

**Functional recovery following regeneration of the damaged retina in the adult newt,
*Notophthalmus viridescens***

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fulfillment of the M.Sc. program in Cellular Molecular Medicine.

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ABSTRACT

A hallmark of retinal diseases is degeneration of neural cells, leading to subsequent vision loss. For such diseases, replenishment of functional neural cells may be an optimal therapy. Unlike humans, the adult red-spotted newt, *Notophthalmus viridescens*, possesses the remarkable ability to regenerate a complete retina following its removal or injury. The purpose of this study was to develop a reproducible model of retinal damage and regeneration in the newt to understand the process of retinal regeneration. Intense light, shown in other organisms to be a relevant model of visual cell loss, was tested in the newt and resulted in variable loss of retinal function, correlating with the appearance of apoptotic cells. Due to the variability of damage observed, surgical removal of the retina was used to complement the light-damage model. A novel and non-invasive protocol using full-field electroretinography was developed to assess retinal function *in vivo* following damage. Measures of retinal function with the electroretinogram protocol successfully showed that photoreceptor function is initially lost and subsequently restored during regeneration. These results enhance our understanding of retinal regeneration in the adult newt and serve as a starting point for further studies aimed at determining the molecular mechanisms involved in the regeneration process.

Table of Contents

AUTHORIZATION.....	ii
ABSTRACT.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	xii
ACKNOWLEDGMENTS.....	xiii
1. INTRODUCTION.....	1
1.1 Visual processing in the vertebrate retina.....	1
1.1.1 Composition of the vertebrate retina.....	1
1.1.2 The phototransduction cascade.....	5
1.2 Electrophysiology as a tool to measure restoration of retinal function.....	8
1.3 Retinal diseases.....	11
1.4 The use of light as a retinal damage model.....	12
1.5 Regeneration.....	14
1.6 The adult red-spotted newt as a vertebrate model for retinal regeneration.....	15
1.7 The comparison of newt to human retinal cells.....	19
1.8 Measuring retinal function in the adult red-spotted newt.....	22
1.9 Thesis goals and hypotheses.....	26
2. Materials and Methods.....	27
2.1 Animal Care and Veterinary Services.....	27
2.2 Electrophysiology of adult red-spotted newts.....	27
2.2.1 Preparation of newts for electrophysiology.....	27

2.2.2 Measurement and amplification of newt ERG recordings.....	28
2.2.3 Optimization of ERG parameters.....	30
2.2.3. i. Dilation time	30
2.2.3. ii Variability of ERG recordings.....	32
2.2.3. iii. Effect of temperature on the ERG	32
2.3 The light damage model.....	35
2.3.1 Light treatment.....	35
2.3.2 ERGs to assess loss of retinal function due to light.....	38
2.4 Retinal Whole Mounts	40
2.5 Tissue processing, cryosectioning and histology	40
2.6 TUNEL	41
2.7 Retinectomies.....	42
2.7.1 The use of in vivo ERGs to measure retinal restoration after retinectomies	43
3. Results.....	45
3.1 Optimization of newt ERGs measured in vivo	45
3.1.1 The effect of pupil dilation time	45
3.1.2. The effect of temperature on the ERG	46
3.1.3 The variability of recording an ERG from the same animal over different days.	46
3.2 The newt electroretinogram	47
3.3 Light-induced damage in the adult newt retina.....	51
3.3.1 Phototoxicity 1 – the use of a halogen light bulb to induce retinal damage	58
3.3.2 Phototoxicity 2 – the use of a mercury arc lamp to induce retinal damage	63
3.3.2.1 MAL Method 1 – Constant light exposure of anaesthetised newts	64

3.4 Retinectomy and regeneration of the newt retina - monitoring restoration of retinal function	92
3.4.1 Retinectomy 1	94
3.4.2 Retinectomy 2	98
4.0 DISCUSSION	105
4.1 Establishment of in vivo newt ERGs	106
4.2 The use of light to induce retinal degeneration.....	110
4.2.1 Light-induced as a potential damage model in the newt.....	111
4.2.2 The use of two different light sources to induce retinal damage in the newt (Phototoxicity 1 vs Phototoxicity 2)	113
4.2.3 Anesthetised versus freely-swimming newts – Method 1 vs Methods 2/3	114
4.2.4 The effect of exposure time and light intensity.....	117
4.2.5 Examining the onset and extent of damage in the newt retina due to light	118
4.2.6 Variability and limitations of the light damage model	120
4.3 Monitoring the restoration of retinal function after surgical removal of the retina ..	122
4.3.1 Electrophysiological and histological assessment post-retinectomy	123
4.3.2 Variability of ERG recovery	124
4.4 Controls and considerations for future experiments	125
4.5 Final Conclusion	127
REFERENCES	129
Appendices.....	139
Appendix 1. Buffers/Reagents	139
Appendix II. Basic protocols.	141

LIST OF TABLES

Table 1. The updated ERG protocol for light-adapted newts using Espion™ Software
Version 4 (Diagnosys).....31

Table 2. The various light-damage conditions tested with the 150 W halogen light source
(Edmund Optics)..... 60

LIST OF FIGURES

Figure 1.	A schematic of the vertebrate eye.....	3
Figure 2.	Schematic of the various layers and cell types found in the vertebrate retina..	4
Figure 3.	The initial steps of the phototransduction cascade in the outer segments of the rod.....	7
Figure 4.	The electroretinogram is the total electrical response of the retina to brief flashes of light.....	9
Figure 5.	Diagram depicting the nine stages of newt retinal regeneration following surgical removal of the retina.....	17
Figure 6.	The retinal pigment epithelium (RPE) and the ciliary marginal zone (CMZ) are 2 main sources of stem cells in the newt eye.....	20
Figure 7.	The human visible light spectrum represents the wavelengths of light (colours) that are perceived by photoreceptor cells found in the back of the retina.....	23
Figure 8.	Experimental setup for recording an electroretinogram in the intact eye of a newt.....	29
Figure 9.	A schematic of the box plot which graphs the distribution of information as a five-number summary.....	34
Figure 10.	Phototoxicity assay setup for the Super High Pressure mercury arc lamp (Nikon).....	39
Figure 11.	Schematic of a retinectomy.....	44
Figure 12.	The effect of dilation on the newt ERG a- and b-wave amplitudes.....	47
Figure 13.	Newt body temperature has an effect on the ERG, as shown by box plot.....	48
Figure 14.	Representative ERG recordings from intact light-adapted newt eyes using optimized parameters.....	50
Figure 15.	The EXFO X-Cite metal halide lamp (120 W) and the mercury arc lamp (100 W) relatively compared in power across different wavelengths of light.....	53
Figure 16.	The spectrum of light emitted by the 150 W halogen bulb found in the	

	MI-150 Fiber Optic Illuminator (Dolan-Jenner), at various irradiances.....	56
Figure 17.	The spectrum of light emitted by the Super High Pressure mercury arc lamp, at maximal intensity (Nikon).....	57
Figure 18.	TUNEL labeling indicated that no apoptosis occurred in the newt retina at 3 and 7 days following 15 mins ultra-intense constant light treatment with the halogen bulb set at a high intensity (>100 000 lux).....	61
Figure 19.	Reduction in ERG a- and b-wave amplitudes of intact newt right eyes 3 days following exposure to 30 and 60 mins of ultra-intense light (>100 000 lux) with the halogen bulb.....	62
Figure 20.	Exposure of intact adult newt retina to ultra-intense light from the mercury arc lamp for 5, 15 and 30 minutes (mins) resulted in no significant change of ERG a- and b-wave amplitudes at 2 days post light treatment, compared to baseline recordings.....	65
Figure 21.	TUNEL-positive adult newt retinal cryosections 2 days following constant ultra-intense light treatment with the mercury arc lamp at various exposure times.....	67
Figure 22.	The TUNEL assay on a newt retinal whole mount revealed apoptosis 2 days after light exposure in a focal region of the central retina, after 30 minutes constant ultra-intense light exposure to the mercury arc lamp while anesthetized (Method 1.....	70
Figure 23.	Adult newts sampled 8 hours post 30 minutes constant ultra-intense light treatment with the MAL, while anesthetized, demonstrated TUNEL-positive cells in all layers of the retina.....	71
Figure 24.	TUNEL-positive retinal cells observed in adult newts 1 day following 30 minutes of ultra-intense light exposure with the MAL, as newts were anesthetised on a dish.....	73
Figure 25.	Newts exposed to ultra-intense light while swimming in a beaker surrounded with aluminum foil for 30 minutes showed a statistically significant decrease of the ERG a- and b-waves at 2 days post light treatment (Experiment 1).....	77

Figure 26.	Apoptotic cells were visible all throughout the retina of newt #7 subjected to 30 mins ultra-intense light (Experiment 1).....	78
Figure 27.	Newts exposed to 30 minutes of ultra-intense light from the MAL while swimming in a fishbowl experienced a slight but non-significant decrease of the averaged ERG a- and b-waves at 2 dpl (Experiment 2).....	80
Figure 28.	Newts swimming in a fishbowl surrounded by ultra-intense light from a mercury arc lamp for 30 minutes experienced a statistically significant decrease of the ERG a- and/or b-waves at 2 days post light treatment.....	81
Figure 29.	Exposure to 30 minutes ultra-intense light while swimming in a fishbowl resulted in the appearance of apoptotic cells all throughout the retina of newt #5 (Experiment).....	84
Figure 30.	Exposure of newt retina to ultra-intense light from the mercury arc lamp for 30 minutes resulted in a slightly delayed decrease in the ERG a- and b-waves of some newts at 4 days post light treatment (Experiment 2).....	85
Figure 31.	TUNEL analysis in the retina of newt #6 at 4 days following 30 mins ultra-intense light exposure (Experiment 2).....	86
Figure 32.	Average ERG results of newts exposed to ultra-intense light from the MAL while swimming in a fishbowl in the presence or absence of a UV filter showed a statistically significant decrease of the ERG a- and b-waves at 2 days post light treatment (Experiment 3).....	88
Figure 33.	Individual results for newts exposed to ultra-intense light from the MAL while swimming in a fishbowl in the presence of a UV filter showed variability of the ERG a- and b-waves at 2 days post light treatment compared to baseline (Experiment 3).....	89
Figure 34.	Variability in the onset and degree of TUNEL-positive cells in the retinas of three freely swimming newts following ultra-intense light exposure to the MAL (Experiment 3).....	91

Figure 35. The progressive recovery of the ERG a- and b-waves by 15 weeks post-retinectomy (wpr) suggest regeneration and restoration of retinal function.....	95
Figure 36. Hematoxylin and eosin staining of retinal cryosections from newts with partial or complete recovery of the ERG, at 15 weeks post - Retinectomy 1.....	97
Figure 37. The progressive recovery of the ERG a- and b-waves over 11 weeks post retinectomy (wpr) suggests regeneration and retinal function restoration in many newts.....	101
Figure 38. Hematoxylin and eosin staining of newt eyes that showed complete or near complete restoration of the ERG amplitudes at 11 weeks post-retinectomy.....	102
Figure 39. Hematoxylin and eosin staining of newt eyes that show partial or no recovery of the ERG amplitudes.....	103
Figure 40. Hematoxylin and eosin staining of two different retinal cryosections from the same newt after retinectomy.....	104
Figure 41. The focal point of a light beam and its effect on maximal illuminance of the retina.....	116

LIST OF ABBREVIATIONS

AMD	age-related macular degeneration
CMZ	ciliary marginal zone
dpl	days post-light
dpr	days post-retinectomy
ERG	electroretinogram
gcl	ganglion cell layer
H and E	haematoxylin and eosin
inl	inner nuclear layer
ipl	inner plexiform layer
IR	infrared
OCT	Optimal Cutting Temperature
onl	outer nuclear layer
opl	outer plexiform layer
PBS	phosphate buffered saline
PCM	pigmented ciliary margin
PFA	paraformaldehyde
RP	Retinitis Pigmentosa
RPE	retinal pigment epithelium
RT	room temperature
TdT	<u>T</u> erminal <u>d</u> eoxy <u>n</u> ucleotidyl <u>T</u> ransferase Enzyme
TUNEL	<u>T</u> erminal deoxynucleotidyl transferase d <u>U</u> TP <u>n</u> ick <u>e</u> nd <u>l</u> abelling
W	Watts
wpr	weeks post-retinectomy

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1. INTRODUCTION

1.1 Visual processing in the vertebrate retina

1.1.1 Composition of the vertebrate retina

The ability to see is a complex process made possible by many components of the eye, such as the cornea, the crystalline lens, and the neural retina (Figure 1). A vital step in this process involves the retina, located in the back of the eye. When light first enters the eye, it is focused mostly by the cornea and partly by the lens onto the retina. The neural retina captures all of the information from light as visible radiant energy and translates the message into nerve signals that are transmitted to the brain for interpretation (Webvision, 2010).

The vertebrate retina is a complex and intricate tissue composed of two layers of synapses, the outer plexiform layer (OPL) and the inner plexiform layer (IPL), and three layers of nerve cell bodies, the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL) (Figure 2). The retinal circuitry intricately runs outwards in, beginning with the photoreceptor cells found in the outer nuclear layer, and ending with ganglion cells found in the innermost portion of the retina. Since the photoreceptor cells are located at the back of the retina, the light that enters must pass through the other retinal layers before reaching and stimulating the photoreceptor cells. The outer nuclear layer is composed of nerve cell bodies of the photoreceptor cells. There are two main types of photoreceptor cells, called rods and cones (Figure 2). The rods are responsible for mediating night vision and our vision in dim light while cones, stimulated

by bright light, are responsible for mediating daylight vision, colour vision and our ability to see fine details.

Adjacent to the photoreceptor cells lies the retinal pigment epithelium (RPE) (Figure 2) attached to the choroid. Cells of the RPE are responsible for not only protecting and recycling the photoreceptor cells but also for providing the chromophores required to absorb light at a specific wavelength (Webvision, 2010). The RPE is attached to the underlying vascular layer, which is known as the choroid. Anterior to the photoreceptor cells is the outer plexiform layer, where the rod and cones synapse to axonal endings of the horizontal cells and bipolar cells. The horizontal cells synapse with the photoreceptor cells and link them to bipolar cells through long connections that run parallel or horizontal to the retinal layers. They relay the message either back to photoreceptors, to bipolar cells, or to both (Hubel, 2010). Bipolar cells receive the input from photoreceptors and transmit the message directly to the ganglion cells. In the inner plexiform layer, bipolar and amacrine cells synapse to ganglion cells (Figure 2). Amacrine cells also make reciprocal synapses to the bipolar cell axons in the inner plexiform layer, similar to the way in which horizontal cells make a feed-back synapse to the photoreceptor cells in the outer plexiform layer (Webvision June 28 2010). Lastly, the message transmitted to the ganglion cells is relayed to the optic nerve and to the brain for interpretation of the image.

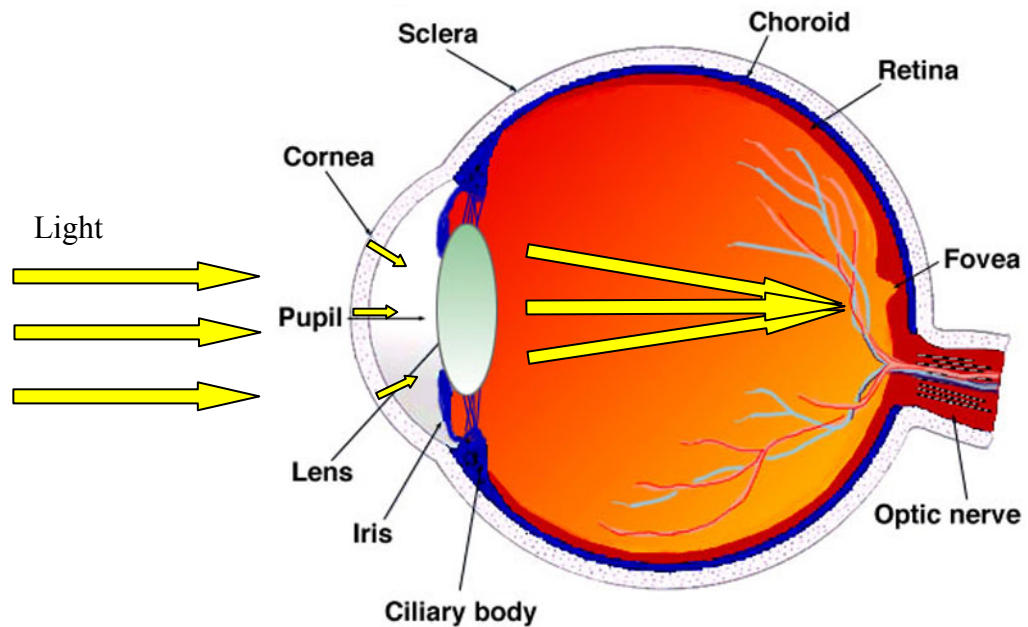


Figure 1. A schematic of the vertebrate eye. Key components of the vision process are the cornea at the front of the eye, the crystalline lens located behind the iris, and the retina located at the back of the eye. Arrows indicate the pathway of light which enters and is refracted (bent) in the transparent cornea directly through the pupil and is further focused onto the retina by the transparent lens (modified from Webvision, 2010).

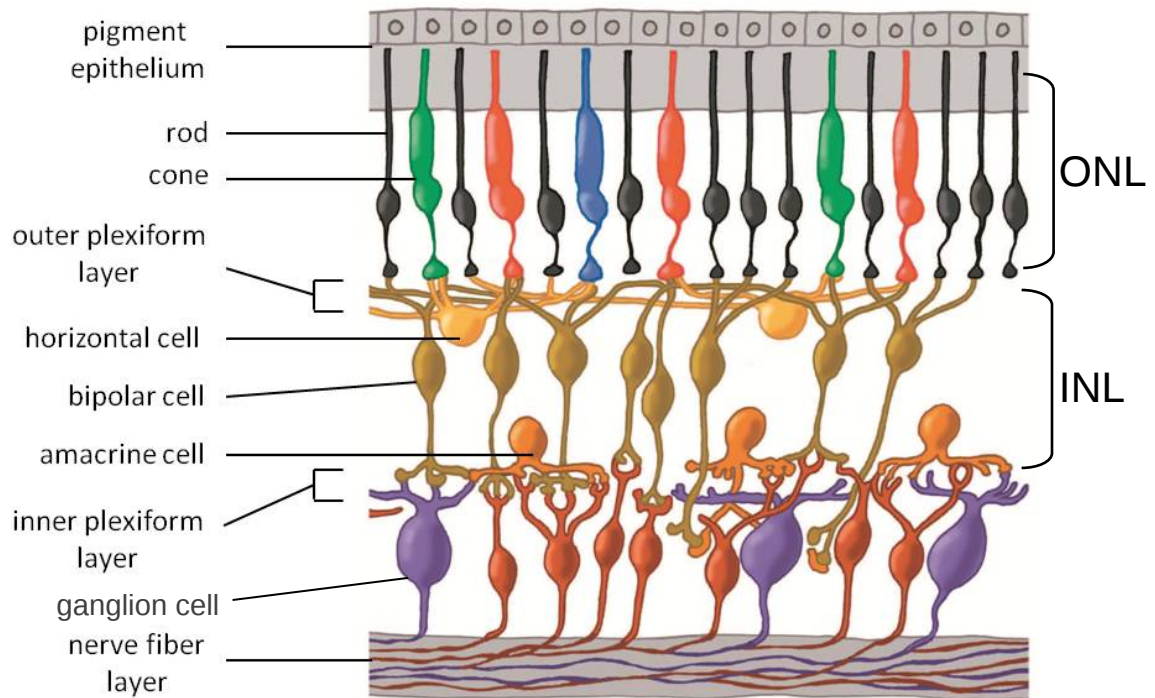


Figure 2. Schematic of the various layers and cell types found in the vertebrate retina. The photoreceptor cells, rods and cones, are found in the outer nuclear layer (ONL) underneath the retinal pigment epithelium, and are the first to capture the photons of light to begin the visual cascade. These synapse through the outer plexiform layer to the horizontal cells and bipolar cells, whose cell bodies are found in the inner nuclear layer (INL). Further synapsis occurs in the inner plexiform layer, where the axons of bipolar cells synapse to the dendrites of ganglion cells. Amacrine cells are found adjacent to the ganglion cells. Impulses from the ganglion cells travel to the brain through the optic nerve fibers, of which there are millions in humans (adapted from Kolb, 2003).

1.1.2 The phototransduction cascade

Phototransduction is the physical process by which retinal photoreceptor cells absorb and translate the energy from photons, the elementary particles of light, into electrical signals which are transmitted throughout the retinal circuitry to the brain (Kolb, 2003). As mentioned above, photoreceptor cells, of which there are over one million in humans, are the first to capture the photons from light, beginning the visual process. Photoreceptors have three main components, the outer segment, the inner segment and the synaptic terminal. The phototransduction machinery is found in the outer segment while metabolic processes occur in the inner segment.

The membranes of the outer segments contain cyclic guanosine 3', 5'-cyclic monophosphate (cGMP)-gated channels, which allow for the passage of ions, mainly sodium, potassium and calcium. In the dark, these channels are open allowing Na^+ and Ca^{2+} to flow freely, leaving the photoreceptors' cell membranes in a depolarized state (Figure 3) (Fain, 2006). Both the G-protein transducin (a trimer) and the tetrameric effector enzyme phosphodiesterase (PDE6) are in their inactive states; and the intracellular concentration of cGMP is relatively high. Therefore, cGMP is able to bind to and open cGMP channels (Figure 3) (Stockman et al., 2006). In the dark, the neurotransmitter glutamate is continually released from the axons of photoreceptor cells and received by the dendrites of neurons in the inner nuclear layer.

When light strikes the retina, visual pigments found in the outer segments of rods or cones absorb the photons of light as the photoreceptors are stimulated. For example, in the rod outer segments, the main visual pigment found is rhodopsin. Rhodopsin is a

member of the G-protein-coupled receptor protein family and consists of an opsin, a bundle of seven transmembrane helices and the photoreactive chromophore, retinal or vitamin A. When light reaches rhodopsin in the rod, retinal undergoes photo-excitation and is isomerised from one conformation to another (11-*cis* retinal to all *trans*-retinal), with all-*trans*-retinal detaching from opsin (Webvision, 2010). The altered rhodopsin molecule activates transducin. This allows guanosine diphosphate (GDP) to be exchanged for guanosine triphosphate (GTP), which causes the separation of activated α -transducin ($G\alpha^*$) from the trimer. Activated transducin in turn activates the phosphodiesterase enzyme (PDE6*) and exposes a site that catalyzes the hydrolysis of cGMP into GMP. This causes cGMP concentrations to be decreased, resulting in the closure of cGMP channels and in turn blocking the inward flow of Na^+ and Ca^{2+} ions (Figure 3) (Kolb, 2003; Stockman et al., 2006; Webvision, 2010). The current decreases, causing hyperpolarization of the membrane voltage (Fishman, 1985). The membrane exists in this state for the duration of light exposure.

The biochemical changes that occur in the photoreceptor cell outer segments initiate a chain of electrochemical events that ensue, with the transmission of chemical signals (neurotransmitters) down the subsequent layers of the retina via the synaptic terminals. The process of breaking down the image into component elements occurs as the photoreceptor cells synapse to the bipolar cells, which in turn synapse to ganglion cells. Each step involves voltage changes in the membrane potential as the neurotransmitter glutamate is released and taken up (Kolb, 2003). Therefore, the rods and cones respond to light and activate graded changes in membrane potential, ultimately producing a fine and focused image that the brain can interpret.

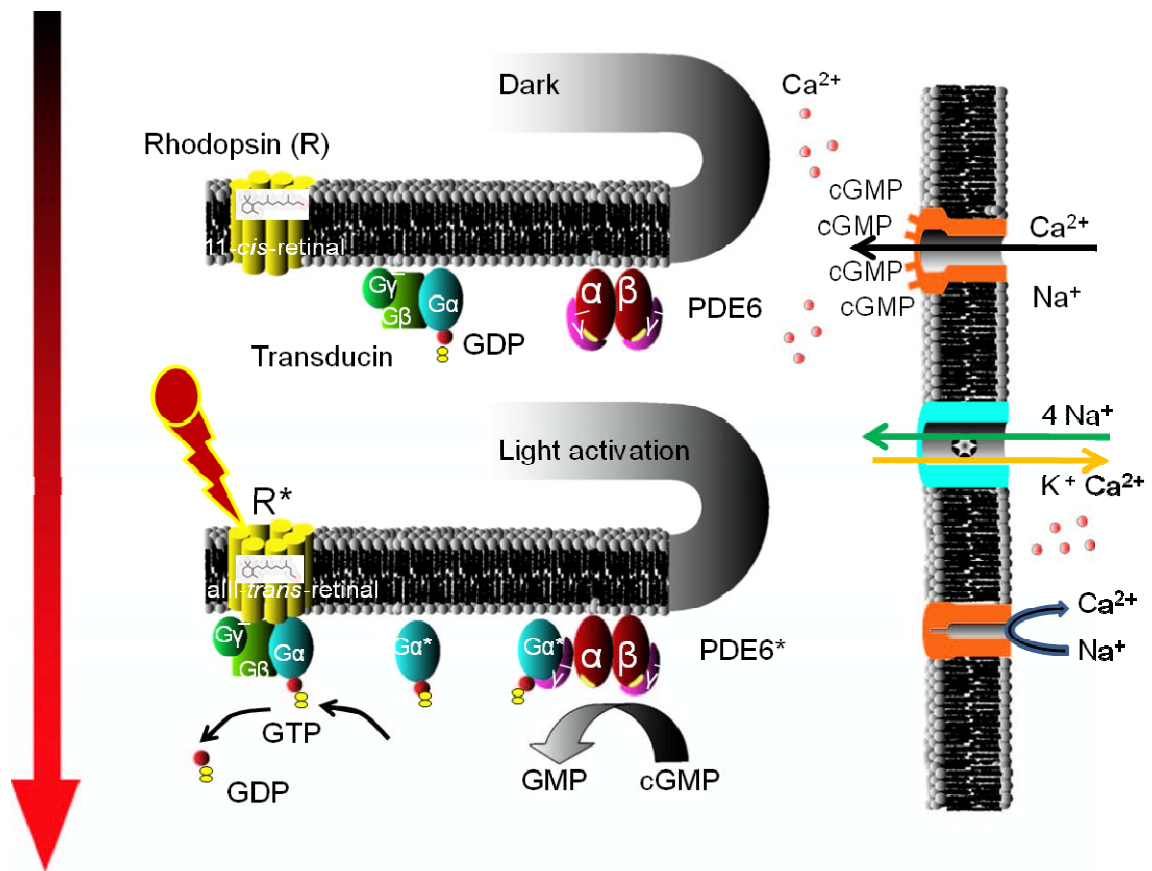


Figure 3. The initial steps of the phototransduction cascade in the outer segments of the rod. In the dark, the segments are depolarized, as the cGMP-gated channels found on the outer membranes allow for the passage of sodium and calcium ions. Membrane hyperpolarization starts on exposure to light. The visual pigment to capture the photons of light in the rod is rhodopsin, consisting of a bundle of seven transmembrane helices and the photoreactive chromophore, retinal. In the dark (top) retinal exists in the 11-*cis* form. When exposed to light (bottom), retinal undergoes photo-excitation resulting in one of the bonds in the molecule to rotate positions to all-*trans*-retinal. The next steps in the vision process involve the activation of the heterotrimeric G-protein transducin ($G\alpha$ -GDP- $G\beta\gamma$) and the subsequent activation of phosphodiesterase 6 which hydrolyzes cGMP into GMP. The reduction of cGMP closes the gated membrane, preventing Na^+ and Ca^{2+} from flowing in. This causes hyperpolarization in the outer segment of the rod, which follows the time course of the light flash and returns to baseline level when the light is off. This is a modification of the figure by Stockman *et al.* (2007) (Stockman *et al.*, 2006; Stockman *et al.*, 2007).

The activation of the membrane potential indicates that the components of the retina are functioning and the proper synapses are forming. Genetic mutations triggering photoreceptor degeneration often affect one or many players of the phototransduction cascade, reviewed by Delyfer (Delyfer et al., 2004). The clinical diagnosis of various retinal degenerations is made possible with electroretinography, which measures the various voltage changes that occur in the retina.

1.2 Electroretinography as a tool to measure restoration of retinal function

Electroretinography is widely used in the clinic to assess retinal function and to diagnose retinal disease. The diagnostic test involves flashing brief pulses of light through the dilated pupil to stimulate the photoreceptor cells and activate the phototransduction cascade. Full-field electroretinography used in this study involves flashes to the entire retina with a mini-ganzfeld (a dome) which emits light at various wavelengths and intensities. A focal flash of light to individual regions of the retina, called multifocal electroretinogram (ERG), is another method of electroretinography commonly used in the clinic (Webvision, 2010). The stimulated electrical signals transmit through the various layers of the retina to the optic nerve and penetrate through the sclera and cornea, where they are ultimately detected with the use of corneal electrodes. The full-field ERG, representing the total electrical response from different components of the retina, is presented as a waveform consisting of both an a-wave and b-wave (Figure 4) (Malmivuo and Plonsey, 1995).

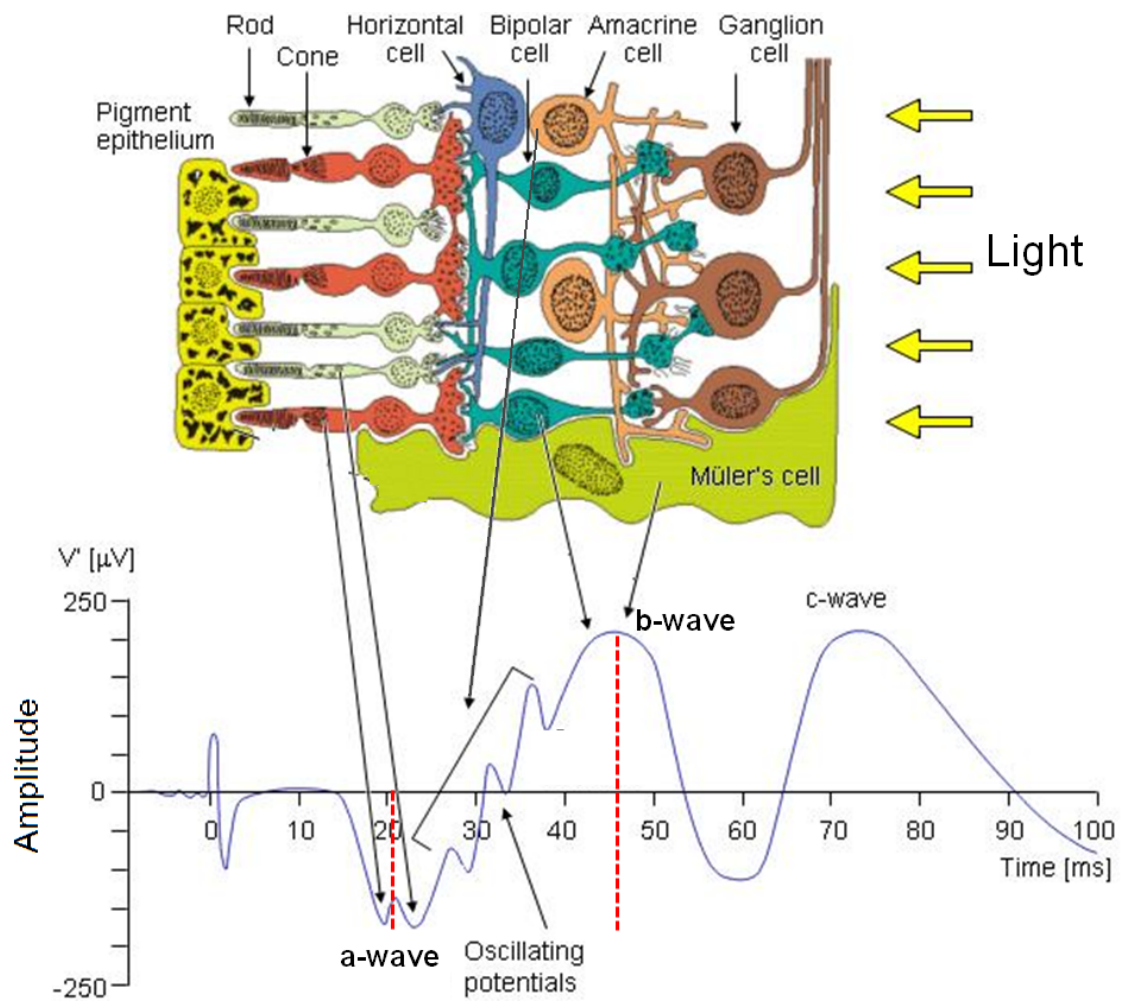


Figure 4. The electroretinogram is the total electrical response of the retina to brief flashes of light. It is represented as a waveform. The various components of the waveform are derived from the different cell types in the retinal circuitry. This study measures the a- and b-waves (red dashes), culminating from a hyperpolarization event in the photoreceptor cells and a depolarization event from the synapsis of photoreceptor cells to Müller and bipolar cells found in the inner nuclear layer, respectively (Malmivuo and Plonsey, 1995).

