Cell death and proliferation characteristics of the retina after optic nerve section in chickens

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.
Abstract

Optic nerve section (ONS) is an experimental model for damage of the optic nerve associated with diseases such as glaucoma and optic neuritis. Damage to the optic nerve causes loss of retinal ganglion cells that are attached, once the cells are damaged, they are not typically replaced. Recently, Fischer and Reh (2003) found that Müller glia have the potential to adopt phenotypes and functional capabilities similar to those of retinal progenitors, a potential source of retinal regeneration. In the chick, when the specific retinal cells are targeted for damage by chemotoxins, there is widespread apoptosis but also mitotically active cells that label with retinal progenitor markers. Fischer and Reh (2002) also discovered that the combination of growth factors FGF2 and insulin is capable of stimulating the regenerative response of the Müller glia to retinal progenitor cells in chick eyes. This study was conducted to analyse damage to the ganglion cells by optic nerve section in chicks to determine the effect of age on the cell death timeline, the proliferative qualities of the retina and to see if injections of growth factors had the ability to increase the proliferation. Histological methods were used to analyse cellular changes and ultrasound to monitor eye growth. Apoptotic activity preceded retinal thinning and ganglion cell loss, indicating that ONS-related cell death is mediated at least in part by apoptotic mechanisms and age did not affect the time course, although, age did affect the eye growth changes, which may be attributed to the plasticity of the younger eyes. ONS damage elicited proliferative activity in the retina as did growth factor injections alone. The combination of ONS damage and growth factor injections increased the proliferative activity and the overall total number of cells in the ganglion cell layer. These findings can potentially lead to the development of therapeutic strategies for the preservation or restoration of retinal cells in diseased eyes.
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List of Abbreviations

°C ........................................................................................................... degrees Celsius
µm .......................................................................................................... micrometer
AC ........................................................................................................... anterior chamber
AION ....................................................................................................... anterior ischemic optic neuropathy
ANOVA ................................................................................................ analysis of variance
ATP ........................................................................................................ adenosine triphosphate
bFGF .................................................................................................... basic fibroblast growth factor
BrdU ...................................................................................................... 5-bromo-2-deoxyuridine
BSA ....................................................................................................... bovine serum albumin
CAD ....................................................................................................... caspase-activated-deoxyribonuclease
CARD .................................................................................................. caspase recruitment domain
caspases ............................................................................................... cysteine-dependent aspartate-cleaving proteases
ced .......................................................................................................... cell-death abnormality
CH ........................................................................................................... choroid
CNS ....................................................................................................... central nervous system
CRA ....................................................................................................... central retinal artery
DAPI ...................................................................................................... 4',6-diamidino-2-phenylindole
dATP .................................................................................................... deoxyadenosine triphosphate
DED ...................................................................................................... death effector domain
DFF ....................................................................................................... DNA fragmentation factor
DISC ...................................................................................................... death-inducing signaling complex
dpf ........................................................................................................... days post fertilisation
E .............................................................................................................. embryological day
EdU ....................................................................................................... 5-ethynyl-2’-deoxyuridine
FGF2 ..................................................................................................... fibroblast growth factor 2
FITC ...................................................................................................... fluorescein isothiocyanate
g/L ......................................................................................................... grams/litre
GCL ....................................................................................................... ganglion cell layer
hES ........................................................................................................ human embryonic stem cells
VC .................................................................vitreous chamber
w/v .................................................................weight/volume
I. LITERATURE REVIEW

1.1 Development of the human eye

In humans, development of the ocular structures begins during the third week of gestation with the formation of an embryonic plate consisting of three primary germ layers: ectoderm, mesoderm and endoderm (Moore, 1989). The ectoderm and the mesoderm, found below it, form the various ocular structures. As the ectoderm thickens it forms the neural plate that serves as the basis of the central nervous system. At day 18 of gestation, a groove develops in the neural plate forming the neural folds and by day 22 the folds grow toward each other to meet then pinch off to form the neural tube (Moore, 1989). Cells of the neural tube are henceforth known as neuroectoderm and remaining ectoderm at the surface of the embryo is known as the surface ectoderm (Moore, 1989). Neural crest cells bud off of the neuroectoderm and are responsible for various structures within the eye (Moore, 1989).

The eyes are extensions of the neural tube, initially known as optic pits (Oyster, 1999) that proliferate and increase in size until they become the optic vesicles on day 25. The connection between the optic vesicles and the neural tube is the optic stalk (Cook et al., 1994; Oyster, 1999). The contact of the optic vesicle to the surface ectoderm on day 27 causes a thickening of the surface ectoderm, forming the lens placode, and an invagination of the optic vesicle, forming the optic cup (Cook et al., 1994; Oyster, 1999). The surface ectoderm, lens placode and resulting bilayered optic cup contain the progenitor cells that form almost all of the ocular structures within the vertebrate eye.

1.1.1 Development of non-retinal ocular structures

The cornea is the transparent layer at the front of the eye that is composed of corneal epithelium, stroma and endothelium, that helps to focus light. The cornea is stimulated to develop by signals from the developing lens at the time that the lens vesicle detaches from the surface ectoderm at day
The surface ectoderm proliferates, resulting in the establishment of multiple epithelial cell layers that eventually become the corneal epithelium. Corneal epithelium development is complete around the fifth or sixth month of gestation (Zinn and Mochel-Pohl, 1975). The corneal endothelium is formed by the neural crest cells that migrate between the corneal epithelium and the lens and by 4 months gestation the endothelium is a flat single row of cells with a basal lamina that gives rise to Descemet’s membrane (Cook et al., 1994). At week 8, another wave of neural crest cells migrate between the epithelium and the endothelium to give rise to the stroma of the cornea made up of fibroblasts, collagen and ground substance (Oyster, 1999; Pearson, 1969; Remington, 1998). The majority of the layers of the cornea are developed in the first 3 months except for Bowman’s layer, which arises from the superficial neural crest cells in the fifth month of gestation (Cook et al., 1994).

Figure 1 Diagram of the corneal layers.
The crystalline lens is a transparent structure in the eye that helps to focus light along with the cornea. Lens formation begins with the lens vesicle, which is a hollow sphere with walls consisting of a monolayer of epithelial cells that is surrounded by a thin basal lamina, the latter of which eventually develops into the lens capsule (Pearson, 1969; Remington, 1998). The posterior cells of the sphere elongate, eventually filling the lumen; these differentiated cells become the primary lens fibres and form the embryonic nucleus at the centre of the adult lens. The anterior epithelial cells of the lens stay in place, while cells near the equator of the lens undergo mitosis before differentiating anteriorly and posteriorly to form the secondary lens fibres that lie around the embryonic nucleus (Remington, 1998). The close proximity of the developing retina aids in inducing the formation and elongation of the lens cells (Remington, 1998).

The uveal or vascular layer that encompasses the eye consists of the choroid, the ciliary body and the iris. The choroid is the vascular structure that surrounds the eye and supplies oxygen and nutrients to the retina and is located between the retina and the sclera. The choroid develops from the mesoderm and neural crest cells and vessels within the choroid appear in the second month of gestation (Mund et al., 1972). The ciliary body is a tissue located behind the iris and its functions include changing the shape of the lens during accommodation and producing aqueous humour that fills the anterior chamber of the eye that provides nourishment to the posterior surface of the cornea. The ciliary body is composed of pigmented and nonpigmented epithelium that is formed from the epithelial layers of the optic cup. In the fourth month of development, the source of blood that courses through the ciliary body is formed and is followed by the formation of the ciliary muscle from neural crest cells in the fifth month (Oyster, 1999; Remington, 1998). The production of aqueous humour begins between 4-6 months of gestation. Development of the iris, or the coloured part of the eye, begins with the migration of mesenchymal cells of the neural crest origin between the cornea and
lens. These cells form the anterior half of the iris, while the posterior half arises from the ends of the optic cup at the end of the third month. During the fifth and sixth months of gestation, the muscles of the iris form from cells that were derived from the optic cup epithelium (Tamura and Smelser, 1973). The vascular parts of the iris form from the vessels of the long posterior and anterior ciliary artery (Oyster, 1999).

The vitreous is the large gel-like substance localised between the lens and the retina. The vitreous is formed from fibrils derived from the lens, retina and degenerating hyaloid system and begins to accumulate at the end of the 7th week of development (Cook et al., 1994; Oyster, 1999). The primary vitreous is composed of collagen and later is largely compressed and replaced by the secondary vitreous, composed of type II collagen produced by the cells of the developing retina (Oyster, 1999). Proteoglycans and hyaluronic acid are thought to be produced by the cells of the hyaloid artery, mesenchyme and/or the neuroectoderm. When the developing eye no longer requires the hyaloid artery system, it begins to break down and disappear (Oyster, 1999). The vitreous humour fills the majority of the eye and is a clear substance to allow for the unimpeded passage of light.

The sclera, the outer tough covering of the eye, also known as the “white of the eye”, develops from neural crest mesenchymal cells that surround the optic cup. These cells condense in an anterior to posterior direction until complete development of the sclera has occurred, usually by the third month, when all of the globe has been encompassed by the differentiated cells (Duke-Elder and Cook, 1963).
1.1.2 Development of the retina, vessels and optic nerve

While the lens is forming, the optic vesicle invaginates to form the optic cup (Oyster, 1999). A deep groove is formed from the joining of the edges of the cup as they are drawn together and is known as the choroidal or embryonic fissure. During this invagination, the hyaloid artery forms to supply nutrients to the developing structures of the eye (Pearson, 1969). It runs along the fissure and optic stalk before entering the fissure posterior to the optic cup. It then extends anteriorly to the rim of the cup as well as to the lens (Oyster, 1999; Pearson, 1969). The hyaloid artery begins to regress when the lens can grow independent of the blood supply, but atrophy ceases at the posterior portion of the eye that eventually becomes the retina. The hyaloid artery, at this time, branches with the retina, eventually giving rise to the central retinal artery (CRA) and its capillary beds (Oyster, 1999; Pearson, 1969). The edges of the fissure eventually fuse, and in the region of the optic stalk, enclose the central retinal artery as it exits the eye (Oyster, 1999; Pearson, 1969). The rim of the cup becomes
the epithelium of the prospective iris (Pearson, 1969). Lens differentiation and the closing of the choroidal fissure both occur around week 5 to 6 of gestation (Oyster, 1999).

At this point, the optic cup is the primitive retina, and the optic stalk is the optic nerve. The eye generally develops from the center to the periphery. The retinal pigment epithelium (RPE) and retina are the first to develop followed by the uveal tract, the cornea and sclera, finally the extraocular muscles and orbital bones (Oyster, 1999).

The RPE is the first retinal layer to differentiate (Warwick, 1976). At week 3 or 4, melanosomes and cellular structures begin to appear in the outer layer of the optic cup allowing for pigmentation. After week 6, the RPE is one cell layer thick, made of up cuboidal and columnar cells (Hollenberg and Spira, 1972; Mund et al., 1972). The base of each cell is adjacent to the developing choroid and the apical ends of the cells face inward into the optic cup. At week 4 or 5, cells begin to proliferate in the inner layer of the optic cup to form the neural retina. Two zones of cells develop in the inner layer of the optic cup; cells accumulate in outer region called the proliferative or germinating zone, while the inner marginal zone (of His) remains anuclear (Remington, 1998). The separation between the marginal zone of the optic cup and the vitreous later becomes the area where the internal limiting membrane is formed (O’Rahilly, 1975). At week 7, the primitive cells in the proliferative zone separate into an inner and an outer neuroblastic layer of cells, which are divided by a transient fibre layer of Chievitz, a nucleus free region (Hollenberg and Spira, 1972; Smelser et al., 1973). The inner neuroblastic layer contains the differentiating retinal ganglion cells (RGC), amacrine cells, and Müller cells (Uga and Smelser, 1973). The ganglion cells migrate forward towards the vitreous to form a third nuclear layer and by week 8 they begin to send out their axonal processes to form the optic nerve within the optic stalk and these axonal processes form the nerve fibre layer (NFL) of the retina (O’Rahilly, 1975). The outer neuroblastic layer gives rise to the
bipolar, horizontal and photoreceptor (PR) cells (O’Rahilly, 1975). The bipolar and horizontal cells migrate from the outer neuroblastic layer towards the Müller and amacrine cells and gradually the layer of Chievitz disappears (O’Rahilly, 1975). By week 12 there is a clear arrangement of the cells consisting of the photoreceptors in the outermost nuclear layer, eventually known as the outer nuclear layer (ONL), the bipolar, horizontal, amacrine and Müller cells in the middle cellular layer, called the inner nuclear layer (INL) and the ganglion cells and displaced amacrine cells form the innermost layer called the ganglion cell layer (GCL). Cytoplasmic processes develop between the cellular layers and the Müller cells extend their radial fibres to form the internal and external limiting membrane (Cook et al., 1994; Pearson, 1969). The inner plexiform layer (IPL) separates the GCL and the INL and the outer plexiform layer (OPL) separates the INL and the ONL. The photoreceptors are responsible for capturing the light or visual input, the information is then sent through their axons to the bipolar cells. The bipolar cells relay the signal through their axons to the ganglion cells. The ganglion cells then send the signal through their axons that traverse the retina and through the optic nerve that to the visual centres of the brain. The amacrine and horizontal cells modulate the bipolar cell activity and the glial cells, known as Müller cells, are also present to act as support cells.

