(1): 283-302.

Davis, R. J., B. C. Tavsanli, C. Dittrich, U. Walldorf and G. Mardon (2003). "Drosophila retinal homeobox (drx) is not required for establishment of the visual system, but is required for brain and clypeus development." Developmental Biology **259**(2): 272-287.
Davison, J. M., C. M. Akitake, M. G. Goll, J. M. Rhee, N. Gosse, H. Baier, M. E. Halpern, S. D. Leach and M. J. Parsons (2007). "Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish." Dev Biol **304**(2): 811-824.
de la Rosa, E. J., C. A. Bondy, C. Hernandez-Sanchez, X. Wu, J. Zhou, A. Lopez-Carranza, L. M. Scavo and F. de Pablo (1994). "Insulin and insulin-like growth factor system components gene expression in the chicken retina from early neurogenesis until late development and their effect on neuroepithelial cells." Eur J Neurosci **6**(12): 1801-1810.
Del Bene, F., A. M. Wehman, B. A. Link and H. Baier (2008). "Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient." Cell **134**(6): 1055-1065.
Del Debbio, C. B., S. Balasubramanian, S. Parameswaran, A. Chaudhuri, F. Qiu and I. Ahmad (2010). "Notch and Wnt signaling mediated rod photoreceptor regeneration by Muller cells in adult mammalian retina." PLoS One **5**(8): e12425.
Deleidi, M. and C. Yu (2016). "Genome editing in pluripotent stem cells: Research and therapeutic applications." Biochem Biophys Res Commun.
Doetsch, F., I. Caille, D. A. Lim, J. M. Garcia-Verdugo and A. Alvarez-Buylla (1999). "Subventricular zone astrocytes are neural stem cells in the adult mammalian brain." Cell **97**(6): 703-716.
Doetsch, F. and C. Scharff (2001). "Challenges for brain repair: insights from adult neurogenesis in birds and mammals." Brain Behav Evol **58**(5): 306-322.
Dong, P. D., E. Provost, S. D. Leach and D. Y. Stainier (2008). "Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas." Genes Dev **22**(11): 1445-1450.
Dullin, J. P., M. Locker, M. Robach, K. A. Henningfeld, K. Parain, S. Afelik, T. Pieler and M. Perron (2007). "Ptf1a triggers GABAergic neuronal cell fates in the retina." BMC Dev Biol **7**: 110.
Dyer, M. A. and C. L. Cepko (2000). "p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina." Development **127**(16): 3593-3605.
Dyer, M. A. and C. L. Cepko (2001). "p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations." J Neurosci **21**(12): 4259-4271.
Easter, S. S., Jr. (2000). "Let there be sight." Neuron **27**(2): 193-195.
Eguchi, G., S. I. Abe and K. Watanabe (1974). "Differentiation of lens-like structures from newt iris epithelial cells in vitro." Proc Natl Acad Sci U S A **71**(12): 5052-5056.
Eivers, E., K. McCarthy, C. Glynn, C. M. Nolan and L. Byrnes (2004). "Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo." Int J Dev Biol **48**(10): 1131-1140.
Faiella, W. and R. Atoui (2016). "Immunotolerant Properties of Mesenchymal Stem Cells: Updated Review." Stem Cells Int **2016**: 1859567.
Fausett, B. V. and D. Goldman (2006). "A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina." J Neurosci **26**(23): 6303-6313.
Fimbel, S. M., J. E. Montgomery, C. T. Burket and D. R. Hyde (2007). "Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish." J Neurosci **27**(7): 1712-1724.
Fischer, A. J. and T. A. Reh (2000). "Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens." Dev Biol **220**(2): 197-210.
Fischer, A. J. and T. A. Reh (2001). "Muller glia are a potential source of neural regeneration in the postnatal chicken retina." Nat Neurosci **4**(3): 247-252.
Fischer, A. J. and T. A. Reh (2003). "Potential of Muller glia to become neurogenic retinal progenitor cells." Glia **43**(1): 70-76.
Fischer, D., Z. He and L. I. Benowitz (2004). "Counteracting the Nogo receptor enhances optic nerve regeneration if retinal ganglion cells are in an active growth state." J Neurosci **24**(7): 1646-1651.

Fleisch, V. C., B. Fraser and W. T. Allison (2011). "Investigating regeneration and functional integration of CNS neurons: lessons from zebrafish genetics and other fish species." Biochim Biophys Acta **1812**(3): 364-380.
Forcales, S. V. (2015). "Potential of adipose-derived stem cells in muscular regenerative therapies." Front Aging Neurosci **7**: 123.
Fraser, B., M. G. DuVal, H. Wang and W. T. Allison (2013). "Regeneration of cone photoreceptors when cell ablation is primarily restricted to a particular cone subtype." PLoS One **8**(1): e55410.
Frohns, F., M. Mager and P. G. Layer (2009). "Basic fibroblast growth factor increases the precursor pool of photoreceptors, but inhibits their differentiation and apoptosis in chicken retinal reaggregates." Eur J Neurosci **29**(10): 1931-1942.
Fujita, S. and M. Horii (1963). "Analysis of cytogenesis in chick retina by tritiated thymidine autoradiography." Arch Histol Jpn **23**: 359-366.
Fujitani, Y., S. Fujitani, H. Luo, F. Qiu, J. Burlison, Q. Long, Y. Kawaguchi, H. Edlund, R. J. MacDonald, T. Furukawa, T. Fujikado, M. A. Magnuson, M. Xiang and C. V. Wright (2006). "Ptf1a determines horizontal and amacrine cell fates during mouse retinal development." Development **133**(22): 4439-4450.
Ganan, Y., D. Macias, M. Duterque-Coquillaud, M. A. Ros and J. M. Hurle (1996). "Role of TGF beta s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod." Development **122**(8): 2349-2357.
Garcia-Verdugo, J. M., S. Ferron, N. Flames, L. Collado, E. Desfilis and E. Font (2002). "The proliferative ventricular zone in adult vertebrates: a comparative study using reptiles, birds, and mammals." Brain Res Bull **57**(6): 765-775.
Garcia, J. M., L. Mendonca, R. Brant, M. Abud, C. Regatieri and B. Diniz (2015). "Stem cell therapy for retinal diseases." World J Stem Cells **7**(1): 160-164.
Gehring, W. J. (2005). "New perspectives on eye development and the evolution of eyes and photoreceptors." J Hered **96**(3): 171-184.
Geiger, P., M. Barben, C. Grimm and M. Samardzija (2015). "Blue light-induced retinal lesions, intraretinal vascular leakage and edema formation in the all-cone mouse retina." Cell Death Dis **6**: e1985.
Gellrich, N. C., R. Schimming, M. Zerfowski and U. T. Eysel (2002). "Quantification of histological changes after calibrated crush of the intraorbital optic nerve in rats." Br J Ophthalmol **86**(2): 233-237.
Ghai, K., C. Zelinka and A. J. Fischer (2010). "Notch signaling influences neuroprotective and proliferative properties of mature Muller glia." J Neurosci **30**(8): 3101-3112.
Giannelli, S. G., G. C. Demontis, G. Pertile, P. Rama and V. Broccoli (2011). "Adult human Muller glia cells are a highly efficient source of rod photoreceptors." Stem Cells **29**(2): 344-356.
Gilbert, E. A., S. L. Payne and M. K. Vickaryous (2013). "The anatomy and histology of caudal autotomy and regeneration in lizards." Physiol Biochem Zool **86**(6): 631-644.
Glass, A. S. and R. Dahm (2004). "The zebrafish as a model organism for eye development." Ophthalmic Res **36**(1): 4-24.
Godinho, L., J. S. Mumm, P. R. Williams, E. H. Schroeter, A. Koerber, S. W. Park, S. D. Leach and R. O. Wong (2005). "Targeting of amacrine cell neurites to appropriate synaptic laminae in the developing zebrafish retina." Development **132**(22): 5069-5079.
Godinho, L., P. R. Williams, Y. Claassen, E. Provost, S. D. Leach, M. Kamermans and R. O. Wong (2007). "Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo." Neuron **56**(4): 597-603.
Goldman, D. (2014). "Muller glial cell reprogramming and retina regeneration." Nat Rev Neurosci **15**(7): 431-442.
Goldman, S. (2005). "Stem and progenitor cell-based therapy of the human central nervous system." Nat Biotechnol **23**(7): 862-871.