As mentioned in the previous section, the light perceived by the photoreceptors in the back of the eye induces a wave of hyperpolarization, or a negative change in intracellular electrical potential, due to the decrease of the inward-directed sodium current across the plasma membrane of photoreceptor outer segments. This is reflected in the ERG as the negative a-wave (Figure 4). Light, by hyperpolarizing the photoreceptor cell, terminates the continuous release of glutamate (which occurs in darkness). Following the hyperpolarization event, the photoreceptor cell returns to a dark state. In the dark, entry of ions into the photoreceptor causes a change in the cell's membrane potential, resulting in the re-release of the neurotransmitter glutamate. Its release, controlled mostly by calcium, induces depolarization, and this change in the intracellular electric potential is detected by the ERG as a positive b-wave (Figure 4) (Webvision, 2010).

The ERG waveform consists of two parameters: the amplitude (in μV) and the implicit time or latency (in ms). The amplitude measures the peak of electrical response in both the hyperpolarization and depolarization events. The a-wave amplitude is the peak of the hyperpolarization event, measured from the baseline to the trough of the a-wave while the b-wave is the peak of the depolarization event, measured from the a-wave trough to the b-wave peak (Figure 4). The latency represents the time it takes to reach the peaks of the a- and b-wave amplitudes.

Since the rods are most sensitive to light, their ERG responses are typically generated after dark adaptation (no exposure to light for at least 20 minutes), using a dim light. Cones are activated using bright flashes of light (Marmor et al., 1991). Stimulation of rods and cones occur after administration of dilation drops to the cornea of the patient.

Dilation enlarges the pupil and allows for the maximal amount of light to enter the eye. Since this is a tool that should be recognized and compared by physicians around the world, there are international standards to follow when creating an electroretinogram protocol, developed by the International Society for Clinical Electrophysiology of Vision (ISCEV) (Marmor et al., 1991). These standards outline the components required to safely measure an ERG.

The electroretinogram is deemed one of the best methods to measure the health of the patient's retina and to ensure the retinal components are properly functioning. This technique is sensitive and allows for detections of lesions or degenerations in the various layers of the retina. If mutations are present in the phototransduction cascade, for example, hyperpolarization and depolarization events would not occur properly and will be reflected on an electroretinogram (ERG).

1.3 Retinal diseases

Retinal degenerative diseases such as retinitis pigmentosa and age-related macular degeneration are a major cause of blindness. Degeneration of neural cells is the common clinical outcome of such diseases. Retinitis pigmentosa (RP) refers to a group of inherited forms of retina degeneration, characterized firstly by a progressive loss of rod photoreceptors, which leads to the loss of peripheral vision, and secondly to the loss of central vision as cone photoreceptors die (Hartong et al., 2006; Webvision, 2010). As of 2006, at least 45 genes have been identified as potentially responsible for retinitis pigmentosa (Hartong et al., 2006). Many of these causative genes associated with RP phenotypes are involved in the phototransduction cascade (Hims et al., 2003), such as

mutations in the visual pigment rhodopsin (Li et al., 1994; Rosenfeld et al., 1992), transducin (Dryja et al., 1996) (McLaughlin et al., 1993), phosphodiesterase (McLaughlin et al., 1993) and the cGMP-gated ion channels (Dryja et al., 1995). Although genetic mutations have been identified in some families, for the greatest portion of RP patients, the underlying genetic causes are unknown. There are currently no effective treatments available. Whereas RP affects primarily the peripheral retina, age-related macular degeneration (AMD) is a disease that primarily affects the central area of the retina, which is essential for colour vision and for detailed visual acuity. It is a major cause of vision loss in the elderly population, with 1 in 9 affected people over the age of 65. Current treatments offer some promise for the treatment of the symptoms of one form of AMD. However, for both AMD and RP, further understanding of the various stages leading to retinal degeneration is necessary to adequately treat these diseases.

1.4 The use of light as a retinal damage model

One component that is considered a final common pathway in the course of different retinal diseases, regardless of whether they are due to genetic mutations or age, is programmed cell death, or apoptosis (Chang et al., 1993). Photoreceptor cell apoptosis has been observed in several different animal models, reviewed by Remé *et al.* (1998).

One model used to acutely induce apoptosis is light. Light models are used as models of visual cell loss associated with retinitis pigmentosa or macular degeneration because there are similarities between the end stages of these retinal diseases and the appearance of the retina after light-induced degeneration (LaVail et al., 1987; Reme et al.,

1998; Wenzel et al., 2005). As well, light models allow for the study of defined time points after experimental induction.

Studies in rodents and zebrafish report photoreceptor cell damage can be induced by exposing the retina to a high intensity of light for a short period of time (minutes or hours) or by exposing the retina to low light for a continual period of time (days or weeks) (Bernardos et al., 2007; Grimm et al., 2000; Grimm et al., 2001; LaVail et al., 1999; Thummel et al., 2008). The notion that exposure to constant light can cause blindness, even at low intensities, was first shown by Noell and colleagues (1966) after leaving lights on in the animal housing facilities where colonies of albino rats were kept. Exposure of rats to 24 hours of moderate-intensity light, by ordinary fluorescent light bulbs, caused irreversible retinal degeneration (Noell et al., 1966). Moreover, light-induced photoreceptor cell death via apoptosis was successfully reported by Bernardos *et al.* (2005, 2007) in albino transgenic zebrafish. Zebrafish were placed in a beaker positioned 5 cm from the tip of a fiber optic liquid light line (3 mm diameter) connected to an EXFO X-Cite 120 W metal halide lamp source (which emits approximately 120 000 lux of light) for 30 minutes. Within 2 days post intense light treatment, photoreceptor cell death was evident in the central retina (Bernardos et al., 2007).

Different apoptotic mechanisms have been suggested to explain how constant light induces retinal damage. Continuous light exposure causes a prolonged activation of the photoreceptors (Lisman and Fain, 1995; Noell, 1980; Noell et al., 1966), which may result in altered cGMP levels and distorted cation channel permeability (Lolley et al., 1980). Also, when light activates the photoreceptor cells, there is no release of the neurotransmitter glutamate to the neurons of the inner nuclear layer, and this persists for

the duration of light exposure. The lack of synapses to stimulate underlying neurons may result in the degeneration of those non-stimulated neurons (Verhage et al., 2000). Another potential mechanism for cell death is oxidative damage to the neurons, caused by the bleaching of the rhodopsin molecule (Ablonczy et al., 2000; Grimm et al., 2001; Vaughan et al., 2003). Antioxidants used to prevent photoreceptor apoptosis implicate a role for reactive oxygen species in retinal apoptosis (Donovan et al., 2001; Lam et al., 1990). While the mechanisms for light-induced apoptosis are not fully understood, the comparison of the effect of light damage to outcomes obtained in animal models of retinal degeneration can allow for further insight into the progression of retinal diseases.

For patients found in the last stages of degenerative diseases such as retinitis pigmentosa, cone-rod dystrophy and macular degeneration, replacement of the damaged or lost retina becomes the only hope for restoring vision to these patients. Regeneration of new retina tissue, through cellular differentiation of existing cells, may help to restore the retinal function. Thus an understanding of the mechanisms involved to restore function during retinal regeneration in the newt holds tremendous promise for the treatment of human retinal diseases.

1.5 Regeneration

Regeneration is defined as the ability to re-grow or repair damaged or lost cells, tissues or organs and is classified according to one of two criteria: 1) morphallaxis regeneration, which occurs in the absence of cell proliferation, and 2) epimorphic regeneration, which requires cell proliferation (Morgan, 1901). While most organisms have at least some ability to regenerate damaged tissues, this capability varies depending

on the species, organ, or developmental stage. The red-spotted newt, *Notophthalmus viridescens*, undergoes epimorphic regeneration. It is deemed a champion model for regeneration because of its remarkable ability to regenerate many parts of its body as an adult, including its limbs, tail, parts of its heart, spinal cord (Borchardt and Braun, 2007; Bryant and Iten, 1977; Echeverri et al., 2001; Hay and Fischman, 1961; Zukor et al., 2011), and parts of its eyes such as its retina (Araki, 2007; Ikegami et al., 2002).

In contrast, mammals have limited regenerative capacity, restricted mostly to the liver (Otu et al., 2007), muscle (Charge and Rudnicki, 2004; Kang and Krauss, 2010) and simple tissue repair. Young children possess the ability to regenerate fingertips if amputated, provided the site is left to heal on its own without any suturing (Illingworth, 1974). However, the level of amputation is important, since children regenerate amputated distal phalanges, but not more proximal phalanges (Borgens, 1982). These cases suggest regeneration occurs in a proximal-distal direction and that during wound healing, communication may exist between the surrounding epithelial cells and the cells underlying the site of the wound to repair the damage. However, this response to certain signals induced by injury is limited to young children until the age of ten suggesting humans lose this ability to regenerate as they age. A clearer understanding of regeneration in the newt might provide new insights for the manipulation of regenerative pathways in the human.

1.6 The adult red-spotted newt as a vertebrate model for retinal regeneration

Intriguingly, the newt retina grows throughout the animal's life, making it a valuable vertebrate model in which to study retinal regeneration. Retinal regeneration can occur up to four times and takes approximately two months to complete. The different

stages of retinal regeneration following surgical removal of the retina are shown in Figure 5 (adapted from Araki, 2007). The process recapitulates the appearance of the retina during development, with the order of appearance being ganglion cells, cone photoreceptors and horizontal cells, amacrine cells, rods, and lastly bipolar and Müller cells (Cheon et al., 1998).

Numerous studies suggest the two sources of regenerative retinal cells in the newt are retinal pigment epithelial (RPE) cells and progenitor cells found in the ciliary marginal zone (CMZ), a peripheral region of the fish and amphibian retina (Keefe, 1973). Upon retinectomy (surgical removal of the retina), the predominant sources of cells for the regenerating retina are cells of the RPE. In the adult newt, RPE cells undergo transdifferentiation to form the neuroepithelial cell layer. The process of transdifferentiation involves a switch of cell fate, whereby the cell first loses its original characteristics, re-enters the cell cycle, proliferates and subsequently redifferentiates into another cell type. This cell conversion is unique to newts and is not typically encountered in mammalian adult tissues. Upon transdifferentiation the neuroepithelial cell layer can then produce all cell types of the neural retina (Araki, 2007). The choroid, attached to the RPE, is also essential for transdifferentiation to occur (Mitsuda et al., 2005). Growth factors such as fibroblast growth factor (FGF), produced as a result of cell-to-cell interactions between the choroid and the RPE, influence active proliferation of the RPE cells and the differentiation of cells in the ganglion, internal and external nuclear layers, and glia (Mitashov, 1996; Mitsuda et al., 2005). The RPE of tadpoles is also capable of

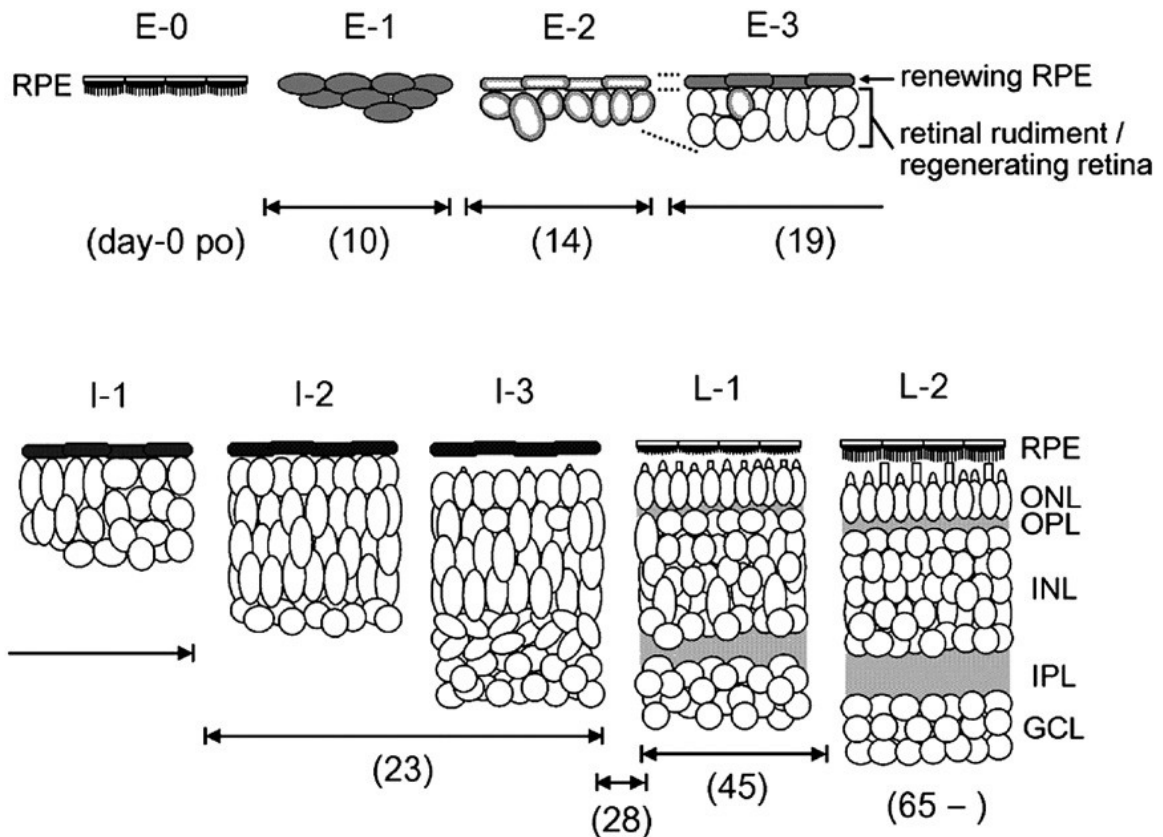


Figure 5. Diagram depicting the nine stages of newt retinal regeneration following surgical removal of the retina. Following retinectomy (**E0**), the pigment epithelium cells are irregularly arranged and have an oval shape. RPE cells undergo gradual depigmentation during **E1**. The cells enter the cell cycle and begin to proliferate, producing retinal stem-like cells around day 5. RPE cells undergo a shift in cell-cell communication between 4 and 5 days (Haruta et al., 2001) suggesting several genes must be activated at this time in order for regeneration to occur in a sequential event (Goto et al., 2006). **E2**: de-pigmentation continues and these retinal cells contribute to the retinal or neuroepithelium rudiment as well as the RPE-layer itself (Mitashov, 1996). **E-3**: the regenerating retina de-pigments almost completely, is 1–2 cells thick and grows to 3–4 cells thick (**I1**). **I-2**: round cell bodies of premature ganglion cells appear in the most proximal region. **I-3**: the regenerating retina is thicker just before formation of the plexiform layers.

L-1: the outer and inner plexiform layers appear but are still immature. **L2**: Depigmented cells have repigmented to reform the initial pigment epithelium layer and the retina has completely regenerated by 2 months post-retinectomy. Abbreviations: RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Source of figure (Nakamura and Chiba, 2007).

undergoing transdifferentiation to regenerate retinal neurons in response to FGF2 *in vitro*, suggesting an important role for FGF in the induction of regeneration in the retina.

While transdifferentiation of the pigmented epithelium is the main source of retinal regeneration, a secondary source of cells comes from the ciliary marginal zone (CMZ). In response to surgical lesions, the CMZ of amphibian retinas is marked by increases in proliferation; however, the CMZ contribution diminishes as the newt ages and the mechanisms are not fully understood (Keefe, 1973; Reh et al., 1987).

Retinal regeneration occurs in a variety of vertebrates including, fish, birds and amphibians; however, the newt, zebrafish and *Xenopus laevis* are the only animals known to regenerate the whole retina after its complete removal, with *Xenopus laevis* losing this regenerative capability after metamorphosis (Mitashov, 1996; Raymond and Hitchcock, 2000; Yoshii et al., 2007). Other reports suggest the possibility that mammals might still retain the ability to regenerate their retina, but only if properly induced. In birds, rodents and humans, RPE cells and ciliary pigmented epithelial cells found in the pigmented ciliary margin (PCM), a region in the retina that compares to the newt CMZ (Figure 6), had the capacity to transdifferentiate into retinal cells under certain cell culture conditions (Ahmad et al., 1999; Ahmad et al., 2000; Fischer et al., 2002; Haruta et al., 2001; Tropepe et al., 2000). Furthermore, RPE transdifferentiation was reported in mammalian embryonic cultures at early stages of mouse development (E13 to E14) (Zhao et al., 1995), while earlier studies in rats showed that RPE cells were also able to fully dedifferentiate *in vitro*, however, this capability was lost at later stages of development (Neill and Barnstable, 1990; Zhao et al., 1995). In addition, transdifferentiation at the retinal margin of chicks was also evident but limited. Amacrine and bipolar cells, but not

photoreceptor, horizontal, or ganglion cells, were produced by progenitors at the retinal margin of postnatal chicks. As well, unlike the progenitors cells in the CMZ of fish and amphibians, those of the chick retina do not increase their rate of proliferation in response to acute damage (Fischer and Reh, 2000; Kubota et al., 2002).

Regeneration of the newt retina appears to be achieved by complex mechanisms involving both transdifferentiation and stem cell differentiation. Mammals appear to be missing the appropriate cues to replace lost or damaged cells of the retina; therefore, further characterization of the regenerative capabilities of the newt may identify novel cell types and pathways involved in the response.

1.7 The comparison of newt to human retinal cells

The ability to study the newt retina is made possible due to the highly conserved anatomical and physiological features of the retina across many species. However, some differences between newt and human retinal cells do exist. One major difference involves the composition of the photoreceptor cell layer throughout the retina. The very central region of the human retina, called the fovea, is composed of only densely-packed cones and has no rods (Hubel, 2010). While the cones dominate the central retina, the rods dominate the peripheral retina. In contrast to humans, the newt rods and cones are distributed throughout the entire retina and no fovea exists (Hollenberg and Dickson, 1971). The proportion of rod to cones in the newt retina is relatively unknown.

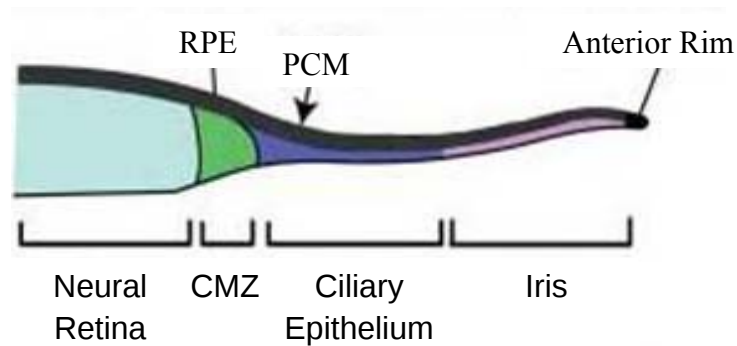


Figure 6. The retinal pigment epithelium (RPE) and the ciliary marginal zone (CMZ) are 2 main sources of stem cells in the newt eye. In mammals, including humans and rodents, the equivalent zone to the CMZ is the pigmented ciliary margin (PCM). The cells of the PCM are capable of transdifferentiating *in vitro* into retinal specific cells including rod photoreceptors, bipolar neurons and even Müller glia cells; however, the limitation *in vivo* is not fully understood (Ahmad et al., 2000; Keefe, 1973; Tropepe et al., 2000).

Humans and newts also differ in the type of photoreceptor cells they possess. Humans have one type of rod cell and three different types of cone cells. Rods contain the visual pigment rhodopsin and are sensitive to the blue-green wavelength of light. Their maximal absorption is in the yellow-green range of light (around 500 nm). Humans are considered to have trichromatic vision because of the possession of 3 different cone cells. The differences between these cones allow for different colours to be distinguished. These are the long (L), medium (M) and short (S) wavelength cones whose maximal absorption of light is 564nm (red), 533nm (green) and 437nm (blue), respectively (Webvision, 2010). In contrast, newts possess two different types of rods called “red” and “green” rods, so called due to the natural glow they emit under the microscope (Stebbins, 1995). The prominent rods in the newt are the “red” rods, which are similar to the human rod in that both contain rhodopsin and their peak absorption of light is around 502 nm, yellow-green light. The “green” rods have a peak absorption of 433 nm and it has been suggested that they contain the same visual pigment as the blue-sensitive cones (Ma et al., 2001). These rods are specific to amphibians and may be involved in hue discrimination in dim light levels. The “green” rods only constitute 1-15% of the total photoreceptor cells in amphibians (Darden et al., 2003).

Newts and humans also differ in the structure of their cone photoreceptors. Newts possess different types of cones, called single and double cones. Single cones are yellow-sensitive and maximally absorb light at a wavelength of 580 nm. The double cone consists of a pair of receptors that are fused at the membranes of their inner segments. Double cones are composed of a “principal” cone with the same pigment as the single cone and an “accessory cone” with the same pigment as the “red” rod (Stebbins, 1995).

Double cones are also found in the retinas of many fish such as teleosts, salmon and trout and in the retinas of frogs and turtles (Ahlbert, 2010; Lyall, 1957; Nishimura and Shimai, 1980). Therefore it appears as though the newt rods and cones contain the same visual pigments.

Though there may be differences in the way that newts and humans perceive light, due to differences in the visual pigments of their photoreceptor cells, the retinal circuitry involved during the vision process of both vertebrates is highly conserved. Moreover, both newt and human retinas have similar photoresponse kinetics. The retina as a light-sensitive tissue perceives white light as a visible spectrum of various wavelengths. While the entire visual spectrum of the red-spotted newts has yet to be elucidated, the visual spectrum of the rough-skinned newt, *Taricha granulosa*, is from 250 to 710 nm, with peak sensitivity falling between 550 and 565 nm (La Touche, 1979). The human visual spectrum is from 390 nm – 780 nm (Henderson, 2010) (Figure 7).

1.8 Measuring retinal function in the adult red-spotted newt

While it is well-known that the newt retina regenerates approximately 65 days after surgical removal (Cheon et al., 1998; Keefe, 1973; Mitashov et al., 1995; Nakamura and Chiba, 2007), studies on the functional restoration of the newt retina are limited (Chiba and Saito, 1995; Kaneko and Saito, 1992; Oi et al., 2003). Most of the studies focused on electrophysiological changes or activity in what they claimed to be ganglion cells, since ganglion cells are the first retinal neurons to differentiate during retinal regeneration (Cheon et al., 1998). Their methods required the preparation of retinal slices for many stages of regeneration.

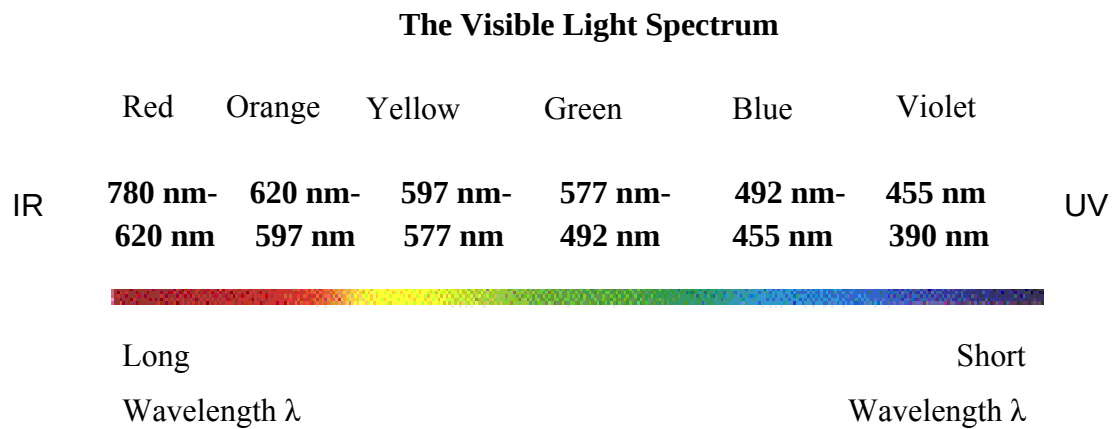


Figure 7. The human visible light spectrum represents the wavelengths of light (colours) that are perceived by photoreceptor cells found in the back of the vertebrate retina. The wavelengths range from approximately 780 nanometer (red) down to 390 nanometer (nm) (violet). The rough-skinned newt visible spectrum, similar in range, is 710 to 250 nm (Henderson, 2010). Ultraviolet light (UV) and infrared (IR) light are not visible to human and newt eyes and fall outside the visible light spectrum. Figure adapted from The Physics Classroom website (Henderson, 2010), accessed on January 5 2010 and found at the following website: <<http://www.physicsclassroom.com/class/light/u12l2b.cfm>>.

The studies largely employed whole-cell patch-clamping to monitor the activity of one or many ion channels in the regenerating retina. Whole-cell patch-clamping involves the use of micropipettes with very small tips (referred to as a patch electrode) that contain patch-electrode filling to perfuse the interior of the neurons of interest. The fluid delivers a pulse of pressure and ruptures the neurons, such that observation and recording of action potentials from the ruptured neurons can be performed (Jackson, 1997; Oi et al., 2003). The use of whole-cell patch-clamps is a more invasive way of measuring the electrical activity of neurons, compared to electroretinography, and is limited to recording electrical responses from a few cells.

In contrast, electroretinography is used to measure the function of several cell types in the retina at once, *in vivo* (Webvision, 2010). There are no previously established protocols for recording ERGs from the intact eyes of red-spotted newts after injury or surgical removal. The only publication available claiming to monitor retinal function restoration in the red-spotted newt was a study conducted in 1977 where newt eyes were removed from their sockets through severing of the artery and optic nerve in order to induce retinal degeneration by temporary devascularisation. The eyes were placed back in the newt head in the same orientation and regeneration was monitored for up to 32 days post-operation. ERG recordings were carried out *ex vivo*, with the newt eyes placed on a dish, freed of their attachment to the optic nerve (Lam, 1977). This study required the use of over 150 isolated eyes from newts during different stages of retinal regeneration. This method was an invasive way to induce retinal damage, with the severing of the optic nerve and artery. Moreover, subsequent retinal regeneration may have been dependent on the regeneration of the optic nerve and the repair of arterial tissue to resupply vasculature

to the choroid and retina, potentially impacting the rate and extent of retinal degeneration and subsequent regeneration. This may have contributed to the variations observed in the time of appearance of the ERG for individual newts (Lam, 1977) for which the authors made no comment. As well, this study required the use of a large number of animals since newts are captured from the wild at various ages and genders, which also contributes to the variability of results.

Therefore, one outstanding question that still needs to be addressed is whether the newt retina is functional after surgical removal or damage and one way to address this question is with the development of a non-invasive way to measure retinal function.

1.9 Thesis goals and hypotheses

A major goal of this thesis is to assess restoration of retinal function after light damage and surgical removal. Full-field electroretinography is utilized as a non-invasive way of measuring function in various layers of the retina in the intact eyes of light-adapted adult red-spotted newts. A novel newt ERG protocol is outlined and the parameters to optimize the protocol are discussed. A major goal of our laboratory is to further our understanding of how retinal regeneration occurs in the newt; therefore, the development of a relevant retinal damage model that would initiate and promote the regeneration response is crucial. In the studies described here, a constant exposure of newt retina to ultra-intense light is used to induce retinal damage. Due to variability in our findings and the lack of reproducibility with the light-induced damage model, surgical removal of the retina is described as an alternative and more effective method to induce retinal regeneration. The optimized newt ERG protocol is used to measure the initial loss of retinal function after surgical removal of the retina and to monitor the subsequent recovery of function as the retina regenerates.

It was hypothesized that both light-induced damage and surgical removal of the newt retina will result in loss of the ERG response; subsequently, recovery of the ERG following surgical removal or damage will be indicative of restoration of retinal function. Our findings suggest that retinal function is restored to photoreceptor cells and cells of the inner nuclear layer after surgical removal of the retina, a significant contribution to the understanding of retinal regeneration in the newt.

2. Materials and Methods

2.1 Animal Care and Veterinary Services

Adult newts were supplied from Charles Sullivan (Nashville, Tennessee) and maintained in large, aerated, flow-through tanks containing approximately 100L of dechlorinated and dechloraminated water. The newts were kept at a mean water temperature of 22-23°C and under a 12 hour light:12 hour dark photoperiod provided by standard fluorescent lighting. After all experiments, newts were maintained under similar conditions in smaller tanks containing dechlorinated and dechloraminated water. Newts were fed live blackworms biweekly (Aquatic Foods) and the entire volume of water was changed biweekly. All procedures were carried out in adherence with protocols approved by the University of Ottawa Animal Care Committee under the auspices of the Canadian Council for Animal Care.

2.2 Electoretinography of adult red-spotted newts

2.2.1 Preparation of newts for electoretinography

Prior to the commencement of the ERG recordings, each light-adapted adult newt was anesthetized for 15 minutes by submerging in warmed 0.05% tricaine methanesulphonate (MS222®, Sigma). Each newt was then placed on a wax dish and covered in MS222®-soaked cheesecloth for the duration of the test. Pupils were dilated with a long-acting dilator, Mydfrin® (2.5%, Alcon). The newt was placed on its side, with its right eye facing up during dilation. Only the right eye was tested during the ERG recording. The body temperature of the newt was maintained constant throughout the test

using the T/Pump® (Gaymar Industries Inc., New York) set to an appropriate temperature. After dilation, a silver loop wire was placed directly on the centre of the newt cornea. Subcutaneous needle electrodes in the head and tail served as a reference and ground, respectively. Prior to the test, the newt eye was kept moist with one drop of lubricating GenTeal® Artificial Tears (Novartis) and the ColorBurst ganzfeld stimulator (Espion™, Diagnosys LLC) was positioned directly over the newt's head (Figure 8).