Although the cells have different functions, it has been shown that there is a single neuroblastic precursor cell that has the ability to develop into at least three types of neurons or two types of neurons and a Müller glial cell (Turner and Cepko, 1987). The retina continues to develop synaptic connections starting with the connections at the centre of the retina and then development continues peripherally. The area that allows for the best visual acuity is known as the fovea and is the last to finish maturing post-natal in humans.
Figure 3 Chick retina labeled with the 3 nuclear and 3 plexiform layers.

The top of the image is closest to the vitreous, the bottom of the image is closest to the choroid and sclera of the eye.

Retinal vessels begin to develop in the fourth month of gestation. Primitive vessels emerge from the previously formed hyaloid artery near the optic nerve and the new vessels insert into the nerve fibre layer. The retinal vessels continue to develop until 3 months after birth. The development of the nasal vessels precede the temporal peripheral vessels (Cook et al., 1994).

The optic stalk is the primitive form of the optic nerve that connects the optic vesicle to the forebrain during development. As the eye develops, the outer layer of the optic stalk becomes a sheath made up of glial tissue that surrounds the optic nerve composed of the axons of the ganglion cells pass as they pass through the lumen to extend to the central nervous system (CNS) along with the central retinal artery (Cook et al., 1994).

1.2 Damage to the retina and optic nerve

Damage to the retina or RGCs and optic nerve can occur in an anterograde or a retrograde fashion (Cowan, 1970). During anterograde damage, the cell or cell body degenerates and cell processes or nerves downstream to the cell can be affected, in the retina, damage to the RGCs results in damage to the RGC axons that comprise the optic nerve. Retrograde damage, which begins behind the eye and
moves to within the retina, can occur if the optic nerve is damaged first, followed by further injury to the attached RGCs. The retina and optic nerve can be mechanically damaged if too much pressure is applied; ischemic damage, where the blood supply is compromised, and/or injury due to tissue inflammation can also be causes for visual dysfunction.

1.2.1  Glaucoma

Glaucoma is a chronic and progressive optic neuropathy that appears as a thinning of the bundle of nerves exiting the optic nerve (Lin and Orengo-Nania, 2012) and can be observed by examination of the retina with direct or indirect ophthalmoscopy when the RGCs and their axons are damaged. Glaucoma is typically associated with a rise in the pressure in the eye, known as intraocular pressure (IOP), but glaucoma can also occur in patients with normal eye pressure or low pressure potentially associated with nerve tissue with high susceptibility to damage. The loss of cells and axons causes significant loss of vision that is currently irreversible and the current treatment for glaucoma is to attempt to reduce the IOP to prevent or to slow the damage to the optic nerve. Glaucoma is classified in a variety of ways, typically based on the anatomy of the angle or the structure where the fluid in the eye drains located between the junction of the cornea and iris. Glaucoma is commonly divided into open angle glaucoma, associated with open drainage and a rise in pressure, or angle closure glaucoma where the drainage is closed, which increases the pressure in the eye. Patients typically have a loss of vision initially in the periphery that slowly encroaches on central vision. In 2002, The Eye Diseases Prevalence Research Group estimated that open angle glaucoma affected 2.22 million citizens in the United States and they predicted the number would rise to 3.36 million in 2020 (Friedman et al., 2004) given the inability to cure the disease.
1.2.2 Optic neuritis

Optic neuritis is acute demyelination of the optic nerve caused by inflammation. Optic neuritis is strongly associated with the onset of multiple sclerosis (MS) and occurs in 14 – 45% of patients with MS (Sorensen et al., 1999). Patients can experience a sudden loss of vision that varies in severity and extent of visual field loss. Color vision abnormalities and contrast sensitivity changes can also be assessed and can have lasting effects but can also potentially improve over time (Beck et al., 1992). Optic neuritis is a condition that can be treated and the Optic Neuritis Treatment Trial (ONTT) examined the difference between treatment with oral prednisone, to treat the inflammation, compared to intravenous methylprednisolone sodium succinate followed by oral prednisone treatment (Beck and Cleary, 1993). It was found that there was short-term acceleration of the rate of recovery for those treated with intravenous plus oral steroids and that oral prednisone alone should not be used as a form of treatment (Beck and Cleary, 1993). The effects of treatment do not guarantee full function of the optic nerve to be restored.

1.2.3 Ischemic optic neuropathy

Ischemic optic neuropathy can cause severe visual impairment or blindness due to lack of blood flow to the retina (Hayreh, 2009). Ischemic optic neuropathy encompasses two main groups of diseases, anterior ischaemic optic neuropathy (AION) and posterior ischaemic optic neuropathy (PION), with the former resulting from the loss of blood supply to the anterior part of the optic nerve, the posterior ciliary artery (PCA), and the latter involving damage to the posterior part of the optic nerve that is supplied by multiple sources but not the PCA (Hayreh, 2009). Typically the treatment of ischemic optic neuropathy is to administer systemic high dose steroids to stop further vision loss in the affected
eye and to prevent vision loss in the other eye although it has not been shown to be successful in the
treatment of PION, which is very difficult to treat (Hayreh, 2011).

1.3 Chick as a model

The chick (*Gallus gallus domesticus*) model is widely used for eye growth, development and
regulation studies. They are readily available, inexpensive and easy to maintain in comparison to
larger animals. The development of the chick eye is very similar to the development of the human
eye but occurs at a faster rate starting within the first 24 hours of incubation of the eggs and the
chicks hatch after 21 days with fully developed eyes. The developmental advantages of using the
chick model include the ability to do short studies (1-2 weeks) and the responses are robust to visual
manipulations.

1.3.1 Development of the chick retina and optic nerve

In the chick retina, all of the retinal cell types are believed to be formed one week before hatching
(Prada et al., 1991). The chick retina, similar to human retina, develops from the centre to the
periphery and contains the same six neuronal cells and one glial cell as humans. The chick retina
develops in three large stages beginning with the ganglion cells, the first cells to form, which appear
very quickly in development, on the fourth day of development before sending their axons through
the space in the optic stalk to connect with neurons in the midbrain. The amacrine and horizontal
cells are the next to develop, forming in the layer external to the ganglion cell layer, in the INL,
followed by differentiation of the cone photoreceptors in the ONL (Fujita and Hori, 1963; Morris,
1973; Prada et al., 1991; Spence and Robson, 1989). The last cells to develop around embryological
day 12 (E12) are the bipolar cells, rod photoreceptors and Müller glia (Kahn, 1974; Prada et al.,
1991). This order of differentiation of the retinal cells varies slightly from that of human
development which occurs in two large cohorts, the first includes the ganglion cells, horizontal cells and cones while the second cohort includes the amacrine cells, bipolar cells, Müller cells and rods (Oyster, 1999). At the time of hatching, around E21, the chick retina differs from humans given its fully functional and post-mitotic state.

1.3.2 Ocular similarities and differences between humans and chicks

The ocular structures of the chick eye have similarities as well as differences compared to the human eye (Walls, 1967). Chicks have laterally placed eyes with only a small degree of binocularity (20 degrees) (Martin, 2009) in contrast to humans who have frontally placed eyes with a large degree of binocularity (114 - 120 degrees) (Howard, 1995; Lens et al., 2008). The outer fibrous scleral layer of the chick is similar to that of humans but the chick also has an inner cartilaginous layer, which does not exist in humans. Like humans, the chick exhibits lenticular accommodation however the chick also exhibits corneal accommodation. The chick changes the shape of the lens using its ciliary muscles, which are skeletal in nature and therefore signaled by nicotinic agonists; in contrast, human ciliary muscle is smooth and relies on muscarinic receptors for signaling. In terms of the retina, the chick has an area centralis, an area of high cone density that is similar to the fovea of humans, although equivalent of a foveal pit is not observed in the chick retina. Chicks also have colour vision, however, unlike humans, who are trichromats, chicks are tetrachromats, having an additional set of ultraviolet-sensitive cones.

1.3.3 Vasculature of the chick eye

The chick retina does not have a central retinal artery that courses through the optic nerve like humans do, having instead, an equivalent structure called pecten oculi that projects into the vitreous chamber to nourish the inner retina. For this reason, the chick has been used in numerous studies to
isolate the retina from the brain by severing the optic nerve without compromising the vascular nourishment to the retina (Wildsoet, 2003). This method thus provides a solely mechanical means of damage to the eye without involving an ischemic form of damage.

1.3.4 **Eye growth and regulation studies**

The chick model has been extensively used in eye growth and regulation studies. Emmetropia describes the normal state of the eye when the eye is relaxed and allows the light from an object to fall on the retina to create a clear image without the aid of spectacles. The opposite of emmetropia is ametropia and consists of myopia (near-sightedness) and hyperopia (far-sightedness). Emmetropisation is the process by which the eye changes from being ametropic to emmetropic. The process of emmetropisation is similar between humans and chicks but the process occurs much faster in chicks (12 – 14 days in chicks vs. 7-9 years in children) (Irving et al., 1992; Pickett-Seltner et al., 1988). Emmetropisation requires the co-ordination of growth of the cornea, anterior segment, lens and vitreous chamber (Troilo, 1992).

The understanding of the emmetropisation mechanism has been primarily investigated using the chick model. One of the first experimental investigations to induce myopia was utilising form-deprivation, which involved degrading the visual environment in a variety of ways, including use of diffusers or by suturing the eyelid shut to prevent vision (Sherman et al., 1977; Wallman et al., 1978; Wiesel and Raviola, 1977; Wilson and Sherman, 1977). It was discovered that the ability to induce myopia was highly susceptible at a young age but decreased with age, which is also true for humans (Wallman and Adams, 1987). The form deprivation myopia studies showed that when there is no visual feedback (lid suture), myopia develops and continues until the experimental treatment was removed. To further investigate the evidence for emmetropisation as a visually guided mechanism,
studies involving lens-induced refractive errors were undertaken. The first report of lens-induced refractive errors was by Schaeffel et al. 1988, where chick eyes compensated for various amounts of defocus they were subjected to. Since then, there have been similar findings in many other species including tree shrews (Siegrwart and Norton, 1998) and monkeys (Hung et al., 1995). The chick model has also been noted as having the ability to compensate quickly for a large range of defocus (Irving et al., 1992; Nevin et al., 1998; Schaeffel and Howland, 1988; Wallman et al., 1995). The mechanism for emmetropisation is still unclear but there are many theories to explain the process (Yackle and Fitzgerald, 1999). The two largest opposing ideas view emmetropisation as either as an active mechanism (Troilo and Wallman, 1991), whereby visual feedback is involved in the process, or as a passive mechanism (Troilo and Wallman, 1991; Troilo, 1992), where the outcome of eye growth is predetermined.

The active emmetropisation mechanism is thought to be regulated by the clarity of the retinal image. The eye detects blur and then adjusts the components of the eye accordingly to create a clear image by making the eye longer or shorter. The most compelling evidence to support the active feedback mechanism of the eye comes from studies where spectacle lenses were used to create myopic or hyperopic defocus on normal chick eyes (Irving et al., 1992; Schaeffel et al., 1988). The defocus resulted in the eyes developing functional emmetropia by changing the shape and length of the eye to meet the new image plane. A plus (biconvex) lens was used to create myopic defocus, where the focal plane of the image was placed in front of the retina, and the retina was found to compensate by first expanding the choroid to push the retina forward to match the image plane, then by slowing ocular elongation to make the eye shorter (Hung et al., 2000; Wildsoet and Wallman, 1995). When a minus (biconcave) lens was used to create hyperopic defocus, to shift the image plane behind the eye, the opposite happened; the eye elongated to compensate for the blur and the retina
was displaced to the same plane as the image. Elongation of the eye occurred by remodeling the sclera at the posterior pole (Gentle and McBrien, 1999) and a backwards movement of the retina as a consequence of the thinning of the choroid and elongation of the vitreous (Wallman et al., 1995; Wildsoet and Wallman, 1995). In the chick model, it has been shown that the refractive changes in the eye are very plastic and chick eyes respond to a large range of lenses from -10D to +15D, although this asymmetry indicates that the mechanisms for myopic and hyperopic defocus may not be the same (Irving et al., 1992). In contrast to the lens induced eye growth changes, continuous excessive eye elongation and myopia occurs when an eye is deprived of a visual stimulus with a diffuser because the images were never brought into focus, resulting in vitreous chambers that were 25% longer than normal but when the visual impediment was removed, the eye still had the ability to recover (Wallman and Adams, 1987).