Gorsuch, R. A. and D. R. Hyde (2014). "Regulation of Muller glial dependent neuronal regeneration in the damaged adult zebrafish retina." Exp Eye Res **123**: 131-140.
Grandel, H., J. Kaslin, J. Ganz, I. Wenzel and M. Brand (2006). "Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate." Dev Biol **295**(1): 263-277.
Grant, P., Rubin, E., and Cima, C. (1980). "Ontogeny of the retina in *Xenopus laevis*. I. Stages in the early development of the retina." J. Comp. Neurol.(189): 593-613.
Grigorian, E. N. and V. A. Poplinskaia (1999). "[Discovery of internal sources of the neural retinal regeneration after its detachment in Pleurodeles. II. The radioautography study]." Izv Akad Nauk Ser Biol(5): 583-591.
Halder, G., P. Callaerts and W. J. Gehring (1995). "Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila." Science **267**(5205): 1788-1792.
Hall, C. M., C. Else and N. Schechter (1990). "Neuronal intermediate filament expression during neurite outgrowth from explanted goldfish retina: effect of retinoic acid." J Neurochem **55**(5): 1671-1682.
Hamon, A., J. E. Roger, X. J. Yang and M. Perron (2015). "Muller glial cell-dependent regeneration of the neural retina: An overview across vertebrate model systems." Dev Dyn.
Hansson, H. A., A. Holmgren, G. Norstedt and B. Rozell (1989). "Changes in the distribution of insulin-like growth factor I, thioredoxin, thioredoxin reductase and ribonucleotide reductase during the development of the retina." Exp Eye Res **48**(3): 411-420.
Harada, T., C. Harada and L. F. Parada (2007). "Molecular regulation of visual system development: more than meets the eye." Genes Dev **21**(4): 367-378.
Harris, W. A., C. E. Holt and F. Bonhoeffer (1987). "Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibres in vivo." Development **101**(1): 123-133.
Haruta, M., M. Kosaka, Y. Kanegae, I. Saito, T. Inoue, R. Kageyama, A. Nishida, Y. Honda and M. Takahashi (2001). "Induction of photoreceptor-specific phenotypes in adult mammalian iris tissue." Nat Neurosci **4**(12): 1163-1164.
Hashimoto, T., X. M. Zhang, B. Y. Chen and X. J. Yang (2006). "VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation." Development **133**(11): 2201-2210.
Hatakeyama, J. and R. Kageyama (2004). "Retinal cell fate determination and bHLH factors." Semin Cell Dev Biol **15**(1): 83-89.
Hatakeyama, J., K. Tomita, T. Inoue and R. Kageyama (2001). "Roles of homeobox and bHLH genes in specification of a retinal cell type." Development **128**(8): 1313-1322.
Hauck, S. M., C. von Toerne and M. Ueffing (2014). "The neuroprotective potential of retinal Muller glial cells." Adv Exp Med Biol **801**: 381-387.
Hayashi, T., J. Huang and S. S. Deeb (2000). "RINX(VSX1), a novel homeobox gene expressed in the inner nuclear layer of the adult retina." Genomics **67**(2): 128-139.
Hayes, S., B. R. Nelson, B. Buckingham and T. A. Reh (2007). "Notch signaling regulates regeneration in the avian retina." Dev Biol **312**(1): 300-311.
Haynes, T., C. Gutierrez, J. C. Aycinena, P. A. Tsonis and K. Del Rio-Tsonis (2007). "BMP signaling mediates stem/progenitor cell-induced retina regeneration." Proc Natl Acad Sci U S A **104**(51): 20380-20385.
He, J., G. Zhang, A. D. Almeida, M. Cayouette, B. D. Simons and W. A. Harris (2012). "How variable clones build an invariant retina." Neuron **75**(5): 786-798.
Herculano-Houzel, S. and R. Lent (2005). "Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain." J Neurosci **25**(10): 2518-2521.
Hernandez, J., L. Matter-Sadzinski, D. Skowronska-Krawczyk, F. Chiodini, C. Alliod, M. Ballivet and J. M. Matter (2007). "Highly conserved sequences mediate the dynamic interplay of basic helix-loop-helix proteins regulating retinogenesis." J Biol Chem **282**(52): 37894-37905.

Hitchcock, P. F., K. J. Lindsey Myhr, S. S. Easter, Jr., R. Mangione-Smith and D. D. Jones (1992). "Local regeneration in the retina of the goldfish." J Neurobiol **23**(2): 187-203.
Hitchcock, P. F., R. E. Macdonald, J. T. VanDeRyt and S. W. Wilson (1996). "Antibodies against Pax6 immunostain amacrine and ganglion cells and neuronal progenitors, but not rod precursors, in the normal and regenerating retina of the goldfish." J Neurobiol **29**(3): 399-413.
Hitchcock, P. F. and P. A. Raymond (1992). "Retinal regeneration." Trends Neurosci **15**(3): 103-108.
Hitchcock, P. F. and P. A. Raymond (2004). "The teleost retina as a model for developmental and regeneration biology." Zebrafish **1**(3): 257-271.
Hochmann, S., J. Kaslin, S. Hans, A. Weber, A. Machate, M. Geffarth, R. H. Funk and M. Brand (2012). "Fgf signaling is required for photoreceptor maintenance in the adult zebrafish retina." PLoS One **7**(1): e30365.
Hollyfield, J. G. (1968). "Differential addition of cells to the retina in Rana pipiens tadpoles." Dev Biol **18**(2): 163-179.
Hollyfield, J. G. (1972). "Histogenesis of the retina in the killifish, Fundulus heteroclitus." J Comp Neurol **144**(3): 373-380.
Holt, C. E., T. W. Bertsch, H. M. Ellis and W. A. Harris (1988). "Cellular determination in the Xenopus retina is independent of lineage and birth date." Neuron **1**(1): 15-26.
Horvitz, H. R. and I. Herskowitz (1992). "Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question." Cell **68**(2): 237-255.
Inoue, T., M. Hojo, Y. Bessho, Y. Tano, J. E. Lee and R. Kageyama (2002). "Math3 and NeuroD regulate amacrine cell fate specification in the retina." Development **129**(4): 831-842.
Jadhav, A. P., K. Roesch and C. L. Cepko (2009). "Development and neurogenic potential of Muller glial cells in the vertebrate retina." Prog Retin Eye Res **28**(4): 249-262.
Jayakody, S. A., A. Gonzalez-Cordero, R. R. Ali and R. A. Pearson (2015). "Cellular strategies for retinal repair by photoreceptor replacement." Prog Retin Eye Res **46**: 31-66.
Jeon, S. and I. H. Oh (2015). "Regeneration of the retina: toward stem cell therapy for degenerative retinal diseases." BMB Rep **48**(4): 193-199.
Jin, K., M. Minami, J. Q. Lan, X. O. Mao, S. Batteur, R. P. Simon and D. A. Greenberg (2001). "Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat." Proc Natl Acad Sci U S A **98**(8): 4710-4715.
Jin, K., A. L. Peel, X. O. Mao, L. Xie, B. A. Cottrell, D. C. Henshall and D. A. Greenberg (2004). "Increased hippocampal neurogenesis in Alzheimer's disease." Proc Natl Acad Sci U S A **101**(1): 343-347.
Jinturkar, K. A. and A. Misra (2011). 2 - Challenges and Opportunities in Gene Delivery. Challenges in Delivery of Therapeutic Genomics and Proteomics. M. Ambikanandan. London, Elsevier**:** 45-82.
Johns, P. R. (1977). "Growth of the adult goldfish eye. III. Source of the new retinal cells." J Comp Neurol **176**(3): 343-357.
Johns, P. R. and R. D. Fernald (1981). "Genesis of rods in teleost fish retina." Nature **293**(5828): 141-142.
Johnson, T. V. and K. R. Martin (2008). "Development and characterization of an adult retinal explant organotypic tissue culture system as an in vitro intraocular stem cell transplantation model." Invest Ophthalmol Vis Sci **49**(8): 3503-3512.
Julian, D., K. Ennis and J. I. Korenbrot (1998). "Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina." J Comp Neurol **394**(3): 271-282.
Jusuf, P., W. A. Harris and L. Poggi (2013). "Preparation of transgenic zebrafish embryos for imaging the developing retina." Cold Spring Harb Protoc **2013**(3).
Jusuf, P. R., A. D. Almeida, O. Randlett, K. Joubin, L. Poggi and W. A. Harris (2011). "Origin and determination of inhibitory cell lineages in the vertebrate retina." J Neurosci **31**(7): 2549-2562.
Jusuf, P. R. and W. A. Harris (2009). "Ptf1a is expressed transiently in all types of amacrine cells in the embryonic zebrafish retina." Neural Dev **4**: 34.