2.2.2 Measurement and amplification of newt ERG recordings

Light of various wavelengths was flashed at a high intensity of 400 cd/m², in a pulse period of 500 ms, to elicit the a- and b-wave responses from the various components of the newt retina. Light emitted by the ColorBurst mini-ganzfeld stimulator were LEDs corresponding to either red (640 nm), green (525 nm) or blue (470 nm) wavelengths of light. The light emitted from the ganzfeld was diffuse and homogeneous. Each recording was an average of 50 pulses, flashed one second apart. Since the electrical signal emanating from the newt's retina was very small, the voltage detected from the electrodes was measured and amplified using a biological amplifier (Espion™). The Espion™ Electrophysiology Software displayed the final averaged (after 50 pulses) ERG recording with the opportunity to browse through each pulse measured. The final waveform was graphed as amplitude (μV) on the y-axis over time (ms) on the x-axis. Recordings were repeated 3-5 times to obtain an average. The manual measurement (scoring) of the a- and b-waves was conducted by visual inspection using the Espion™ Electrophysiology Software. The a-wave amplitude was scored from the baseline to the

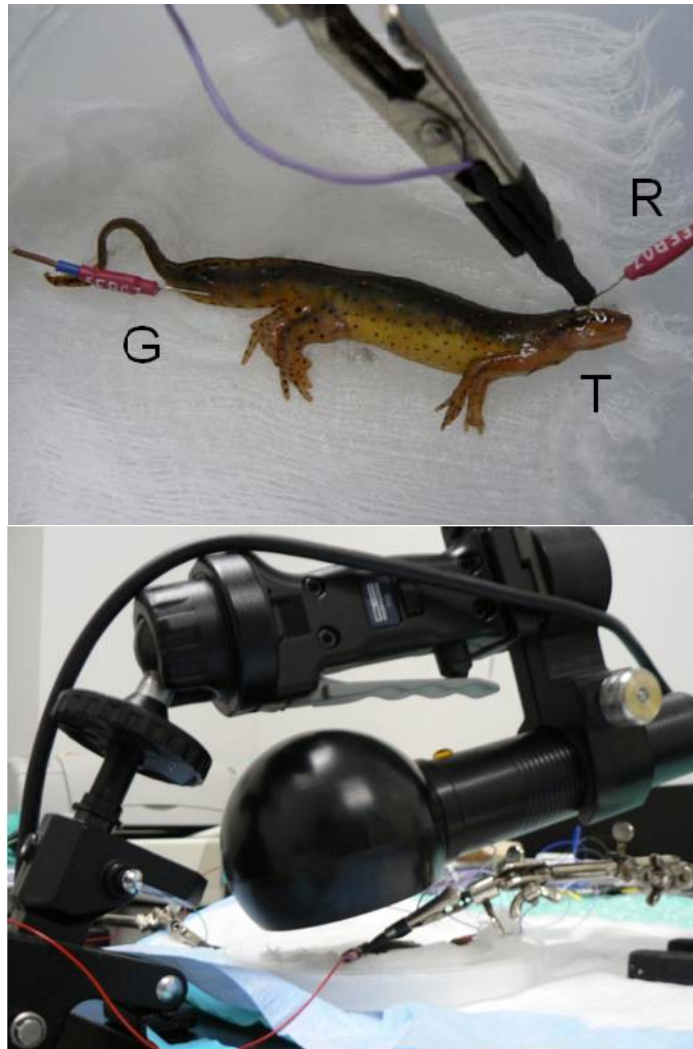


Figure 8. Experimental setup for recording an electroretinogram in the intact eye of a newt. A silver loop wire, the test electrode (T) to receive electrical input from the retina, is placed directly on the centre of the newt cornea. Subcutaneous needle electrodes in the head and tail served as a reference (R) and ground (G), respectively (top). The newt is placed on a petri dish covered with an MS222®-soaked cheesecloth. The ColorBurst ganzfeld stimulator (Espion™, Diagnosys LLC), from which brief flashes of light are emitted to stimulate the various layers of the retina, is positioned directly over the newt's head prior to the commencement of the test (bottom).

negative peak (the trough) and the b-wave amplitude was scored from the negative peak to the positive peak. The implicit time was measured from the onset of the stimulus at 0 ms to the peak of the a- or b-wave. Below is a table summarizing the principal parameters established for the newt ERG protocol.

2.2.3 Optimization of ERG parameters

The parameters optimized for *in vivo* ERG testing on newt eyes included maximal dilation times, variability of recording an ERG from the same animal over different days, and the effect of temperature.

2.2.3. i. Dilation time

Dilation tests were carried out on two separate days (Experiment 1 and Experiment 2) using a total of eight different newts. Four newts were measured on each day. All newts were anesthetised with 0.05% MS222[®] 15 minutes prior to dilation, and were continually anesthetised until the ERGs were completed. We measured ERG a- and b-waves pre-dilation (baseline) and after dilation of 5, 15, 25, 35, 40, and 45 minutes with Mydfrin[®]. Yellow, red, and two shades of blue light with an intensity of 400 cd/m² over a 500 ms pulse period were flashed into non-dilated and dilated newt right eyes. For each wavelength of light, the averaged values at each time point were divided over the corresponding baseline values (non-dilated eyes) and presented as a fold change. The temperature of the newt bodies throughout the tests ranged from 17.7°C-19.0°C during Experiment 1 and 16.5-18.1°C during Experiment 2.

Parameters	Newton
Eye Tested	Channel 1 (one eye)
STIMULUS	
Pulse Period (ms)	500
Pulse Intensity (cd/m ²)	400
Pulse Colour	Red (640 nm)
AQUISITION	
Sample Frequency (Hz)	1000
Trials per result	50
Trial Duration (ms)	500
Trial Pre-trigger (ms)	10
Auto Pre-trigger (ms)	17
CHANNELS	
Y-axis scaling (µV)	20
Filter Low cut-off (Hz)	1.25
Filter Hi cut-off (Hz)	30

Table 1. The updated ERG protocol for light-adapted newts using Espion™ Software Version 4 (Diagnosys). Only one eye is tested at a time. The Stimulus parameter refers to the flash of light emitted by a small monocular ganzfeld, based on LED technology. The Acquisition parameters define the length of time light is measured, trigger times and delay times in between tests. The Channels parameters allow for the scaling of the amplitudes to be designated, and filters out a very low or high frequency of electricity that can be attributed to background noise or artifact.

2.2.3. ii Variability of ERG recordings

The variability of recording an ERG on the same newt on separate days was measured. Nineteen different newts each had ERGs recorded on three separate days. For each test, the ERG a- and b-waves were measured 40 mins post-dilation time using bright red light flashes as these conditions yielded optimal ERG responses. After each day of recording, a- and b-waves were averaged for each newt, yielding three final averages over three days. For each of the 19 newts, the standard deviation was also calculated. The % variability on each individual newt's a- and b-wave over three separate days of recording was determined as follows:

$$1. \quad \% \text{ variability/newt} = \frac{\text{Standard deviation}}{\text{Final average of each newt a- and b-wave over three days}} \times 100\%$$

$$2. \quad \text{Final averaged \% variability (19 newts)} = \% \text{ variability of each animal} / 19$$

The temperature measured during the three days of testing ranged from 21.7°C - 23.4°C.

2.2.3. iii. Effect of temperature on the ERG

We measured the effect of the newt's external body temperature on the ERG amplitudes. This was carried out by changing the temperature of the newt's surrounding environment, including warmed MS222[®] and altered room temperature. We tested the following three external body temperatures: 18.5°C, 25°C and 30°C. Each of these temperatures was tested on separate days; for example, on day 1, ERGs were recorded with the newt's body temperature maintained at 18.5°C. MS222[®] and water temperature were measured with a standard thermometer and body temperature was measured using a

FLUKE® digital thermometer (John Fluke Manufacturing Company, Inc). The anesthetic was heated to the desired temperature using a water bath and maintained at a constant temperature using a mini oil heater (Lancaster) and the T/Pump® (Gaymar Industries Inc), set at 30°C or 35°C. For experiments where the newts were kept at a constant temperature of 18.5°C, the mini oil heater was not turned on and the T/Pump® was set to its lowest temperature.

We recorded the ERG a- and b-waves for a total of six newts at each temperature. We used the optimized ERG parameters and the test began at 40 minutes post-dilation. Each a- and b-wave was plotted against temperature and graphed using box plots (SigmaPlot 11.0). Box plots graph the distribution among many samples of one group (Sigma Plot), for example the distribution of the a-waves for animals kept at a body temperature of 25°C. This is useful for graphing data from animals whose natural heterogeneity contributes to a high variability in the ERG a- and b-waves. Each box plot graphs five data points, as shown in Figure 9. Any data that is considered an outlier is plotted as a dot above the whiskers. All statistical analyses were performed using SPSS 10.0 and/or SigmaPlot 11.0. The datasets were analyzed with a one-way ANOVA on ranks (significance level, $p < 0.05$ with SigmaPlot; $p < 0.001$ with SPSS) and post hoc multiple comparisons (Bonferroni).

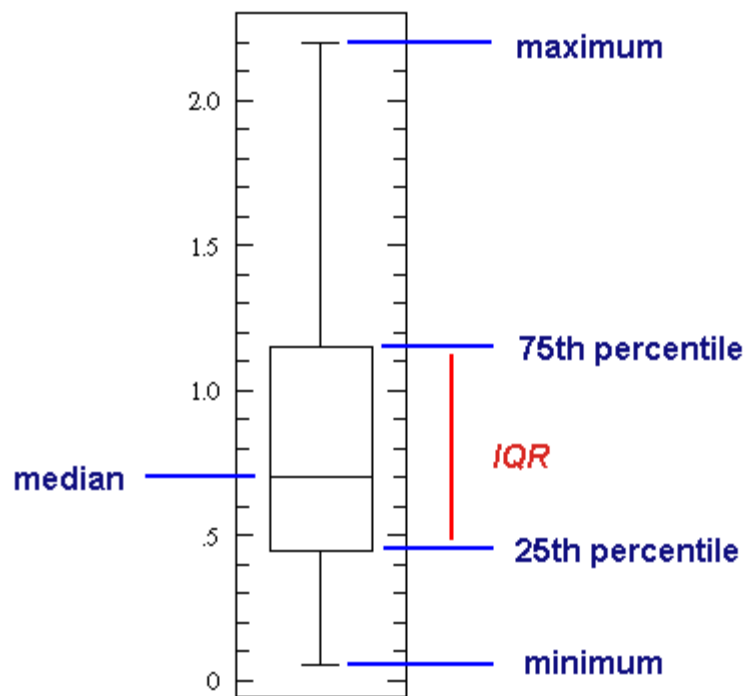


Figure 9. A schematic of the box plot which graphs the distribution of information as a five-number summary. The box plot graphs the 25th percentile (the lower hinge), the 75th percentile (the upper hinge), the median (the line across the box indicating the 50th percentile, or the centre of the dataset), and the minimum and maximum values, depicted by SigmaPlot as the 5th and 95th percentile (top and bottom whiskers). The interquartile range (*IQR*) spans 50% of the data set, showing the spread between the upper and lower percentiles (Kirkman, 2006).

2.3 The light damage model

2.3.1 Light treatment

Newt retinal damage by light, also termed phototoxicity, was induced using two light sources:

- 1) A Fiber-Lite MI-150 Illuminator (Edmund Optics) containing a 150 W tungsten-halogen bulb (Phototoxicity 1) and
- 2) A Super High Pressure mercury arc lamp (Nikon) containing a 100 W mercury arc lamp (Phototoxicity 2).

At maximal setting, both sources generated >100 000 lux at a water temperature approximately 24°C. The spectrum of light emitted from each light source was measured using the Ocean Optics spectrophotometer (USB4C00478) and the data was analyzed using the SpectraSuite software (Ocean Optics).

For the halogen bulb source, the three main light intensities tested were 15 000 lux, 20 000 lux, and > 100 000 lux. These intensities were measured with a hand-held photometer. Light-adapted adult newts were first anesthetized with MS222® for at least 20 minutes and their pupils dilated for 10 minutes. Newts were placed in a radiation box and positioned 5.5 cm high on various stacked plastic and pyrex dishes. The right eyes of newts were angled directly towards the illuminator, 5 or 10 cm from the tip of the MI-150 Illuminator, at a height of 5.5 cm from the benchtop. Newts were covered with MS222®-soaked cheesecloths to ensure they were anesthetized during the procedure. When dilation was complete, the MI-150 Illuminator was turned on, and placed at the appropriate setting (6th setting for 15 000 lux, 10th setting for 20 300 lux and maximal

setting for > 100 000 lux). The illuminator and light box were covered with black felt to shield the path of light from the surrounding environment. Newts were exposed to various constant light exposure times: 15, 30, 60 and 95 minutes.

For the mercury arc lamp, **two main methods** were used to expose newt eyes to constant and intense light (Figure 10). In the first method, newts were anesthetized and placed directly in the path of light. For the second method, newts were allowed to swim freely (either in a 500 mL beaker or a fishbowl containing 300 mL distilled water) while exposed to constant light. For the duration of the thesis, each method will be referred to individually with its corresponding experiments. The mercury arc lamp had one maximal output and was turned on 30 minutes prior to use for proper heating. No red cut-off filter was used for any experiments involving the mercury arc lamp.

Method 1: The first method involved placing anesthetized newts on a petri dish with their eye facing the lamp. Newt eyes were dilated for at least 5 minutes prior to light exposure. To avoid burning the skin and to shield any intense light possibly directed around the newt eye, the newt's body and face were shielded from the light using aluminum foil. A two mm pinhole, the approximate diameter of a newt's eye, was created in the aluminum foil to allow for light to reach the right eyes of the newts. The IR, red cut-off filter and condenser were set up 24.5 cm, 11.5 cm, and 6 cm from the newt's eye, respectively. For this set of experiments, a red light cut-off filter (580 nm) was used to allow for mostly blue light to reach the retina, as blue is a damaging wavelength, and since the literature suggests that the predominant newt cones absorb blue-green wavelengths of light (Stebbins, 1995). The newts remained immobile throughout the entire experiments and only one newt was treated at a time. Newts were exposed to 5, 15,

and 30 minutes of constant ultra-intense light. The eyes were fixed with PFA after light treatment and subjected to the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for apoptosis. A variety of experimental conditions were also tested including various distances from the light source, various dilation times, and various durations of light exposure. The newts from these experiments were followed up by ERG to test whether damage incurred. For those in which reductions in the ERG were seen, a subset of animals was sampled for TUNEL and/or retinal whole mounts. In one experiment, newts were subjected to 30 minutes constant ultra-intense light and a subset of these samples were fixed one or two days post-light treatment for tissue processing. Three eyes were subjected to retinal whole mounts while three eyes were assayed for the appearance of apoptotic cells using TUNEL. For the newts in which no reductions in the ERG were seen, it was assumed that the optimized conditions for the light assay were not met.

Method 2: Method 2 involved newts swimming freely in a 500 mL beaker or fishbowl as they were exposed to 30 minutes of constant light. The beaker and fishbowl were each placed approximately 55 cm from the mercury arc lamp. There were 3 experiments conducted using Method 2. For all experiments, newts were first dark-adapted overnight. The following day, newts were anesthetized for 20 minutes, their pupils dilated for 20 minutes and they were then revived in water (approximately 20 minutes). This was all carried out in the dark under safe red light. Newts swam freely for 30 minutes in a bowl or beaker, individually or in pairs. A light box fully surrounded the beaker and was covered in aluminum foil to allow for light to reflect off of it and to reach the retina at various angles. Experiment 1 involved 5 newts individually swimming in a beaker.

Experiment 2 involved 9 newts swimming alone or in pairs in a fishbowl while Experiment 3 involved 8 newts swimming alone in a fishbowl, with 6 newts exposed to a second UV filter (cut off of 480 nm).

2.3.2 ERGs to assess loss of retinal function due to light

ERGs were recorded 20 minutes (Phototoxicity 1) or 40 minutes (Phototoxicity 2) post-dilation with Mydrin[®] using bright red flashes of light, as per the optimized protocol discussed in Section 2.2. ERG a- and b-waves were first conducted prior to light treatment and subsequently at various time points after light treatment. Intact right eyes from separate adult newts served as controls. ERGs were recorded from controls on the same day as those recorded from the treated newts. The fold changes of both a- and b-waves amplitudes pre- and post-light treatment time points were measured and averaged. All graphs plotted amplitudes (μV) over time (ms). The standard error was calculated. Statistical comparison between treated and baseline ERG amplitudes was made using Student's t-test, with a p-value < 0.05 considered to be significant. When the populations did not follow normal distribution, which occurs with small samples, the t-test was not valid. In those cases, the Mann-Whitney Rank Sum test was used as an alternative to measure statistical significance between the ERG amplitudes of light-treated and non-treated retinas.

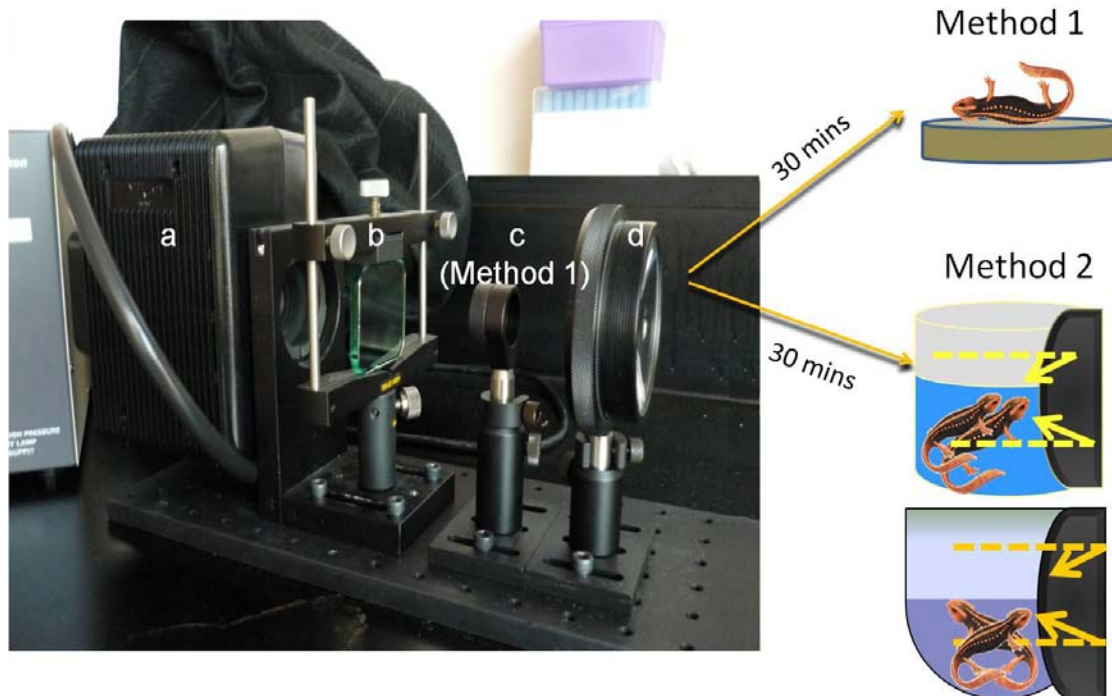


Figure 10. Phototoxicity assay setup for the Super High Pressure mercury arc lamp (Nikon). The second light source consisted of (a) a Super High Pressure mercury arc lamp containing an internal filter for ultraviolet (UV) light, (b) an infrared filter, (c) a cut-off filter for red light, as well as (d) a condenser to focus light directly into the newt eye. The red light filter, filtering 580 nm light, was only used for Method 1. Newt eyes were positioned 52 cm away from the light source in Method 1 while the centre of the beaker or fishbowl was positioned 55 cm away from the light source in Method 2.

2.4 Retinal Whole Mounts

Newt retinal whole mounts were conducted 2 days post 30 mins constant light treatment in order to determine the full extent of the damaged area. Newts were anesthetized and the eyes were enucleated with fixed 4% PFA for 30 mins. Under the Zeiss dissecting microscope, a small incision was made near the optic nerve at the back of the eye with microscissors, creating a cut site. The sclera and RPE were carefully dissected out from that cut site, starting from the back of the eye and working around towards the cornea on each side of the cut site. This exposed the retina which was attached to the lens. The lens and retina were carefully transferred to a new dish containing a 13 mm spherical 0.8 μ m Nucleopore Track-Etch Membrane (Whatman®) with one drop of fresh 1x PBS. The retina was carefully dissected off the lens and spread onto the membrane, with ganglion cells facing up. To allow for adhesion of the whole mount to the membrane, the PBS was carefully aspirated off. The whole mount was then placed in a 6- or 12-well dish for further fixation with 4% paraformaldehyde (PFA).

2.5 Tissue processing, cryosectioning and histology

Newt eyes were fixed with 4% PFA for 30 minutes at 4°C while rocking. Fixed eyes were washed at least three times with 1x PBS and saturated with 30% sucrose overnight at 4°C. Newt eyes were then equilibrated with a 1:1 solution of 30% sucrose/1X PBS: embedding medium (OCT) and frozen over liquid nitrogen. Samples were frozen and stored at -80°C until ready for sectioning. Please see Appendix II for a complete protocol. All eyes were sectioned at a thickness of 10 μ m using a Shandon

Cryotome®, FSE series (Thermo). Newt eyes are sectioned serially. Selected sections were stained with hematoxylin and eosin as per the standard protocol found in Appendix II.

2.6 TUNEL

A full protocol for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) on retinal cryosections is found in Appendix II. This assay relies on the appearance of nicked DNA strands which are found on the 3' hydroxyl terminal end after DNA fragmentation. The enzyme terminal deoxynucleotidyl transferase (TdT, a DNA polymerase) catalyzes the addition of digoxigenin-labelled dUTPs to the nicked end of the strand. The location of the dUTPs, and therefore of DNA fragmentation, is recognized by an antibody against digoxigenin which is tagged with the fluorescent marker Rhodamine. This assay was used to measure cell death on light-treated and control samples. This homemade kit is a modification of the protocols by IHC World (IHC World, 2008) and the ApopTag® Fluorescein Direct *In Situ* Apoptosis Detection Kit (Millipore). Sections represent eyes that were either exposed or not exposed to ultra-intense light. Cryosectioned newt eyes treated with diluted DNase I (Sigma) served as a positive control, and a negative control consisted of cryosectioned newt eyes treated with DNase I but not subjected to end labeling with the TdT enzyme. All slides were counterstained with DAPI (1:8500- 1:10000) to stain nuclei.

2.7 Retinectomies

Two separate retinectomies, surgical removal of the retina, were conducted involving five animals in Retinectomy 1 and ten animals in Retinectomy 2. For both experiments, newts were anesthetized with MS222[®] for at least 30 minutes, placed on a petri dish and covered with MS222[®]-soaked cheesecloth for the duration of the surgery. Only the right eye of each test newt underwent retinectomy. All surgeries were performed using the Zeiss MDU Ophthalmology Microscope (with S22 stand). Retinectomies involved first making an incision directly beneath the corneal/scleral junction with a 27 G 1/2 needle (Becton Dickinson). The newt eye was held carefully in place with a pair of micro-fixation forceps that contain a 0.3mm notched O-ring (Instrumentarium). Care was taken to avoid puncturing a blood vessel. The sclera was cut further parallel to the corneal/scleral junction using a pair of fine-angled microscissors (Fine Science Tools) to create a 2-3 mm wide flap (Figure 11a). The flap was lifted and the vitreal chamber of the eye was gently flushed with ddH₂O to cause a retinal detachment (Figure 11b). The retina was then removed using 5 CC forceps (Fine Science Tools), while avoiding removal of the retinal pigment epithelium (Figure 11c). The scleral/corneal flap was reattached and if necessary, slightly tucked under the eyelids. The newts were then placed in a recovery tank with a low level of dechlorinated and dechloraminated tap water, enough to cover their bodies and to ensure the eyes remain dry as long as possible to allow wound closure. The newts were returned to the animal care facility for proper light cycling and feeding until further testing.

2.7.1 The use of in vivo ERGs to measure retinal restoration after retinectomies

ERGs were recorded on treated and control newts using optimized conditions pre- and post-surgery for both experiments (40 minutes post-dilation with Mydfrin[®] using bright red flashes of light). ERG a- and b-waves were first conducted prior to surgical removal of the retina and subsequently at various time points after retinectomy. The fold change of both a- and b-waves amplitudes pre- and post-surgery were measured and averaged to calculate standard error. All graphs plotted amplitudes (μV) over time (ms). Intact right eyes from separate adult newts serve as ERG controls. ERGs were recorded from controls on the same day as those recorded from the treated newts.

For Retinectomy 1, ERGs were recorded approximately every two weeks and up to 15 weeks post-retinectomy (wpr), and conducted with the newt body temperature maintained at 22.3-26.2°C. The eyes were sampled at 106 days post-retinectomy (15 wpr) and stained with hematoxylin and eosin (H and E).

For Retinectomy 2, the ERG recordings were recorded up to 11 weeks post-retinectomy and conducted with the newt body temperature maintained at 24.0-28.0°C. This experiment included a sham control which involved anesthesia and incision of the corneal/scleral junction but no removal of retina. To verify the histology of any partially regenerated newts, two newts (#5 and #8) were sampled at 8 weeks post-retinectomy and stained with H & E. All other samples were fixed at 78 days post-retinectomy (11 wpr) and processed for sectioning.

Statistical comparison between groups was made using Two-Way Analysis of Variance (ANOVA) and the Holm-Sidak method for multiple pair-wise comparisons, with $p < 0.05$ considered to be significant.

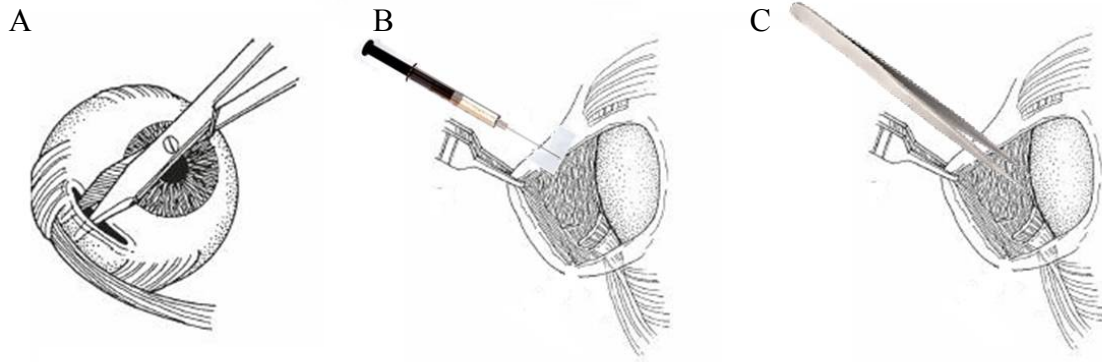


Figure 11. Schematic of a retinectomy. A small incision was first made around the scleral/corneal junction of the newt eye with a 27 Gauge $\frac{1}{2}$ inch needle (Becton-Dickinson). A pair of microscissors was used to create a flap (A) into which water was injected to cause a retinal detachment (B). The retina was teased out using a fine pair of forceps (C) and caution was exercised to ensure that the retinal pigment epithelium remained intact and was not removed as well.

3. Results

3.1 Optimization of newt ERGs measured in vivo

To date, there are no published *in vivo* ERG recordings from the adult red-spotted newts. In order to establish an ERG protocol to be used to monitor the retinal function of regenerated retinas, the following three ERG parameters were optimized:

- Pupil dilation time
- The effect of temperature
- Variability of recording an ERG from the same animal over different days

This protocol was developed in light-adapted newts as a non-invasive method to record functionality of the newt retina.

3.1.1 The effect of pupil dilation time

In order to record the maximal ERG amplitudes, it is necessary to first dilate the pupils of the newts. This allows for the maximal amount of light to reach and stimulate the photoreceptor cells, which are located at the back of the retina. Two dilation tests were carried out on separate days using a total of 8 newts. A drop of the long-acting pupil dilator Mydfrin® was administered to the cornea, and the amplitudes of the ERG a- and b-waves were measured at 5-minute intervals, up to a maximum of 45 minutes. The results showed that maximal amplitudes were achieved by 35-40 minutes post-Mydfrin® treatment, suggesting that the newt pupils are maximally dilated at this time (Figure 12). Without dilation, the ERG amplitudes remain low and constant with time (results not

shown). The external body temperatures of the newts were 17.7°C-19.0°C for experiment 1 (Figure 12 a, b) and 16.5-18.1°C for experiment 2 (Figure 12 c,d).

3.1.2. The effect of temperature on the ERG

From studies in other organisms, it has been suggested that there is a temperature-dependent effect on the amplitudes of ERGs (Adolph, 1985; Kong and Gouras, 2003; Mizota and Adachi-Usami, 2002; Ookawa and Tateishi, 1970). To determine whether temperature fluctuations influence the ERG amplitudes in newts, a- and b-waves were recorded from 6 newts with external body temperatures of 18.5°C, 25°C and 30°C. Box plots (Figure 13) were used to show that the maximum amplitudes of the a- and b-waves varied with temperature. An external body temperature rise from 18.5°C to 25°C resulted in the a-wave increasing from a median of 6.97 μ V to 11.09 μ V. At 30°C, the median of the a-wave was 17.56 μ V. Statistically significant differences were observed by ANOVA ($p < 0.001$) when comparing the a-wave amplitudes at 18.5°C and 30°C. Similarly, an external body temperature rise resulted in significant increases in the b-wave from 18.5°C to 30°C (ANOVA, $p < 0.001$). The median of the b-wave amplitude increased from 20.75 μ V (at 18.5°C) to 36.39 μ V (at 25°C) to 45.44 μ V (at 30°C).

3.1.3 The variability of recording an ERG from the same animal over different days

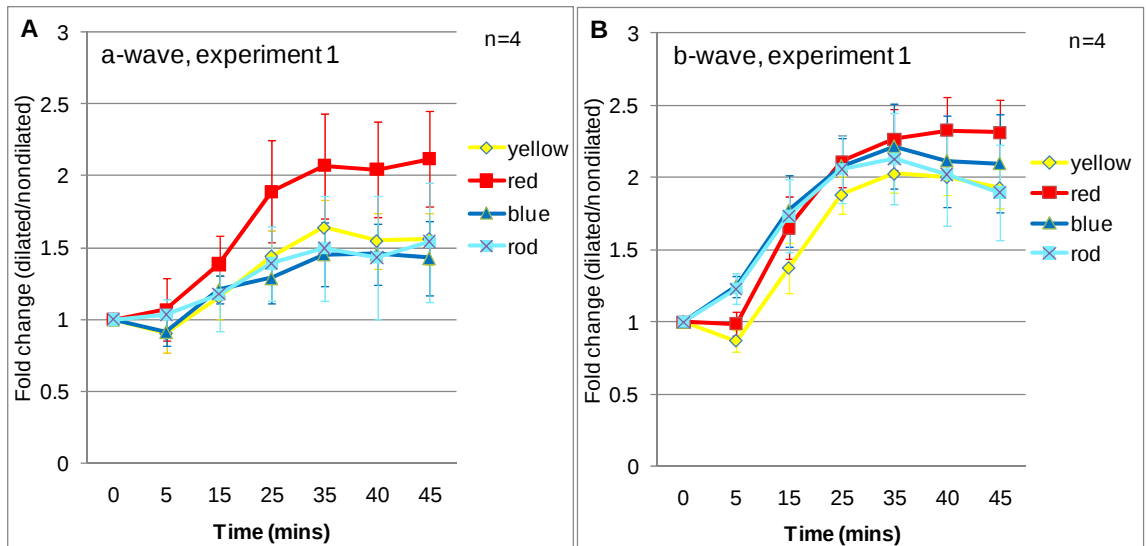
Studies that examine recovery of retinal function over time require repeated recording of ERGs from the same animal; therefore, it is necessary to establish the technical variability in the ERG protocol. ERGs of nineteen different newts were recorded on three separate days, and at the same time of the day in order to control for the possible effect of day and night light cycles. For each newt, the variability was

determined across the three different days. The average variability for all 19 newts was calculated to be 21.2% for the a-wave and 25.1% for the b-wave.

3.2 The newt electroretinogram

Full-field ERGs were successfully recorded from the intact eyes of light-adapted newts, using the optimized parameters determined above. A typical newt ERG response was elicited by bright flashes of red light after 15 minutes of anesthesia followed by 40 minutes of dilation. Two prominent waveforms were measured, the a- and b-waves, whose implicit times (ie, the period from stimulus onset to the peak of the respective component of the response) were approximately 75 ms and 130 ms, respectively, over a 500 ms pulse period (Figure 14). The average a- and b-waves for 19 animals were 17.1 μV $\pm$ 1.6 and 58.4 μV $\pm$ 8.6, respectively. The newt body temperature was kept constant around 23°C throughout the tests. The c-wave, which reflects the integrity of the ganglion cells, was not observed or measured during the 500 ms pulse period.