The passive theory of emmetropisation suggests that genetics and control from coordinated growth of all the other ocular structures is what directs the eye to change shape. It has been shown that there is a 42% chance that a child will be myopic if both parents are myopic, a 22.5% chance of myopia if one parent has myopia, and an 8% chance of being myopic if neither parents have myopia (Gwiazda et al., 1993). It has also been suggested that emmetropisation can be coordinated with lens and anterior segment changes during development without visual input (Yackle and Fitzgerald, 1999).

A recent study used the chick to investigate the interaction between passive and the active forms of emmetropisation, specifically genetics and the environment (Chen et al., 2011). Chicks were selectively bred for high susceptibility or low susceptibility to form deprivation myopia and then treated with different lenses to assess the refractive error development. Compared to the lowly susceptible birds, highly susceptible birds developed a larger degree of myopia when treated with minus lenses, proving that environmentally induced myopia has a strong genetic component. The
degree of change with the plano (no refractive power) lens and the plus lens groups did not differ between the high and low-susceptibility birds. These findings suggest that high myopia and lens-induced myopia have a similar molecular component in their pathways that is not found in the visually guided regulatory system for plus lenses (Chen et al., 2011). It also shows that the mechanisms guiding myopia and hyperopia are different, and that the interaction between genetics and environment may exist for myopia but not for hyperopia.

The idea that emmetropisation is locally regulated is supported by several studies. The first study involved using minus lenses and diffusers to cover only half of the eye/retina, and resulted in elongation or myopia of only the half of the eye that was subjected to the visual manipulation (Hodos and Kuenzel, 1984). A similar experiment with positive lenses resulted in less eye growth in the half of the eye exposed to the lenses. (Diether and Schaeffel, 1997; Hodos and Kuenzel, 1984; Wallman et al., 1987). Another study involving optic nerve section (ONS) to isolate the retina from the brain showed that that central nervous system played a role in the emmetropisation process. After ONS, myopia in chicks was still able to be altered by form deprivation (Troilo et al., 1987) and ONS did not prevent the chick eye’s ability to recover (Troilo and Wallman, 1991) accomplished by the local control of the retina, choroid and sclera in response to blur. Although these findings support local control of emmetropisation, there are also findings that support that the control is altered. After ONS, eyes tend to show a hyperopic state compared to normal eyes (Troilo et al., 1987; Wildsoet and Wallman, 1995) and the eyes recovering from form deprivation tend to over-shoot emmetropia (Troilo and Wallman, 1991). In addition, hyperopic defocus compensation is altered as well as the growth responses of the fellow eye of ONS-treated plus lens-wearing eyes (Wildsoet and Wallman, 1995), where the responses tend to be attenuated.
1.3.5 Retinal and optic nerve damage studies

In the chick retina, anterograde degeneration can be induced by injection of colchicine, a microtubule depolymeriser, in hatching chick eyes, leading to ganglion cell death and amacrine cell dysfunction or loss as well (Fischer et al., 1999; Morgan, 1981). The physiological effects of colchicine on chick eye growth include longer axial lengths (myopic shift), larger vitreous chamber and equatorial diameters, increased eye weight and myopic refractive error (Fischer et al., 1999). Another experimental model of damage to target ganglion cells is transecting the optic nerve, and this method represents a retrograde form of damage. Presumably, optic nerve transection leads to ganglion cell loss without affecting the amacrine cells, although secondary degenerative effects are unknown. The physiological effects of ONS include an initial hyperopic shift due to smaller vitreous chamber depths and thickening of the choroid (Choh and Wildsoet, 2002). Although the targets of destruction are the same (ganglion cells) for anterograde and retrograde models of damage, distinct eye growth patterns exist for the specific dysfunctional retinal states. Since the retinal cell death and proliferation characteristics of the retina have yet to be explored, the difference in eye growth patterns warrants investigation of ONS-treated eyes.

1.4 Cell Death (Apoptosis)

Apoptosis is a cell death mechanism that is programmed in the cell, activated naturally during development and possibly also in response to injury. In one of the first studies to examine apoptosis (Kerr, 1971) it was shown that ligation of the portal vein branches supplying the rat liver resulted in atrophy of the hepatic lobes and the presence of Councilman bodies (apoptotic bodies) containing intact unharmed mitochondria and lysosomes. Moreover, the cells had shrunk in size and were characterised by condensed chromatin fragments that were at times surrounded by membranes. The
cells did not become degraded but were eventually ingested by macrophages or surrounding cells. The phenomenon was thought to be complementary to mitosis and was later termed “apoptosis”, referencing the Greek term for leaves falling from trees (Kerr et al., 1972).

The main enzymes involved in apoptosis are the caspases (cysteine-dependent aspartate-cleaving proteases) or procaspases (the inactive form) (Taatjes et al., 2008). Two distinct classes of caspases have been identified, initiators, which are activated through protein-protein interactions, and effectors, which are activated later in the enzymatic cascade (Hengartner, 2000). The first caspase identified, cell-death abnormality-3 (ced-3), found in the nematode, closely resembled interleukin-1ß converting enzyme (ICE) now known as caspase-1 in humans (Fan et al., 2005). Presently, 14 caspases have been identified and they are divided into three subgroups based on their homology of amino acid sequence (Fan et al., 2005). The first subgroup has the role of activating apoptosis, and is also known as initiator caspases (-2, -8, -9 and -10). The other 2 groups involve the roles of apoptosis executioner (caspase-3, -6, -7) and inflammatory mediator (caspase-1, -4, -5, -11, -12, -13, and -14) respectively, and fall under the category of effector caspases (Fan et al., 2005). The activation of cell death involves the interaction of protein domains such as the death effector domain (DED) or caspase recruitment domain (CARD) (Fan et al., 2005). Initiator caspases are cleaved, resulting in their activation, which then triggers a cascade of events including the activation of the effector caspases by initiator caspase dependent cleavage of their respective procaspases, finally ending in cell death.

1.4.1 Apoptotic pathways

There are two common pathways to activate caspases: the extrinsic and the intrinsic. The extrinsic pathway is also referred to as the death receptor-mediated pathway and the intrinsic referred to as the mitochondrion-mediated pathway (Fan et al., 2005). The extrinsic pathway is initiated by the binding
of an extracellular ligand to either the Fas or tumour necrosis factor receptor (TNFR1); the ligand is also known as the death ligand or the cell death signal (Fan et al., 2005; Riedl and Shi, 2004). The recruitment and aggregation of cytosolic factors FADD (Fas-associated death domain) and TRADD (TNFR-associated death domain), then activate initiator procaspases such as procaspase-8 (Fan et al., 2005; Riedl and Shi, 2004). Typically, effector caspase-3 activation follows. Caspase-3 then cleaves and activates DNA fragmentation factor (DFF) to fragment DNA.

The intrinsic pathway is commonly activated by caspase-8 and caspase-9. The caspase-9 intrinsic pathway involves the opening of mitochondrion permeability transition pores (MPTPs) to allow for the release of cytochrome c from the mitochondria to the cytosol (Fan et al., 2005). The combination of cytosolic procaspase-9, deoxyadenosine triphosphate (dATP), cytochrome c, and oligomerised Apaf-1 result in the formation of a complex called an apoptosome (Fan et al., 2005) that activates caspase-9. Procaspses-3 and -7 are the effector caspases associated with caspase-9 (Fan et al., 2005). Unlike caspase-9, caspase-8 only requires cytochrome c for activation. Cytosolic caspase-6 is recruited and is the only caspase that can cleave procaspase-8 to caspase-8, in effect enhancing the caspase activity. Caspase-8 then activates caspase-3 to cause DNA fragmentation.

Bcl-2 family of proteins are regulators of apoptosis and are commonly found in the intrinsic pathway of apoptosis and can either increase or suppress apoptosis (Hengartner, 2000). In humans, there are 20 Bcl-2 proteins found so far to date (Reed, 2000). Most pro-apoptotic Bcl-2 proteins such as Bax, Bak and Bid are inserted into the mitochondrial membrane (Fan et al., 2005; Gross et al., 1999) while other pro-apoptotic proteins such as Bad are moved from the cytosol to enter the mitochondria (Reed, 2000). The proteins that become integral to the mitochondrial membrane act by controlling the permeability of the MPTPs. In contrast, the Bcl-2 proteins that enter the mitochondria associate with other Bcl-2 proteins already present to alter their function to either increase or decrease
the apoptotic activity (Gross et al., 1999). Bcl-2/Bcl-XL are anti-apoptotic and they have the ability to stop apoptosis by inhibiting pro-apoptotic proteins such as Bax from inserting into mitochondrial membrane (Gross et al., 1999). Ultimately, when the Bcl-2 family proteins reach the mitochondria they can increase or decrease apoptosis by regulating the release of cytochrome c and other proteins including caspases or caspase-activating proteins by forming channels/holes or by altering the permeability of MPTPs (Hengartner, 2000; Reed, 2000).

Once the apoptotic cascade has been initiated, the degradation of DNA soon follows. One way to fragment DNA is with the apoptotic endonuclease known as the DNA fragmentation factor (DFF) also known as caspase-activated-deoxyribonuclease (CAD). Studies have shown that caspase-3 can cleave the CAD inhibitor (ICAD) off of the DFF/CAD to activate the endonuclease function to cleave DNA. Caspase-9 causes damage to the nuclear pores to allow for caspase-3 to enter the nucleus to cause DNA degradation. The high-mobility group (HMG) proteins and histone H1 have the ability to increase the endonuclease activity of DFF/CAD (Fan et al., 2005). The fragmentation of the DNA is a key step in apoptosis but the cell must then undergo vital morphological changes including the breakdown of nuclear lamins to cause nuclear shrinkage, breakdown of the cytoskeletal proteins and cleavage of the cell to allow for cell blebbing.

Condensation of chromatin is a key feature in apoptosis that is mediated by nuclear lamins. Nuclear lamins are intermediate filament proteins that make up the underlying inner nuclear membrane and they also function to help organise the chromatin (McKeon, 1991). In apoptosis, protease cleavage causes the disassembly of lamins (Earnshaw, 1995; Lazebnik et al., 1995) and in mutant lamin cells where cleavage is not found, there is a delay in nuclear shrinkage, nuclear breakdown is not complete, the chromatin is prevented from condensing and is not packed into apoptotic bodies (Rao et al., 1996). The formation of the apoptotic bodies and the regulation of
cytoskeletal changes have been identified to be linked to gelsolin and p21-activated kinases (PAKs). In vitro, gelsolin is an actin-binding protein that is cleaved by caspases to cause cells to become round and detach themselves from the dish and cause nuclear fragmentation (Kothakota, 1997). Gelsolin is found widely in the mammalian tissues (Kothakota, 1997) and is identified as a substrate for caspase-3, a key mediator in the apoptosis of mammalian cells, and may be a key player in effecting morphological change (Kothakota, 1997). In vitro, when apoptosis was induced, PAK2 cleavage also occurred by caspase activation (Rudel, 1997). Cells that had PAK2 that were not cleaved showed no apoptotic bodies when apoptosis was induced but the cells were still intact with fragmented DNA (Rudel, 1997). It was determined that since PAKs are common in mammalian tissues, caspase activation of PAK2 may also be essential for the formation of apoptotic bodies and blebbing in the apoptotic cascade.

1.4.2 Apoptosis detection

Apoptosis can be detected in a variety of ways. Currently the most common cytochemical/spectroscopic method for detection is the use of the TUNEL assay or terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick end labeling. During apoptosis, endonucleases cause DNA strand breaks leaving free 3’-OH ends. TUNEL detects and labels the free 3’-OH terminal ends of single and double-stranded DNA breaks. TUNEL is used to detect apoptosis at the late stage and the advantage of 3’OH labeling is the ability to tag the initial DNA breaks (Sgonc and Gruber, 1998). The procedure requires pre-treatment of the sample with protease, then a labeled dUTP is incorporated into the DNA breaks (Vecino et al., 2004). The Apostain is a newer method that is similar to TUNEL but was developed as a more specific probe for apoptotic cells that is not
easily affected by the method of tissue fixation but is not yet commonly used (Prochazkova et al., 2003).

Electron microscopy is another way of detecting apoptosis by identifying apoptotic characteristics including cell shrinkage, increased cytoplasmic density, chromatin condensation, circumscribed masses in the nuclear membrane, blister-like formations and finally membrane-bound apoptotic bodies (Kerr, 1971). The advantage of electron microscopy is the high spatial resolution but due to costs associated with this technique, it is no longer routinely used (Taatjes et al., 2008). More commonly used nowadays is light microscopy with various DNA and cellular stains. A common histological method is Feulgen staining (Kuo et al., 1998; Vecino et al., 2004) where Schiff reagent is used to stain aldehyde groups on acid exposed deoxyribose sugars and the amount of stain is directly proportional to the amount of DNA. The apoptotic cells are easily identified by the dark purple compacted nucleus (Vecino et al., 2004).