Kaneko, H., K. M. Nishiguchi, M. Nakamura, S. Kachi and H. Terasaki (2008). "Retardation of photoreceptor degeneration in the detached retina of rd1 mouse." Invest Ophthalmol Vis Sci **49**(2): 781-787.
Karl, M. O., S. Hayes, B. R. Nelson, K. Tan, B. Buckingham and T. A. Reh (2008). "Stimulation of neural regeneration in the mouse retina." Proc Natl Acad Sci U S A **105**(49): 19508-19513.
Karl, M. O. and T. A. Reh (2010). "Regenerative medicine for retinal diseases: activating endogenous repair mechanisms." Trends Mol Med **16**(4): 193-202.
Kaslin, J., J. Ganz and M. Brand (2008). "Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain." Philos Trans R Soc Lond B Biol Sci **363**(1489): 101-122.
Kaslin, J., J. Ganz, M. Geffarth, H. Grandel, S. Hans and M. Brand (2009). "Stem cells in the adult zebrafish cerebellum: initiation and maintenance of a novel stem cell niche." J Neurosci **29**(19): 6142-6153.
Kassen, S. C., V. Ramanan, J. E. Montgomery, T. B. C, C. G. Liu, T. S. Vihtelic and D. R. Hyde (2007). "Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish." Dev Neurobiol **67**(8): 1009-1031.
Kassen, S. C., R. Thummel, C. T. Burket, L. A. Campochiaro, M. J. Harding and D. R. Hyde (2008). "The Tg(ccnb1:EGFP) transgenic zebrafish line labels proliferating cells during retinal development and regeneration." Mol Vis **14**: 951-963.
Kassen, S. C., R. Thummel, L. A. Campochiaro, M. J. Harding, N. A. Bennett and D. R. Hyde (2009). "CNTF induces photoreceptor neuroprotection and Muller glial cell proliferation through two different signaling pathways in the adult zebrafish retina." Exp Eye Res **88**(6): 1051-1064.
Kay, J. N., K. C. Finger-Baier, T. Roeser, W. Staub and H. Baier (2001). "Retinal ganglion cell genesis requires lakritz, a Zebrafish atonal Homolog." Neuron **30**(3): 725-736.
Kay, J. N., B. A. Link and H. Baier (2005). "Staggered cell-intrinsic timing of ath5 expression underlies the wave of ganglion cell neurogenesis in the zebrafish retina." Development **132**(11): 2573-2585.
Keefe, J. R. (1973). "An analysis of urodelian retinal regeneration. I. Studies of the cellular source of retinal regeneration in Notophthalmus viridescens utilizing 3 H-thymidine and colchicine." J Exp Zool **184**(2): 185-206.
Keeley, P. W., I. E. Whitney, N. R. Madsen, A. J. St John, S. Borhanian, S. A. Leong, R. W. Williams and B. E. Reese (2014). "Independent genomic control of neuronal number across retinal cell types." Dev Cell **30**(1): 103-109.
Kikuchi, K. (2015). "Dedifferentiation, Transdifferentiation, and Proliferation: Mechanisms Underlying Cardiac Muscle Regeneration in Zebrafish." Curr Pathobiol Rep **3**(1): 81-88.
Kim, H. S., J. Shin, S. H. Kim, H. S. Chun, J. D. Kim, Y. S. Kim, M. J. Kim, M. Rhee, S. Y. Yeo and T. L. Huh (2007). "Eye field requires the function of Sfrp1 as a Wnt antagonist." Neurosci Lett **414**(1): 26-29.
Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann and T. F. Schilling (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.
Kimura, Y., Y. Okamura and S. Higashijima (2006). "alx, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits." J Neurosci **26**(21): 5684-5697.
Kishimoto, N., K. Shimizu and K. Sawamoto (2012). "Neuronal regeneration in a zebrafish model of adult brain injury." Dis Model Mech **5**(2): 200-209.
Knapp, D. and E. M. Tanaka (2012). "Regeneration and reprogramming." Curr Opin Genet Dev **22**(5): 485-493.
Knight, J. K. and P. A. Raymond (1990). "Time course of opsin expression in developing rod photoreceptors." Development **110**(4): 1115-1120.
Koeberle, P. D. and M. Bahr (2004). "Growth and guidance cues for regenerating axons: where have they gone?" J Neurobiol **59**(1): 162-180.
Kohwi, M. and C. Q. Doe (2013). "Temporal fate specification and neural progenitor competence during development." Nat Rev Neurosci **14**(12): 823-838.