Experiment 1



Experiment 2

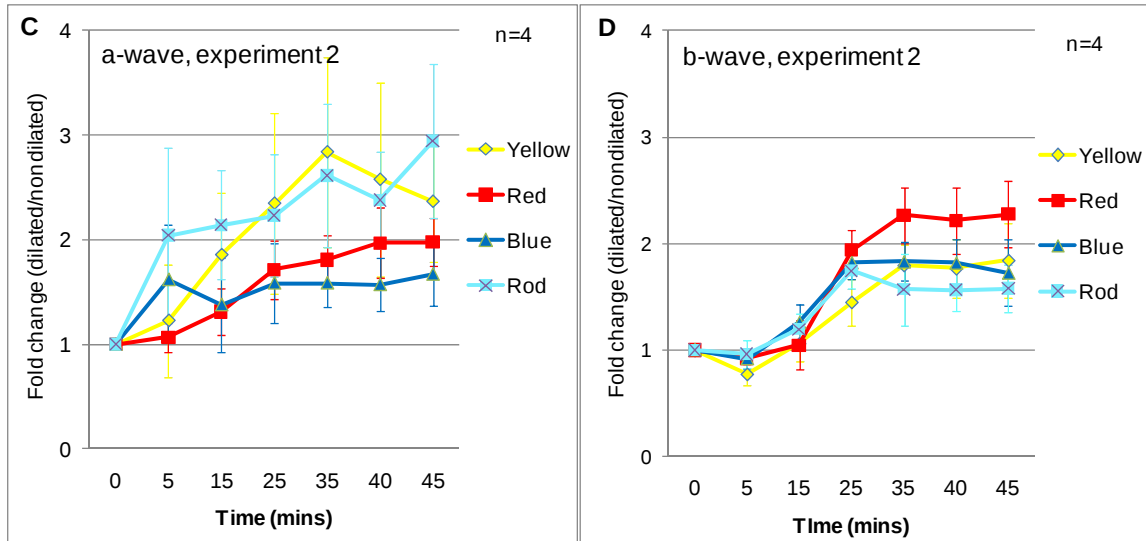


Figure 12. The effect of dilation on the newt ERG a- and b-wave amplitudes. The a-wave (A,C) and b-wave (B, D) responses from newt retinas (Alcon) were measured pre-dilation and over a 45 minute time period post-Mydfrin® administration. Dilation tests were carried out on two separate days using a total of 8 newts, with 4 animals measured on each day. The results represent a fold change of ERG amplitudes from retinas post-dilation over pre-dilation, after stimulation from 4 different wavelengths of light. The results of the 2 experiments concur and show that pupils appear to be maximally dilated 35-40 minutes after exposure to Mydfrin®. All newts were anaesthetised 15 minutes prior to dilation, and continually anaesthetised until the ERGs were completed. The temperatures throughout the tests were 17.7°C-19.0°C (A, B) and 16.5°C -18.1°C (C, D). Standard Error bars are shown.

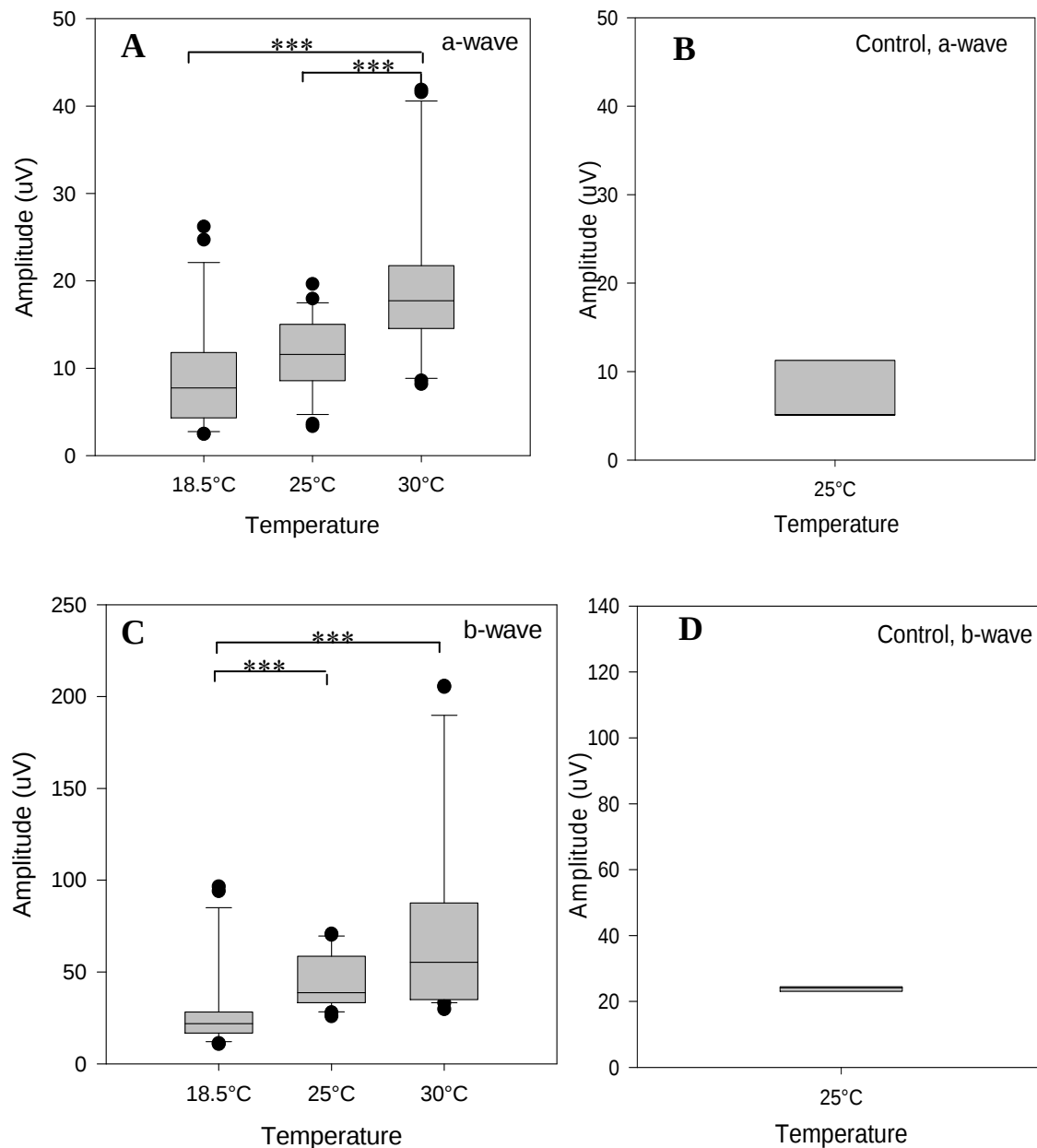


Figure 13. Newt body temperature has an effect on the ERG, as shown by box plot. ERG a- (A) and b-waves (C) of six newts were measured on 3 separate days, maintaining the newt body temperature at approx 18.5°C, 25°C or 30°C for each day. A separate control animal (B,D) was maintained at 25°C on each day to ensure that all other test parameters were relatively constant throughout the 3 days. These results highlight the importance of maintaining the newts at a constant body temp throughout the test. Plots demonstrate the median (50th percentile), 5th, 25th, 75th, and 95th percentiles. Significant differences exist among groups as shown by asterisks (ANOVA on ranks, $p < 0.001$).

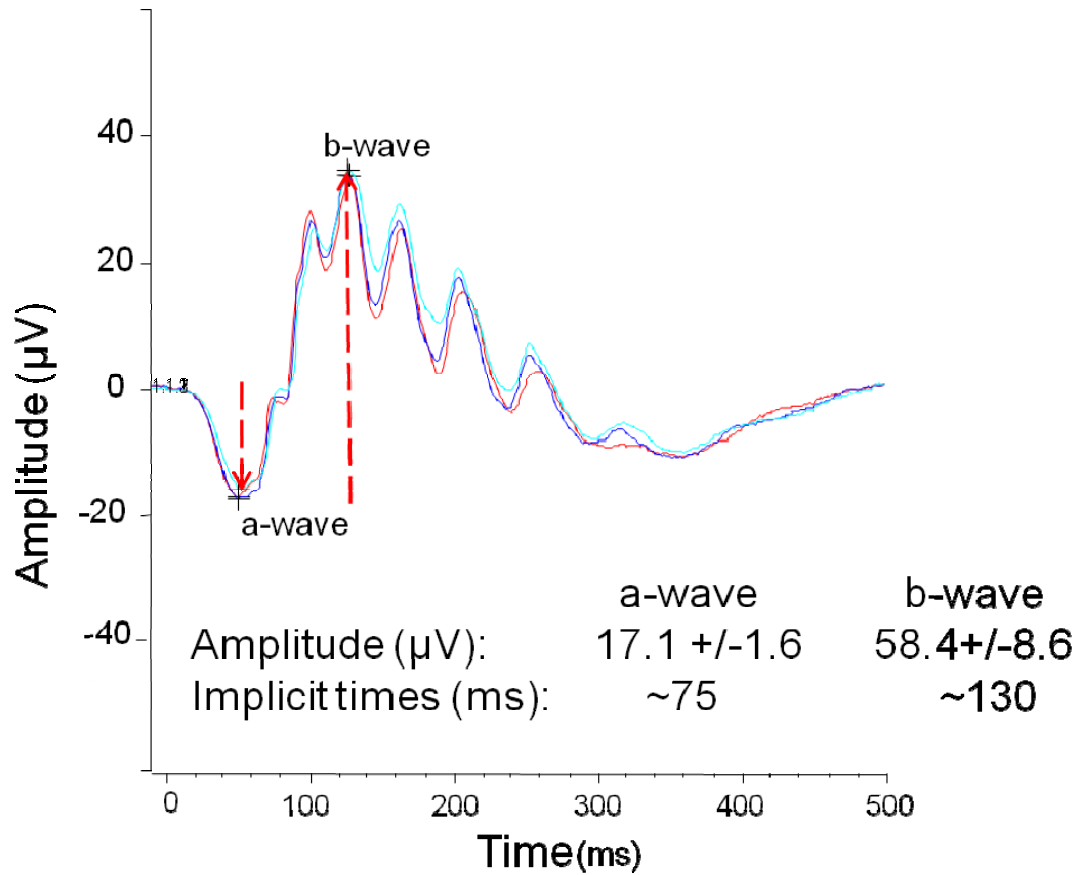


Figure 14. Representative ERG recordings from intact light-adapted newt eyes using optimized parameters. Each ERG trace is an average of 50 flashes of light with a- and b-waves indicated (red arrows). Note the oscillatory potentials descending the b-waves. The newt a-wave peaks at approximately 75 ms while the b-wave peaks at around 130 ms, representing the implicit times.

3.3 Light-induced damage in the adult newt retina

A major goal of our laboratory is to provide insight into how retinal regeneration occurs in the newt; therefore, it is necessary to consider a relevant retinal damage model that would initiate and promote the regeneration response. The light-induced damage model was shown in other organisms, including zebrafish and rodents (Bernardos et al., 2007; Grimm et al., 2000; Grimm et al., 2001; Thummel et al., 2008), to be an acute model of visual cell loss. These previous studies reported that photoreceptor cell damage was induced by continuously exposing the retina to high intensity light, also termed phototoxicity.

Ultra-intense light is a novel way of inducing retinal damage in the red-spotted newt. The powerful metal halide lamp, at a short exposure time of 30 minutes, was successful in specifically damaging the photoreceptor cells of albino transgenic zebrafish (Bernardos et al., 2007; Thummel et al., 2008). The zebrafish were placed in a beaker positioned 5 cm from the tip of a fiber optic liquid light line (3 mm diameter) connected to an EXFO X-Cite 120 W metal halide lamp source (which emits approximately 120 000 lux of light) for 30 minutes. Two days following intense light treatment, photoreceptor cell death was evident in the central retina (Bernardos et al., 2007). This light-induced damaging method was therefore applied to the newts, since there was a potential that this model would cause damage to the newt retina in a similar fashion. Newts and zebrafish possess retinas that are conserved in structure and function; both vertebrates are capable of regenerating their retinas, both possess double cones, and both are aquatic animals that have acquired the ability to focus light onto the retina under water.

Various conditions have been tested in other organisms to induce photoreceptor cell death, such as different wavelengths and intensities of light, and different durations of light exposure. Therefore, it was necessary to first optimize the conditions required to stimulate apoptosis in the newt retina. The various conditions tested included source of light, duration of light exposure and distance from the light source. Other experimental factors considered were dilation, dark-adaptation prior to light exposure, filtration of UV light, and filtration of red light.

Two different light sources were used in this study: the MI-150 Fiber Optic Illuminator (Dolan-Jenner) which contains a 150 W halogen light bulb and the Super High Pressure mercury arc lamp (Nikon) which contains a 100 W mercury arc lamp. It was of interest to predict the intensity of light that likely reached the photoreceptor cells. A 100 W mercury arc lamp irradiates more light in the visible spectrum compared to a 100 W tungsten-halogen bulb (Nolte, 2010). The EXFO X-Cite 120 W metal halide lamp, used in the zebrafish experiments (Bernardos et al., 2007), and the mercury arc lamp are comparable in terms of irradiance over the human visible light spectrum (Figure 15). In the laboratory, the density or “amount” of light that falls on a surface and that is perceived by the eye can be measured using a photometer (Ltd., 2006; Wulfinghoff, 1999). The lux is the SI derived unit of illuminance. Light at the highest intensity was emitted from both the halogen bulb and the mercury arc lamp, and the illuminance that was detected by a handheld photometer was $> 100\,000$ lux. Since the two light bulbs saturated the handheld photometer, the halogen light bulb was first chosen as it was readily available and could be set to different intensities.

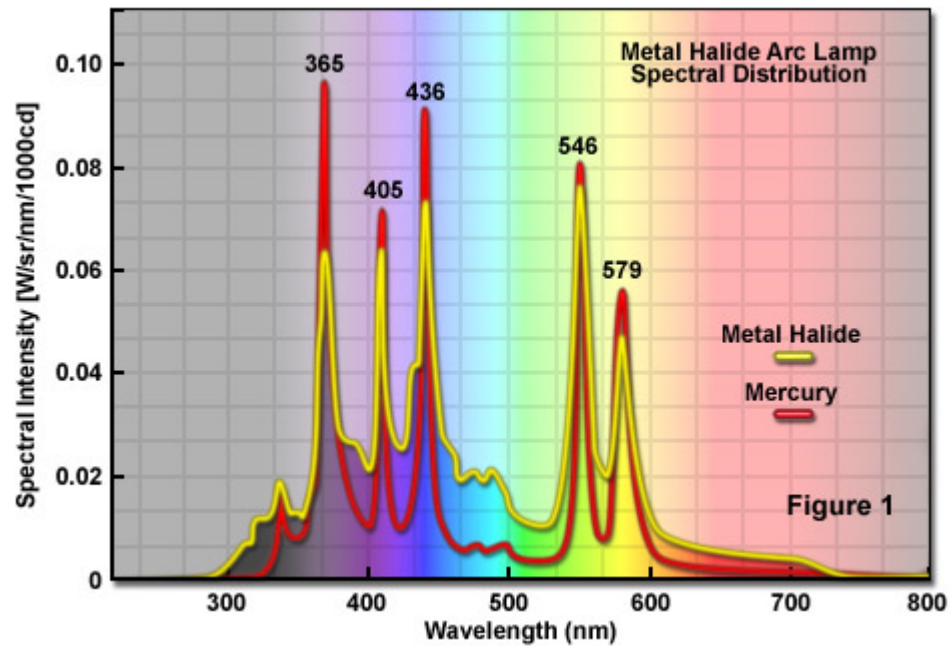


Figure 15. The EXFO X-Cite metal halide lamp (120 W) and the mercury arc lamp (100 W) relatively compared in power across different wavelengths of light. The peaks represent the intensity of light that reach the photoreceptor cells at a particular wavelength. This figure, accessed on January 3 2010, was found on the Zeiss website (Davidson, 2010).

< <http://zeiss-campus.magnet.fsu.edu/articles/lightsources/mercuryarc.html> >

We wanted to determine the threshold by which the halogen light bulb could induce retinal damage. In contrast, the mercury arc lamp could only be set to one maximal intensity (>100 000 lux). A light intensity of 100 000 lux is equivalent to a bright sunny day and exposure to direct sunlight is damaging to the retina.

Another difference between the halogen and the mercury arc lamps was the spectral distribution of light (the wavelengths of light) emitted by each source. The wavelength of light is a factor that was considered to have an effect on retinal damage (Gorn and Kuwabara, 1967; Grimm et al., 2001); however the efficiency of the wavelength to produce light damage has been shown to be dependent on the animal's spectral sensitivity (Gorn and Kuwabara, 1967; Lanum, 1978). As well, the degree to which a wavelength of light was considered damaging was dependent upon its ability to bleach the photopigments, the visual pigments found in the photoreceptors of the retina (Noell et al., 1966). In the case of rodents, whose retinas are predominantly rods, the only photopigment to bleach is rhodopsin. Blue light has been shown to bleach rods of rodents, leading to apoptosis of photoreceptor cells (Grimm et al., 2001; Noell et al., 1966). There have been no studies on the wavelengths of light that are known to be damaging to the newt retina.

The wavelengths of light emitted from the mercury arc lamp and halogen light bulbs were measured with the Ocean Optics spectrometer and analyzed using the Ocean Optics SpectraSuite software. Each lamp emitted each wavelength of light at a particular intensity. The percent intensities for peak wavelengths emitted were calculated. The band width of each peak wavelength was defined according to the visible spectrum available

on The Physics Classroom website (Henderson, 2010) (section 1.7, Figure 7). The MI-150 halogen source allowed for light to be set at several different intensities.

At maximal intensity of light, the halogen lamp emitted mostly long wavelengths of red light (a band width of 620-780 nm), with an intensity of 49.6% (Figure 16). At maximal intensity of light (>100 000 lux), the most power emitted by the mercury arc lamp corresponded to the green-yellow spectrum, with a total intensity of 51.4% for the full length green and yellow wavelengths (492– 597nm). An intensity of 14.9% corresponded to light in the purple spectrum (390-455nm) while only 2% of the power corresponded to UV light (350-400 nm) (Figure 17).

For both light sources, the conditions optimized were duration of light exposure, distance from the light source, and number of animals exposed at once. In the case of the halogen light bulb, experiments were conducted exposing only one newt at a time to the light source whereas in the case of the mercury arc lamp three separate experiments were conducted exposing either one or two newts to the light source simultaneously. For studies using the mercury arc lamp, additional factors were tested such as pupil dilation time, filtration of red light, dark adaptation, and subsequent UV filtration. The results from various phototoxicity methods are separately discussed in the following sections. While there were many preliminary studies conducted on each light source, the data represents the results from the most optimized conditions.

Since electroretinography is one of the best methods of measuring survival and loss of retinal cells in an unbiased way, the newt ERG protocol was used to assess the degree of functional damage.

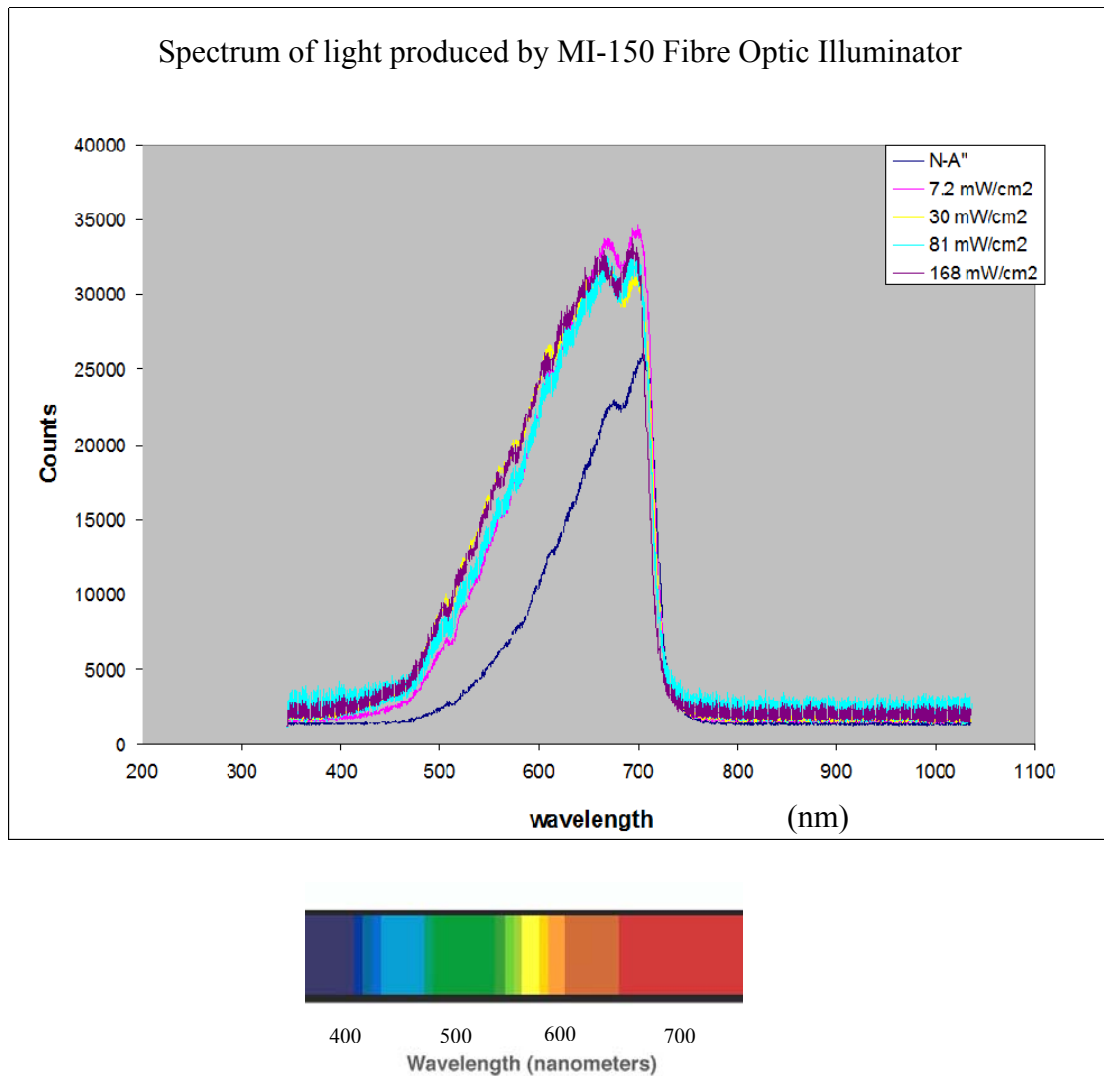


Figure 16. The spectrum of light emitted by the 150 W halogen bulb found in the MI-150 Fiber Optic Illuminator (Dolan-Jenner), at various irradiances. The Ocean Optics spectrophotometer detects the quantity of light (displayed as counts) emitted by the 150 W Halogen bulb at wavelengths of light ranging from 345 nm to 1036 nm. Five different irradiance settings, measured in mW/cm², were tested: the lowest setting possible (N-A, power not detectable by the spectrophotometer), 7.2, 20, 81 and 168 mW/cm². The data was analyzed using the SpectraSuite (Ocean Optics) software and displayed as a spectrum of light. The predominant wavelength (colour) at the high power settings was 650-700nm, corresponding to the red wavelength of light. Spectrum of light image adapted from Webvision (2010).

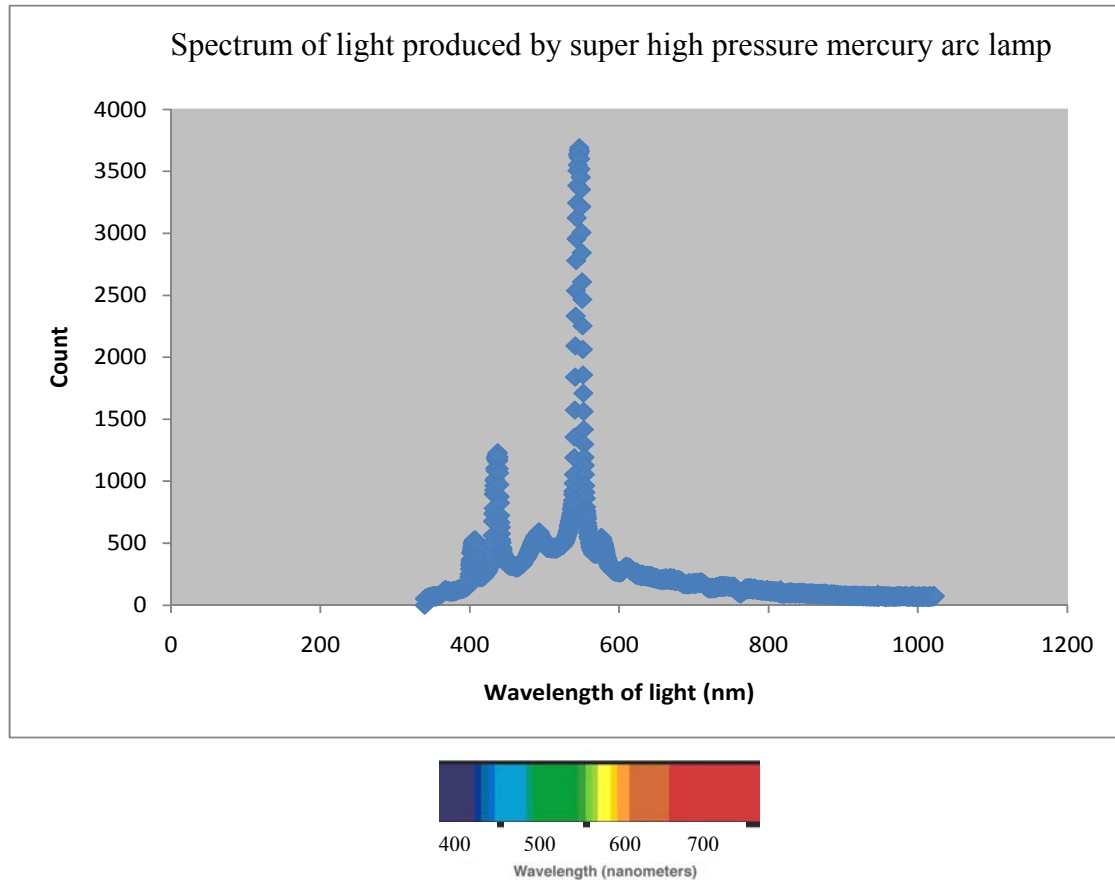


Figure 17. The spectrum of light emitted by the Super High Pressure mercury arc lamp, at maximal intensity (Nikon). The Ocean Optics spectrophotometer measures the intensity of light (displayed as counts) emitted by the 100 W mercury arc lamp at wavelengths of light ranging from 339 nm to 1022 nm. The experimental setup involved a built-in UV filter, an infrared filter (cut-off 700 nm) and a red light cut-off filter, all placed in between the mercury arc lamp and the spectrophotometer to filter out infrared and red light. Literature suggested that red was not a predominant wavelength of light absorbed by the newt retina; therefore, red light was filtered out to allow for the presence of the more predominantly-absorbed wavelength of light (yellow) (Stebbins, 1995). With the red-cut off filter in place, the lamp predominantly emits light in a line width of 540-554 nm which borders the green/yellow wavelengths of light. The arc lamp emitted both green and yellow light (a line width of 492 nm – 577) at an intensity of 51.4% while 14.9% of the lamp’s power corresponded to light in a line width of 390 nm - 455 nm (the purple spectrum). Although the Super High Pressure mercury arc lamp was equipped with a UV filter, a residual amount of UV light (a line width of 339.31 nm - 399.79 nm) was emitted at an intensity of 2%. The data was analyzed using the SpectraSuite (Ocean Optics) software.

3.3.1 Phototoxicity 1 – the use of a halogen light bulb to induce retinal damage

In this first set of experiments, white light from a 150 W halogen bulb was shone directly into the newt eye at three different intensities: 15 000 lux, 20 300 lux and an ultra-high-intensity of > 100 000 lux. The light was emitted from a fiber optic beam (1 mm in diameter) attached to the source of light containing the 150 W halogen bulb, the MI-150 Fiber Optic Illuminator (Dolan-Jenner). Adult newts were anesthetized with MS222®, their pupils dilated for 10 minutes with Mydfrin® and their right eyes positioned 5 or 10 cm from the tip of the MI-150 Fiber Optic Illuminator. At various light intensities, up to four exposure times were tested while the newts remained anesthetized: 15, 30, 60 and 95 minutes constant light exposure (Table 2).

Viability of the photoreceptors after exposure to various intensities of light was assessed using ERGs in *in vivo* eyes at 3, 4 and/or 7 days post-light exposure (dpl) prior to fixation, processing and TUNEL analysis. Pupils were dilated for 15 minutes prior to electroretinogram recording. There was no significant difference in the a- and b-wave amplitudes of animals exposed to 15 000 lux or 20 300 lux for various exposure times, compared to baseline levels (data not shown).

None of the conditions were sufficient to induce photoreceptor cell death, verified by TUNEL and histology. Eight newts positioned 5 cm away from the light source, and exposed to 15 minutes of ultra-intense light (20 300 lux) were sampled at 3 and 7 dpl (with 4 newts sampled on each day). The TUNEL assay was conducted on retinal cryosections found in the middle of the eye and there were no apoptotic (TUNEL-positive) cells detected in any of the 4 newt eyes sampled (Figure 18). A total of four

newts exposed to ultra-intense light ($>100\ 000$ lux) for 30 and 60 minutes showed decreased ERG a- and b-wave amplitudes at 3 dpl, compared to baseline amplitudes. However this difference was not statistically significant by t-test (Figure 19). This may have been due to a low number of animals tested. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on the histology sections of a sampling of newts from various conditions to identify any damage that might not have been detectable by ERG. No apoptotic cells were found (data not shown).

Overall, the most promising conditions to yield light-induced retinal damage with the halogen light source involved ultra-intense ($>100\ 000$ lux) at a minimum of 30 minutes exposure. While conditions led to decreases in ERG amplitudes, there were no detectable apoptotic cells with TUNEL. These results suggested that at 3 days post-light, the dead cells were either already cleared, leaving no cells for TUNEL analysis, or that the decrease in ERG, in combination with error of recording ERGs on the same animal over time, was simply not a significant decrease. This led to the conclusion that the light-damage conditions were not optimal.

For the next set of experiments, it was necessary to not only achieve a minimal 50% reduction in retinal function, but to correlate this loss of ERG amplitude to apoptosis of retinal cells. Therefore, the conditions of the experiment were changed to include a different light source, the mercury arc lamp (MAL).

		Time (mins) Number of newts (E= ERGs conducted, T=TUNEL conducted)			
Distance from source	Intensity (lux)	15	30	60	95
5 cm	15 000		1 (E)	2 (E)	
	20 300	8 (E, T)			3(E, T)
	> 100 000	5 (E)	2(E, T)	2 (E, T)	
10 cm	20 300			3 (E, T)	

Table 2. The various light-damage conditions tested with the 150 W halogen light source (Edmund Optics). A variety of anaesthetised newts were subjected to light at three different light intensities (15 000 lux, 20 300 lux, >100 000) for up to four different exposure times (15, 30, 60 or 95 minutes) while placed on a dish located either 5cm or 10 cm from the light source. The number of newts used for each condition is shown along with the method used to assess retinal damage, either full-field electroretinography (E), TUNEL analysis (T), or both. All newts were subjected to electroretinography. There was no significant difference in the a- and b-wave amplitudes of animals exposed to 15 000 lux or 20 300 lux yet certain newts exposed to 20 300 lux were analyzed with TUNEL to identify any apoptosis possibly undetected by full-field ERG.