Flow cytometry is a common laser-based method of counting cells along with the treatment of specific reagents (Sgonc and Gruber, 1998) but does not always allow for imaging of structural changes (Taatjes et al., 2008). Dyes highlight the change in size and granularity of the cells and apoptotic cells cause light scattering properties to be altered. In apoptosis, early morphological changes such as water loss, cell shrinkage and fragmentation cause light scatter patterns that are not found in cells dying by other cell death mechanisms. Apoptotic cells have lower forward scattering and higher side scatter than viable cells because of their smaller size and the change in the cytoplasm. Late apoptotic changes, including fragmentation and a decreased amount of DNA, can be easily identified because of their low affinity for stains. The changes in the plasma membrane that occur during late apoptosis can also be detected using a combination of one dye that diffuses through intact membranes, Hoeschst, and a dye that does not such as Propidium Iodide, allowing for discrimination
between dying and viable cells (Sgonc and Gruber, 1998). Annexin V staining is another method of detecting the apoptotic pathway and can be used in both flow cytometry and light microscopy to identify early- to mid-stage apoptosis by binding to the phosphatidylserine (PS) on the surface of the cell membrane that gets flipped from the internal to the external side of the cell.

Gel electrophoresis is another way to identify the apoptotic process. The degradation of DNA by nucleosomes causes cleavage of the DNA in certain locations, the size of the fragments produced during apoptosis is approximately 185bp (Martinez et al., 2010; Wyllie et al., 1980) and can be observed with gel electrophoresis and is typically referred to as the DNA-ladder configuration (Sgonc and Gruber, 1998). Western blotting is also a common technique for protein-based analyses of the release of cytochrome c, regulation of apoptotic proteins and the activation of caspases (Martinez et al., 2010). Western blotting detection of cytochrome c release is faster than detection by immunoelectron microscopy. Antibodies to tag caspase-cleaved proteins or “neoepitopes” (formed after the enzymatic cleavage of proteins) are now commercially available. The neoepitopes are used as antigens to produce reagents against caspase-mediate apoptosis components. The most common antibodies are raised against neoepitopes for cytokeratin 18 and caspase-3. Since caspase-3 is common in almost all apoptotic cells, this reagent is typically the reagent of choice to label caspase-mediated apoptotic cell death (Taatjes et al., 2008).

### 1.4.3 Other types of cell death

Necrosis was once thought to be the only cell death mechanism until 1951, when apoptosis was characterised (Glücksmann, 1951). Unlike apoptosis, necrosis is a pathological and unregulated process that occurs in response to severe forms of environmental changes, hypoxia, and toxins and results in inflammation of a mass of cells. The characteristics of necrosis are cell swelling, as a result
of a loss of selective permeability of the cell membrane, which ultimately causes damage to the
ganelles including the mitochondria. Other cellular ganelles are also affected (Hawkins et al.,
1972). The nucleus remains intact while the cytoplasmic components are altered as a result of
changes to the ganelles of the cell. The end stages involve the breakdown of the DNA into
fragments (Hawkins et al., 1972).

Autophagy is another programmed cell death pathway but unlike apoptosis, apoptotic bodies
are not formed. Instead, materials for degradation are packaged into autophagosomes which are
double-membraned vesicles containing cytosolic components. The autophagosome fuses with
membrane of a lysosome resulting in the release of a single membraned autophagic body into the
lumen. The autophagic body degrades by lysosomal hydrolases resulting in the recycling of the
cytoplasmic components. Autophagy has been referred to as type II PCD and apoptosis as
programmed cell death type I (type I PCD) (Wang and Klionsky, 2003). The two programmed cell
death mechanisms can interact, where apoptosis can activate autophagy and autophagy can facilitate
 efficient apoptosis (Xue et al., 1999). Defective autophagy has been implicated in diseases such as
cancer, Huntington’s and Parkinson’s (Klionsky and Emr, 2000).

Necroptosis is also known as programmed necrosis (Vandenabeele et al., 2010). Similar to
necrosis, necroptosis involves rapid dysfunction of the mitochondria, development of reactive oxygen
species, cell-swelling, rupture of the plasma membrane and spilling of the cellular contents (Galluzzi
and Kroemer, 2008). The cell is able to disintegrate by oxidative burst, membrane polarisation of the
mitochondria, lysosomal and membrane permeabilisation. Necroptosis is defined as programmed cell
death because it follows a program, requiring the activation of caspases and serine-threonine kinase
receptor-interacting protein 1 (RIP1) (Vanden Berghe et al., 2010).
1.5 Cell Death Processes in the Vertebrate Eye

Apoptosis also plays a vital role in the development of the vertebrate visual system. Apoptosis occurs in the early morphogenesis and embryological stages of the eye including invagination, fusion and the separation of tissue (Wride, 1996). In the later stages of eye development, apoptosis regulates the number of cells and determines the size and shape of the various ocular structures (Bozanić et al., 2003). Changes in apoptotic cell distribution have the potential to lead to malformations of the eye (Bozanić et al., 2003).

Apoptosis is a key component of normal development especially during early stages in the formation of the parts of the optic cup, stalk, epithelium of the lens pit and the ectoderm where the lens detaches (Bozanić et al., 2003). The highest amount of apoptotic activity has been identified in areas where morphogenetic events to shape the eye occur (Bozanić et al., 2003). The other large area of apoptosis is the remodeling of the optic nerve (Bozanić et al., 2003).

In the lens, apoptosis has been identified using embryos with mutant genes. Ishizaki et. al. showed that apoptosis is needed during the lens epithelial cell differentiation into lens fibres process because machinery that is common in programmed cell death mechanisms, including caspases, were involved (Ishizaki et al., 1998). To create the clarity of the lens in a variety of species including bovine (Dahm et al., 1998), monkey (Bassnett, 1997), rat (Richardson and McAvoy, 1986) and aves (Bassnett and Mataic, 1997; Chaudun et al., 1994; Wride and Sanders, 1998), apoptosis occurs to remove the nuclei and other organelles from the lens fibres (Wride, 1996). Over-expression of Bcl-2 (anti-apoptosis), resulted in the development of disorganised fibres (Wride, 1996), while an increase in apoptosis led to smaller eyes (Hettmann et al., 2000).
Cell death is important for the development of the retinal layers and occurs in at least two phases, an early phase during neurogenesis, cell migration and cell differentiation and a late phase when connections or synapses are being formed. The pattern of cell death is common amongst different vertebrates but the time and the amount of cell death varies (Vecino et al., 2004). In general, retinas undergo 2 or 3 waves of apoptosis with each wave causing degeneration of cells. The number of waves differ with amphibians (Glücksmann, 1940) showing three waves and the rest (fish, mammals, rodents, reptiles, humans) occur in two waves (Bozanić et al., 2003; Cole and Ross, 2001; de la Rosa and de Pablo, 2000; Francisco-Morcillo et al., 2004; Galli-Resta and Ensini, 1996).

1.6 Cell Proliferation

Cell proliferation is the sequence of events that occur when cells that are stimulated to reproduce by entering the cell cycle. Cells at rest are initially in G0 phase. Upon entering the cell cycle, cells enter the first period of growth G1. This stage prepares the cell for DNA synthesis/replication or S phase. At the end of G1, there is a restriction point (R), the point where the cell commits to complete the cell cycle. When there are two copies of the cell’s chromosomes, the cell enters G2, the second period of growth where the cell prepares for mitosis (M). M phase is when the cell divides into two daughter cells and this phase is divided into a series of steps: prophase, prometaphase, metaphase, anaphase and finally telophase including cytokinesis allowing the cell to divide into two parts (Berridge, 2012).

Mitosis begins with prophase, with the pair of centrosomes in the cell separating and migrating to opposite poles of the cell. The pairs of chromosomes condense and form sister chromatids and the nuclear membrane then begins to break down (Berridge, 2012). Spindles begin to form in the next phase, prometaphase, including astral microtubules, polar microtubules and kinetochore spindles. The astral and polar microtubules overlap with each other and aid in pushing
the poles apart, while the kinetochore spindles attach to the kinetochores on the sister chromatids. Metaphase involves the aligning of the chromatids at the metaphase plate and separation is delayed until all the chromatids are in place. Once all the cells are aligned, the cell moves into anaphase where separation of the chromatids begins. Finally, telophase involves the separation of the chromosomes to either pole of the cell and cytokinesis allows the cell to divide by way of a contractile ring of actomyosin, which marks the end of the mitotic process until the cells are stimulated to repeat the process.

1.6.1 Proliferation detection
The most common analogue of the DNA bases is bromodeoxyuridine (BrdU) that gets incorporated into the proliferation DNA during S phase. BrdU is detected indirectly with anti-BrdU along with a fluorescent tag. The development of BrdU assays eliminated the need of radioisotopes, which was needed to detect 3H-thymidine, a radioactive thymidine nucleoside that was commonly used in the past. A newer method, similar to BrdU includes the use of 5-ethynyl-2’-deoxyuridine (EdU) incorporation. EdU can be used to label cells in S-phase of the cell cycle and typically uses an alkyne modified dUTP that is detected by click chemistry that fluoresces the azide attached to the alkyne. The small size of the EdU regent allows detection without the use of harsh denaturation steps as is needed for BrdU treatment. Although it is not commonly used at the moment, EdU is expected to increase in popularity (Zeng et al., 2010).

Proliferating cells can be detected using antibodies to specific antigens such as monoclonal antibody Ki-67 that is a commonly used antigen to label human cells in S, G2 and M phase (Gerdes et al., 1983). The MCM2-7 complex, the replicative helicase needed for DNA replication initiation and elongation that is essential for entry into S phase of the mitotic cycle, is another marker for mitosis.
Antibodies for specific phosphorylation sites of histone H3, which play a role in chromosome condensation, have also been used to mark mitotic cells (Goto et al., 1999).

Other methods to detect proliferation take advantage of the by-products resulting from the process of mitosis. The use of tetrazolium salts and AlamarBlue reagent can be reduced by metabolic intermediates during the proliferative process, the increase in metabolic intermediates is measured by a shift in colours, with tetrazolium salts changing in color from yellow to purple and AlamarBlue changing from a non-fluorescing indigo blue to a fluorescent pink colour (Caviedes et al., 2002). The ViaLight assay uses bioluminescence levels to measure the amount of intracellular ATP which increases with cells proliferation (Jiang et al., 2008). The oxygen biosense system uses oxygen-sensitive ruthenium-based fluorescence levels, which are directly proportional to the amount of cellular or enzymatic oxygen consumption (Wang et al., 2005).

1.7 Cell Regeneration in the Retina

In light of the many sight threatening eye diseases, stem cell researchers have been searching for ways to replace cells that are lost. Three methods have been developed, the use of human embryonic stem cells (hES), induced pluripotent stem cells (iPS) and the stimulation of retinal progenitor cells. hES cells isolated from blastocysts are pluripotent and have the ability to replicate indefinitely (Evans and Kaufman, 1981; Martin, 1981). Difficulties with the use of hES cells are ethical problems associated with the use of human embryos and the possibility of tissue rejection. The discovery that iPS cells, derived from adults cells, could be induced by transcription factors to become pluripotent, removed the need for embryonic tissue (Takahashi and Yamanaka, 2006) but the ability to transplant cells in vivo still posed another difficulty. Retinal progenitor cells are multipotent, having the capability to differentiate into the various neurons and glial cells types of the retina; their use
eliminates the need for transplantation of cells (Xiang, 2013). It has been long recognised that cold-blooded, lower vertebrates retain retinal progenitor cells that have the ability to generate new neurons throughout life at the retinal margin. In amphibians and fish, the growth of the eye is coordinated with the growth of new neurons (Perron and Harris, 2000; Reh and Levine, 1998). In warm-blooded vertebrates, it was generally accepted that after the early stages of development, the retina does not have the ability to generate new neurons. In birds and mammals, the ocular growth that occurs post-natally or post-hatch is not coordinated with new neurons but by the stretching of the retina to compensate for eye growth (Teakle et al., 1993). While it was generally thought that progenitors were limited to lower vertebrates, at least three sources of progenitor cells have been identified in the chick retina including (1) the cells at the peripheral edge of the retina, similar to the ciliary marginal zone in lower vertebrates as well as the ora serrata in humans (Fischer and Reh, 2000), (2) the non-pigmented ciliary epithelial cells of the ciliary body (Fischer and Reh, 2003b) and (3) the Müller glial cells of the retina (Fischer and Reh, 2003a). In adult chicken retina there is a potential source of progenitor cells in the area of the pigmented cells at the pars plana of the ciliary body (Fischer and Reh, 2001a). Mammalian equivalents of progenitor cells have also been reported, although they show a much more reduced neurogenic ability (Nickerson et al., 2007).