Kostadinov, D. and J. R. Sanes (2015). "Protocadherin-dependent dendritic self-avoidance regulates neural connectivity and circuit function." Elife **4**.
Kostyk, S. K., P. A. D'Amore, I. M. Herman and J. A. Wagner (1994). "Optic nerve injury alters basic fibroblast growth factor localization in the retina and optic tract." J Neurosci **14**(3 Pt 2): 1441-1449.
Kragl, M., D. Knapp, E. Nacu, S. Khattak, M. Maden, H. H. Epperlein and E. M. Tanaka (2009). "Cells keep a memory of their tissue origin during axolotl limb regeneration." Nature **460**(7251): 60-65.
Kuhrt, H., M. Gryga, H. Wolburg, B. Joffe, J. Grosche, A. Reichenbach and H. R. Noori (2012). "Postnatal mammalian retinal development: quantitative data and general rules." Prog Retin Eye Res **31**(6): 605-621.
La Vail, M. M., D. H. Rapaport and P. Rakic (1991). "Cytogenesis in the monkey retina." J Comp Neurol **309**(1): 86-114.
Lahne, M., J. Li, R. M. Marton and D. R. Hyde (2015). "Actin-Cytoskeleton- and Rock-Mediated INM Are Required for Photoreceptor Regeneration in the Adult Zebrafish Retina." J Neurosci **35**(47): 15612-15634.
Lamba, D., M. Karl and T. Reh (2008). "Neural regeneration and cell replacement: a view from the eye." Cell Stem Cell **2**(6): 538-549.
Lawrence, J. M., S. Singhal, B. Bhatia, D. J. Keegan, T. A. Reh, P. J. Luthert, P. T. Khaw and G. A. Limb (2007). "MIO-M1 cells and similar muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics." Stem Cells **25**(8): 2033-2043.
Lee, H. S., Y. S. Bong, K. B. Moore, K. Soria, S. A. Moody and I. O. Daar (2006). "Dishevelled mediates ephrinB1 signalling in the eye field through the planar cell polarity pathway." Nat Cell Biol **8**(1): 55-63.
Lee, S. C., E. J. Cowgill, A. Al-Nabulsi, E. J. Quinn, S. M. Evans and B. E. Reese (2011). "Homotypic regulation of neuronal morphology and connectivity in the mouse retina." J Neurosci **31**(40): 14126-14133.
Lenkowski, J. R., Z. Qin, C. J. Sifuentes, R. Thummel, C. M. Soto, C. B. Moens and P. A. Raymond (2013). "Retinal regeneration in adult zebrafish requires regulation of TGFbeta signaling." Glia **61**(10): 1687-1697.
Levine, E. M., P. F. Hitchcock, E. Glasgow and N. Schechter (1994). "Restricted expression of a new paired-class homeobox gene in normal and regenerating adult goldfish retina." J Comp Neurol **348**(4): 596-606.
Levine, E. M., M. Passini, P. F. Hitchcock, E. Glasgow and N. Schechter (1997). "Vsx-1 and Vsx-2: Two Chx10-like homeobox genes expressed in overlapping domains in the adult goldfish retina." Journal of Comparative Neurology **387**(3): 439-448.
Lie, D. C., H. Song, S. A. Colamarino, G. L. Ming and F. H. Gage (2004). "Neurogenesis in the adult brain: new strategies for central nervous system diseases." Annu Rev Pharmacol Toxicol **44**: 399-421.
Lillien, L. and C. Cepko (1992). "Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha." Development **115**(1): 253-266.
Lima, L., B. Drujan and P. Matus (1990). "Spatial distribution of taurine in the teleost retina and its role in retinal tissue regeneration." Prog Clin Biol Res **351**: 103-112.
Lin, J. W., A. V. Biankin, M. E. Horb, B. Ghosh, N. B. Prasad, N. S. Yee, M. A. Pack and S. D. Leach (2004). "Differential requirement for ptf1a in endocrine and exocrine lineages of developing zebrafish pancreas." Dev Biol **274**(2): 491-503.
Lindmark, D. G. and M. Muller (1976). "Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles." Antimicrob Agents Chemother **10**(3): 476-482.
Liu, Q., R. L. Londraville, E. Azodi, S. G. Babb, C. Chiappini-Williamson, J. A. Marrs and P. A. Raymond (2002). "Up-regulation of cadherin-2 and cadherin-4 in regenerating visual structures of adult zebrafish." Exp Neurol **177**(2): 396-406.
Liu, W., Z. Mo and M. Xiang (2001). "The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development." Proc Natl Acad Sci U S A **98**(4): 1649-1654.

Livesey, F. J. and C. L. Cepko (2001). "Vertebrate neural cell-fate determination: lessons from the retina." Nat Rev Neurosci **2**(2): 109-118.
Livesey, F. J., T. L. Young and C. L. Cepko (2004). "An analysis of the gene expression program of mammalian neural progenitor cells." Proc Natl Acad Sci U S A **101**(5): 1374-1379.
Livne-Bar, I., M. Pacal, M. C. Cheung, M. Hankin, J. Trogadis, D. Chen, K. M. Dorval and R. Bremner (2006). "Chx10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina." Proc Natl Acad Sci U S A **103**(13): 4988-4993.
Locker, M., W. El Yakoubi, N. Mazurier, J. P. Dullin and M. Perron (2010). "A decade of mammalian retinal stem cell research." Arch Ital Biol **148**(2): 59-72.
Lorenc, V. E., J. R. Jaldin-Fincati, J. D. Luna, G. A. Chiabrando and M. C. Sanchez (2015). "IGF-1 Regulates the Extracellular Level of Active MMP-2 and Promotes Muller Glial Cell Motility." Invest Ophthalmol Vis Sci **56**(11): 6948-6960.
Lorenzetto, E., M. Ettorre, V. Pontelli, M. Bolomini-Vittori, S. Bolognin, S. Zorzan, C. Laudanna and M. Buffelli (2013). "Rac1 selective activation improves retina ganglion cell survival and regeneration." PLoS One **8**(5): e64350.
Ma, W., R. T. Yan, W. Xie and S. Z. Wang (2004). "A role of ath5 in inducing neuroD and the photoreceptor pathway." J Neurosci **24**(32): 7150-7158.
Maaswinkel, H. and L. Li (2003). "Spatio-temporal frequency characteristics of the optomotor response in zebrafish." Vision Res **43**(1): 21-30.
MacDonald, R. B., O. Randlett, J. Oswald, T. Yoshimatsu, K. Franze and W. A. Harris (2015). "Muller glia provide essential tensile strength to the developing retina." J Cell Biol **210**(7): 1075-1083.
Mader, M. M. and D. A. Cameron (2004). "Photoreceptor differentiation during retinal development, growth, and regeneration in a metamorphic vertebrate." J Neurosci **24**(50): 11463-11472.
Maier, W. and H. Wolburg (1979). "Regeneration of the goldfish retina after exposure to different doses of ouabain." Cell Tissue Res **202**(1): 99-118.
Mangrum, W. I., J. E. Dowling and E. D. Cohen (2002). "A morphological classification of ganglion cells in the zebrafish retina." Vis Neurosci **19**(6): 767-779.
Mann, I. (1964). " The Development of the Human Eye." Grune and Stratton Inc: 1-45.
Marchioro, M., J. M. Nunes, A. M. Ramalho, A. Molowny, E. Perez-Martinez, X. Ponsoda and C. Lopez-Garcia (2005). "Postnatal neurogenesis in the medial cortex of the tropical lizard Tropidurus hispidus." Neuroscience **134**(2): 407-413.
Marquardt, T., R. Ashery-Padan, N. Andrejewski, R. Scardigli, F. Guillemot and P. Gruss (2001). "Pax6 is required for the multipotent state of retinal progenitor cells." Cell **105**(1): 43-55.
Martinez-De Luna, R. I., L. E. Kelly and H. M. El-Hodiri (2011). "The Retinal Homeobox (Rx) gene is necessary for retinal regeneration." Developmental Biology **353**(1): 10-18.
Masland, R. H. (2012). "The neuronal organization of the retina." Neuron **76**(2): 266-280.
Mata, N. L., R. A. Radu, R. C. Clemmons and G. H. Travis (2002). "Isomerization and oxidation of vitamin a in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight." Neuron **36**(1): 69-80.
Mathers, P. H., A. Grinberg, K. A. Mahon and M. Jamrich (1997). "The Rx homeobox gene is essential for vertebrate eye development." Nature **387**(6633): 603-607.
McMahon, D. G. (1994). "Modulation of electrical synaptic transmission in zebrafish retinal horizontal cells." J Neurosci **14**(3 Pt 2): 1722-1734.
Mensinger, A. F. and M. K. Powers (2007). "Visual function in regenerating teleost retina following surgical lesioning." Vis Neurosci **24**(3): 299-307.
Meyers, J. R., L. Hu, A. Moses, K. Kaboli, A. Papandrea and P. A. Raymond (2012). "beta-catenin/Wnt signaling controls progenitor fate in the developing and regenerating zebrafish retina." Neural Dev **7**: 30.
Ming, G. L. and H. Song (2011). "Adult neurogenesis in the mammalian brain: significant answers and significant questions." Neuron **70**(4): 687-702.