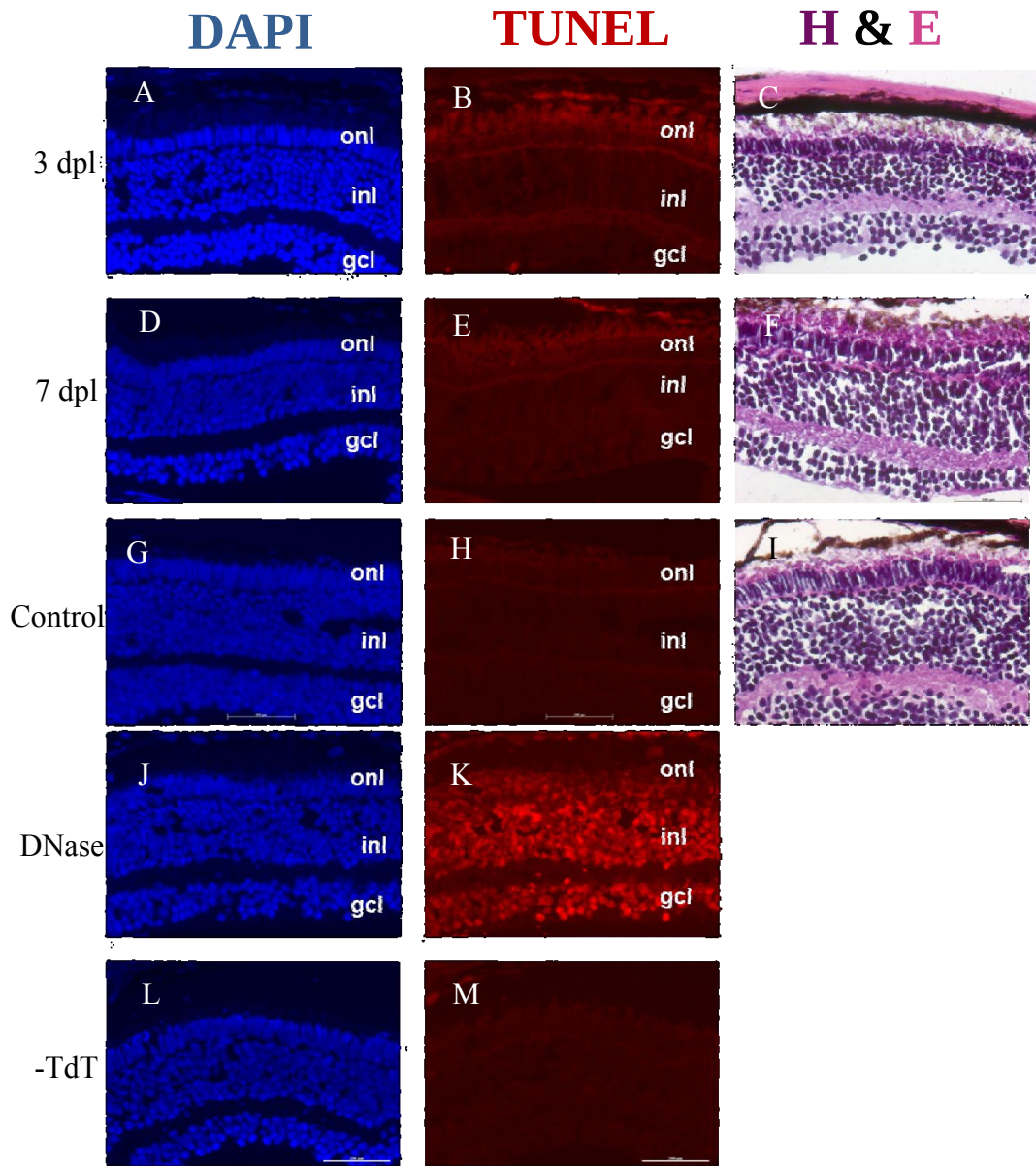


Figure 18. TUNEL labeling indicated that no apoptosis occurred in the newt retina at 3 and 7 days following 15 mins ultra-intense constant light treatment with the halogen bulb set at a high intensity (>100 000 lux). Newts were anesthetized during the test with their pupils directed to the light. Cryosections from the central region of the newt retina at 3 (A-C) and 7 (D-F) days post-light (dpl) were labeled with DAPI nuclear staining (blue), TUNEL (nuclear red), which labels DNA-nicked ends, hematoxylin (H) (nuclear purple-blue) and eosin (E) (cytoplasmic pink/red) along with the control (not exposed to light) (G-I). There were no TUNEL-positive cells, compared to the positive control, a DNase I treatment (25 μ g/ml) for 15 minutes on an untreated retinal cryosection (J-K). These results suggested that the halogen lamp did not generate sufficient widespread damage. The absence of the TdT enzyme during the TUNEL assay served as a negative control (M, N).

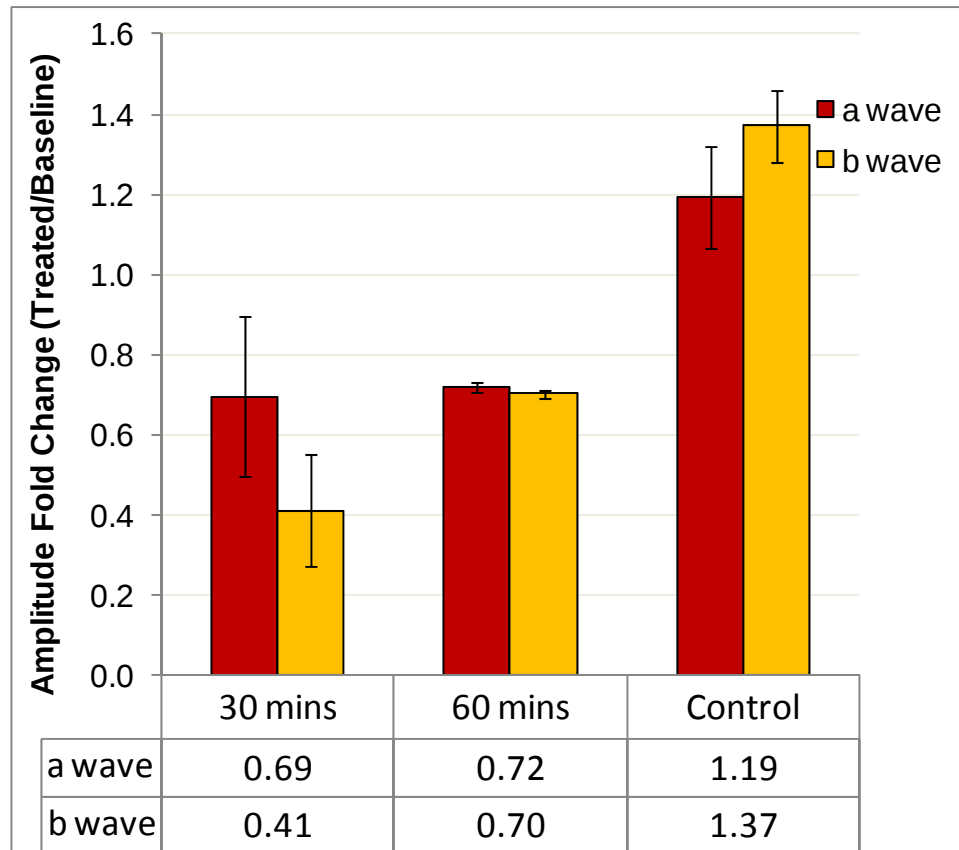


Figure 19. Reduction in ERG a- and b-wave amplitudes of intact newt right eyes 3 days following exposure to 30 and 60 mins of ultra-intense light (>100 000 lux) with the halogen bulb. Newts were anesthetised during exposure to intense light. The graph shows a fold change of amplitudes from the treated eye over the baseline amplitudes (pre-treatment) and represents the average of 2 treated animals and 3 control newts. While there was a reduction in the ERG amplitudes of eyes exposed to light for 30 and 60 mins, this result was not significant. Controls represent newts whose eyes were not exposed to light. The newt ERG protocol was not yet fully optimized; therefore, the newt pupils were dilated with Mydfrin® for only 15 minutes (as opposed to the later optimized 40 minutes). Standard error bars are shown.

3.3.2 Phototoxicity 2 – the use of a mercury arc lamp to induce retinal damage

It was hypothesized that the mercury arc lamp (MAL) would be more effective at inducing acute retinal damage compared to the halogen bulb because it not only irradiates light at a higher intensity compared to the halogen bulb, but it does so at wavelengths of light that are readily absorbed by the newt retina photoreceptor cells. This second phototoxicity setup involved focusing ultra-intense light ($> 100\,000$ lux) emerging from the Super High Pressure mercury arc lamp (Nikon) directly into the pupil of the adult newt eye for exposure times of 5, 15 or 30 minutes. In contrast to the halogen lamp, the Super High Pressure mercury arc lamp had only one power setting which was referred to as maximal intensity for the purposes of this thesis. The illuminance of this lamp was measured by a photometer to be $> 100\,000$ lux, which is a saturation of the photometer. This is comparable to the metal halide lamp used to efficiently damage the photoreceptor cells of zebrafish (Bernardos et al., 2007; Thummel et al., 2008). One third of the MAL output resided in the visible spectrum, about half of the output represented ultraviolet emission while the remainder of the output was dissipated as heat in the form of infrared radiation (Davidson, 2010). Therefore, to ensure that only light in the visible spectrum reached the newt eyes, for all experiments using the MAL, light was passed through a UV filter, an infrared (IR) filter, and a condenser (to further focus the light) prior to reaching the newt's retina (Methods 2.3.1, Figure 10).

Two different newt placement methods were attempted, along with various factors (such as light exposure time, dilation, dark-adaptation) until optimal results were achieved. The two different placement methods involved exposure of the retina to

constant light while the newt was either anesthetized in an MS222®-soaked dish or swimming around in a beaker or fishbowl (Figure 10). The two different methods and their subsequent experiments will be described in detail in the following sections.

3.3.2.1 MAL Method 1 – Constant light exposure of anesthetised newts

Each adult newt was first anesthetised with 0.05% MS222 (Sigma) for 15 minutes and its pupil subsequently dilated with Mydfrin® (Alcon) 10 minutes prior to the phototoxicity test. There was only one newt tested at a time. Each newt was positioned up to approximately 52 cm away from the light source to ensure that maximal light was directed into the pupil. Aluminum foil with a 2 mm hole, a sufficient diameter for light to enter the newt's pupil, was placed in front of the newt eye to shield any intense light from reaching and burning the newt's eyelid. A cheese cloth soaked with MS222® was wrapped around the newt to keep the newt both hydrated and anesthetized during the phototoxicity test. For these experiments, a red light cut-off filter (580 nm) was used to allow for mostly yellow light to reach the retina, the wavelength of light that is predominantly absorbed by the cones of the newt retina (Stebbins, 1995).

Three different exposure times were first tested on a total of eight newts: 5, 15, and 30 minutes constant ultra-intense light. Baseline ERG recordings (recordings prior to light treatment) of newt a- and b-waves were first conducted prior to light treatment. At 2 dpl, newt ERGs were measured, averaged and compared to the baseline a- and b-waves amplitudes (Figure 20). The results were compiled as an average of two newts at the 5 minute exposure time and 3 newts each at the 15 and 30 minute exposure times. There were no significant differences in the averaged ERG amplitudes at 2 dpl compared to

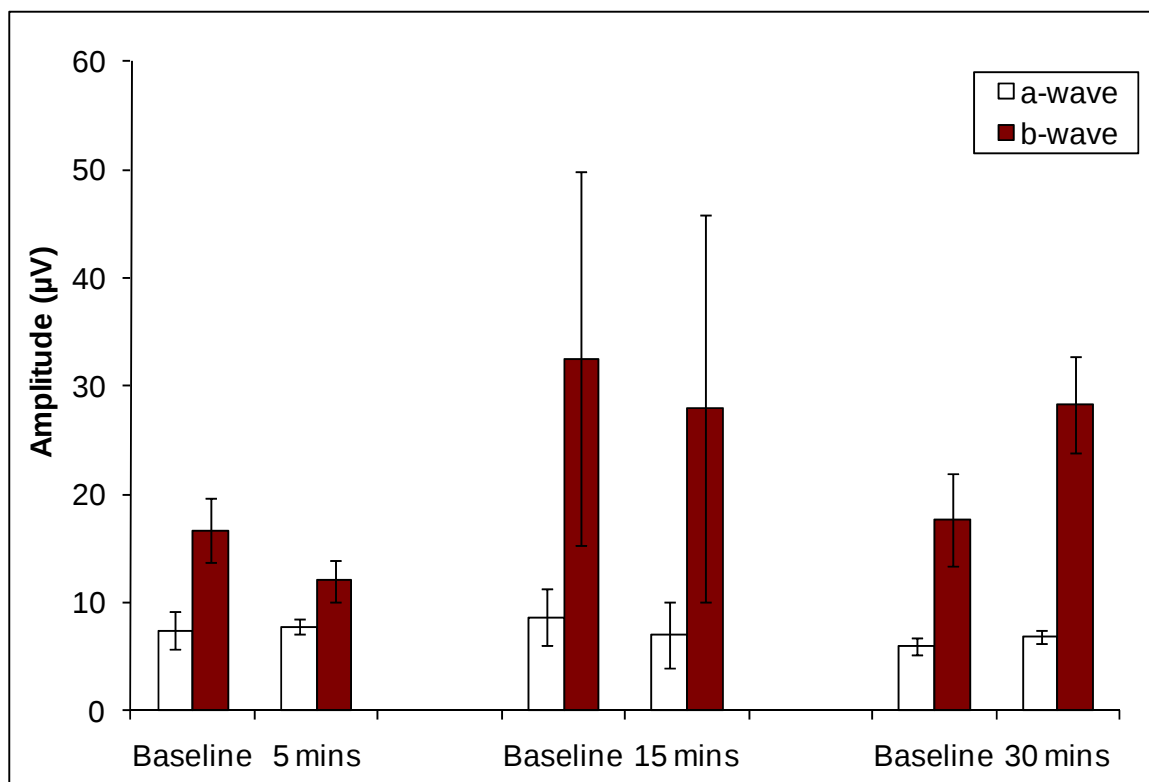
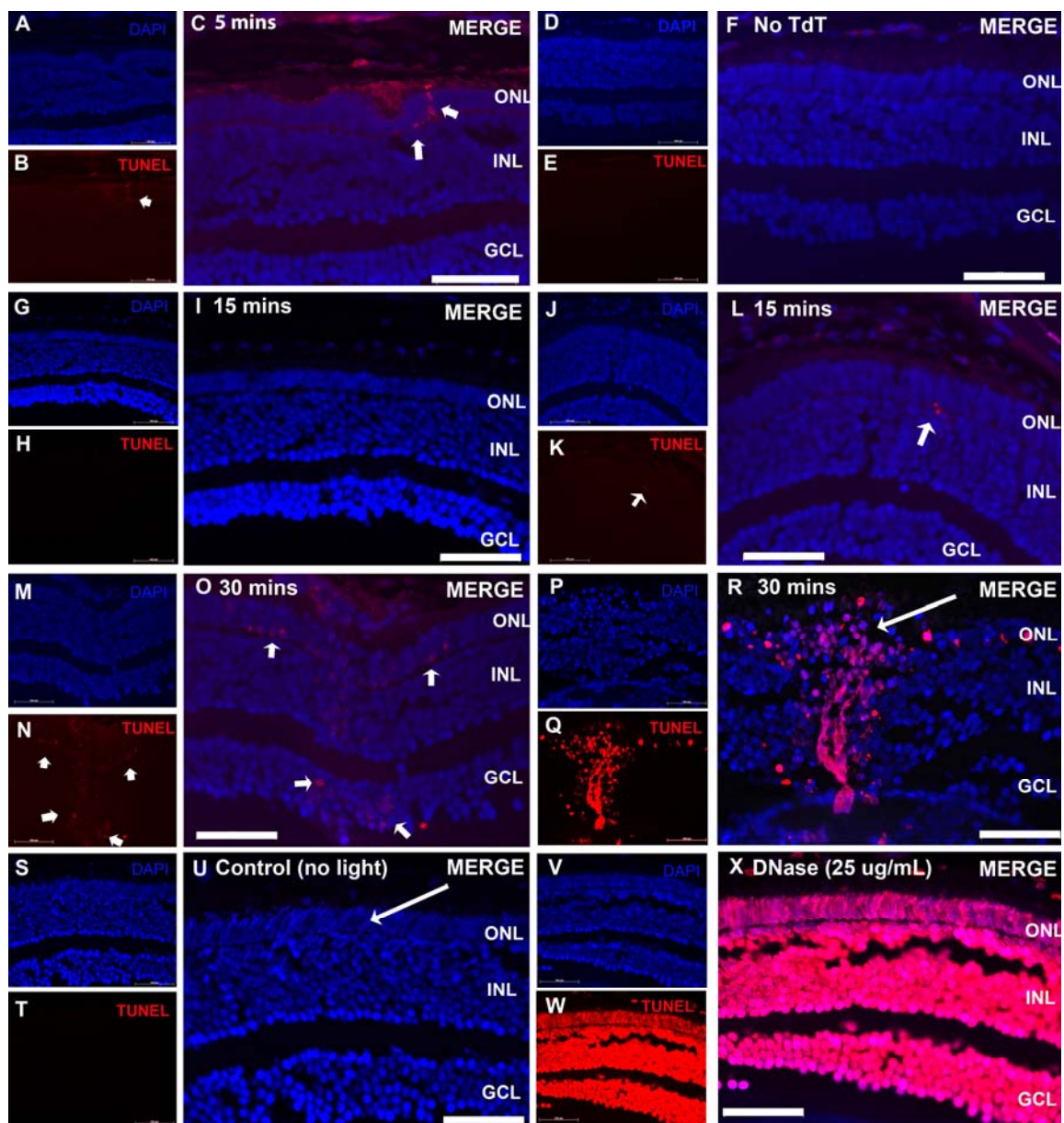


Figure 20. Exposure of intact adult newt retina to ultra-intense light from the mercury arc lamp for 5, 15 and 30 minutes (mins) resulted in no significant change of ERG a- and b-wave amplitudes at 2 days post-light treatment, compared to baseline recordings. Newts were anaesthetised during the tests. Each result was an average of 2 newts (5 mins) or 3 newts (15 and 30 mins) with standard error bars shown.

averaged baseline amplitudes. Since there was a lot of variability in the ERG amplitudes among different newts, subsequent ERG experiments were reported as a fold change from treated over baseline ERGs for each animal tested, rather than an average of results. The samples were fixed at 2 dpl, and the onset of apoptosis due to light was verified with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

TUNEL-positive cells were observed in the retinas of some newts from all three time exposures; however, there were very few TUNEL-positive cells observed for retinas exposed to 5 and 15 minutes of ultra-intense light. Only one of two newts exposed to 5 minutes of constant light (Figure 21 C) and one of three newts exposed to 15 minutes of light (Figure 21 I vs Figure 21 L) demonstrated TUNEL-positive cells. Moreover, the TUNEL-positive cells found in these cases were not clearly overlapping with DAPI staining. All three newts exposed to a constant 30 minute exposure of light displayed varying degrees of TUNEL-positive cells (e.g. Figure 21 O and R). Results from TUNEL assays conducted on serial sections indicated that the damage was predominantly concentrated to a focal region in the central retina (Figure 21 N- R). This focal damage may have been too small to be detected by full-field ERGs, which might explain the lack of change in the ERG amplitudes after light treatment.

Figure 21. TUNEL-positive adult newt retinal cryosections 2 days following constant ultra-intense light treatment with the mercury arc lamp at various exposure times. Anesthetised newt were exposed to light emitted by the Super High Pressure mercury arc lamp (Nikon) for 5 (A-C), 15 (G-L), and 30 (M-R) minutes. Cryosections were labelled with DAPI (blue) and TUNEL (red). Very few TUNEL-positive cells were observed with five minutes of constant light exposure and they appeared restricted to the photoreceptor cells (A-C, arrow). Fifteen minutes of constant light induced no TUNEL-positive cells in one animal (G-I) and very few in another (J-L, arrow) which did not appear to colocalize with DAPI. Thirty minutes of constant light induced maximal DNA damage, with several TUNEL-positive nuclei observed but restricted to the central region of the retina (O, P). However, variability is shown in the results from each animal (M-O compared to P-R). In one animal, it was observed that cell bodies changed morphologically at 2 days and the nuclei no longer appear organized in layers (arrow, R) compared to the control, eyes not exposed to ultra-intense light (arrow, U). The positive control was a 25 µg/ml DNase treatment to induce strand breaks (V-Y). The negative control for TUNEL (terminal deoxynucleotidyl transferase enzyme substituted for ddH₂O) (D-F) and controls not exposed to intense light treatment (S-U) showed no TUNEL positive cells. 100 µm scale bars shown. Abbreviations: onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer



Retinal whole mounts were also conducted to verify the extent of the light-induced damage in the retina. The retina was spread onto a 0.8 μm Nucleopore Track-Etch Membrane (Whatman®), with ganglion cells facing up. TUNEL assays were then immediately conducted on the fixed retinal whole mounts. TUNEL-positive cells were found clustered in a diameter approximately 100 μm in width (Figure 22), which represented roughly 10% of the entire retina. Damage to an area this small would likely not be detected by full-field ERG.

Unlike in zebrafish, our results suggested that retinal damage due to light is not photoreceptor-cell specific in the newt. It was of interest to look at time points earlier than 2 days, in order to determine whether or not photoreceptor cells were the first to undergo apoptosis. If the activation of photoreceptor cells was prolonged due to a long exposure of light, this may have resulted in their apoptosis, leading to their decreased synapsis to underlying neurons of the retina. If the neurons of the inner nuclear layer and the ganglion cell layer were not receiving adequate neurotransmission, this could consequently result in their apoptosis. A TUNEL assay was therefore conducted on newt retinas exposed to only 8 hours of light to determine the onset of apoptosis shortly after light exposure. Preliminary results in the central retina indicated that retinal damage occurs both in the photoreceptor cell layers and ganglion cells 8 hours post-light damage (Figure 23). Therefore, the light damage was not restricted to the photoreceptor cells, as was the case with the zebrafish study (Bernardos et al., 2007), suggesting that the properties of newt retinal cells may differ from those of zebrafish in their increased sensitivity to light.

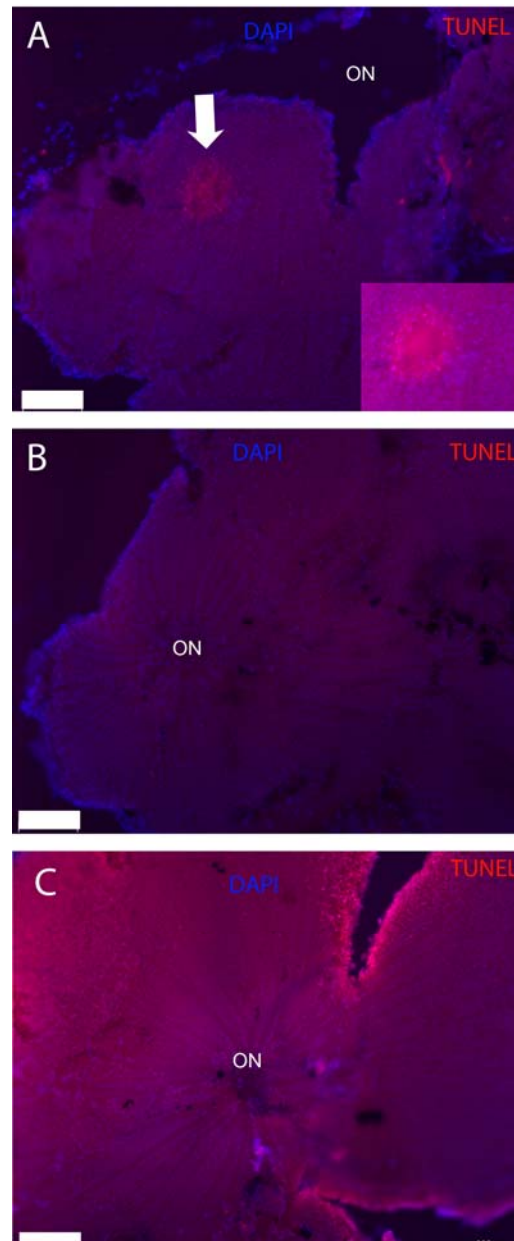


Figure 22. The TUNEL assay on a newt retinal whole mount revealed apoptosis 2 days after light exposure in a focal region of the central retina, after 30 minutes constant ultra-intense light exposure to the mercury arc lamp while anesthetized (Method 1). Whole mounts are oriented with ganglion cells up and stained for DAPI (blue). A) In a specific region (arrow) over 300 μm away from the optic nerve (ON), an area approximately 100 μm in diameter, running deep into the photoreceptor cell layer appeared TUNEL-positive. Inset shows magnification of the damage site. B) The control represents a separate animal not exposed to ultra-intense light. The positive control for TUNEL (C) was a DNase of the entire retinal whole mount. Scale bars shown for the large images (100 μm). Abbreviation: dpl, days post-light.

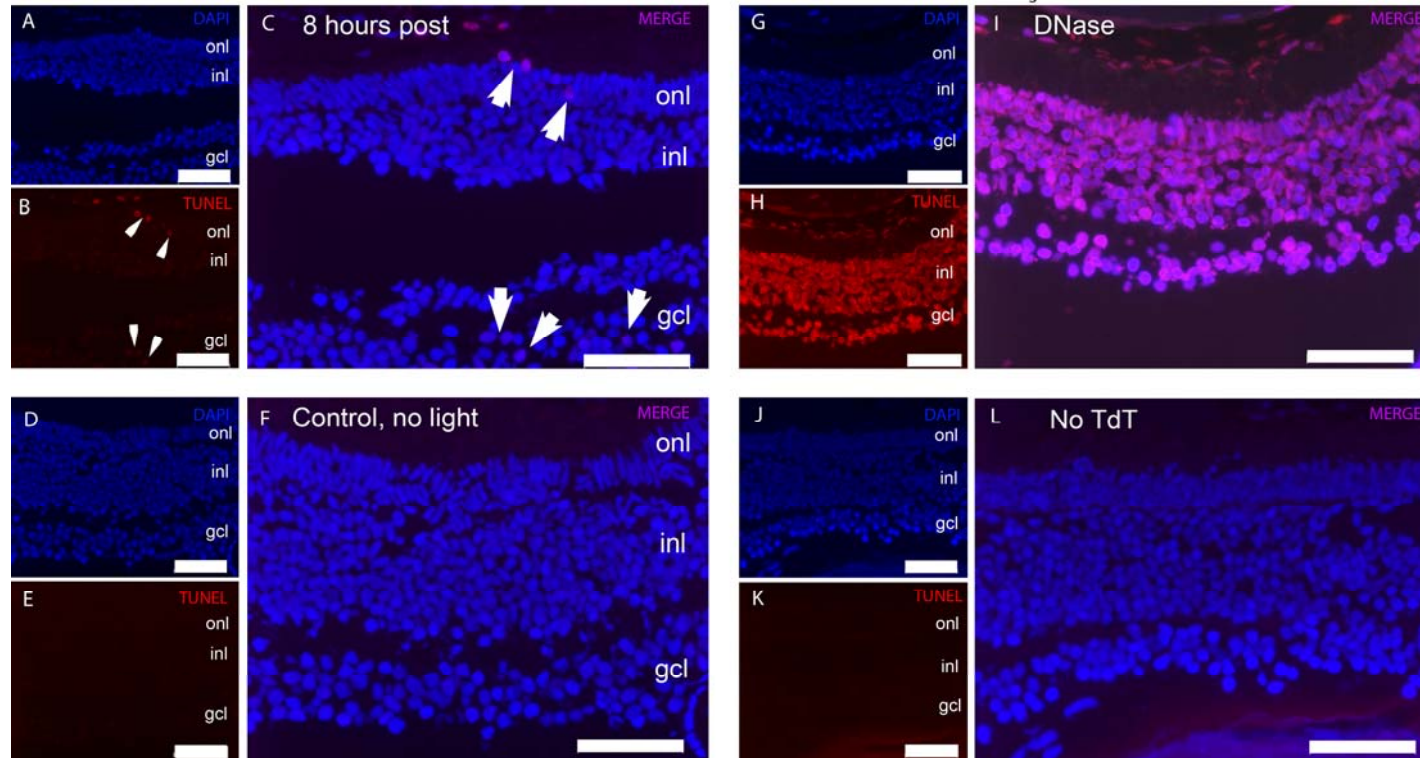


Figure 23. Adult newts sampled 8 hours post 30 minutes constant ultra-intense light treatment with the MAL, while anesthetized, demonstrated TUNEL-positive cells in all layers of the retina. Eight hours post-light exposure (A-C) a few TUNEL-positive cells (red) appeared in the photoreceptor cells and ganglion cells of the central retina (arrowheads), whereas no TUNEL-positive cells were observed in the control, the contralateral left eye not exposed to light (D-F). This suggested that unlike in zebrafish, light-induced retinal damage in the newt was not restricted to the photoreceptor cells. The positive control for TUNEL (G-I) was a DNase treatment (25 $\mu\text{g}/\text{ml}$ for 10 minutes) on a retinal cryosection. The negative control for the TUNEL assay involved the substitution of terminal deoxynucleotidyl transferase (TdT) enzyme with ddH₂O (J-L). DAPI is stained blue. Scale bars shown (100 μm). Abbreviations: onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer.

A similar TUNEL assay was conducted on newts subjected to 30 minutes of ultra intense light exposure and assayed at 1 dpl. The damage was still restricted to the central region of the retina; however there was a cone-like damage effect observed, with the most TUNEL-positive cells in the photoreceptor cells compared to significantly fewer TUNEL-positive ganglion cells (Figure 24). This was an interesting observation because phototransduction involves the cascade-like activation of several photoreceptor cells that synapse to a fewer number of neurons in the inner nuclear layer which in turn synapse to an even fewer number of ganglion cells. Therefore, although this type of damage pattern was not observed at 8 hours post-light, there may still be a cascade effect, whereby the photoreceptor cells were first affected by light damage and their inability to synapse to underlying nuclear layers resulted in the subsequent apoptosis of neurons in the inner nuclear and ganglion cell layers. Since this was observed in a very small sample size, it would be of interest to repeat these assays in time points earlier than 24 hours to see if such an effect exists.

3.3.2.2 MAL Method 2 – Constant light exposure of freely swimming newts

In Method 1, ultra-intense light from the mercury arc lamp caused focal damage to anesthetized newts, which was not sufficient to induce significant changes to the ERG. In order to monitor retinal cell loss and subsequent regeneration using ERGs, it was therefore necessary to optimize the conditions of the experiment to ensure that light reached more regions of the retina, causing sufficient damage to abrogate the ERG.

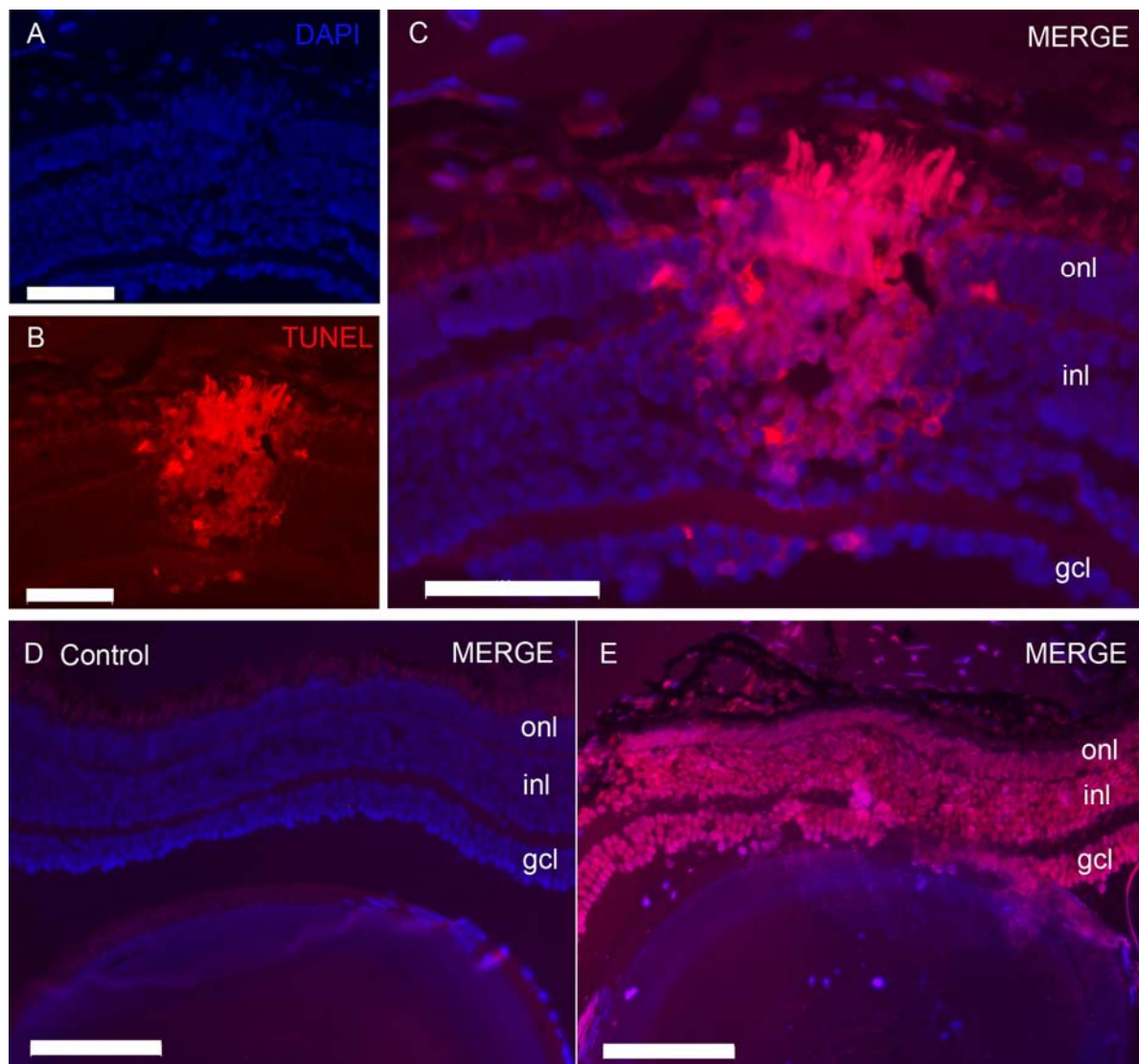


Figure 24. TUNEL-positive retinal cells observed in adult newts 1 day following 30 minutes of ultra-intense light exposure with the MAL, as newts were anaesthetised on a dish. At 1 day (A-C) following light exposure, TUNEL-labelled (red) cells appear restricted to the central retina and span all retinal layers in a funnel-like fashion, with photoreceptor cells mostly affected by light while only a few ganglion cells undergoing apoptosis. As for all other TUNEL experiments, the negative controls were not exposed to light (D) and the positive control (E) was a DNase treatment (25 $\mu\text{g}/\text{ml}$ for 10 minutes) on a retinal cryosection. Scale bars shown (100 μm). Abbreviations: onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer.