There are several sources of evidence that indicate that Müller glia share a similar lineage and precursor with the retinal neurons (Holt et al., 1988; Turner and Cepko, 1987). This relationship between retinal neurons and Müller glia has led to the idea that proliferating glia may be a target for retinal regeneration. In mammals, Müller cells can re-enter the cell cycle and continue to survive in vitro after the retina has been damaged (Lewis et al., 1992; Sarthy, 1985). The relationship between neural progenitors, stem cells and glia has recently been investigated and it has been discovered that in the chick retina, Müller glia have the potential to adopt phenotypes and functional capabilities
similar to those of retinal progenitors, which may allow them to serve as a source of retinal regeneration (Fischer and Reh, 2003a). When the chick retina is subject to extensive damage by neurotoxin injection, there is widespread apoptosis, but surprisingly there is also a large number of mitotically active cells that remains in the retina, as indicated by BrdU incorporation, Phospho-Histone H3 and proliferating cell nuclear antigen (PCNA) labeling (Fischer and Reh, 2001b). The newly generated cells initially labeled with a marker glutamine synthetase (GS) for Müller cells but after a few days began to express markers for embryonic retinal progenitors including Pax6 and Chx10 (Fischer and Reh, 2001b). Newly generated cells distributed throughout the inner and outer nuclear layers of the retina and were able to survive for a few weeks post-damage. Overall, the fates of the BrdU-labeled cells included a small percentage (less than 4%) that differentiated into retinal neurons expressing Hu, calretinin or cellular retinoic acid-binding protein (CRABP), a larger percentage (~20%) that differentiated into Müller glia and the majority of the cells (~80%) that remained undifferentiated and continued to express the embryonic markers, Pax6 and Chx10. When damage to the retina occurred within the first week post-hatch, the Müller glia proliferated in the central regions of the retina, whereas treatment after the first week post-hatch resulted in Müller glial cell proliferation in the peripheral regions of the retina (Fischer and Reh, 2003a).

It has been determined that the type of neuron targeted for destruction may be the neuron promoted for regeneration by the Müller glia-derived progenitor cells. This conclusion was drawn from studies (Fischer et al., 2002a) using kainate used to damage bipolar, amacrine and ganglion cells, colchicine to selectively target ganglion cells and N’-methyl-D-aspartate (NMDA) to destroy amacrine and bipolar cells. With the use of kainate or colchicine, the newly generated cells expressed markers of ganglion cells that were not observed with NMDA-induced damage, in general indicating that the cell targeted for destruction was the cell type replaced (Fischer and Reh, 2003a).
Chick embryos have shown the ability to regenerate neural retina from surgically removed RPE after insertion into embryonic chick otocyst (Coulombre and Coulombre, 1965). The regeneration was determined to be growth factor driven, specifically basic fibroblast growth factor (bFGF) (Park and Hollenberg, 1989). FGFs are produced by retinal cells in response to damage (Cao et al., 2001; Kostyk et al., 1994; Wen et al., 1995), to promote the development of ganglion cells in cultures of chick and rodent (Guillemot and Cepko, 1992; McCabe et al., 1999; Pittack et al., 1991; Zhao and Barnstable, 1996), to stimulate the proliferation of embryonic retinal progenitors (Anchan and Reh, 1995; Lillien and Cepko, 1992), and to enhance the survival of RGCs targeted by optic nerve section in frogs (de la Rosa et al., 1994). FGF2 prevents apoptosis by increasing the amount of anti-apoptotic proteins and by reducing the apoptotic effectors (Ríos-Muñoz et al., 2005). During retinal development, the Müller glia and pigmented epithelial cells also express insulin-like growth factors (IGFs) (de la Rosa et al., 1994; Hansson et al., 1989). IGF stimulates proliferation of retinal progenitors cells and works synergistically with FGF2 (Jiang et al., 2001). The combination of FGF2 and insulin alone was sufficient to stimulate the regenerative response of the Müller glia of chick eyes (Fischer et al., 2002b). The Müller glia have shown the ability to dedifferentiate and proliferate but they seem to be limited to the progenitor state, rarely re-differentiating into specific neurons. Limitations may be attributed to the retina not having the signals or support for neuronal differentiation of embryonic progenitors into post-mitotic neurons (Fischer and Reh, 2003a).

1.7.1 Gliosis

There are three types of glial cells in the retina, microglia, astrocytes and Müller cells (Bringmann et al., 2006). Gliosis is a process stimulated in response to damage to physically replace the cells that are damaged, although the function of the previously damaged cells is not restored. Glial cell
proliferation is stimulated to protect and repair neurons by releasing neurotrophic factors and antioxidants but it is also been associated with the release of apoptotic factors such as nitric oxide synthase (Bringmann et al., 2006). As the principal glial cells of the vertebrate retina, Müller cells are important for the maintenance of the structure and function of the retina by providing nutrients (Tsacopoulos and Magistretti, 1996), regulating retinal blood flow (Paulson and Newman, 1987), recycling neurotransmitters for neural signaling (Matsui et al., 1999), maintaining homeostasis of the retina (ion, water and pH) (Newman and Zahs, 1998; Newman, 1996) and controlling excitability of neurons (Newman and Zahs, 1998; Stevens et al., 2003). During pathological conditions, Müller cells can respond by activating microglia, altering vasculature and promoting the migration of defense mechanisms to the retinal tissue. This process is referred to as Müller cell gliosis (Bringmann et al., 2006). The Müller glial cells, in contrast to the other retinal neurons, are surprisingly resistant to various forms of damage including ischemia, anoxia or hypoglycemia (Silver et al., 1997).

Müller cell gliosis has both non-specific and specific responses depending on the mechanism of damage. Non-specific responses to injury include the up-regulation of glial fibrillary acidic protein (GFAP) as well as the activation of the extracellular signal-regulated kinases (ERKs) (Bringmann and Reichenbach, 2001; Dahl, 1979; Eisenfeld et al., 1984). An example of a specific gliotic response includes the alteration of the expression of glutamine synthetase that is used for neurotransmitter recycling. In the event of optic nerve crush, there is an excess of glutamate released in the area of the ganglion cells (Chen and Weber, 2002). Gliosis has both neuroprotective and detrimental effects.
II. INTRODUCTION

Damage to the retina is a common cause of blindness. The retina is the light-sensitive tissue in the eye that captures light and sends the visual information to the brain. Destruction to the retina can occur in many different ways. Harm to the optic nerve and ganglion cells (RGCs) can occur in diseases such as optic neuropathy and glaucoma, whereas alteration to the retinal pigment epithelium (RPE) and photoreceptors can occur in diseases such as age-related macular degeneration and retinitis pigmentosa. Once the cells are lost, they are not typically replaced.

Recent studies have also shown that in birds and rodents, specific retinal cells can be stimulated to regenerate (Fischer and Reh, 2001b). Regeneration of retinal cells can be elicited by chemotoxic injury to the retina as well as exposure to growth factors. Damage to the retina with neurotoxin injection into the vitreous and injections of exogenous growth factors, insulin and fibroblast growth factor-2 (FGF-2), resulted in de-differentiation and proliferation of the glial support cells (Fischer et al., 2002b). Neurons were also promoted for regeneration by the Müller glia-derived progenitor cells, and the type of neuron initially targeted for destruction was the neuron promoted for regeneration by the Muller glia-derived progenitor cells. Injections of colchicine, a toxin that targets ganglion cells, resulted in newly generated cells that express ganglion cell markers (Fischer and Reh, 2001b).

Chemical injection is not the only method of inducing damage to the ganglion cells. Optic nerve section (ONS) or RGC axotomy is a mechanical procedure that simulates an extreme form optic neuropathy. Both colchicine injections and RGC axotomies have been shown to lead to altered eye growth patterns that differ, despite both methods targeting the same cells (RGCs). Colchicine treated eyes showed an enlarged vitreous chamber, whereas eyes treated with ONS showed the opposite
trend, an initial hyperopic shift due to thickening of the choroid and shallowing of the vitreous chamber (Choh and Wildsoet, 2002; Fischer et al., 1999). The distinct eye growth patterns are likely associated with the specific dysfunctional retinal states, dependent on how the retina was disturbed and how the cells were lost. Given the differences in the in eye growth patterns, this project was undertaken to determine whether ONS-treated eyes can induce proliferative activity in the retina, to see if growth factors can increase the proliferation and whether the mitotic cells can be induced to differentiate. The time course and cell death characteristics following ONS were also analysed.
III. MATERIALS AND METHODS

3.1 Animals

White leghorn hatchling chicks (gallus gallus domesticus) were obtained from Maple Leaf Poultry, New Hamburg, Ontario, Canada. The chicks were housed in a galvanised metal brooder and were fed medicated chick starter ad libitum. The chicks were subjected to a cycle of 14-hours lights on and 10-hours lights off.

3.2 Experiments

There were two different experiments carried out. The first experiment was carried out to characterise the time course of degenerative cellular events and to determine whether age was a factor. The second experiment examined the proliferative cellular events post-ONS and whether growth factor injections could affect proliferative activity.

3.2.1 Experiment 1: Effect of age

1-day-old chicks (n = 28) and 1-week-old chicks (n = 35) underwent ONS and sham surgery. Ultrasound readings were taken prior to ONS and on days 1, 3, 5, and 7 post-ONS. Chicks were sacrificed and eyes enucleated on days 3, 5, 7, 10 and 14 post-ONS (1-day-old: n = 10 for all time points; 1-week-old: n = 3 for all time points). Tissues were analysed for cell death using TUNEL.

3.2.2 Experiment 2: Effect of growth factors

An initial experiment was undertaken to determine prevalence of BrdU labeling across time points post-ONS. 1-day-old chicks underwent ONS and sham surgery. Ultrasound readings were taken prior to ONS and on days 1, 2, 3, 4, 5, 6, 7 and 8 post-ONS. Chicks were sacrificed and eyes
enucleated on days 1, 2, 3, 4, 5, 6, 7, and 8 post-ONS (n ≥ 4 for all time points) and retinas were analysed for proliferative activity. BrdU was injected 4 hours prior to enucleation.

To determine whether growth factors could affect proliferative activity, 1-day-old chicks underwent ONS and sham surgery, and ultrasound readings taken prior to ONS and on days 1, 3, 5, and 7 post-ONS (n = 28). Chicks were injected with growth factors immediately after the ONS procedure and every 3 days thereafter (days 0, 3, 6, 9, and 12 post-ONS) and sacrificed on days 3, 5, 7, 10 and 14 post-ONS (n ≥ 3 for all time points). BrdU was injected 4 hours prior to enucleation. Eyes were enucleated and retinas were analysed for proliferative activity.

3.3 Optic nerve section and sham procedures

One-day or one-week old chicks were treated with ONS surgery on one eye and sham surgery on the other eye. The eye that received ONS treatment alternated between right and left eye. Chicks were wrapped in a latex blanket to prevent movement during the procedure and to provide warmth, and then were placed on their side with their beak placed in a nose cone attached to an anaesthetic machine (Benson Medical #975-0800-000). The chicks were anaesthetised with isoflurane (CDMV #108737; 2 – 2.5% in oxygen at 1 litre/minute) in a few minutes. Once anaesthetised, feathers on the lateral canthus of the eye were disinfected with an alcohol swab. Scissors were used to trim the feathers and alcohol swabs used to remove feathers. An incision was made on the outer canthus through the skin and then the thin inner fascia layer. The slit was enlarged with forceps and the optic nerve was located. The ONS procedure consisted of severing the optic nerve with a blade and forceps. The sham procedure consisted of exposing the optic nerve but not severing the axons. The incisions in the inner fascial tissue and skin were then closed using a suture (Johnson & Johnson #7733G). Antibacterial topical gel anaesthetic (Polysporin® with lidocaine) was applied to the
surgical area. The birds were removed from the anaesthetic and allowed to recover under a heat lamp. The tools were cleaned in an ultrasonicator then sterilised with a glass bead steriliser between each bird. Each surgery was approximately 20 minutes per bird.

3.4 Ultrasound Procedure

High frequency A-scan ultrasonography was used to measure the eye growth changes over time. The head of the chick was placed into a head holder, with its beak inserted into a nose cone attached to the anaesthetic machine. The birds were anaesthetised (1.5% isoflurane in oxygen) and custom-made lid retractors were used to hold the eye open. A 35 MHz polymer transducer (Panametric 135-2-R 1.00, Pulsecho, Port Hope, Ontario) attached to a pulser-receiver (Panametrics 5073PR, Pulsecho, Port Hope, Ontario). Ultrasound readings were recorded with a digital oscilloscope (LeCroy WaveRunner 64Xi, ACA TMetrix Inc, Mississauga, Ontario) then transferred to a computer for analysis. Each ultrasound procedure took approximately 5 - 10 minutes per bird.

The ultrasound readings consisted of 7 peaks that represent the interfaces between the ocular components. In order, the interfaces include the anterior corneal surface, anterior and posterior lens surface, vitreal-retinal interface, retinal-choroid interface and anterior and posterior sclera. Locations of each peak were determined and the average inter-peak differences in time were calculated using a MatLab- (MathWorks, Natick, Massachusetts, U.S.A.) based custom program. The time intervals, representing twice the distance traveled by sound, were then multiplied by the speed of light passing through the anterior chamber, lens, vitreous, retina, choroid and sclera (1.6078 mm/μsec for lens and 1.534 mm/μsec for the rest of the eye; Wallman and Adams, 1987) and divided by 2 to yield anterior chamber (AC) depth, lens (LN) thickness, vitreous chamber (VC) depth, retinal (RT) thickness,
choroidal (CH) thickness and scleral (SC) thickness. The lengths were statistically analysed using repeated measures analysis of variance (ANOVA) and values were considered significant at \( p \leq 0.05 \).