Mohamed, T., A. Shakeri and P. P. Rao (2016). "Amyloid cascade in Alzheimer's disease: Recent advances in medicinal chemistry." Eur J Med Chem **113**: 258-272.
Montague, P. R. and M. J. Friedlander (1991). "Morphogenesis and territorial coverage by isolated mammalian retinal ganglion cells." J Neurosci **11**(5): 1440-1457.
Montgomery, J. E., M. J. Parsons and D. R. Hyde (2010). "A novel model of retinal ablation demonstrates that the extent of rod cell death regulates the origin of the regenerated zebrafish rod photoreceptors." J Comp Neurol **518**(6): 800-814.
Moore, K. B., K. Mood, I. O. Daar and S. A. Moody (2004). "Morphogenetic movements underlying eye field formation require interactions between the FGF and ephrinB1 signaling pathways." Dev Cell **6**(1): 55-67.
Morris, A. C., T. Scholz and J. M. Fadool (2008). "Rod progenitor cells in the mature zebrafish retina." Adv Exp Med Biol **613**: 361-368.
Morrow, E. M., M. J. Belliveau and C. L. Cepko (1998). "Two phases of rod photoreceptor differentiation during rat retinal development." J Neurosci **18**(10): 3738-3748.
Moshiri, A., C. R. McGuire and T. A. Reh (2005). "Sonic hedgehog regulates proliferation of the retinal ciliary marginal zone in posthatch chicks." Dev Dyn **233**(1): 66-75.
Nagashima, M., L. K. Barthel and P. A. Raymond (2013). "A self-renewing division of zebrafish Muller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons." Development **140**(22): 4510-4521.
Nakhai, H., S. Sel, J. Favor, L. Mendoza-Torres, F. Paulsen, G. I. Duncker and R. M. Schmid (2007). "Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina." Development **134**(6): 1151-1160.
Nash, B. M., D. C. Wright, J. R. Grigg, B. Bennetts and R. V. Jamieson (2015). "Retinal dystrophies, genomic applications in diagnosis and prospects for therapy." Transl Pediatr **4**(2): 139-163.
Nawabi, H., K. Zukor and Z. He (2012). "No simpler than mammals: axon and dendrite regeneration in Drosophila." Genes Dev **26**(14): 1509-1514.
Nawrocki, L., R. BreMiller, G. Streisinger and M. Kaplan (1985). "Larval and adult visual pigments of the zebrafish, Brachydanio rerio." Vision Res **25**(11): 1569-1576.
Nelson, C. M., R. A. Gorsuch, T. J. Bailey, K. M. Ackerman, S. C. Kassen and D. R. Hyde (2012). "Stat3 defines three populations of Muller glia and is required for initiating maximal muller glia proliferation in the regenerating zebrafish retina." J Comp Neurol **520**(18): 4294-4311.
Nelson, C. M. and D. R. Hyde (2012). "Muller glia as a source of neuronal progenitor cells to regenerate the damaged zebrafish retina." Adv Exp Med Biol **723**: 425-430.
Newman, E. and A. Reichenbach (1996). "The Muller cell: a functional element of the retina." Trends Neurosci **19**(8): 307-312.
Ng Chi Kei, J., S. Dudczig, P. D. Currie and P. R. Jusuf (2016). "Feedback from each retinal neuron population drives expression of subsequent fate determinant genes without influencing the cell cycle exit timing." J Comp Neurol.
Ng, J. C., P.; Jusuf, P. (2013). "The Regenerative Potential of the Vertebrate Retina - Lessons from the Zebrafish." Springer Book Series - Regenerative Biology of the Eye.
Noctor, S. C., A. C. Flint, T. A. Weissman, R. S. Dammerman and A. R. Kriegstein (2001). "Neurons derived from radial glial cells establish radial units in neocortex." Nature **409**(6821): 714-720.
Norden, C., S. Young, B. A. Link and W. A. Harris (2009). "Actomyosin is the main driver of interkinetic nuclear migration in the retina." Cell **138**(6): 1195-1208.
Novikova Iu, P., V. A. Poplinskaia, K. S. Aleinikova and E. N. Grigorian (2008). "[A study of the localization and accumulation of S-phase cells in the retina of newt Pleurodeles waltl after experimental pigment epithelial detachment]." Ontogenez **39**(2): 143-150.
O'Brien, E. E., U. Greferath, K. A. Vessey, A. I. Jobling and E. L. Fletcher (2012). "Electronic restoration of vision in those with photoreceptor degenerations." Clin Exp Optom **95**(5): 473-483.

Ohnuma, S., S. Hopper, K. C. Wang, A. Philpott and W. A. Harris (2002). "Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina." Development **129**(10): 2435-2446.
Ohsawa, R. and R. Kageyama (2008). "Regulation of retinal cell fate specification by multiple transcription factors." Brain Res **1192**: 90-98.
Okawa, H., M. Hoon, T. Yoshimatsu, L. Della Santina and R. O. Wong (2014). "Illuminating the multifaceted roles of neurotransmission in shaping neuronal circuitry." Neuron **83**(6): 1303-1318.
Onuma, Y., S. Takahashi, M. Asashima, S. Kurata and W. J. Gehring (2002). "Conservation of Pax 6 function and upstream activation by Notch signaling in eye development of frogs and flies." Proc Natl Acad Sci U S A **99**(4): 2020-2025.
Organization, W. H. (2012). "Global Data on Visual Impairment 2010."
Otteson, D. C., A. R. D'Costa and P. F. Hitchcock (2001). "Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish." Dev Biol **232**(1): 62-76.
Page, A., P. Paoli, E. Moran Salvador, S. White, J. French and J. Mann (2016). "Hepatic stellate cell transdifferentiation involves genome-wide remodeling of the DNA methylation landscape." J Hepatol **64**(3): 661-673.
Park, C. M. and M. J. Hollenberg (1989). "Basic fibroblast growth factor induces retinal regeneration in vivo." Dev Biol **134**(1): 201-205.
Paunesku, T., S. Mittal, M. Protic, J. Oryhon, S. V. Korolev, A. Joachimiak and G. E. Woloschak (2001). "Proliferating cell nuclear antigen (PCNA): ringmaster of the genome." Int J Radiat Biol **77**(10): 1007-1021.
Pearson, R. A. (2014). "Advances in repairing the degenerate retina by rod photoreceptor transplantation." Biotechnol Adv **32**(2): 485-491.
Pei, Y. F. and J. A. Rhodin (1970). "The prenatal development of the mouse eye." Anat Rec **168**(1): 105-125.
Penn, J. S. (1985). "Effects of continuous light on the retina of a fish, Notemigonus crysoleucas." J Comp Neurol **238**(1): 121-127.
Perlman, H., L. Maillard, K. Krasinski and K. Walsh (1997). "Evidence for the rapid onset of apoptosis in medial smooth muscle cells after balloon injury." Circulation **95**(4): 981-987.
Perron, M. and W. A. Harris (2000). "Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors." Cell Mol Life Sci **57**(2): 215-223.
Peterson, R. E., J. M. Fadool, J. McClintock and P. J. Linser (2001). "Muller cell differentiation in the zebrafish neural retina: evidence of distinct early and late stages in cell maturation." J Comp Neurol **429**(4): 530-540.
Phillips, M. I. (2012). "Gene, stem cell, and future therapies for orphan diseases." Clin Pharmacol Ther **92**(2): 182-192.
Pilz, G. A., A. Shitamukai, I. Reillo, E. Pacary, J. Schwausch, R. Stahl, J. Ninkovic, H. J. Snippert, H. Clevers, L. Godinho, F. Guillemot, V. Borrell, F. Matsuzaki and M. Gotz (2013). "Amplification of progenitors in the mammalian telencephalon includes a new radial glial cell type." Nat Commun **4**: 2125.
Pisharath, H. (2007). "Validation of nitroreductase, a prodrug-activating enzyme, mediated cell death in embryonic zebrafish (Danio rerio)." Comp Med **57**(3): 241-246.
Pittack, C., M. Jones and T. A. Reh (1991). "Basic fibroblast growth factor induces retinal pigment epithelium to generate neural retina in vitro." Development **113**(2): 577-588.
Poggi, L., M. Vitorino, I. Masai and W. A. Harris (2005). "Influences on neural lineage and mode of division in the zebrafish retina in vivo." J Cell Biol **171**(6): 991-999.
Pollak, J., M. S. Wilken, Y. Ueki, K. E. Cox, J. M. Sullivan, R. J. Taylor, E. M. Levine and T. A. Reh (2013). "ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors." Development **140**(12): 2619-2631.
Prasov, L., T. Masud, S. Khaliq, S. Q. Mehdi, A. Abid, E. R. Oliver, E. D. Silva, A. Lewanda, M. C. Brodsky, M. Borchert, D. Kelberman, J. C. Sowden, M. T. Dattani and T. Glaser (2012). "ATOH7