The method employed by Bernardos *et al.* (2007) on albino zebrafish involved placing the zebrafish together in a beaker for 30 minutes, where they continually swam in the path of light emitted from a fiber optic liquid light line (3 mm diameter) attached to the metal halide lamp. This published method was modified to involve newts swimming freely alone or in pairs, for 30 minutes, in a fishbowl or beaker whose centre was placed approximately 55 cm away from the light source. At this position, the light beam was most intense just inside the fishbowl or beaker, representing the focal point of the light beam. Beyond this focal point, light was scattered throughout the beaker or fishbowl, illuminating all areas where the newts would swim. Rather than using a fiber optic line, the light beam was focused using a condenser.

It was determined from Method 1 that the 30 minute light exposure caused retinal cell death, observed with TUNEL staining 2 days following light exposure. Three experiments were conducted using Method 2 on newts exposed to 30 minutes ultra-intense light while swimming freely in a beaker or fish bowl. These are as follows:

Experiment 1) Newts swimming in a beaker whose centre was placed approximately 55 cm away from the light source. Aluminum foil was wrapped around three quarters of the beaker.

Experiment 2) Newts swimming in a fishbowl whose centre was placed approximately 55 cm away from the light source. The fishbowl was surrounded by a light box (cardboard box covered in aluminum foil).

Experiment 3) Newts swimming in a fishbowl surrounded by a light box with or without a second UV filter used to cut off light at 480 nm.

All three experiments had the following conditions in common, in order to maximize light damage. Firstly, all newts were dark-adapted overnight to induce a greater sensitivity of the photoreceptor cells to ultra-intense light. Secondly, the dilation time was increased from 10 to 20 minutes, to ensure that maximal dilation was reached during the light assay, to allow for stimulation of the maximal number of photoreceptor cells. Thirdly, the red cut-off filter was no longer used since data from Phototoxicity 1 suggested that the halogen bulb, which predominantly emits red light, did not induce retinal damage in the newt. Therefore, red light did not appear to be damaging to the newt retina at >100 000 lux. Also, since the mercury arc lamp emitted light at other wavelengths (yellow, green, violet) it was not necessary to filter out red light.

In addition, for all experiments in Method 2, recordings of newt ERG a- and b-waves were first conducted prior to light treatment (baseline) and at 2 or 4 days after light treatment. Prior to ERG recordings, all animals were anesthetised with MS222® for 15 minutes and their pupils dilated for 40 minutes. The fold change of both a- and b-wave amplitudes post-treatment over baseline were measured and averaged. Intact right eyes from separate adult newts served as controls. ERGs were recorded from these animals on the same day as those recorded from the treated animals. After ERGs from treated animals were recorded, the samples were fixed, processed, and frozen for sectioning and detection of apoptosis with the TUNEL assay.

3.3.2.2i Method 2, Experiment 1 – newts swimming in a beaker

Five newts were separately exposed to 30 minutes of ultra-intense light while swimming around in a 500 mL beaker whose centre was placed approximately 55 cm away from the light source. Aluminum foil was wrapped around three quarters of the

beaker to allow for light to reflect off the foil and onto the newt retina at various angles. Two days following light treatment, there was a significant decrease of the a- and b-waves of only two newts treated with 30 minutes ultra intense light, compared to baseline levels (Student's T-Test, $p < 0.001$ or $p < 0.05$) (Figure 25). There was no statistically significant difference in the control a- or b-waves in comparison to baseline time points (Mann-Whitney Rank Sum Test, $p = 0.318$ for a-waves $p = 0.301$ for b-waves). For the control animals, the amplitudes of four newts were averaged and since there was a lot of variability among the amplitudes of these animals, the data did not follow a normal distribution.

The ERG amplitude reductions correlated to a significant appearance of TUNEL-positive cells in all layers of the retina of the two animals, following ultra-intense light exposure (Figure 26). Since there was no reduction in the a- or b-wave of the three other newts (data not shown), this indicated that the light-damaging conditions were not yet optimal. It was observed that the aluminum foil created shady spots in the inside edges of the beaker. Newts may have swum to these areas and thus were not continually exposed to intense light. A new setup was therefore needed.

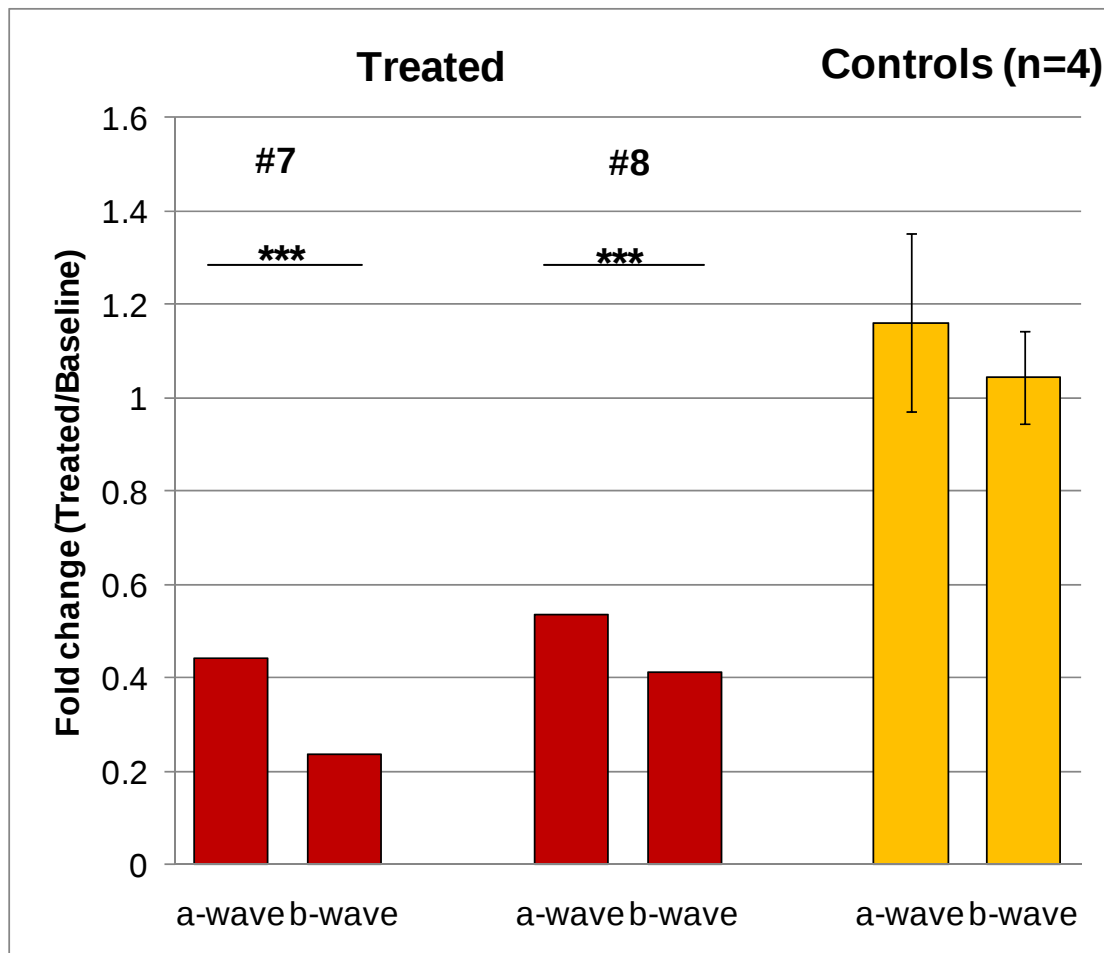


Figure 25. Newts exposed to ultra-intense light while swimming in a beaker surrounded with aluminum foil for 30 minutes showed a statistically significant decrease of the ERG a- and b-waves at 2 days post-light treatment (Experiment 1). The results represent a fold change of ERG amplitudes of treated animals or control animals over the baseline recordings of the same respective animal prior to light treatment. After dark-adaptation, newts swam in a beaker surrounded by ultra-intense light. Light exposure resulted in a statistically significant loss of the ERG a- and b-wave amplitudes for newt #7 and #8 (T-Test, *** $p < 0.001$ for a- and b-waves). Three other newts did not show a reduction in ERG a- and b-waves compared to baseline (data not shown). Controls are four newts that were not exposed to light and there was no statistical significance in the a-waves ($p = 0.318$) or b-waves ($p = 0.301$) of these newts between their baseline recordings and their ERGs at the later time point. Standard error bars shown for the control.

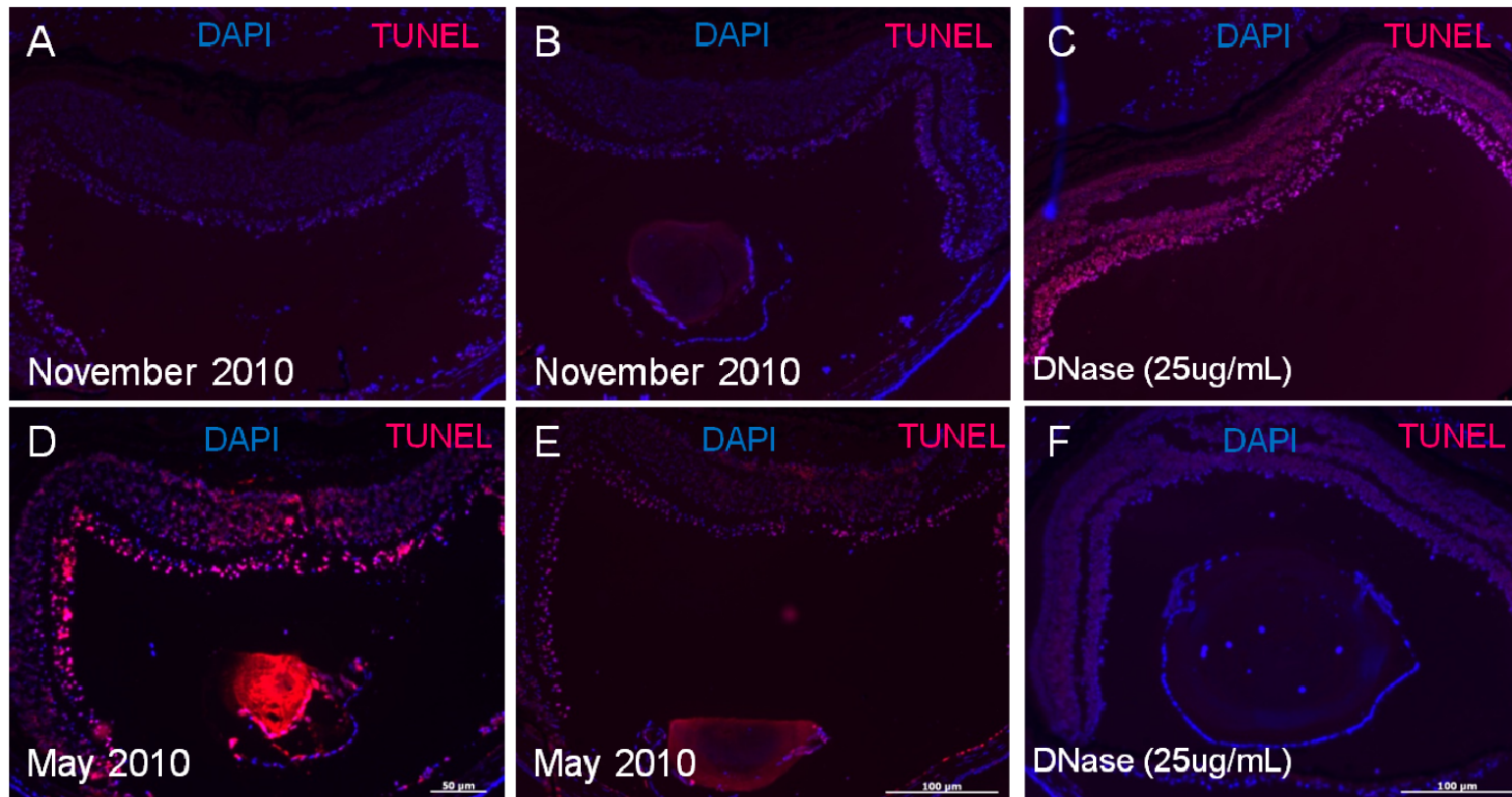


Figure 26. Apoptotic cells were visible all throughout the retina of newt #7 subjected to 30 mins ultra-intense light (Experiment 1). There was variability in the extent of retinal damage observed in sections from the same newt but assayed on different months. Figures A-C corresponded to results from a TUNEL assay conducted in November 2010 on the right eyes of light-induced newts. Figures D-F corresponded to results from a TUNEL assay carried out in the same animal (Newt #7, experiment 1) but in May 2010. There was variability observed in the number and intensity of apoptotic cells from one experiment to the next, indicating that the assay must be carried out on fresh slides with fresh reagents. Figures A and B represent sections that are 120 μm apart in the central area of the newt eye, while Figures D and E, sections that surround A and B, are 300 μm apart. Positive controls (Figures C & F) for the TUNEL assay consisted of sections incubated with 25 μg/mL DNase for 10 minutes at room temperature. The slides were all frozen on the same day. Scale bars represent 100 μm.

3.2.2.2.ii- Method 2 Experiment 2 – newts swimming in a fishbowl

In this experiment, a fishbowl was used because it contained round edges which did not allow for any shady areas to form inside, as was the case with the beaker in Experiment 1. In order for apoptosis to occur, photoreceptors needed to be over-stimulated, therefore it was crucial that the newt retinas be continuously exposed to light. The retinas of 9 newts were exposed to 30 minutes of ultra-intense light while swimming either individually or while in pairs around in the fishbowl filled with approximately 300 mL of dechlorinated water. In addition, a thermometer was placed in the fishbowl to measure the temperature to ensure that heat was not dissipating in the water, and potentially contributing to the apoptosis of the retinal cells. Thirty minutes of light exposure caused a change in water temperature of 1.5°C. The fishbowl was placed approximately 55 cm away from the light source. To ensure that light was directed to various areas of the retina, a light box, covered with aluminum foil, was placed around the fishbowl. The fishbowl was also placed over a styrofoam box also covered with aluminum foil. This setup was used to ensure that light stimulated a sufficient number of photoreceptors in the retina to be detected by ERG. ERGs were monitored up to 14 days post-light to see if there was an effect on the ERG amplitudes beyond 2 dpl.

Two days following a 30 minute light exposure, ERGs results showed a reduction in the a- and b-waves of newts treated with light compared to baseline levels. However, the reduction was not significant when the results were averaged for all nine newts (Figure 27). The results were re-graphed to show ERG amplitude differences for each animal (Figure 28). A variability in results was observed as 1/9 newts demonstrated a reduction of the ERG amplitudes that was greater than 50% (#8) compared to baseline,

while 3/9 newts had a reduction in ERG amplitudes greater than 20% (#5, 11, 13). The reductions in ERGs of four newts were statistically significant compared to baseline values (Mann-Whitney Rank Sum, $p < 0.001$) while there was no statistical significance in the ERG amplitudes of all controls. There was variability of ERG a- and b-waves among the different treated and control animals, therefore the data did not represent equal variance among all newts. This was largely due to the sampling of newts that were captured from the wild at different ages and genders. Individual comparisons of amplitudes were conducted with Student's t-test.

Three of the nine newts that showed the greatest reductions in ERG amplitudes (newts #5, #8 and #13) were sampled for TUNEL analysis, along with controls (which did not show any changes in the ERG amplitudes compared to baseline levels). TUNEL-positive cells were observed throughout all retinal layers of newts #5 and #8 (Figure 29). In contrast to Method 1, where exposure of light to anaesthetised newts resulted in damage to a focal region of the retina, a greater number of retinal cells were damaged by light using Method 2, when newts swam freely and the light reached various regions of the retina. Apoptotic cells were observed in both the central and peripheral regions of newts #5 and #8 using Method 2. These results indicated that there was a correlation between a reduction in ERG amplitudes (of at least 50%) and apoptosis in a vast region of the newt retina. However, as with Experiment 1 (freely swimming newts in a beaker), light damage was not observed for all animals in the fishbowl; therefore, these conditions were not optimal to produce consistent results.

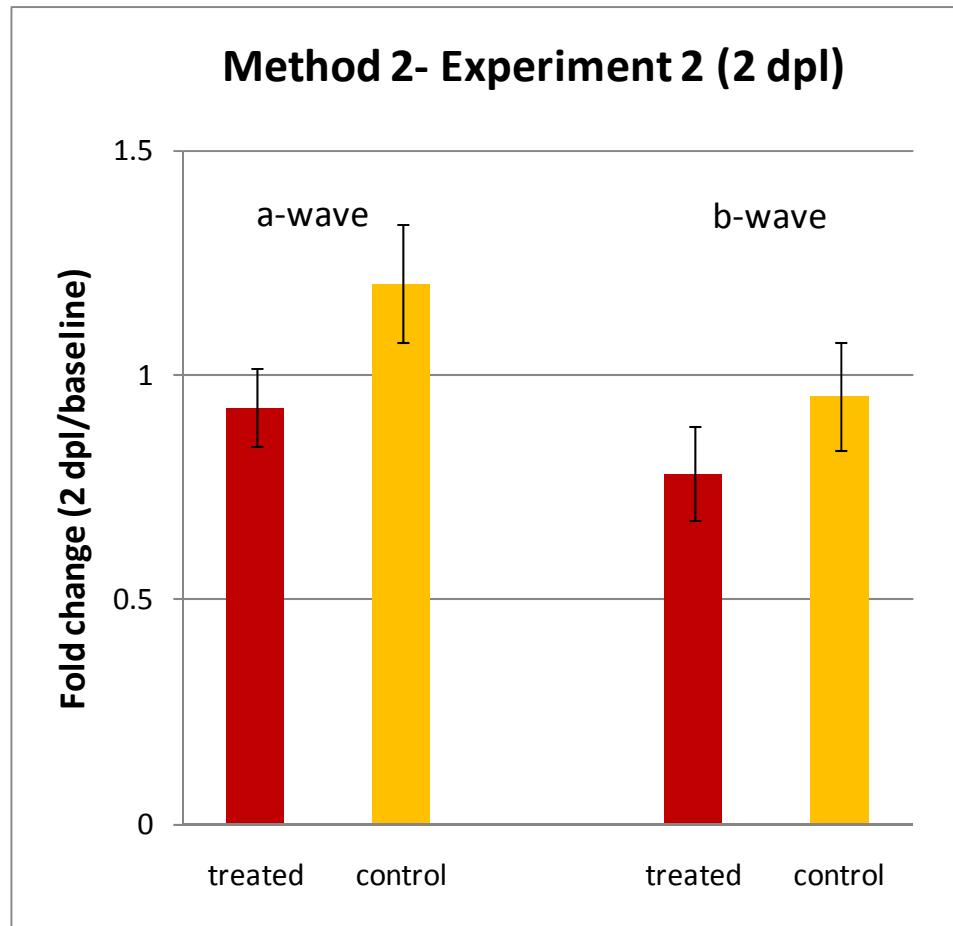


Figure 27. Newts exposed to 30 minutes of ultra-intense light from the MAL while swimming in a fishbowl experienced a slight but non-significant decrease of the averaged ERG a- and b-waves at 2 dpl (Experiment 2). The results represented a fold change over the baseline recordings of the averaged ERG amplitudes of nine treated and five control newts. Control newts were not exposed to ultra-intense light. Newts were dark-adapted prior to the test. Standard error bars are shown.

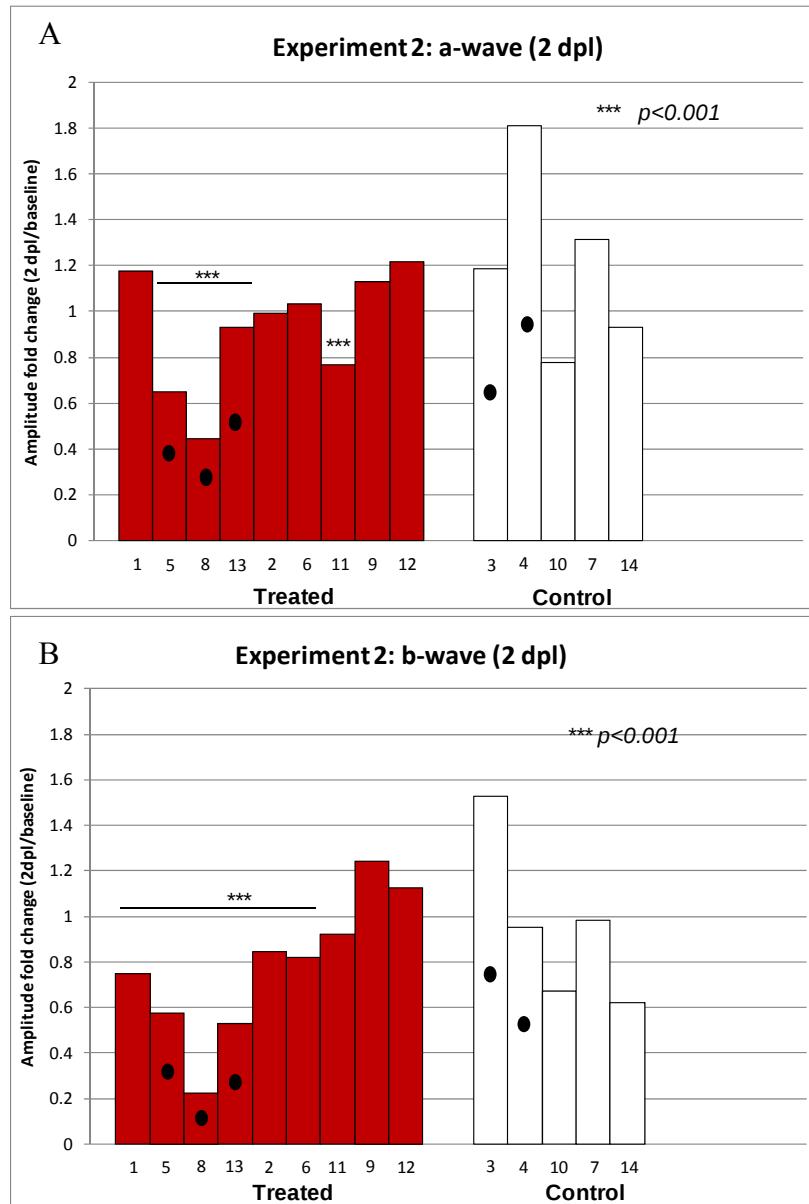


Figure 28. Newts swimming in a fishbowl surrounded by ultra-intense light from a mercury arc lamp for 30 minutes experienced a statistically significant decrease of the ERG a- and/or b-waves at 2 days post-light treatment. Four of nine light-treated newts showed a significant decrease in the ERG a-waves (A) while six of nine light-treated newts showed a significant decrease in the ERG b-waves (B) compared to baseline (Student's T-Test, *** $p<0.001$). The results represented a fold change of ERG amplitudes of treated or control newts over the baseline recordings of the same respective newt. Control newts were not exposed to light. Circles indicated newts that were sampled at 2 days post-light for subsequent study with TUNEL. Newts were dark-adapted prior to treatment. Newts that were paired together were #1 with #9, #5 with #11, #8 with #6, and #2 with #12. #13 was alone in the fishbowl throughout the test.

At four days following light treatment, ERGs were conducted on the remaining five newts. All other previous experiments only measured ERGs for up to 2 dpl, as it was assumed that apoptosis would have occurred by this time point. At 4 dpl, there was a significant reduction in the ERG a- and b-waves of newt #6, not observed at 2 dpl, as well as a significant reduction in the b-wave of newt #2, demonstrating a delayed response to light damage (Figure 30). Due to the small number of newts at this time point, no statistical tests were conducted. TUNEL analysis on retinal cryosections for newt #6 revealed apoptotic cells in many layers of the retina (Figure 31), similar to the results for newts #5 and #8.

Therefore in this experiment, 4/9 newts that were exposed to 30 minutes of ultra intense light after swimming in a fish bowl demonstrated a significant loss of ERG amplitudes after treatment. These results correlated with the observation of apoptotic cells in both the central and peripheral regions of the retina.

A limitation of this method was the lack of specificity of damage. We observed apoptosis not only in the central and peripheral regions but in the epithelial cells of the lens and cornea (Figure 30) indicative of off-target effects. To minimize the off-target effects of light damage, the conditions of this experiment were slightly modified in Experiment 3 with the addition of a UV filter.

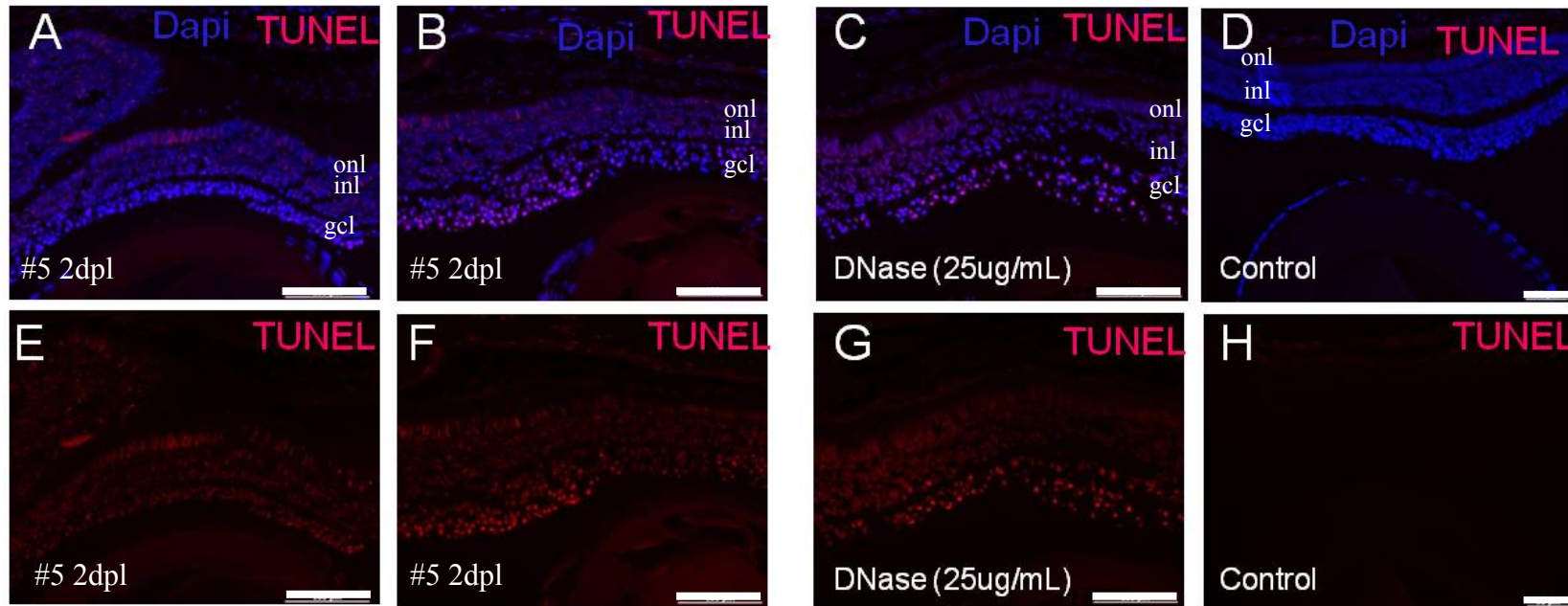


Figure 29. Exposure to 30 minutes ultra-intense light while swimming in a fishbowl resulted in the appearance of apoptotic cells all throughout the retina of newt #5 (Experiment 2). TUNEL-positive cells marking apoptosis were observed throughout all retinal layers of 2 sections (A and B) that are 360 μm apart. These sections were roughly from the central regions of the newt eye. Panels A-D correspond to merged results of TUNEL-positive (red) cells and nuclei stained with DAPI. The apoptotic assay was conducted on the right eye of the newt. No TUNEL-positive cells were observed in the control newt (D and H), that was not exposed to light. Figures E and F are showing signals from the rhodamine channels only, and clearly demonstrate the wide distribution of TUNEL-positive cells throughout all layers of the retina. The positive control for the TUNEL assay (C and G), are retinal cryosections incubated with 25 $\mu\text{g}/\text{mL}$ DNase for 10 minutes, at room temperature. The slides were all frozen on the same day and employed the same reagents. Scale bars represent 100 μm in all sections but D and H (50 μm scales). Abbreviations: onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; dpl, days post-light.

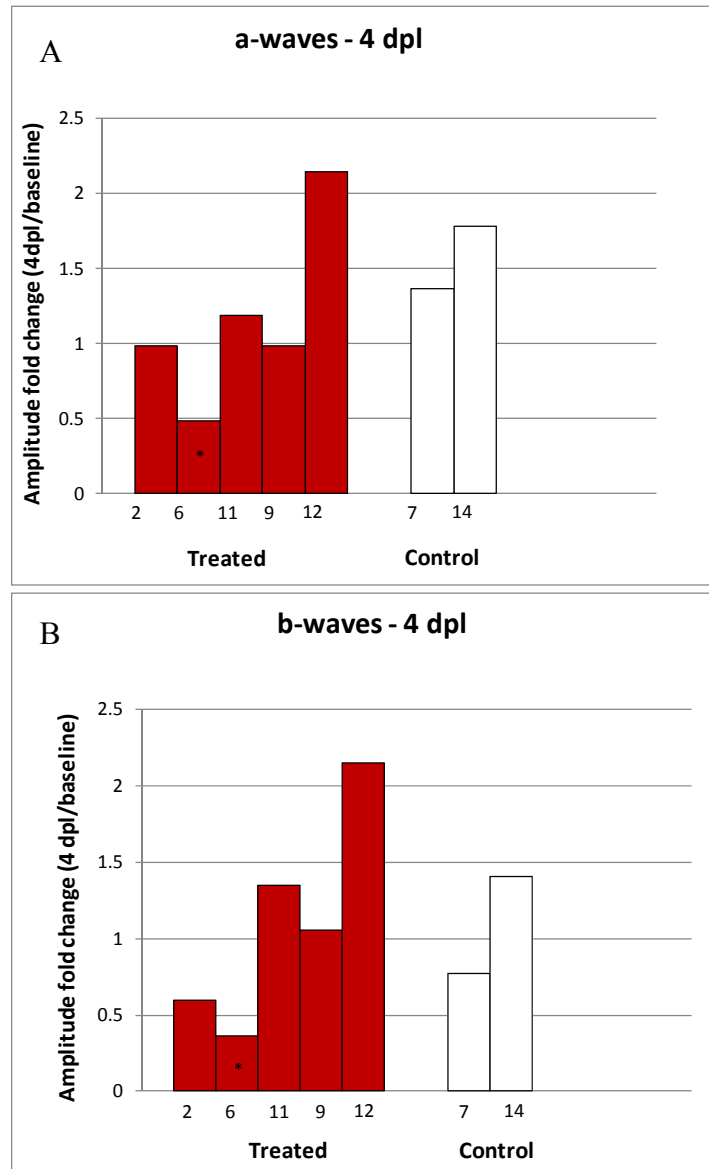


Figure 30. Exposure of newt retina to ultra-intense light from the mercury arc lamp for 30 minutes resulted in a slightly delayed decrease in the ERG a- and b-waves of some newts at 4 days post-light treatment (Experiment 2). One newt (newt #6) demonstrated a significant reduction of both a- (A) and b- (B) waves compared to baseline levels and newt #2 demonstrated a significant reduction of its b-wave (B). No reduction of ERG amplitudes was observed in these animals at 2 dpl (Figure 28). This data suggests that variability existed in the onset of apoptosis after ultra-intense light exposure. While other animals demonstrated a reduction in ERG a- and b-waves at 2 dpl, newt #6 showed decrease of both a- and b-waves at 4 dpl. Newt #2 showed a decrease of only the b-wave at 4 dpl. Only 2 controls were used in this case as other controls newts were sampled at 2 days post-light treatment. Newt #6 was sampled along with control #14 for TUNEL analysis.