### 3.5 Injections

Prior to injections, birds were anaesthetised and the area around the orbit was disinfected. All injections (10 µL) were made into the vitreous chamber. Intravitreal injections consisted of either 5-bromo-2-deoxyuridine (BrdU from Sigma-Aldrich, B5002, 100 µg/mL) alone, a marker for mitosis or a combination of growth factors, insulin (Cedarlane MAB1417, 2 µg per injection) and fibroblast growth factor-2 (FGF-2, Cedarlane 133-FB-025/CF, 100 ng per injection) followed by BrdU. The injection procedure took approximately 3–5 minutes per bird.

### 3.6 Dissection, preparation and staining of retinal sections

Chicks were sacrificed at various time points and their eyes enucleated. The anterior portion of the eye including the anterior chamber, lens and vitreous were removed leaving the posterior eyecup. The eyecup was fixed in 4% (w/v) paraformaldehyde solution in 0.1M Sorensen’s sodium buffer (SB: 23.996 g/L NaH₂PO₄, 28.392 g/L Na₂HPO₄, in deionised water, pH 7.5) with 3% sucrose for 20 mins. The eyecups were then washed (3 x 5 mins) in a 0.1M SB solution and cryoprotected overnight in 30% (w/v) sucrose in SB. Eyecups were then embedded in Optimal Cutting Temperature Embedding medium (VWR CA27900-246; embedding molds 22x22 mm, Fischer Scientific 38 104 18) before being frozen. Tissues were sectioned at 12 µm (Leica CM 1900 UV) and mounted onto Superfrost Plus glass slides (Fischer Scientific 12 550-15). Sections were air-dried before being stored at -20°C. Central sections of the retina of the chicks were used for immunohistochemical staining.
3.6.1 **TUNEL**

Retinas were treated with a TUNEL kit from Roche Diagnostics (1 684 795). Slides were treated according to the instructions provided with the kit. Sections were washed (3 x 5 mins) in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 101 mM Na₂HPO₄, 2 mM KH₂PO₄, in deionised water), followed by treatment with 3% (v/v) H₂O₂ in methanol (10 mins). Sections were washed again (3 x 5 mins PBS) then permeabilised with 0.1% (v/v) Triton-X in PBS (30 mins) at room temperature. The slides were washed again (3 x 5 mins PBS) and blocked with 4% (w/v) bovine serum albumin (BSA) in Tris HCl (30 mins). The TUNEL mixture (label and enzyme) was then applied for a 2-hour incubation at 37°C before being washed (3 x 5 mins PBS) and counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen D1306; 5 mins) to label cell nuclei. Washed slides were mounted under a coverslip with Prolong Antifade Gold (Invitrogen P36930).

Positive controls were treated with DNase (Roche Diagnostics 10 104 159 001) prior to the TUNEL protocol. Negative controls were slides treated according to the TUNEL protocol except that the TUNEL mixture contained the label and no enzyme.

3.6.2 **BrdU**

Birds received BrdU injections 4 hours prior to being sacrificed. Sections were washed (3 x 5 mins PBS) and treated with 4N HCl (10 mins at room temperature) to unwind the DNA and allow for antibody tagging. Slides were washed again (3 x 5 mins PBS) then incubated with anti-BrdU (Developmental Studies Hybridoma Bank (DSHB) G3G4; 1:80 in 0.3% (v/v) Triton-X in PBS, 37 °C, 2 hours) to tag BrdU incorporation into the retina. Slides were washed again (3 x 5 mins PBS) then incubated with fluorescein isothiocyanate (FITC) anti-mouse IgG (Fab specific, Sigma F5262; 1:200 in 0.3% (v/v Triton-X in PBS, 37 °C, 1 hour). Following washing (3 x 5 mins PBS), slides were
counterstained with DAPI (5 mins) before being washed again (3 x 5 mins PBS) and mounted under a coverslip with Antifade.

### 3.7 Imaging, Cell Counts and Analysis

Microscopic analysis of the tissue was performed using an upright fluorescence deconvolution microscope (Zeiss Axio Imager.Z2). Z-stack images of central regions of the retina were taken at 20x magnification.

For each time point, the total number of cells in the ganglion cell layer was counted (DAPI). Cells labeled with TUNEL and BrdU were also counted and compiled per time point. Cells were considered to be localised to the GC layer if they lay within, touching or within 1 cell away from the GCL. All cells were calculated as a value per mm of retina. The average total number of cells in the GC layer and the average number of immuno-labeled cells were analysed for differences with two-way ANOVAs followed by Tukey or Bonferroni post-hoc tests. Values were considered significant at $p \leq 0.05$. 
IV. Results

4.1 Experiment 1: Effect of age

4.1.1 Ocular components showing similar growth patterns but different magnitudes

To determine the effect of age on ocular growth patterns, the ocular components were measured at various time points post-surgery, for both sets of birds. Overall, the anterior chamber depth, lens thickness, choroidal thickness and scleral thickness changes exhibited similar growth patterns between the younger and the older chicks.

The younger chicks had smaller anterior chambers (ACs) overall (Fig 4A, day 7: ONS 1.27 ± 0.02 mm, sham 1.31 ± 0.01 mm) compared to the older chicks (Fig 4B, day 7: ONS 1.45 ± 0.02 mm, sham 1.44 ± 0.01 mm). The AC depths increased over time in both younger and older chicks (p < 0.0001 for both; but only the younger chicks showed significant expansion differences between the eyes (p = 0.0185, Fig 4A). Specifically, AC expansion in ONS-eyes of the younger chicks (Fig 4A) was slightly slower than that of sham eyes, with significant changes relative to baseline occurring 5 days post-ONS (p < 0.0001) compared to 3 days post-sham (p = 0.0001) resulting in significantly deeper AC depths beginning at 3 days post-ONS (p < 0.0106). Expansion of ACs in older chicks (Fig 4B) was significant relative to baseline starting at day 3 (p ≤0.0010).

The younger chicks had thinner lenses (LNs) overall (Fig 4C, day 7: ONS 2.06 ± 0.02 mm, sham 2.01 ± 0.01 mm) compared to the older chicks (Fig 4D, day 7: 2.17 ± 0.14, sham 2.23 ± 0.01 mm). The lens became thicker in both the younger and older chicks, although lens thickening was faster in the younger chicks (Fig 4C) beginning at 1 day post-ONS (p = ≤0.0001), compared to that in older chicks (Fig 4D) beginning at 3 days post-surgery (p = 0.0004). Lens thicknesses were not
different between the eyes for both older and younger chicks (p = 0.1088, p = 0.2530, respectively) for all time-points except for day 7 post-ONS, where lenses of ONS-treated eyes of the older chicks were thinner at day 7 (p = 0.0017; Fig 4D).
Figure 4  AC, LN, CH, and SC changes as a function of time of younger and older chicks.

Normalised mean AC depth for younger (A) and older (B) chicks, LN thickness for younger (C) and the older (D) chicks, CH thickness for younger (E) and older (F) chicks, SC thickness for younger (G) and older (H) chicks, as a function of time. (*) significance from baseline, (†) significance between treated and control, (**) significant changes of both eyes from baseline.
The dashed vertical lines on the graphs indicate the time of the ONS and sham procedure.
For both younger and older chicks, expansion pattern of the choroids (CHs) in the ONS-eyes were similar (Fig 4E and F), showing significant increases beginning at day 1 (p = 0.0029, p < 0.0001, respectively) until day 3 (day 3 thickness: younger 0.50 ± 0.04 mm, older 0.34 ± 0.02 mm), at which time, expansion of the choroids stopped (p ≥ 0.1267, p ≥ 0.1979, respectively). Sham-treated eyes showed no differences as a function of time (p = 1.0000 for all time points) of the choroidal thickness, for either younger or older chicks.

Scleral (SC) patterns (Fig 4G and H) of growth for both younger and older birds were similar to the lens, showing expansion over time (p < 0.0001 for both), but no overall difference between the eyes (p = 0.9401, p = 0.1727, respectively). Like the growth of the lenses, only older birds (Fig 4H) showed slight differences between the eyes, with scleras in ONS-eyes both expanding faster (day 3, p < 0.0001) than those in their fellow eyes (day 5, p = 0.0001) and becoming significantly thicker (mean difference ± s.e.m.: 0.016 ± 0.007 mm) than fellow eyes at day 7 (p = 0.0005). Younger chicks (Fig 4G) showed no differences between the eyes over time (p = 0.4440); significant increases in scleral thicknesses began at day 3 (p = 0.0060).

4.1.2 Ocular components showing different growth patterns

The ultrasound data was again used to determine the ocular components showing different growth patterns. Overall, the vitreous chamber (VC) depth and the retinal thickness (RT) changes exhibited different growth patterns between the younger and the older chicks.

Two phases of growth patterns were observed for vitreous chambers for both the younger (Fig 5A) and older (Fig 5B) chicks, however changes within each phase were different between the two groups. Overall, vitreous chambers in ONS-treated eyes were more shallow compared to their
fellow eyes \((p < 0.0001\) for both younger and older chicks). However in younger chicks (Fig 5A), VC depth in ONS-eyes decreased over the first three days \((p < 0.0001)\), followed thereafter by a progressive expansion resulting in significantly deeper VCs at day 7 relative to day 3 \((p = 0.0143)\). In contrast, in older chicks (Fig 5B), VC depth in ONS-eyes did not change over the first three days \((p = 1.0000)\), although, similar to the younger chicks, VC expansion was also observed after 3 days post-ONS. The rate of vitreous expansion after day 3 post-ONS was also faster in the older chicks (Fig 5A), with significant differences detected at day 5 \((p < 0.0001\) vs. day 3) compared to significance occurring at day 7 in the younger chicks (Fig 5B, \(p = 0.0143\)). VCs in the sham-treated eyes also differed between the two groups. VCs of sham-eyes in younger chicks (Fig 5A) showed biphasic development with no change over the first 3 days followed by expansion between days 3 and 5. In contrast, the VCs in sham-eyes of the older chicks (Fig 5B) showed a progressive increase, resulting in earlier significant changes \((day 3: p < 0.0001\) relative to baseline), compared to later significant changes in younger chicks \((day 7: p < 0.0001)\). Despite the differences in growth patterns for the eyes, in both younger and older chicks, significantly shallower VC depths were observed in the ONS-eyes starting at day 3 post-ONS \((p \leq 0.0001, p \leq 0.0036,\) respectively).

Like the vitreous chamber, growth pattern differences of the retina were different between the 2 groups. Retinas of ONS-treated eyes in younger chicks (Fig 5C) showed a significant thickening starting at 1 day \((p = 0.0001)\) and peaking at day 3 \((p = 0.0001)\), before thinning significantly relative to peak thickness \((p < 0.0001\) for both day 5 and 7). Sham-eyes (Fig 5C) showed a similar but slower thickening of retinas that became significant \((p = 0.0142)\) at day 3. Retinal thicknesses after day 3 did not change over time \((p = 1.0000\) for both day 5 and 7). Since retinal thickness changes in ONS-treated eyes were faster, retinas in these eyes were thicker at day 1 \((p = 0.0347)\) and day 3 \((p < 0.0001)\) and significantly thinner at day 7 \((p < 0.0001)\) compared to sham-treated eyes. The time
course for retinal thickness changes in the ONS-eyes of the older chicks (Fig 5D) appeared to follow the same biphasic pattern, except that no significant differences were detected until day 7 post-ONS when retinas were significantly thinner compared to all other time points ($p \leq 0.0001$) and compared to sham-treated eyes at the same time point ($p \leq 0.0001$). RT changes of the sham-treated eyes in these birds (Fig 5D) did not change as a function of time ($p \geq 0.5265$).

![Graphs showing VC and RT changes as a function of time for younger and older chicks.](image)

**Figure 5** VC and RT changes as a function of time of younger and older chicks.

Normalised mean VC depth in the younger (A) and older (B) chicks and RT thickness in the younger (C) and older (D) chicks, as a function of time. (*) indicates significance from baseline and (†) indicates significance between treated and control eyes. The dashed vertical lines on the graphs indicate the time of the ONS and sham procedure.
4.1.3 Correlation between structures

To determine whether vitreous chamber depths were inversely related to retinal and choroidal thickness changes, vitreous chambers were correlated to retina plus choroid and choroid alone. The data in the younger chicks (Figure 6A and B) did not show a strong correlation when retinal values were included ($r^2 = 0.2659$) or when excluded ($r^2 = 0.3095$). The data in the older chicks (Figure 6C and D) showed no correlation when retina values were included ($r^2 = 0.0134$) or when excluded ($r^2 = 7.1444 \times 10^{-8}$).