mutations cause autosomal recessive persistent hyperplasia of the primary vitreous." Hum Mol Genet **21**(16): 3681-3694.
Purves D, A. G., Fitzpatrick D, Katz LC, LaMantia A, McNamara JO, Williams SM (2001). "Neural Circuits." Neuroscience. 2nd edition.
Qin, Z., L. K. Barthel and P. A. Raymond (2009). "Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish." Proc Natl Acad Sci U S A **106**(23): 9310-9315.
Qin, Z., A. R. Kidd, 3rd, J. L. Thomas, K. D. Poss, D. R. Hyde, P. A. Raymond and R. Thummel (2011). "FGF signaling regulates rod photoreceptor cell maintenance and regeneration in zebrafish." Exp Eye Res **93**(5): 726-734.
Rakic, P. (2002). "Neurogenesis in adult primate neocortex: an evaluation of the evidence." Nat Rev Neurosci **3**(1): 65-71.
Ramachandran, R., B. V. Fausett and D. Goldman (2010). "Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway." Nat Cell Biol **12**(11): 1101-1107.
Ramsden, C. M., M. B. Powner, A. J. Carr, M. J. Smart, L. da Cruz and P. J. Coffey (2013). "Stem cells in retinal regeneration: past, present and future." Development **140**(12): 2576-2585.
Randlett, O., R. B. MacDonald, T. Yoshimatsu, A. D. Almeida, S. C. Suzuki, R. O. Wong and W. A. Harris (2013). "Cellular requirements for building a retinal neuropil." Cell Rep **3**(2): 282-290.
Rapaport, D. H., J. T. Fletcher, M. M. LaVail and P. Rakic (1992). "Genesis of neurons in the retinal ganglion cell layer of the monkey." J Comp Neurol **322**(4): 577-588.
Rapaport, D. H., L. L. Wong, E. D. Wood, D. Yasumura and M. M. LaVail (2004). "Timing and topography of cell genesis in the rat retina." J Comp Neurol **474**(2): 304-324.
Raymond, P. A., L. K. Barthel, R. L. Bernardos and J. J. Perkowski (2006). "Molecular characterization of retinal stem cells and their niches in adult zebrafish." BMC Dev Biol **6**: 36.
Raymond, P. A., L. K. Barthel, M. E. Rounsifer, S. A. Sullivan and J. K. Knight (1993). "Expression of rod and cone visual pigments in goldfish and zebrafish: a rhodopsin-like gene is expressed in cones." Neuron **10**(6): 1161-1174.
Raymond, P. A. and P. K. Rivlin (1987). "Germinal cells in the goldfish retina that produce rod photoreceptors." Dev Biol **122**(1): 120-138.
Reese, B. E., P. W. Keeley, S. C. Lee and I. E. Whitney (2011). "Developmental plasticity of dendritic morphology and the establishment of coverage and connectivity in the outer retina." Dev Neurobiol **71**(12): 1273-1285.
Reese, B. E., B. D. Necessary, P. P. Tam, B. Faulkner-Jones and S. S. Tan (1999). "Clonal expansion and cell dispersion in the developing mouse retina." Eur J Neurosci **11**(8): 2965-2978.
Reh, T. A. (1987). "Cell-specific regulation of neuronal production in the larval frog retina." J Neurosci **7**(10): 3317-3324.
Reh, T. A., M. Jones and C. Pittack (1991). "Common mechanisms of retinal regeneration in the larval frog and embryonic chick." Ciba Found Symp **160**: 192-204; discussion 204-198.
Reh, T. A., T. Nagy and H. Gretton (1987). "Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin." Nature **330**(6143): 68-71.
Reh, T. A. and T. Tully (1986). "Regulation of tyrosine hydroxylase-containing amacrine cell number in larval frog retina." Dev Biol **114**(2): 463-469.
Reichenbach, A. and A. Bringmann (2013). "New functions of Muller cells." Glia **61**(5): 651-678.
Reis, L. M., A. Khan, A. Kariminejad, F. Ebadi, R. C. Tyler and E. V. Semina (2011). "VSX2 mutations in autosomal recessive microphthalmia." Mol Vis **17**: 2527-2532.
Rhyu, M. S., L. Y. Jan and Y. N. Jan (1994). "Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells." Cell **76**(3): 477-491.
Richard-Parpaillon, L., C. Heligon, F. Chesnel, D. Boujard and A. Philpott (2002). "The IGF pathway regulates head formation by inhibiting Wnt signaling in Xenopus." Dev Biol **244**(2): 407-417.
Roesch, K., A. P. Jadhav, J. M. Trimarchi, M. B. Stadler, B. Roska, B. B. Sun and C. L. Cepko (2008). "The transcriptome of retinal Muller glial cells." J Comp Neurol **509**(2): 225-238.

Romero-Aleman, M. M., M. Monzon-Mayor, C. Yanes and D. Lang (2004). "Radial glial cells, proliferating periventricular cells, and microglia might contribute to successful structural repair in the cerebral cortex of the lizard Gallotia galloti." Exp Neurol **188**(1): 74-85.
Sadler, T. W. (2005). "Embryology of neural tube development." Am J Med Genet C Semin Med Genet **135C**(1): 2-8.
Sagasti, A., M. R. Guido, D. W. Raible and A. F. Schier (2005). "Repulsive interactions shape the morphologies and functional arrangement of zebrafish peripheral sensory arbors." Curr Biol **15**(9): 804-814.
Sakami, S., P. Etter and T. A. Reh (2008). "Activin signaling limits the competence for retinal regeneration from the pigmented epithelium." Mech Dev **125**(1-2): 106-116.
Santos, E., M. Monzon-Mayor, M. M. Romero-Aleman and C. Yanes (2008). "Distribution of neurotrophin-3 during the ontogeny and regeneration of the lizard (Gallotia galloti) visual system." Dev Neurobiol **68**(1): 31-44.
Santos, G. W. (1971). "Application of marrow grafts in human disease." Am J Pathol **65**(3): 653-668.
Saszik, S., J. Bilotta and C. M. Givin (1999). "ERG assessment of zebrafish retinal development." Vis Neurosci **16**(5): 881-888.
Scaravilli, F. (1984). "The influence of distal environment on peripheral nerve regeneration across a gap." J Neurocytol **13**(6): 1027-1041.
Scharff, C., J. R. Kirn, M. Grossman, J. D. Macklis and F. Nottebohm (2000). "Targeted neuronal death affects neuronal replacement and vocal behavior in adult songbirds." Neuron **25**(2): 481-492.
Schimmer, J. and S. Breazzano (2015). "Investor Outlook: Significance of the Positive LCA2 Gene Therapy Phase III Results." Hum Gene Ther Clin Dev **26**(4): 208-210.
Schmidt, R., U. Strahle and S. Scholpp (2013). "Neurogenesis in zebrafish - from embryo to adult." Neural Dev **8**: 3.
Schmitt, E. A. and J. E. Dowling (1994). "Early eye morphogenesis in the zebrafish, Brachydanio rerio." J Comp Neurol **344**(4): 532-542.
Schneider, M. L., D. L. Turner and M. L. Vetter (2001). "Notch signaling can inhibit Xath5 function in the neural plate and developing retina." Mol Cell Neurosci **18**(5): 458-472.
Schook, P. (1980a). "Morphogenetic movements during the early development of the chick eye. A light microscopic and spatial reconstructive study." Acta Morphol Neerl Scand **18**(1): 1-30.
Schook, P. (1980b). "Morphogenetic movements during the early development of the chick eye. An ultrastructural and spatial reconstructive study. B. Invagination of the optic vesicle and fusion of its walls." Acta Morphol Neerl Scand **18**(23): 159-180.
Scott, E. K. and H. Baier (2009). "The cellular architecture of the larval zebrafish tectum, as revealed by gal4 enhancer trap lines." Front Neural Circuits **3**: 13.
Scott, E. K., L. Mason, A. B. Arrenberg, L. Ziv, N. J. Gosse, T. Xiao, N. C. Chi, K. Asakawa, K. Kawakami and H. Baier (2007). "Targeting neural circuitry in zebrafish using GAL4 enhancer trapping." Nat Methods **4**(4): 323-326.
Senut, M. C., A. Gulati-Leekha and D. Goldman (2004). "An element in the alpha1-tubulin promoter is necessary for retinal expression during optic nerve regeneration but not after eye injury in the adult zebrafish." J Neurosci **24**(35): 7663-7673.
Shahinfar, S., D. P. Edward and M. O. Tso (1991). "A pathologic study of photoreceptor cell death in retinal photic injury." Curr Eye Res **10**(1): 47-59.
Sharma, S. C. and F. Ungar (1980). "Histogenesis of the goldfish retina." J Comp Neurol **191**(3): 373-382.
Sherpa, T., S. M. Fimbel, D. E. Mallory, H. Maaswinkel, S. D. Spritzer, J. A. Sand, L. Li, D. R. Hyde and D. L. Stenkamp (2008). "Ganglion cell regeneration following whole-retina destruction in zebrafish." Dev Neurobiol **68**(2): 166-181.
Shimazaki, T. (2016). "Vertebrate Neural Stem Cells: Development, Plasticity,and Regeneration." Keio J Med.