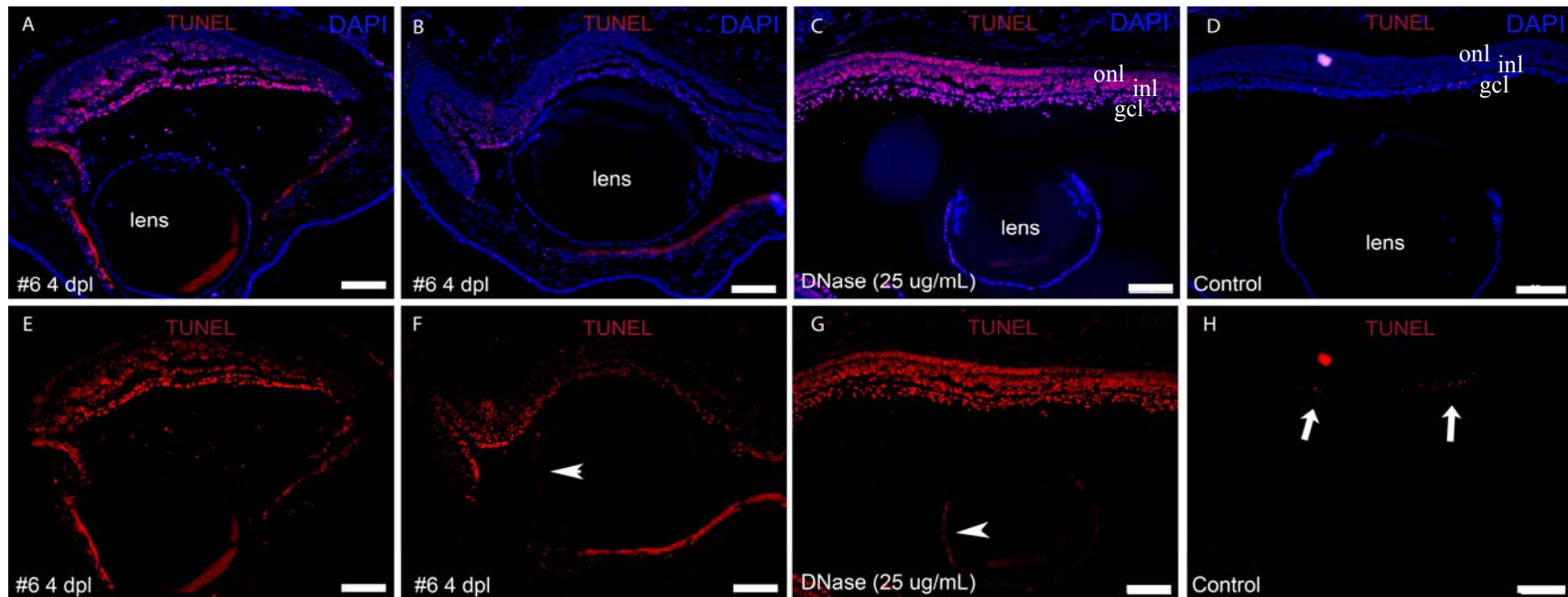


Figure 31. TUNEL analysis in the retina of newt #6 at 4 days following 30 mins ultra-intense light exposure (Experiment 2). Apoptotic cells were observed throughout all retinal layers, as shown in 2 cryosections (A and B) that are 360 μm apart. Panels A-D represent the merged results of TUNEL-positive (red) cells and nuclei stained with DAPI while Panels E-F represent only the TUNEL-positive cells seen through the Rhodamine (red) fluorescence channel on the Zeiss Axiovert. The apoptotic assay was conducted on the right eye of the newt and TUNEL-positive cells were observed in all retinal layers. Sections are from the central regions of the newt eye. TUNEL-positive cells were also seen in the epithelial cells of the lens (arrowhead). A few TUNEL-positive cells were observed in the control newt (H, arrows) which was not exposed to light. This damage was confined to a focal area of the eye (as examined by TUNEL assays on many sections of this control) and was only seen in ganglion cells. Scale bar shown is 100 μm . DNase served as a positive control (C, G). Abbreviations: onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; dpl, days post-light.

3.2.2.2.iii Method 2 Experiment 3 – newts swimming in a fishbowl, testing effect of UV

Experiment 3 replicated the use of conditions from Experiment 2, newts swimming in a fishbowl with constant light from the MAL exposing the retina at various angles. In addition, in order to determine whether the residual UV emitted by the MAL (approximately 2% of the total power) (Figure 17) had a damaging effect on the retina, a second UV filter (cut-off of 480 nm) was used. According to the visual spectrum, this also cut off the violet wavelength of light (Figure 7). Six newts were tested with the UV filter in place and two newts were tested without the additional UV filter. No newts were paired during the light treatments in order to prevent one newt from obstructing the pathway of light from the other.

ERGs were recorded prior to light treatment (baseline) and at 2 days following light treatment, with the results of the a- and b-waves represented as fold changes of treated over baseline amplitudes. Figure 32 presents the averaged ERG amplitudes of six newts subjected to ultra-intense light exposure in the presence of the UV filter (480 nm) and two newts subjected to ultra-intense light exposure in the absence of the UV filter. A statistically significant reduction in the ERG a- and b-waves was observed with the presence or absence of the UV filter (Mann-Whitney Rank Sum test, $p < 0.001$), suggesting that the damaging effect of the MAL on the newt retina was likely due to wavelengths of light emitted outside of the UV and violet light spectrums. In the case where there was no UV filter, both light-treated newts had reductions of the ERG a- and b-waves that were less than 50%.

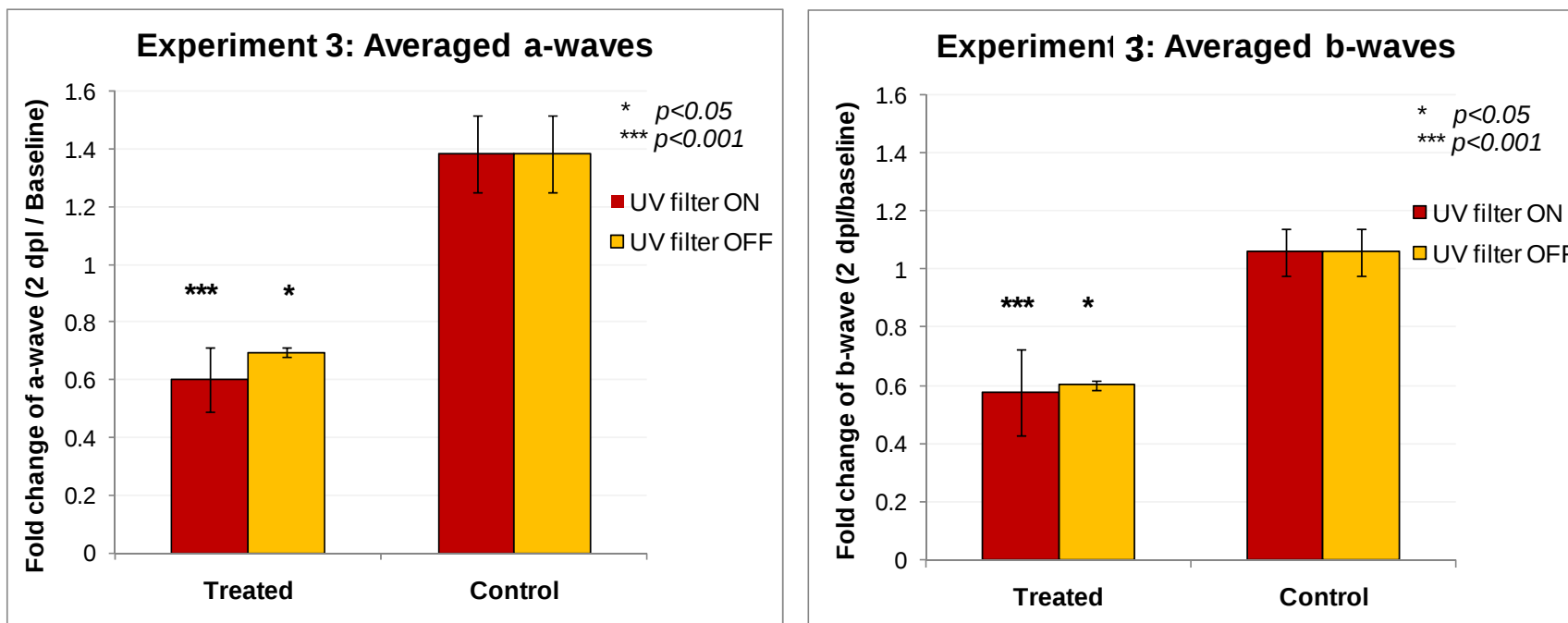


Figure 32. Average ERG results of newts exposed to ultra-intense light from the MAL while swimming in a fishbowl in the presence or absence of a UV filter showed a statistically significant decrease of the ERG a- and b-waves at 2 days post-light treatment (Experiment 3). These results suggested that residual UV light was not the cause of damage to retinal cells, since filtering of UV light (480 nm cut-off) still resulted in a significant decrease of ERG a- (A) and b- (B) waves over baseline (Mann-Whitney Rank Sum test, $p<0.05=*$, $p<0.001=***$). Controls were newts that were not exposed to ultra-intense light. Newts were dark-adapted prior to treatment. Standard error bars shown.

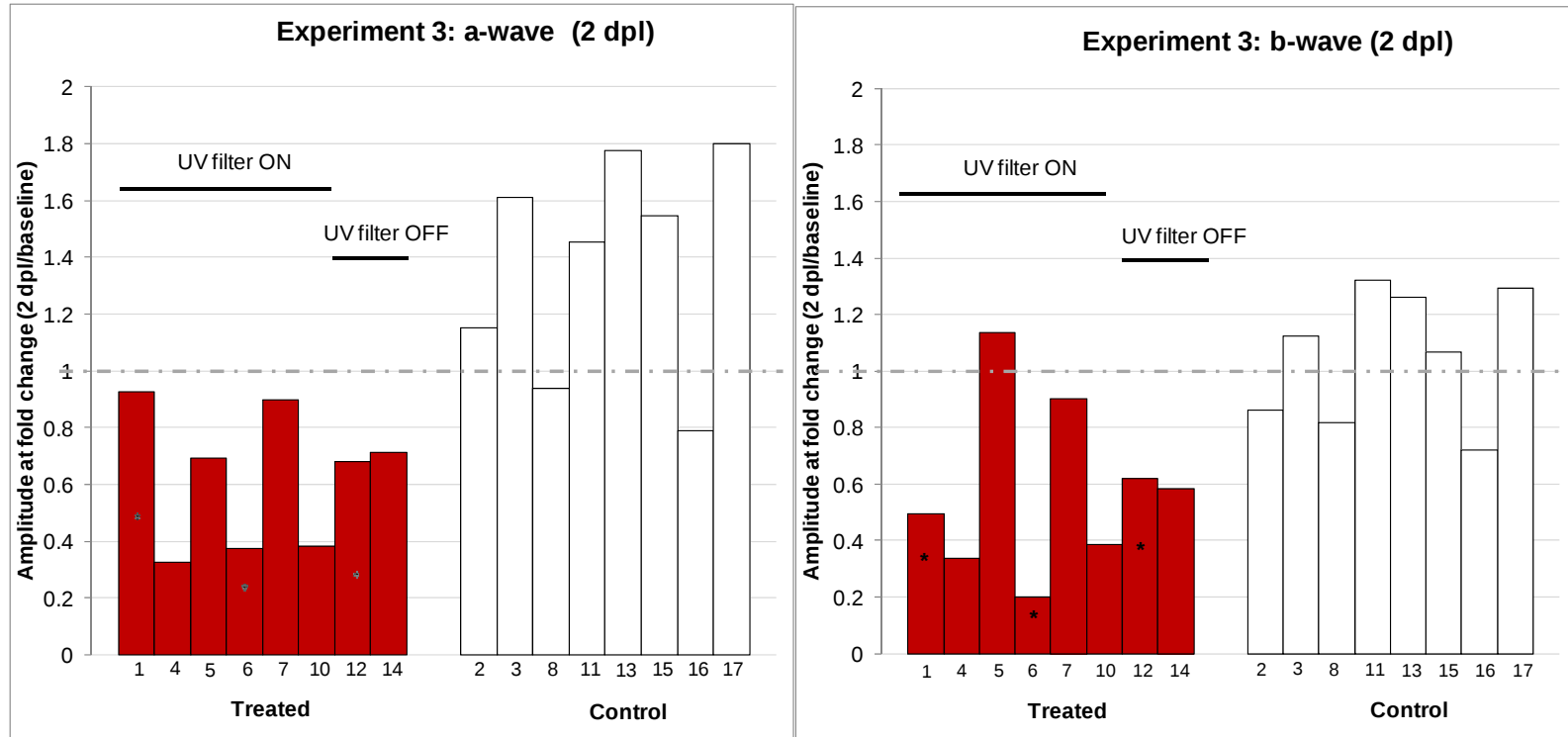


Figure 33. Individual results for newts exposed to ultra-intense light from the MAL while swimming in a fishbowl in the presence of a UV filter showed variability of the ERG a- and b-waves at 2 days post-light treatment compared to baseline (Experiment 3). Six newts were light-treated in the presence of a UV filter, and 3/6 newts demonstrated ERG a- and b-wave amplitude decreases greater than 50%. Two newts (#12 and #14) were light-treated in the absence of a UV filter and showed near equal reductions of the a-wave (roughly 30%) and similar reductions of the b-wave (38.1% for #12 and 46% for #14). Controls were newts that were not exposed to light. Newts were dark-adapted prior to treatment. Newts sampled for TUNEL are shown (*).

As was the case in previous experiments involving the MAL, there was variability in the effect on the ERG after light treatment in the presence of the UV filter (Figure 33). Three of six newts showed a significant reduction in the ERG amplitudes of greater than 50%, in both ERG a- and b-waves. One of six newts had a 30.8% reduction in only the a-wave and no significant change with the b-wave (newt #5). This newt was not further sampled because the animal died shortly after the electroretinogram test. One of the six newts showed only an 8% reduction of the a-wave but a 50% reduction of the b-wave (newt #1). Lastly, one of six newts showed only a slight reduction (around 10%) of the ERG a- and b-wave (newt #7) (Figure 33).

While there was variability observed among the animals, there did appear to be a relationship between a significant reduction of ERG amplitudes (50% or greater) and the appearance of apoptotic cells in various regions of the newt retina (e.g. newt #6, Figure 34 A). When the difference of the ERG was less than 50%, very few TUNEL-positive cells were observed. In the case of newt #12 (a slight reduction in both the ERG amplitudes), a few apoptotic cells were observed in the ganglion and photoreceptor cells in the outer nuclear layer (Figure 34 B) but not to the same extent as newt #6 (Figure 34 A). In the case of newt #1, the reduction in only the ERG b-wave correlated to the presence of a few TUNEL positive cells in the ganglion cells and no apoptotic cells in the photoreceptor cells (Figure 34 C).

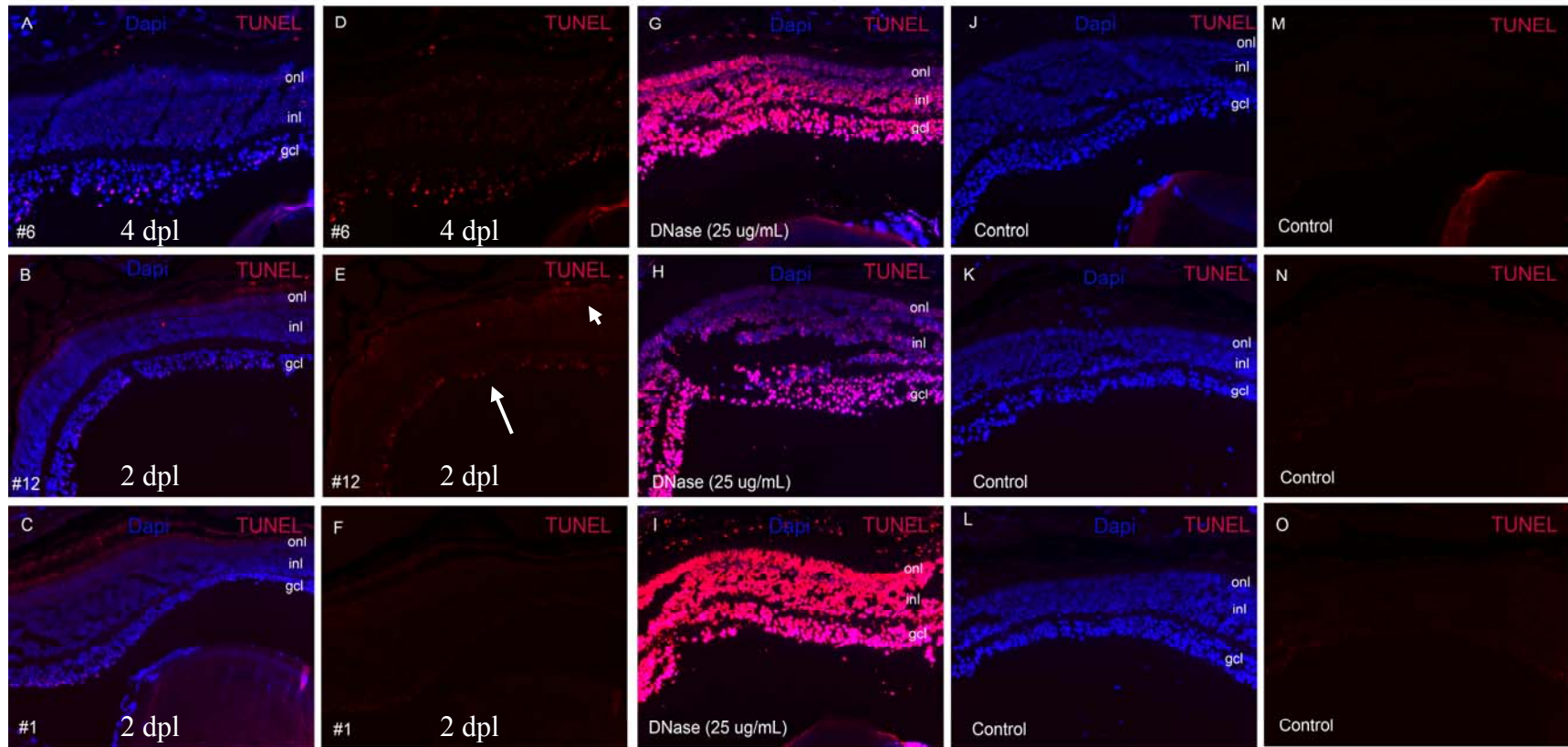


Figure 34. Variability in the onset and degree of TUNEL-positive cells in the retinas of three freely swimming newts following ultra-intense light exposure to the MAL (Experiment 3). Newts exposed to 30 minutes of light while freely swimming in a fishbowl experienced a variable degree of apoptosis in their retinas, with newt #6 (A,D) showing TUNEL-positive cells (red) in the inl and gcl at 4 dpl, newt #12, (B, E) showing TUNEL-positive cells mostly restricted to the ganglion cell layer (arrow) and a few cells in the photoreceptor cell layer (arrowhead) at 2 dpl and newt #1 (C,F) experiencing no apoptosis at 2 dpl. No TUNEL-positive cells were observed in the control newts (J-O). DNase served as a positive control for all TUNEL assays (G,H,I). Abbreviations: onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; dpl, days post-light.

3.3.3 Summary of light- induced retinal damage in the newt

In summary, many different methods were attempted to induce retinal damage in the adult newt using light. The establishment of a reproducible damage model has proven to be challenging in the newt, with variability of damage observed with the various light sources and factors tested. While the optimal conditions have not yet been developed (or may not exist) to induce retinal damage in a consistent manner, the newt ERG protocol, in conjunction with the TUNEL assay, have proven to be valuable tools to measure loss of retinal function in the adult newt retina.

3.4 Retinectomy and regeneration of the newt retina - monitoring restoration of retinal function

Retinectomy has been widely used as a surgical method to initiate and to study regeneration of the newt retina. Retinectomies involved a surgical incision into the corneal/scleral junction of the newt's right eye and careful removal of the retina through the incision site using fine forceps. No surgery or incisions were carried out on the left eye. Since the cells of the retinal pigment epithelium, found adjacent to the photoreceptor cells at the back of the retina, are essential for retinal regeneration to occur, careful attention was made not to remove any RPE from the eye. Due to the technical challenges associated with the retinectomy, only a portion of the retina was removed.

Electroretinography was used to determine the initial loss of retinal function due to surgical removal of the retina and to monitor the subsequent recovery of function as the retina regenerated. Recovery of the a-wave is indicative of photoreceptor-cell

restoration while recovery of the b-wave implies the restoration of neurons found in the inner nuclear layer, such as the bipolar and horizontal cells.

Two separate retinectomy experiments were conducted on a total of 15 newts (five newts in Retinectomy 1 and ten newts in Retinectomy 2) and retinal function was monitored until full functionality was attained for a sufficient number of animals. Retinal function was monitored up to 15 weeks for Retinectomy 1, and up to 11 weeks for Retinectomy 2. Data showed promising recovery of the a- and b-waves of 6/15 newts to baseline levels, one as early as 8 weeks post-retinectomy (wpr) (Retinectomy 2, newt #15). Four out of 15 newts had partially recovered their ERG amplitudes by endpoint (Retinectomy 1, newt #12; Retinectomy 2, newts #10, 14, 8). Newts with partial ERG recovery were sampled and stained with H & E to determine if the ERG status correlated with histological recovery of the retina. Three of the 15 newts showed an initial decrease of ERG amplitudes after surgery, indicating sufficient retinal removal; however they failed to show any recovery of the ERG throughout the duration of the experiment (Retinectomy 1, newt #13; Retinectomy 2, newt #1, 5). Of these newts, only the eye from newt #5 was sampled as the others showed either a hole in the sclera that had not properly healed (newt #1) or a loss of structural integrity likely due to major loss of vitreal fluid in the eye during surgery (newt #13). Due to these technical limitations, these samples could not be fixed or sectioned. Lastly, 2/15 newts died after surgery (Retinectomy 1, newt #3; Retinectomy 2, newt #4). The results from each experiment are further reported below.

Right eyes from independent animals served as controls for the electroretinograms, due to technical challenges of recording ERGs from both left and

right eyes of the same animals. These also served as controls for histological assessment along with the left eyes of animals that underwent surgery. After surgery and in between time points, all newts were housed in the animal care facility at 23-24°C with 12 hour light: 12 hour dark photoperiods.

3.4.1 Retinectomy 1

The first experiment was a preliminary study that involved removing the retina from the right eye of five animals and monitoring the recovery of the ERG a- and b-waves at 2 days post-retinectomy (dpr), 1, 2, 4, 6, 8, 11 and 15 wpr along with controls (right eyes of animals that did not undergo retinectomy in either eye) (Figure 35). The external body temperatures of the newts throughout this experiment were 22.3-26.2°C. Recovery of the ERG amplitudes was achieved if the a- and b-wave amplitudes at endpoint were comparable to the a- and b-waves measured from the same animal prior to surgery (baseline), factoring in a technical variability of 21.2% for the a-wave and 25.1% for the b-wave. These values were previously established as the variability of recording ERGs from the same animals on different days.

Two newts, #2 and #6 (Figure 35), showed complete recovery of the ERG to baseline levels by 15 wpr. By 11 wpr, the b-waves were completely restored to baseline levels, while the a-waves were over 60% restored. Though the trends of initial loss and recovery appeared evident, the initial losses of the a- and b-waves, from baseline to 2 dpr, were not statistically significant for the group (Two-Way ANOVA, Holm-Sidak method). Also, the differences in ERG amplitudes between 2 dpr and 15 wpr of retinectomized newts were not considered statistically significant compared to the difference seen in the

amplitudes of control newts at these time points (Two-Way ANOVA, Holm-Sidak method). The lack of significance may have been due to the low number of animals that showed initial loss and subsequent recovery of the ERG a- and b-waves to baseline levels.

Partial recovery was seen for newt #12, which initially lost the ERG but recovered to approximately 50% of baseline level by 2 wpr (Figure 36 E). Reasons for partial recovery are further explained in the Discussion. The promising recovery of the ERG amplitudes for Newt #3 was observed; however, this animal died prior to the termination of the experiment.

Cryosections from newts #2, #6 and #12 were stained for hematoxylin and eosin (H & E) at 15 wpr (Figure 36 A,C) along with contralateral (left eye) controls and right eyes from animals that did not undergo retinectomy. H & E staining of various sections across these eyes showed that all retinal layers were present, including plexiform layers. The appearance of plexiform layers marks the end stages of retinal regeneration (see Figure 5). In the case of newts #6 and #12, there are some regions where the nuclei appear more loosely packed compared to the contralateral untreated eye, indicating the potential sites where the retina was removed (Figure 36).

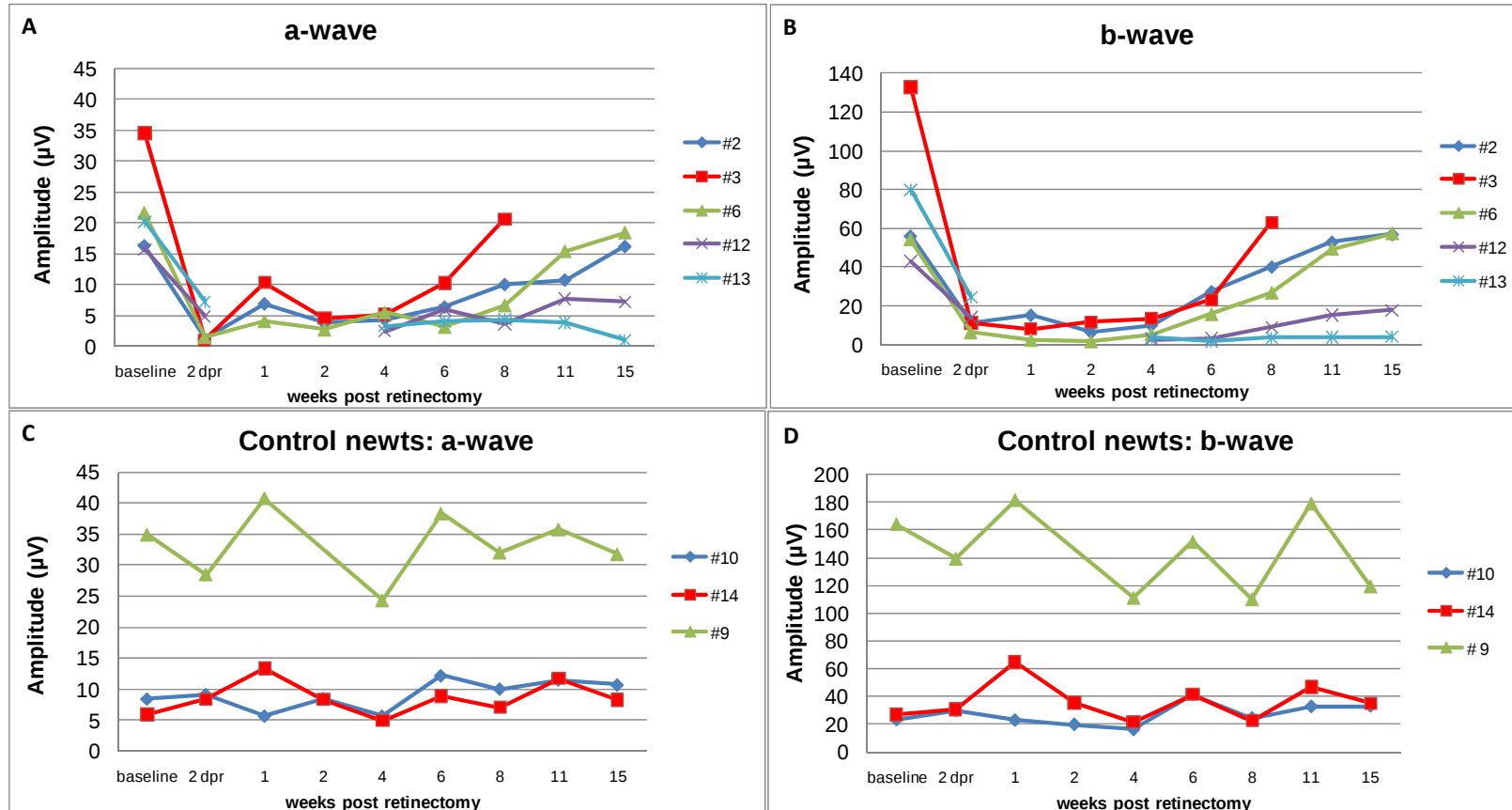


Figure 35. The progressive recovery of the ERG a- and b-waves by 15 weeks post-retinectomy (wpr) suggest regeneration and restoration of retinal function. The retinas of five newts were surgically removed, leaving the RPE intact. ERG a- (A, C) and b-wave (B, D) amplitudes (μV) were monitored pre-surgery (baseline), at two days post-retinectomy (dpr) and up to 15 wpr. Newts #2 and #6 showed initial loss and subsequent recovery of both the ERG a- and b-waves while newt #3 showed promising recovery of the ERG amplitudes but died prior to termination of the experiment. Control newts did not undergo retinal surgery (C,D).