![Figure 6](image-url)

**Figure 6** Linear correlation between RT+CH vs. VC and CH vs. VC of younger and older chicks.

Linear correlation between normalised retina and choroid (RPC) values against normalised VC values in younger (A) and older (C) chicks and between normalised CH values against normalised VC values in younger (B) and older (D) chicks.
4.1.4  Cell death activity in the GCL

To determine the cell death activity in the GCL over time and to compare the activity between the younger and the older chicks, retinal images were stained with TUNEL and DAPI, and cell counts performed. Images of retinas for younger (Fig 7) and older chicks (Fig 8) were relatively similar, images showing GCL, INL and ONL. ONS-treated eyes in both the younger and older chicks showed progressively decreasing numbers of GCL cells starting from day 3, with significantly lower number of cells at day 5 (relative to day 3; p = 0.0272; Fig 9A) in younger birds, and at day 7 (c.f. p ≤ 0.0061; Fig 9B) in older birds. Cells in the GCL of sham-treated eyes in both groups of birds did not change over time (younger p ≥ 0.4651, older p ≥ 0.9792; Fig 9A and B).

The younger and the older chicks also showed the same trends in apoptotic activity (Fig 9C and D); apoptotic activity increased to a significant peak at day 5 post-ONS (peak values ± s.e.m.: younger, 20.50 ± 2.83% cells/mm, p ≤ 0.0002; older, 15.76 ± 4.24% cells/mm, p = 0.0182), before decreasing thereafter. Little to no activity was detected at day 14 post-ONS (younger: 1.23 ± 0.02% cells/mm; older: 0% cells/mm). There was little to no apoptotic activity in the sham-treated eyes of either the younger or older chicks (images: Fig 6 and 7, graph: Fig 9C and D).
Figure 7  TUNEL and DAPI stained retinal cell images of younger chicks

Retinal cell nuclei in the younger chicks stained with DAPI (blue) and TUNEL (green) in the ONS- (left) and sham-treated (right) eyes for each time point. GCL at the top, followed by the INL and ONL.
Figure 8  TUNEL and DAPI stained retinal cell images of older chicks
Retinal cell nuclei in the older chicks stained with DAPI (blue) and TUNEL (green) in the
ONS- (left) and sham-treated eyes for each time point. GCL, at the top followed by the
INL and ONL.
Figure 9 Total number of cells and apoptotic cell numbers in the GCL as a function of time of the younger and older chicks.

Total number of cells in the GCL as a function of time in the younger (A) and older (B) chicks. Apoptotic cells in the GCL in the younger (C) and older (D) chicks. (*) indicates significance from baseline and (†) indicates significance between treated and control eyes.

4.2 Experiment 2: Effect of growth factors

4.2.1 Ocular components showing similar growth patterns but different magnitudes

To determine the effect of growth factors on ocular growth patterns, the ocular components were measured using ultrasound at various time points post-surgery, for both sets of birds. Overall, the lens thickness, vitreous chamber depth, retinal thickness, and choroidal thickness changes exhibited
similar growth patterns between the chicks that did receive GF (+GF) compared to those that did not (-GF).

Like the eyes of day old birds that did not receive growth factor injections (-GF birds, Fig 10A), the lens thicknesses of +GF birds (Fig 10B) did not differ between the eyes, although they increased, with significant changes also occurring at 1 day post-ONS. A slight attenuation of lens thickening of the +GF birds between days 5 and 7 (p = 0.6226, compared to p < 0.0001 for –GF birds).

The vitreous changes (Fig 10C and D) were also very similar; ONS-eyes (+GF, Fig 10D) showed biphasic growth patterns with time course for an initial decrease in VC depth also occurring between day 0 and 3 (p < 0.0001) followed by an expansion between days 3 and 5 that was slightly faster than for –GF birds, with the former showing earlier significant changes at day 5 (p = 0.0242 compared to p = 1.0000 for -GF birds). Sham-eyes (Fig 10D) were also slightly different, expansion was faster in the +GF group with significant changes occurring at day 5 (p < 0.0001) compared to day 7 for –GF group (p < 0.0001). Interocular differences in VC depths in +GF group from time points 3, 5 and 7, as was observed for -GF birds (p < 0.0001 for all time points).

The initial thickening of retinas (Fig 10E and F) observed in ONS-eyes of +GF birds was slightly slower than for -GF birds with significant changes occurring at day 3 (p = 0.0010) rather than at 1 day post-ONS (p < 0.0001). The rate of decrease in retinal thickness in ONS-eyes was similar in both groups, although, at day 7 the +GF retinas (Fig 10F) were thicker than those of the –GF group (+GF: 0.25 ± 0.003 mm, -GF: 0.24 ± 0.003 mm). Unlike the sham-eyes of –GF birds, no changes in retinal thickness as a function of time were detected in sham-eyes of +GF birds (p = 1.0000). Unlike in –GF birds, interocular retinal thickness differences were not significant at days 1 and 3 (p = 0.3137, p = 1.0000, respectively) for +GF birds but were at day 7 (p = 0.0119, Fig 10F).
Like retina, initial thickening of choroid (Fig 10G and H) observed in ONS-eyes was also slower in +GF birds, showing significantly greater thickening later, at day 3 (p < 0.0001), compared to at day 1 as observed for –GF (p = 0.0029, Fig 10G), which was reflected by the larger increase in choroidal thickness at day 3 in –GF chicks compared to the +GF chicks (Fig 10H: +GF 0.32 ± 0.04 mm, Fig 10G: -GF 0.50 ± 0.04 mm). Choroidal thicknesses of sham-eyes in both groups of birds did not change with respect to time (p = 1.0000 for both). Interocular differences were also significant at the same time points, days 3, 5, and 7 (p < 0.0001 for all time points).
Figure 10 LN, VC, RT and CH changes as a function of time of –GF and +GF chicks

Normailised mean LN thickness –GF (A) and +GF (B), VC depth –GF (C) and +GF (D), retinal thickness –GF (E) and +GF (F), and CH thickness –GF (G) and +GF (H) as a function of time. (*) significance from baseline, (†) significance between treated and control eyes, (**) significant changes of both eyes from baseline. The dashed vertical lines on the graphs indicate the time of the ONS and sham procedure.
4.2.2 Ocular components showing different growth patterns

To determine the ocular components showing different growth patterns, the ultrasound data collected was again analysed. Overall, the anterior chamber depth and scleral thickness changes exhibited different growth patterns between the –GF and +GF chicks. The anterior chamber (Fig 11A and B) and sclera (Fig 11C and D) both showed differences in growth between the +GF and the –GF groups. The anterior chamber of the ONS-treated eyes differed the most, with the +GF ONS-eyes (Fig 11B) showing none of the expansion observed for the –GF birds (Fig 11A), although the anterior chamber of sham-treated eyes showed the same pattern of increasing growth (significantly thicker ACs at days 3, 5 and 7; p < 0.001 for all time points). Interocular differences varied slightly with differences occurring later at day 5 (p < 0.0001) in +GF birds (Fig 11B) compared to day 3 (p = 0.0106) in –GF birds (Fig 11A).

Unlike scleras of the –GF group, the scleras in ONS-eyes of the +GF group (Fig 11D) were thicker overall (p = 0.0007; compared to p = 0.9401 for –GF group). Scleral thickening was similar, with the +GF group showing significant thickness increase at day 3 (p < 0.0001, Fig 11D).
Figure 11  AC and SC changes as a function of time of –GF and +GF chicks

Normalised mean AC depths –GF (A) and +GF (B) and SC thickness –GF (C) and +GF (D) as a function of time. (*) indicates significance from its respective baseline, (†) indicates significance between treated and control eyes, (***) indicate significant changes of both eyes from baseline. The dashed vertical lines on the graphs indicate the time of the ONS and sham procedure.
4.2.3 Correlation between structures

To determine whether vitreous chamber depths were inversely related to retinal and choroidal thickness changes after treatment with growth factors, vitreous chambers were correlated to retina plus choroid and choroid alone. Correlations of vitreous chamber depths to retinal and choroidal thicknesses were again analysed for +GF birds (Fig 12A and B). Similar to –GF birds, the correlation between the vitreous and the retina plus the choroid was quite similar to that of the vitreous against the choroid alone, although the correlation was slightly stronger in the +GF (+GF: $r^2 = 0.5005$ and $r^2 = 0.4234$, respectively; -GF $r^2 = 0.2659$ and $r^2 = 0.3095$).

Figure 12 Linear correlation between RT+CH vs. VC and CH vs. VC in +GF chicks

Linear correlation between normalised RT+CH values against normalised VC values (A) and between CH values against VC values (B).
4.2.4  **Cellular activity in the GCL**

The total number of cells in the GCL decreased in both the –GF and +GF groups (Fig 13A and B; images Fig 14 and 15) but to different degrees in the ONS-treated eyes, with no differences over the first 3 days (p = 1.0000 for all time points), followed by a significant decrease in cell numbers starting day 5 post-ONS (relative to day 3: -GF p ≤ 0.0026, Fig 13A; +GF p ≤ 0.0070, Fig 13B). When comparing the overlapping time points (days 3, 5 and 7 post-ONS, Fig 13C), no difference between the two groups of birds was found (p = 0.6218). The sham-treated eyes for both groups did not show any statistically significant changes over time (+GF p ≥ 0.9989, -GF p ≥ 0.4961).

**Figure 13 Total number of cells in the GCL of –GF and +GF chicks**

Total number of cells in the GCL of –GF chicks (A), +GF chicks (B) and overlapped graphs (C). (*) indicates significance from baseline and (†) indicates significance between treated and control eyes.
Figure 14 BrdU labeled retinal cell images of –GF chicks

Retinal cells of the –GF chicks stained with DAPI (blue) and BrdU (green) in the ONS- (left) and sham-treated (right) eyes for each time point. GCL at the top, followed by the INL and ONL.
Figure 15  BrdU labeled retinal cell images of +GF chicks

Retinal cells of the +GF chicks stained with DAPI (blue) and BrdU (green) in the ONS- (left) and sham-treated (right) eyes for each time point. GCL at the top, followed by the INL and ONL.
The –GF chicks (images Fig 14 and cell counts Fig 16A) showed proliferative cells in only the ONS-treated eyes starting at 3 days post-ONS (2.23 ± 0.71 cells/mm) that continued until 7 days post-ONS (12.5 ± 1.66 cells/mm average of days 4 – 7 post-ONS), with values at 5 days post-ONS statistically greater than 1 day post-ONS (p = 0.0453, 13.77 ± 3.02 cells/mm). No activity on days 1, 2 or 8 post-ONS, nor at any time point in the sham-treated eyes. The +GF chicks (images Fig 15 and cell counts Fig 16B) showed proliferative cells in both the ONS- and sham-treated eyes at all time points. The ONS-treated eyes showed proliferative activity at day 3 post-ONS (7.82 ± 1.22 cells/mm) that increased to a peak at day 5 (17.88 ± 2.91 cells/mm, p = 0.0058), followed by a decrease thereafter. Activity was still present at 14 days post-ONS (1.68 ± 1.07 cells/mm). In contrast to the –GF birds, the sham-treated eyes in the +GF group showed proliferative activity at every time point (3, 5, 7, 10 and 14 post-ONS) although values did not change significantly over time (p ≥ 0.6645 for all time points).

Figure 16 Proliferative activity in the GCL of –GF and +GF chicks

Proliferative activity in the GCL of –GF chicks (A), +GF chicks (B) and overlapping graphs (C). (*) indicates significance from baseline and (†) indicates significance between treated and control eyes.

The –GF chicks (images Fig 14 and cell counts Fig 16A) showed proliferative cells in only the ONS-treated eyes starting at 3 days post-ONS (2.23 ± 0.71 cells/mm) that continued until 7 days post-ONS (12.5 ± 1.66 cells/mm average of days 4 – 7 post-ONS), with values at 5 days post-ONS statistically greater than 1 day post-ONS (p = 0.0453, 13.77 ± 3.02 cells/mm). No activity on days 1, 2 or 8 post-ONS, nor at any time point in the sham-treated eyes. The +GF chicks (images Fig 15 and cell counts Fig 16B) showed proliferative cells in both the ONS- and sham-treated eyes at all time points. The ONS-treated eyes showed proliferative activity at day 3 post-ONS (7.82 ± 1.22 cells/mm) that increased to a peak at day 5 (17.88 ± 2.91 cells/mm, p = 0.0058), followed by a decrease thereafter. Activity was still present at 14 days post-ONS (1.68 ± 1.07 cells/mm). In contrast to the –GF birds, the sham-treated eyes in the +GF group showed proliferative activity at every time point (3, 5, 7, 10 and 14 post-ONS) although values did not change significantly over time (p ≥ 0.6645 for all time points).
4.2.5  Proliferative activity in the GCL + NFL

The proliferative activity was not limited to the GCL; many cells were also detected in the NFL.