Silver, J. and J. Sapiro (1981). "Axonal guidance during development of the optic nerve: the role of pigmented epithelia and other extrinsic factors." J Comp Neurol **202**(4): 521-538.
Silvestroff, L., S. Bartucci, E. Soto, V. Gallo, J. Pasquini and P. Franco (2010). "Cuprizone-induced demyelination in CNP::GFP transgenic mice." J Comp Neurol **518**(12): 2261-2283.
Slack, J. M., G. Lin and Y. Chen (2008). "The Xenopus tadpole: a new model for regeneration research." Cell Mol Life Sci **65**(1): 54-63.
Song, M. J. and K. Bharti (2015). "Looking into the future: Using induced pluripotent stem cells to build two and three dimensional ocular tissue for cell therapy and disease modeling." Brain Res.
Spear, P. C. and C. A. Erickson (2012). "Apical movement during interkinetic nuclear migration is a two-step process." Dev Biol **370**(1): 33-41.
Spence, J. R., J. C. Aycinena and K. Del Rio-Tsonis (2007). "Fibroblast growth factor-hedgehog interdependence during retina regeneration." Dev Dyn **236**(5): 1161-1174.
Spence, J. R., M. Madhavan, J. D. Ewing, D. K. Jones, B. M. Lehman and K. Del Rio-Tsonis (2004). "The hedgehog pathway is a modulator of retina regeneration." Development **131**(18): 4607-4621.
Stamm, C., B. Westphal, H. D. Kleine, M. Petzsch, C. Kittner, H. Klinge, C. Schumichen, C. A. Nienaber, M. Freund and G. Steinhoff (2003). "Autologous bone-marrow stem-cell transplantation for myocardial regeneration." Lancet **361**(9351): 45-46.
Stenkamp, D. L. (2007). "Neurogenesis in the fish retina." Int Rev Cytol **259**: 173-224.
Stenkamp, D. L. (2015). "Development of the Vertebrate Eye and Retina." Prog Mol Biol Transl Sci **134**: 397-414.
Stern, J. and S. Temple (2015). "Retinal pigment epithelial cell proliferation." Exp Biol Med (Maywood) **240**(8): 1079-1086.
Stevens, C. F. (1998). "Neuronal diversity: too many cell types for comfort?" Curr Biol **8**(20): R708-710.
Stiemke, M. M. and J. G. Hollyfield (1995). "Cell birthdays in Xenopus laevis retina." Differentiation **58**(3): 189-193.
Stone, J. S. and E. W. Rubel (2000). "Cellular studies of auditory hair cell regeneration in birds." Proc Natl Acad Sci U S A **97**(22): 11714-11721.
Stone, L. S. and H. Steinitz (1957). "Regeneration of neural retina and lens from retina pigment cell grafts in adult newts." J Exp Zool **135**(2): 301-317.
Sullivan, S. A., L. K. Barthel, B. L. Largent and P. A. Raymond (1997). "A goldfish Notch-3 homologue is expressed in neurogenic regions of embryonic, adult, and regenerating brain and retina." Dev Genet **20**(3): 208-223.
Sun, Q., S. Bahri, A. Schmid, W. Chia and K. Zinn (2000). "Receptor tyrosine phosphatases regulate axon guidance across the midline of the Drosophila embryo." Development **127**(4): 801-812.
Sundermeier, T. R. and K. Palczewski (2015). "The impact of microRNA gene regulation on the survival and function of mature cell types in the eye." FASEB J.
Sustar, M., D. Perovsek, I. Cima, B. Stirn-Kranjc, M. Hawlina and J. Brecelj (2015). "Electroretinography and optical coherence tomography reveal abnormal post-photoreceptoral activity and altered retinal lamination in patients with enhanced S-cone syndrome." Doc Ophthalmol **130**(3): 165-177.
Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-676.
Takeda, M., A. Takamiya, J. W. Jiao, K. S. Cho, S. G. Trevino, T. Matsuda and D. F. Chen (2008). "alpha-Aminoadipate induces progenitor cell properties of Muller glia in adult mice." Invest Ophthalmol Vis Sci **49**(3): 1142-1150.
Tan, M. M. and A. R. Harvey (1997). "A comparison of postlesion growth of retinotectal and corticotectal axons after superior colliculus transections in neonatal rats." J Comp Neurol **386**(4): 681-699.
Tanaka, E. M. and P. W. Reddien (2011). "The cellular basis for animal regeneration." Dev Cell **21**(1): 172-185.