When the values of the proliferative cells from both layers were combined (Fig 17), the number of proliferative cells in –GF birds (Fig 17A) increased for every time point that activity was found (days 3 – 7 post-ONS) and days 4 and 5 were significant from baseline (day 4: 15.65 ± 3.04 cells/mm vs. GCL only 12.29 ± 3.23 cells/mm, day 5: 19.71 ± 2.79 cells/mm vs. GCL only 13.77 ± 3.02 cells/mm, p ≤ 0.0339). The +GF chicks (Fig 17 B) showed increases in proliferative cells for all time points in the ONS-treated eyes except at day 14, and in sham-treated eyes the number of proliferative cells was also greater, with the most striking difference occurring at day 5 post-ONS (31.61 ± 5.36 cells/mm vs. GCL only 17.88 ± 2.91 cells/mm, p ≤ 0.0002).

![Figure 17 Proliferative activity in the GCL of –GF and +GF chicks](image)

Proliferative activity in the –GF (A) and +GF (B) comparing the values from the GCL only to the GCL + NFL. (*) indicates significance from baseline and (†) indicates significance between treated and control eyes.
V. Discussion

5.1 Experiment 1: Effect of age

The difference in the size of the ocular components, between the younger and older chicks, was likely a result of the overall ocular growth of the chick between 1 day and 7 days post-hatch. The difference in size was most clearly demonstrated prior to surgery in the anterior chamber and the lens (Fig 3, AC: A vs. B, LN: C vs. D); age-related increases in growth of the eye were previously documented (Avila and McFadden, 2010).

The younger chicks also appeared to show more robust responses. Specifically, a decrease in vitreous chamber depth and a larger increase in choroidal thickness were observed for the younger chicks. In contrast, the older chicks exhibited no change in the vitreous chamber depth along with a smaller increase in choroidal thickness at the same time points. Together these differences led to a distinct overall growth pattern with the observation of no growth for the older chicks in the first 3 days, in contrast to the younger chicks that demonstrated a significant decrease in vitreous depth and increase in retinal thickness. The changes in growth patterns observed for the younger birds is likely associated with the early phase to compensate for myopic defocus as well as post-ONS (Wildsoet and Wallman, 1995; Wildsoet, 2003), while, changes in growth patterns of the older birds are similar to previous demonstrations of the eye’s ability to compensate for myopic defocus by slowing the growth of the vitreous chamber in response to positive lenses and the cessation of form-deprivation (Norton, 1990; Sivak et al., 1990; Wallman and Adams, 1987; Wallman et al., 1995). Differences between the age groups highlight the ocular plasticity of the younger birds. Note that in addition to the changes to the retina and vitreous, the choroids of younger chicks became thicker in comparison to the older chicks.
The changes of the different ocular components seen in this study post-ONS follow the development of hyperopia that has been previously documented and attributed to the significantly shorter vitreous chamber (Troilo et al., 1987). The authors of the study raised the idea that the brain regulates eye growth because hyperopia developed without an intact optic nerve. Another hypothesis was the need for both a functional central and peripheral mediated neural mechanisms that may be disrupted with the death of the ganglion cells as a result of the ONS (Fischer et al., 1999). The development of hyperopia in chicks has also been associated with a decrease in anterior chamber depth, which was demonstrated in the ONS-eyes of the chicks in our study (Fig 3A and B), along with a thinning of the lens (Fig 3C and D) (Avila and McFadden, 2010). There was no change post-ONS in the lens thicknesses of our birds, which was similar to the study by Schaeffel and Howland, who showed the typical hyperopia induced in chicks using plus-lenses (Schaeffel and Howland, 1991), although other studies have shown lens thinning (0.003%) (Wildsoet, 2003).

Retinal thickness was of interest because of the suspected loss of the RGCs, but interestingly, the retina was found to thicken during the first 3 days. This has not been previously shown by other authors, either it has not been documented or it has not been looked at. However, retinal thickening was limited to younger chicks; this phenomenon may be attributed to the dramatic increase in the thickness of the choroid that was much more pronounced in the younger chicks (Fig 3E and F). As the increase in choroidal thickness allows the retina to be placed at the plane of focus, there is a decrease in the surface area of the retina, resulting in an apparent retinal thickening. If the opposite were to occur, the choroid would thin, creating an increase in surface area and a thinning of the retina as was previously observed by other investigators (Troilo et al., 2000).

Control of the choroid is postulated to occur locally, partially by the retina. The retina has been identified as having the ability to detect image defocus and in turn directing the choroid to
compensate accordingly as a form of local feedback (Wildsoet and Wallman, 1995). Although the choroid demonstrates local feedback, it may also continue to be controlled by the central nervous system because of the innervation from the ciliary ganglion, pterygopalatine, superior cervical ganglia and the oculomotor, trigeminal and facial nerves (Bill, 1985), also, the choroidal bloodflow is highly stimulated by the Edinger-Westphal nucleus (Fitzgerald et al., 1990), all of which are unharmed after ONS. Interestingly, diffusers (Shih et al., 1993) cause choroidal blood flow to be altered but not the thickness (Wallman et al., 1995). The local regulation of the choroid may be accomplished by the RPE. This idea is supported by the evidence that shows the choriocapillaris is strongly under the influence of the RPE (Korte et al., 1989). Separation or damage of the choriocapillaris and/or RPE results in concurrent atrophy in various diseases including age-related macular degeneration and retinitis pigmentosa.

The choroid has also been linked to scleral changes and eye development. The choroid is thought to be the first to respond to the myopic signal by bringing the retina as close to the focal plane as possible, then a signal is sent to the sclera to complete emmetropisation, also allowing the choroid to return to normal thickness (Wildsoet and Wallman, 1995). The implication is that there is local feedback where the retina/RPE signals the choroid and the choroid in turn signals the sclera (Wildsoet and Wallman, 1995). I believe that the increase in scleral thickness as was demonstrated in the scleras of the older chicks should have also been found in the scleras of younger chicks because it has been previously shown that after ONS treatment, the eye decreases the ability to elongate and in turn the scleral growth is also affected via signaling mechanisms described above (Wildsoet and Wallman, 1995). Typically, axial growth is associated with scleral thinning (Vurgese et al., 2012), which was demonstrated in this study in the sham-treated eyes of the older chicks (Fig 3H). It is unclear why the scleral changes were not also seen in the younger chicks and may be attributed to procedural error.
Although the vitreous depth has been linked to the simultaneous change in the choroid (Wildsoet and Wallman, 1995), the correlation between the structures was not very strong in this study (Fig 5). The older birds did not show any correlation whereas the younger chicks did, which may reflect the large and dramatic changes in the vitreous and choroid.

This is the first study to examine the ONS cell death timeline in chick eyes. Apoptotic cells were stained with TUNEL but were also smaller in size compared to the other cells in the retina. The apoptotic activity timeline and the overall change in the total number of cells over time in the GCL were very similar between the younger and the older chicks; the implication is that ONS-related cell death processes were at least mediated in part by apoptosis and that the processes was dependent on time of surgery (or acute damage) but not the age of the chicks. Although the apoptotic activity timelines were very similar, the number of apoptotic cells was consistently higher in the younger group of chicks, which may be attributed to the younger eyes being more susceptible to injury. Apoptosis has been extensively studied in the rat (Berkelaar et al., 1994; García-Valenzuela et al., 1994), mouse (Germain et al., 2012), monkey (Quigley et al., 1995) and rabbit (Quigley et al., 1995).

In the adult rat model, it has been shown that with intracranial optic nerve transection the ganglion cells survived for 5 days and then diminished rapidly with only 10% of RGCs at day 14 (Berkelaar et al., 1994). In mice, 6 days after axotomy, the density of the RGCs decreased to 49% of the original population and by 14 days the density decreased to 15% (day 5 was not assessed) (Germain et al., 2012). This study showed a decrease of RGC population of the younger chicks to 63% at day 5 to 31% at day 14 (compared to the fellow eyes). In contrast, the older birds showed a decrease to 64% at day 5 to 25% at day 14. Note that apoptotic activity in chicks was induced by a mechanical model without ischemia and may account for slight differences in the timeline. It is widely accepted that after transection of the optic nerve, there are apoptotic changes that occur and the typical timeline has
shown that the peak of activity occurs very rapidly around 5 – 6 days post-ONS. The apoptosis of the RGCs in turn decreases the total number of cells in the GCL and the cells that are left over are thought to be displaced amacrine cells. Future studies should include characterisation of the cells remaining in the GCL.

5.2 Experiment 2: Effect of growth factors

The eye growth patterns overall were similar between the –GF and +GF groups, which was to be expected because ONS was performed at 1 day post-hatch for both groups. The variations in the speed of growth may be attributed to the stimulation of the eye with the growth factors, especially at the later time points. The variation was noted in the vitreous chamber depth changes of both the +GF ONS- and sham-treated eyes, where a faster increase in depth was seen after 3 days post-ONS, indicating a faster rate of recovery. Also, the retinas of the +GF group were slightly thicker than those of the -GF group at day 7, which may be attributed to a growth factor dependent increase in proliferative cells and/or a delay resulting from inhibition of the apoptotic mechanism (Ríos-Muñoz et al., 2005). It has not been determined which of the two mechanisms occurred in these experiments. Further work analysing the amount of apoptotic activity and the specific timeline would yield this information, however, the number of apoptotic cells in the retinal sections from the –GF group and the +GF group were not analysed in this study. The increase in choroidal thickness of the +GF group was not as large as was seen in the -GF group and this difference may be due to the growth factors diminishing the hyperopic response of ONS treatment. Insulin is associated with anti-inflammatory effects (Jeschke et al., 2008), and if the large change in the choroidal thickness seen in the younger birds was due to inflammation, the growth factors used may explain less drastic change of the choroid in the +GF group, although ONS has not been shown to involve severe inflammation (Kreutzberg,
The anterior chambers of the +GF ONS-treated eyes showed no increase in thickness over time whereas the –GF ONS-treated eyes did, which may be the result of the injections potentially causing an increase in size equatorially and preventing axial elongation. The growth factor injections did not have a large effect on the lens or the sclera. Traditionally, the lens does not show large changes in myopia or the changes are not detectable, which may be the case in this study. FGF is known to play a role in lens development but the concentrations used in this study were likely not the concentration at which lens cells are known to proliferate (McAvoy et al., 1991). Like a previous study showing that FGF was does not play a role in scleral remodeling during the development and recovery of myopia (Gentle and McBrien, 2002), no changes to the sclera were found with FGF injection.

The proliferative activity in the GCL without the addition of growth factors indicated that the acute damage caused by ONS was sufficient to stimulate cells of the retina to re-enter the cell cycle and show proliferative labeling. This idea was further supported by the lack of proliferation in the sham-treated eyes. The slight increase in the proliferative activity was also reflected in the small change in the overall number of cells in the GCL. The use of growth factors allowed for a greater number of cells in the GCL to survive for a longer time (Fig 12C). The proliferative activity in the GCL of the ONS-treated eyes of the +GF group was initially a synergistic (result of acute damage and growth factors) at day 3 (7.82 cells/mm vs. the expected 2.61cells/mm), followed by an additive effect at day 5 (17.88 cells/mm vs. the expected 17.91 cells/mm), and finally a less than additive effect at day 7 (7.79 cells/mm vs. the expected 15.38 cells/mm). The decrease in the synergistic proliferative effects over time may be the result of the extensive damage at the later time points.

Only days 3, 5 and 7 were available for comparison and it could not be determined if growth factors could delay the apoptotic timeline or if there was an inhibition of a component of the apoptotic
cascade and presumably a longer timeline would give better insight. The appearance of proliferative activity in the GCL layer as well as in the NFL may indicate the possibility of Müller cell gliosis contributing to the proliferation. Proliferative cellular activity (between 0 and 5 cells) was seen in the other cellular layers although not at all time points (data not shown). The localisation of the proliferation in the GCL and NFL indicate that the stimulation was a result of the damaged cells from the ONS treatment. The sham-treated +GF chicks also showed proliferative activity, confirming that the combination of FGF and insulin is sufficient to stimulate cells of the retina to proliferate without damage. Although the proliferative activity was limited, the activity was seen in every layer of the retina (data not shown) in the +GF chicks. Proliferation in all layers of the retina may reflect that injury to specific cells is the stimulus to drive proliferation in specific areas, and without the stimulus, proliferation is wide-spread. Future studies should include characterising the type of cells labeled with BrdU. Injury to a specific cell type seems to be the trigger for regeneration of the cells that are lost (Fischer and Reh, 2003a), regardless of the form of damage (colchicine vs. ONS).

The identification of apoptotic mechanisms after ONS damage, as well as the determination of the apoptotic timeline and apoptosis being independent of age, can potentially lead to further investigations of ways to block or inhibit parts of the apoptotic cascade in hopes of preventing cell damage and loss. Time course for cell death is not affected by age but eye growth patterns are affected by age and presumably effects are due to increased plasticity. The ability for the chick retina to exhibit (1) proliferation from acute ONS damage alone, (2) an increase in proliferation with the addition of growth factors and (3) the ability for growth factors to stimulate proliferation alone without damage, can potentially lead to the development of therapeutic strategies for the preservation or restoration of retinal cells in diseased eyes.
Bibliography


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