Thomas, J. L., C. M. Nelson, X. Luo, D. R. Hyde and R. Thummel (2012). "Characterization of multiple light damage paradigms reveals regional differences in photoreceptor loss." Exp Eye Res **97**(1): 105-116.
Thummel, R., J. M. Enright, S. C. Kassen, J. E. Montgomery, T. J. Bailey and D. R. Hyde (2010). "Pax6a and Pax6b are required at different points in neuronal progenitor cell proliferation during zebrafish photoreceptor regeneration." Exp Eye Res **90**(5): 572-582.
Thummel, R., S. C. Kassen, J. M. Enright, C. M. Nelson, J. E. Montgomery and D. R. Hyde (2008). "Characterization of Muller glia and neuronal progenitors during adult zebrafish retinal regeneration." Exp Eye Res **87**(5): 433-444.
Thummel, R., S. C. Kassen, J. E. Montgomery, J. M. Enright and D. R. Hyde (2008). "Inhibition of Muller glial cell division blocks regeneration of the light-damaged zebrafish retina." Dev Neurobiol **68**(3): 392-408.
Tropepe, V., B. L. Coles, B. J. Chiasson, D. J. Horsford, A. J. Elia, R. R. McInnes and D. van der Kooy (2000). "Retinal stem cells in the adult mammalian eye." Science **287**(5460): 2032-2036.
Tyler, M. J., L. H. Carney and D. A. Cameron (2005). "Control of cellular pattern formation in the vertebrate inner retina by homotypic regulation of cell-fate decisions." J Neurosci **25**(18): 4565-4576.
Udvadia, A. J., R. W. Koster and J. H. Skene (2001). "GAP-43 promoter elements in transgenic zebrafish reveal a difference in signals for axon growth during CNS development and regeneration." Development **128**(7): 1175-1182.
Ueki, Y. and T. A. Reh (2013). "EGF stimulates Muller glial proliferation via a BMP-dependent mechanism." Glia **61**(5): 778-789.
Ueki, Y., M. S. Wilken, K. E. Cox, L. Chipman, N. Jorstad, K. Sternhagen, M. Simic, K. Ullom, M. Nakafuku and T. A. Reh (2015). "Transgenic expression of the proneural transcription factor Ascl1 in Muller glia stimulates retinal regeneration in young mice." Proc Natl Acad Sci U S A **112**(44): 13717-13722.
Valente, R. C., L. S. Capella, R. Q. Monteiro, V. M. Rumjanek, A. G. Lopes and M. A. Capella (2003). "Mechanisms of ouabain toxicity." FASEB J **17**(12): 1700-1702.
Valter, K., J. Maslim, F. Bowers and J. Stone (1998). "Photoreceptor dystrophy in the RCS rat: roles of oxygen, debris, and bFGF." Invest Ophthalmol Vis Sci **39**(12): 2427-2442.
Van Houcke, J., L. De Groef, E. Dekeyster and L. Moons (2015). "The zebrafish as a gerontology model in nervous system aging, disease, and repair." Ageing Res Rev **24**(Pt B): 358-368.
Van Raay, T. J. and M. L. Vetter (2004). "Wnt/frizzled signaling during vertebrate retinal development." Dev Neurosci **26**(5-6): 352-358.
Verma, I. M. and N. Somia (1997). "Gene therapy -- promises, problems and prospects." Nature **389**(6648): 239-242.
Vihtelic, T. S. and D. R. Hyde (2000). "Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (Danio rerio) retina." J Neurobiol **44**(3): 289-307.
Vihtelic, T. S., J. E. Soverly, S. C. Kassen and D. R. Hyde (2006). "Retinal regional differences in photoreceptor cell death and regeneration in light-lesioned albino zebrafish." Exp Eye Res **82**(4): 558-575.
Vitorino, M., P. R. Jusuf, D. Maurus, Y. Kimura, S. Higashijima and W. A. Harris (2009). "Vsx2 in the zebrafish retina: restricted lineages through derepression." Neural Dev **4**: 14.
Wallace, V. A. (2008). "Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina." Brain Res **1192**: 61-75.
Walls, G. I. (1967). "The Vertebrate Eye and Its Adaptive Radiation." New York: Hafner.
Walmsley, G. G., Z. N. Maan, V. W. Wong, D. Duscher, M. S. Hu, E. R. Zielins, T. Wearda, E. Muhonen, A. McArdle, R. Tevlin, D. A. Atashroo, K. Senarath-Yapa, H. P. Lorenz, G. C. Gurtner and M. T. Longaker (2015). "Scarless wound healing: chasing the holy grail." Plast Reconstr Surg **135**(3): 907-917.
Walsh, N., K. Valter and J. Stone (2001). "Cellular and subcellular patterns of expression of bFGF and CNTF in the normal and light stressed adult rat retina." Exp Eye Res **72**(5): 495-501.

Wan, J., R. Ramachandran and D. Goldman (2012). "HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration." Dev Cell **22**(2): 334-347.
Wan, J., H. Zheng, Z. L. Chen, H. L. Xiao, Z. J. Shen and G. M. Zhou (2008). "Preferential regeneration of photoreceptor from Muller glia after retinal degeneration in adult rat." Vision Res **48**(2): 223-234.
Wan, J., H. Zheng, H. L. Xiao, Z. J. She and G. M. Zhou (2007). "Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina." Biochem Biophys Res Commun **363**(2): 347-354.
Wang, S. W., B. S. Kim, K. Ding, H. Wang, D. Sun, R. L. Johnson, W. H. Klein and L. Gan (2001). "Requirement for math5 in the development of retinal ganglion cells." Genes Dev **15**(1): 24-29.
Wassle, H., C. Puller, F. Muller and S. Haverkamp (2009). "Cone contacts, mosaics, and territories of bipolar cells in the mouse retina." J Neurosci **29**(1): 106-117.
Weber, I. P., A. P. Ramos, P. J. Strzyz, L. C. Leung, S. Young and C. Norden (2014). "Mitotic position and morphology of committed precursor cells in the zebrafish retina adapt to architectural changes upon tissue maturation." Cell Rep **7**(2): 386-397.
Wen, R., Y. Song, T. Cheng, M. T. Matthes, D. Yasumura, M. M. LaVail and R. H. Steinberg (1995). "Injury-induced upregulation of bFGF and CNTF mRNAS in the rat retina." J Neurosci **15**(11): 7377-7385.
Wen, R., W. Tao, Y. Li and P. A. Sieving (2012). "CNTF and retina." Prog Retin Eye Res **31**(2): 136-151.
Wilkinson-Berka, J. L. and A. G. Miller (2008). "Update on the treatment of diabetic retinopathy." ScientificWorldJournal **8**: 98-120.
Wilson, S. G., W. Wen, L. Pillai-Kastoori and A. C. Morris (2015). "Tracking the fate of her4 expressing cells in the regenerating retina using her4:Kaede zebrafish." Exp Eye Res **145**: 75-87.
Wong, L. L. and D. H. Rapaport (2009). "Defining retinal progenitor cell competence in Xenopus laevis by clonal analysis." Development **136**(10): 1707-1715.
Wong, R. O. and L. Godinho (2003). "Development of the vertebrate retina." The Visual Neurosciences **Chalupa LM Werner JS eds.** (MIT Press Cambridge, MA): 77–93.
Wu, D. M., T. Schneiderman, J. Burgett, P. Gokhale, L. Barthel and P. A. Raymond (2001). "Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina." Invest Ophthalmol Vis Sci **42**(9): 2115-2124.
Yang, E. V., L. Wang and R. A. Tassava (2005). "Effects of exogenous FGF-1 treatment on regeneration of the lens and the neural retina in the newt, Notophthalmus viridescens." J Exp Zool A Comp Exp Biol **303**(10): 837-844.
Young, R. W. (1985). "Cell proliferation during postnatal development of the retina in the mouse." Brain Res **353**(2): 229-239.
Yurco, P. and D. A. Cameron (2005). "Responses of Muller glia to retinal injury in adult zebrafish." Vision Res **45**(8): 991-1002.
Yurco, P. and D. A. Cameron (2007). "Cellular correlates of proneural and Notch-delta gene expression in the regenerating zebrafish retina." Vis Neurosci **24**(3): 437-443.
Zaghloul, N. A., B. Yan and S. A. Moody (2005). "Step-wise specification of retinal stem cells during normal embryogenesis." Biol Cell **97**(5): 321-337.
Zhang, R., P. Han, H. Yang, K. Ouyang, D. Lee, Y. F. Lin, K. Ocorr, G. Kang, J. Chen, D. Y. Stainier, D. Yelon and N. C. Chi (2013). "In vivo cardiac reprogramming contributes to zebrafish heart regeneration." Nature **498**(7455): 497-501.
Zhang, R., Z. Zhang, C. Zhang, L. Zhang, A. Robin, Y. Wang, M. Lu and M. Chopp (2004). "Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat." J Neurosci **24**(25): 5810-5815.
Zhao, X. F., S. Ellingsen and A. Fjose (2009). "Labelling and targeted ablation of specific bipolar cell types in the zebrafish retina." BMC Neurosci **10**: 107.
Zhu, M., M. Li, D. Ye, W. Jiang, T. Lei and K. Shu (2016). "Sensory symptoms in Parkinson's disease: Clinical features, pathophysiology, and treatment." J Neurosci Res.
Zolessi, F. R., L. Poggi, C. J. Wilkinson, C. B. Chien and W. A. Harris (2006). "Polarization and orientation of retinal ganglion cells in vivo." Neural Dev **1**: 2.

Zou, S. Q., W. Yin, M. J. Zhang, C. R. Hu, Y. B. Huang and B. Hu (2010). "Using the optokinetic response to study visual function of zebrafish." J Vis Exp(36).
Zuber, M. E., G. Gestri, A. S. Viczian, G. Barsacchi and W. A. Harris (2003). "Specification of the vertebrate eye by a network of eye field transcription factors." Development **130**(21): 5155-5167.
Zupanc, G. K. and R. F. Sirbulescu (2011). "Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish." Eur J Neurosci **34**(6): 917-929.