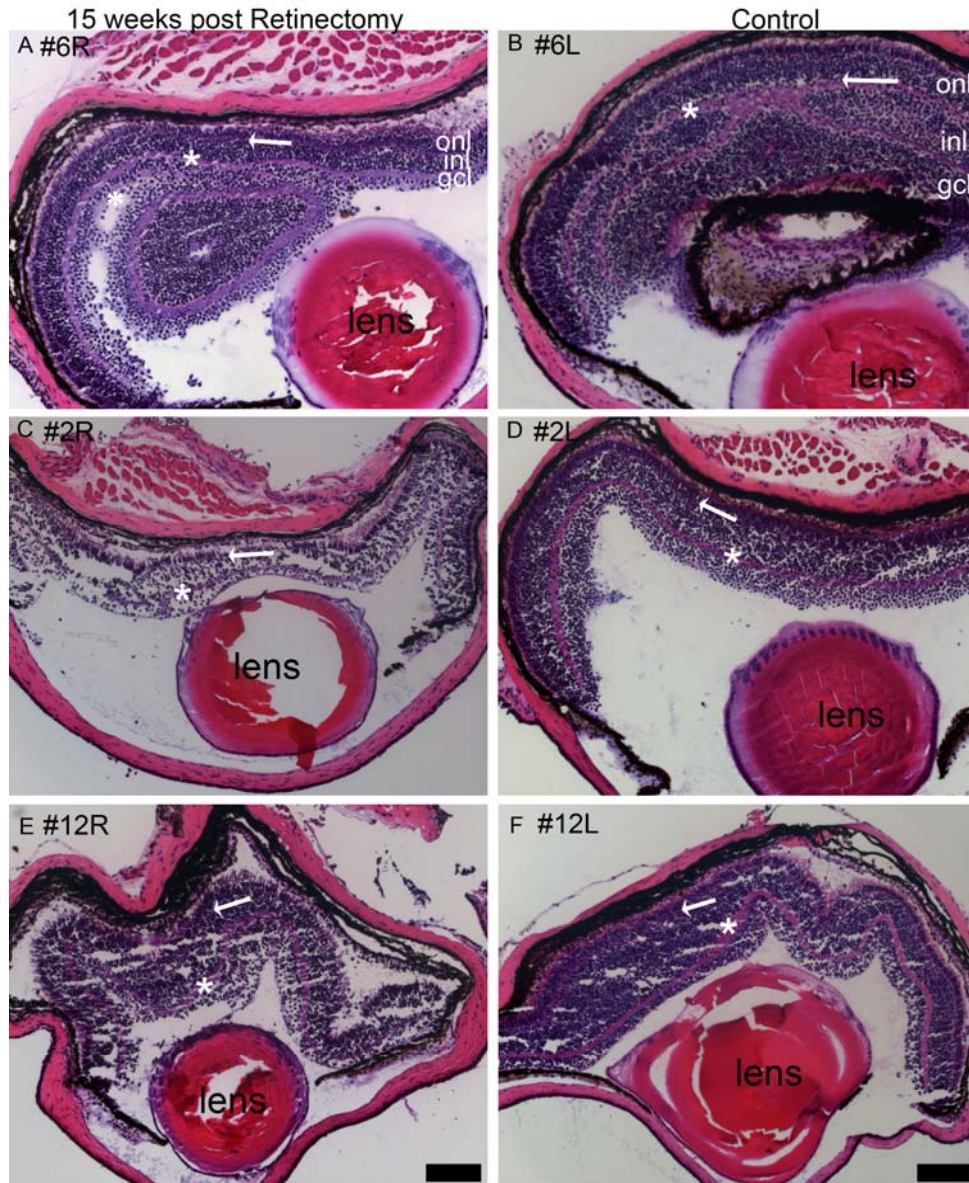


Figure 36. Hematoxylin and eosin staining of retinal cryosections from newts with partial or complete recovery of the ERG, at 15 weeks post Retinectomy 1. The right eyes (R) of newts #6 and #2 (A, C) showed complete restoration of the ERG amplitudes to baseline levels, while newt #12 (E) did not. There are no apparent histological differences that would correlate with differences in the ERG recovery. No surgery was performed on controls, which are the left eyes (L) of newts that underwent retinectomy (B, D, F). Note that the retina of each animal has been segregated into distinct synaptic (plexiform) layers. Asterisks indicate the inner plexiform layer between the ganglion cell layer (gcl) and the cells of the inner nuclear layer (inl) while the arrows indicate outer plexiform layers between the inl and the outer nuclear layer (onl) (where photoreceptor cells are found). Scale bars shown (100 μ m).

3.4.2 Retinectomy 2

A second experiment was conducted using a larger sample size of newts compared to Retinectomy 1 (10 newts versus 5 newts) and the ERG amplitudes were monitored at 2, 4, 8 and 11 wpr (Figure 37). The external body temperatures of the newts throughout this experiment were 23.5-27.2°C. Electroretinography was not performed prior to 2 wpr to allow for the incision sites to fully heal. At 2 wpr, there was a statistically significant loss of ERG a- and b-waves (greater than 50% loss) compared to baseline, indicating a substantial loss of retinal function due to surgical removal of the retina (Two-Way ANOVA, Holm-Sidak method, $p < 0.001$). At 2 wpr, newt #5 lost 42% of the a-wave and 38 % compared to baseline; however, this newt demonstrated continual loss of ERG amplitudes until 8 wpr, indicating there was no regeneration occurring.

By 8 wpr, the ERG a- and b-waves of treated newts were significantly increased compared to at 2 and 4 wpr. In this group, 2/10 newts did not show any recovery (newts #1, #5, Figure 37). At this time point, one newt with partially recovered ERG amplitudes (newt #8) was sampled along with one newt that was not showing any restoration of ERG a- and b-waves (newt #5), for comparison of histology. Also at 8 wpr, newt #1 showed a marked decrease in the ERG a- and b-waves compared to baselines. Upon enucleating the eye for fixation and processing, it was observed that the incision site had not closed. A large hole was still present in the corneal/scleral junction and this caused the eye to lose structural integrity. Therefore, this newt was omitted from analysis. One experimental newt died prior to the termination of the experiment (newt #4).

The endpoint of 11 weeks was chosen since full recovery of ERG amplitudes to baseline levels was attained for four of the remaining six newts (newts #6, #15, #16,

#20). This result suggested full functionality of retina due to regeneration. The increased ERG amplitudes at 11 wpr were considered statistically significant compared to ERG amplitudes at 2 and 4 wpr (Two-Way ANOVA, Holm-Sidak method, $p < 0.01$), while there was no statistical significance between ERG amplitudes at baseline and 11 wpr (Two-Way ANOVA, Holm-Sidak method, $p = 0.768$), suggesting full recovery of function. There was no statistical significance in the ERG amplitudes of controls across all time points, except at 8 wpr where an outlier (newt #22) may have contributed to a high difference in ERG amplitudes compared to baseline (Two-Way ANOVA, Holm-Sidak method, $p = 0.041$). While the ERG amplitudes of newts #10 and #14 were increasing at 11 wpr compared to at 2 wpr, they had not fully recovered to baseline levels by 11 wpr (Figures 37). This suggested that there was variability in the recovery of the ERG among animals. Fully regenerated newts #6, #15, #16, and #20 as well as partially regenerated newts #10 and #14 were all fixed and sampled at 11 wpr.

Samples that showed both partial (Figure 39, newts #8, #10, and #14) and full recovery (Figure 38, newts #6, #15, #16, #20) of the ERG were stained for H and E, along with control newts, for comparison. In addition, serial sections from different regions within the same newt eye were stained for H & E, to look for regions where extensive removal of the retina may have occurred. The overall lamination and morphology of the fully regenerated retinas of newts #6, #15, #16 and #20 appeared normal; however, the nuclei were generally not as compact in each layer, compared to the controls (Figure 38). These regions containing retinal layers that are looser represent regions in which retina was likely removed. Histology assessment of two different

cryosections from the same animal showed a difference in morphological compaction of the retina (Figure 40), indicative of the region in which retina was removed.

Certain regions of partially regenerated samples (at 8 wpr) were even less compact compared to 11-week regenerates and controls (Figure 39). In the case of newt #5, one region of the retina contained fewer nuclei than the region on the other side of the optic nerve, in the same retina (Figure 39 A). Animals that did not show densely packed nuclei in their retinas compared to controls generally did not have ERG amplitudes that were fully recovered to baseline levels, indicating a potential correlation between nuclear density and full functionality of the retina.

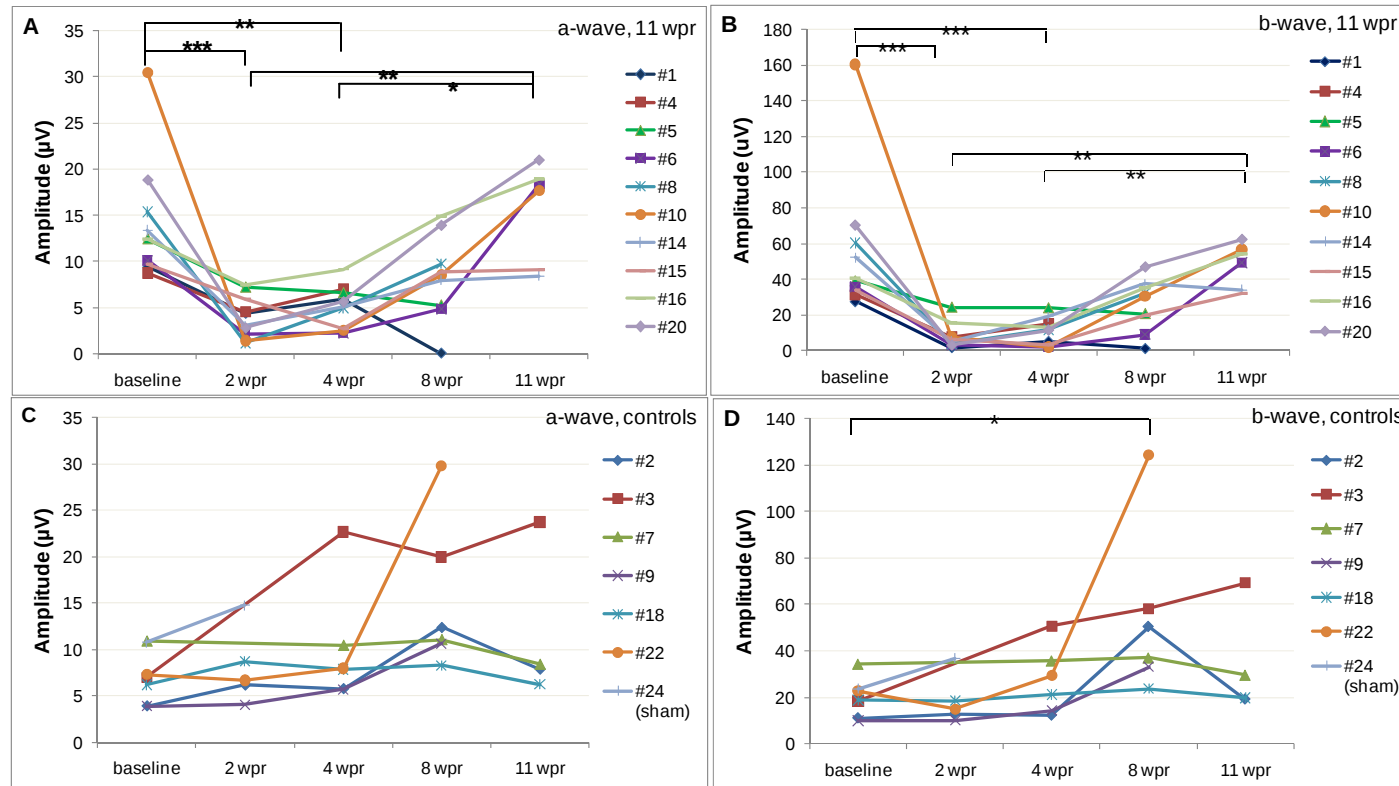


Figure 37. The progressive recovery of the ERG a- and b-waves over 11 weeks post-retinectomy (wpr) suggests regeneration and retinal function restoration in many newts. The retinas of 10 newts were surgically removed, leaving the RPE intact. ERG a- (A, C) and b-waves (B, D) were monitored pre-retinectomy (baseline) and up to 11 wpr. For both a- and b-waves, the amplitudes at baseline and 11 wpr were considered statistically significant compared to ERG amplitudes at 2 and 4 wpr (Two-Way ANOVA, Holm-Sidak method, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Control newts did not undergo retinal surgery (C,D) and a sham control (#24) was a newt that underwent scleral incision but no retinal removal (C, D). The sham was monitored up to 2 weeks post-incision. Newt #1 died shortly after the 8 week time point.

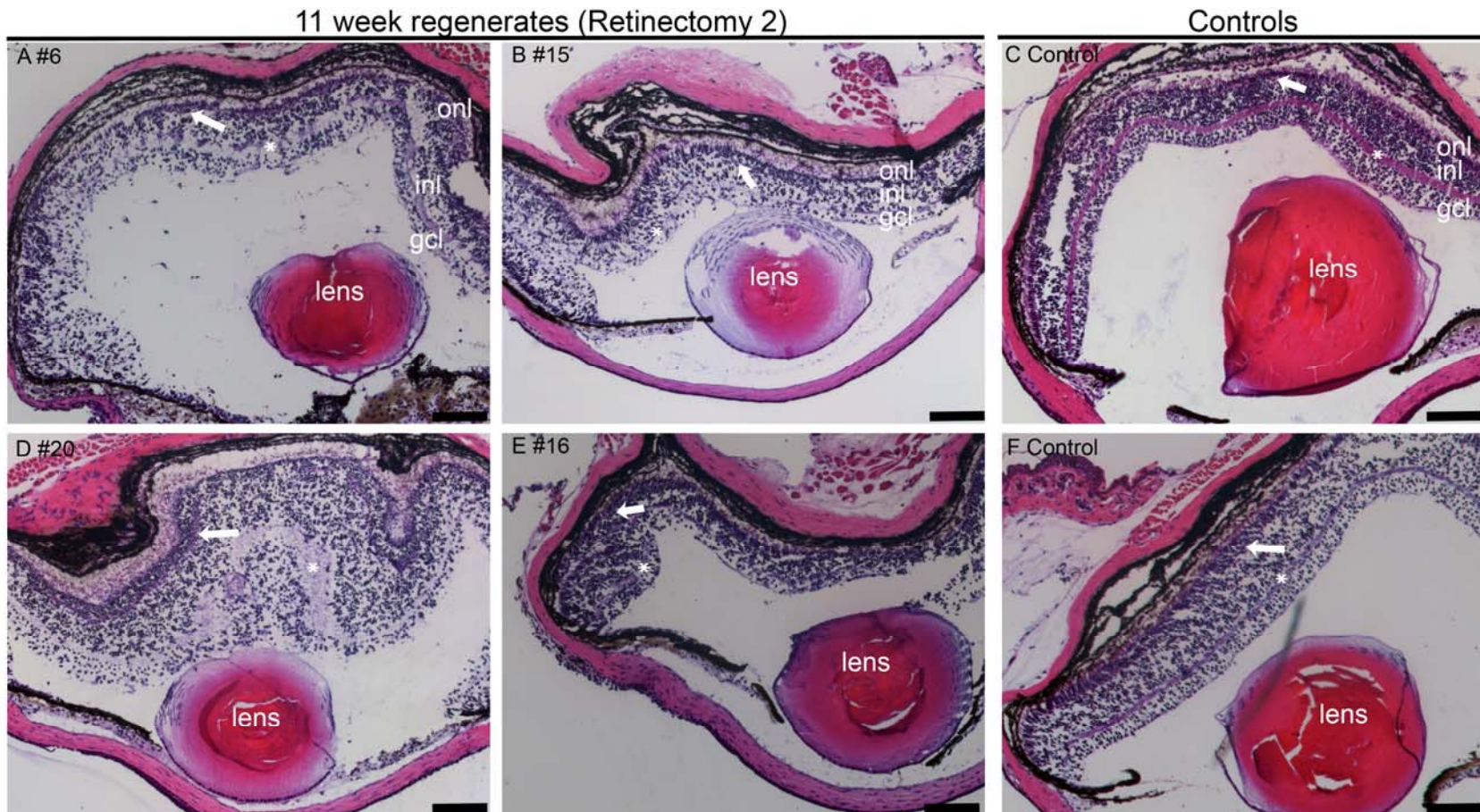


Figure 38. Hematoxylin and eosin staining of newt eyes that showed complete or near complete restoration of the ERG amplitudes at 11 weeks post-retinectomy. Both regenerates 6 (A), 15 (B), 20 (D), and 16 (E) and controls (C,F) possess retinas that have segregated into distinct synaptic layers. Asterisks indicate the inner plexiform layer between the ganglion cell layer (gcl) and the cells of the inner nuclear layer (inl) while the arrows indicate outer plexiform layers between the inl and the outer nuclear layer (onl). Scale bars shown (100 μ m).

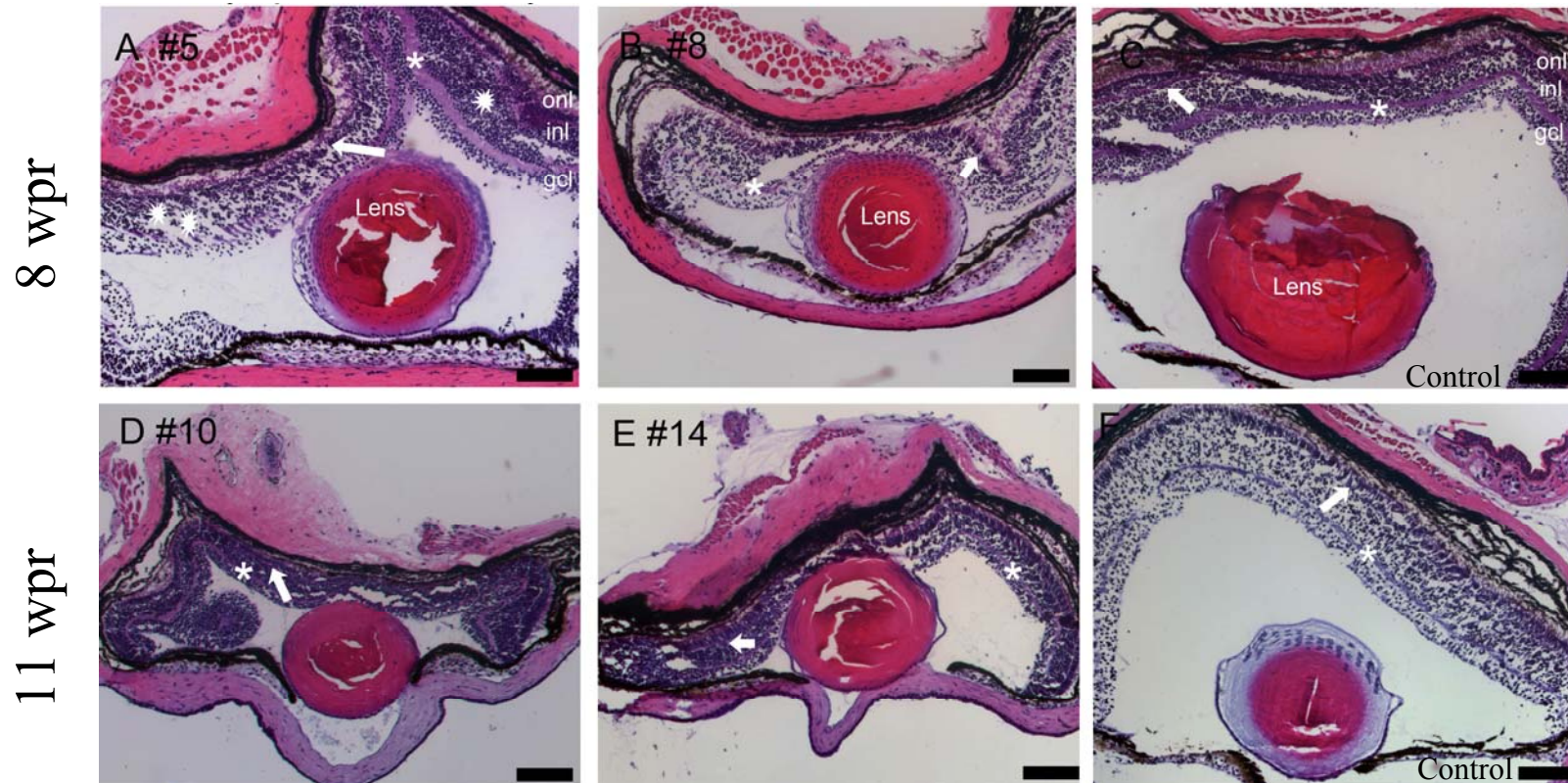


Figure 39. Hematoxylin and eosin staining of newt eyes that show partial or no recovery of the ERG amplitudes. Newts 5 (A) and 8(B) were sampled at 8 weeks post-retinectomy (wpr). Newt #5 showed no recovery of the ERG. Histologically, this newt's retina contained fewer nuclei in one region (double star burst) than the other side of the optic nerve (single start burst). Newt 8 (B) showed partial recovery of the ERG. Newts 10 (D) and 14 (E) were sampled at 11 wpr and showed partial recovery of the ERG amplitudes. Controls represent retinal cryosections from newts that did not undergo surgery (C, F). All plexiform layers are visible for each cryosection sampled. Asterisks indicate the inner plexiform layer between the ganglion cell layer (gcl) and the cells of the inner nuclear layer (inl) while the arrows indicate outer plexiform layers between the inl and the outer nuclear layer (ont). Scale bars represent 100 μm in diameter.

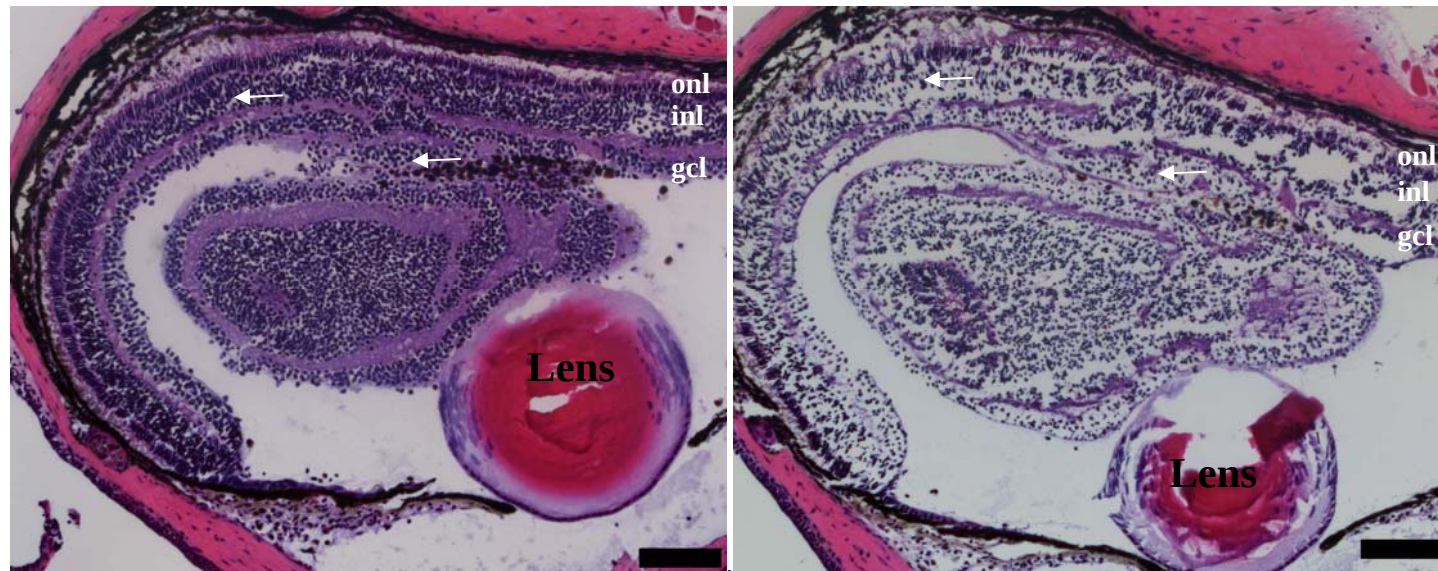


Figure 40. Hematoxylin and eosin staining of two different retinal cryosections from the same newt after retinectomy. The two images represent sections from the same newt that are 140 μm apart but centered near the middle of the eye. The figure on the right shows an area of the eye that may have undergone substantial retinal removal, as the cells of the various layers are loosely packed (arrows) compared to those in the figure on the left, from the same newt. Scale bars shown (50 μm). Abbreviations indicate the ganglion cell layer (gcl), inner nuclear layer (inl) and outer nuclear layer (onl).

4.0 DISCUSSION

The newt has retained the exceptional ability to regenerate its retina as an adult and is reported to do so approximately 65 days after surgical removal (Cheon et al., 1998; Keefe, 1973; Mitashov et al., 1995; Nakamura and Chiba, 2007). While this phenomenon has been known since the 1800s, there are still many outstanding questions in the field, such as: Why is the newt one of only a few organisms that has retained this regenerative capability as an adult? Is the newt retina fully functional after regeneration? What molecular mechanisms or cues are absent or repressed in organisms that cannot regenerate their retina, such as humans?

Studies on the functional restoration of the newt retina are limited (Chiba and Saito, 1995; Kaneko and Saito, 1992; Oi et al., 2003) with previous studies largely employing whole-cell patch-clamping to monitor the appearance and activity of one or many ion channels in the regenerating retina. This invasive method is limited to recording electrical responses from a few cells at a time and does not measure the retinal circuit as a whole.

In contrast, electroretinography is a non-invasive method used to measure the electrical responses from various cell types in the retina as they respond to light (Webvision, 2010). The electroretinogram specifies which retinal layers have been functionally restored based on the responses from either the a- or b-wave. This method can be used to confirm the timing and location of functionally restored neurons. The electroretinography protocol particularly examines whether or not the photoreceptor cells

and the neurons of the inner nuclear layer (mainly bipolar and horizontal cells) are functionally restored as a whole.

Therefore, the ultimate goals of this study were to contribute to our understanding of the regenerative process in the adult newt, by establishing both a reproducible damage and regeneration model and a non-invasive method to monitor the restoration of retinal function after damage. This will further our understanding of the timeline and process that occurs during the regeneration of the newt retina following damage.

4.1 Establishment of *in vivo* newt ERGs

We have successfully established a non-invasive *in vivo* method to measure retinal function in the adult red-spotted newt. We met this objective by optimizing the conditions to record full-field ERGs from light-adapted adult newt eyes using the Espion™ electrodiagnostic system (Diagnosys LLC). The optimal conditions involved employing long red flashes of light with an intensity of 400 cd/m² (over a pulse period of 500 ms), dilating the newt pupils with Mydrin® for at least 35 minutes, and maintaining a constant newt body temperature. We used a bright flash of light to elicit large a- and b-waves and to ensure light enters the tiny newt pupil. Weak or moderately intense light do not yield an apparent ERG a- and b-wave (Fishman, 1985). We monitored light-adapted newts since preliminary observations showed similar ERG traces in both dark-adapted and light-adapted newts (data not shown). Since it is challenging to accurately place the electrode on the centre of the cornea in the dark, we resolved to only record ERGs in light-adapted newt eyes.

We also observed that body temperature contributes to variability of the ERG recordings, indicating the newt retina is sensitive to changes in temperature (section 3.1.2, Figure 14). There were six different animals in each group and since each showed a slightly different profile (due to variability among different animals), the box plot appeared to be the most reliable method to report our findings. Furthermore, our results suggested that elements of the retinal network are differentially affected by temperature. For instance, a rise from 18.5°C to 25°C appeared to affect the b-wave more dramatically than the a-wave while a change from 18.5°C to 30°C had a significant effect on both the a- and b-waves.

Previous studies have reported an effect of body temperature on the ERG in other animals, including mice (Kong and Gouras, 2003; Mizota and Adachi-Usami, 2002) and turtles (Adolph, 1985). In the chick, the b-wave decreases with decreasing body temperature, while the amplitude of the a-wave is unaffected (Ookawa and Tateishi, 1970). Changes in temperature may result in changes in the functional properties of the inner components of the retina or the phototransduction cascade. The increase in the ERG a- and b-waves at a higher temperature may be due to increased intraretinal responses in the photoreceptors and among the photoreceptors and neurons of the inner nuclear layer. Changes in temperature may affect the reaction rates of enzymes or electrochemical events required to transduce the light-induced responses that normally occur when the retina is exposed to light. However, further experiments will be required to validate this theory.

These results indicate that during electroretinography measurements, care must be taken to maintain the ambient room temperature and body temperature of the newt as

constant as possible. On each test date, ambient room temperature was maintained using a mini oil heater while body temperature of the animal was kept constant using a water-based heater, the T/Pump®.

Due to its non-invasive nature, our method allows for multiple ERG recordings in the same animal over time with a measured variability of 21.2% for the a-wave and 25.1% for the b-wave. The likely source of variability is electrode placement. Studies have shown that deviation from the centre of the cornea can result in a decrease in the ERG amplitudes (Cringle et al., 1986; Dzendolet, 1960). The silver-loop electrode is normally placed in the centre of the newt cornea. The diameter of the loop is about 1 mm, which is approximately the diameter of a newt pupil. Therefore, while careful attention was directed to ensuring that the loop electrode was placed in the centre of the cornea, and that the loop did not cover the pupil, it is possible the loop electrode obstructed some of the light from entering the pupil or that it was not placed in the exact position on the newt cornea for all tests done on various days. Moreover, due to the small size of the newt eye and the proximity of the cornea to the upper or lower lids, the tester must be meticulous in ensuring the electrode does not touch the newt's skin. Contact with the eyelid yields lower ERG amplitudes, likely due to the skin being more resistant to electrical impulses in comparison to the cornea. When used on humans and other organisms, the Espion™ electrodiagnostic system contributes a variability of 10-15% (personal communication with Dr. Stuart Coupland); therefore electrode placement may contribute to a higher variability observed in newts.

One limitation of this protocol is the inability to record ERGs from both eyes of the same animal. The newt eye was not sturdy and it receded into the eye socket upon

touch. Therefore, it was necessary to place the newt on its side, then insert a plastic tip in through the newt mouth and directed to the back of the eye where the tip could rest against the eye socket. Gentle pressure was placed on the tip inserted into the mouth to prevent the eyes from falling back. Therefore, it was also necessary to ERG separate newts as controls. It was necessary to compare the baseline (pre-treatment) and post-treatment ERGs of each newt, rather than to compare the ERGs of the treated animals to the controls, since high interanimal variability has been observed (data not shown).

ERG studies on this species of newt were previously conducted on enucleated eyes placed on a dish using older ERG systems not found in the clinic (Lam, 1977). The reported invasive method would not allow for recording retinal regeneration in the same animal over time. In addition, removal of the eye from the eye cup is likely inducing optic nerve damage and subsequent regeneration, and there is potential mechanical damage to the eye resulting from this surgery which may compromise the results. In this earlier study, a large number of newts were necessary to obtain a representative pool of data. Newts are normally captured from the wild at unknown ages and different genders; thus, these factors would contribute to variable ERG a- and b-waves among different animals. For this reason, we chose to follow the same animals over time, using the same conditions pre-treatment and post-treatment. This would allow for a smaller sample size (compared to 100 newts used in the aforementioned study). Moreover, Lam (1977) did not refer to maintenance of constant temperature during the ERG tests, which we have reported is a cause of variability.

The establishment of a non-invasive *in vivo* newt ERG protocol will be an important tool for the monitoring of retinal function after retinal removal or damage.

Future studies can make use of this tool in order to further elucidate the molecular mechanisms that occur during retinal regeneration, with the hopes of one day applying this knowledge towards the betterment of patient care.

4.2 The use of light to induce retinal degeneration

One of our main goals was to design a simple, reproducible method of inducing photoreceptor-cell damage in the intact newt eye, to gain an understanding of how the newt would regenerate its damaged photoreceptor cells and to determine if this knowledge could one day be applied to the treatment of retinal disease. Genetic- or age-related retinal degenerations, such as macular degeneration and retinitis pigmentosa, primarily involve photoreceptor cells being damaged over a longer period of time throughout the lifetime of a patient. The common end result is apoptosis, or programmed cell death, of these retinal cells, leading to blindness. The rationale for using light as a damaging agent was that there were morphological similarities shown between these end stages of retinal disease or degeneration and the appearance of the retina after experimental damage using light (Organisciak, 2003). Moreover, light damage is an acute way of simulating the various stages of chronic retinal diseases. Different animal models have been used to understand the mechanism behind light-induced damage and there is evidence suggesting that constitutive phototransduction can cause retinal degeneration (Fain and Lisman, 1999; Lem and Fain, 2004). Also, constant light exposure is known to acutely induce oxidative stress in the retina which may trigger apoptosis (Organisciak, 2003). Since light-induced cell death can be achieved in a shortened time course, and since there are commonalities between retinal degeneration due to light and due to

disease, a light-induced damage model was therefore a promising model to explore in order to study the events leading to retinal degeneration. It was hypothesized that ultra-intense light would induce photoreceptor-cell specific damage in the newt retina that would result in loss of the ERG response.

It must be noted that the quantity and intensity of light used to induce retinal degeneration differs from the quantity and intensity of light used to measure retinal function with the ERG. ERGs involve exposing retinas to brief flashes of bright light to stimulate photoreceptor cells. The brief flashes typically only last up to 500 ms, therefore no damage is caused. This is distinct from the methods used to induce retinal damage, which generally involved light at a high intensity and continual exposure to the retina for a long period of time, continuously activating the visual cascade and likely resulting in the inability of the photoreceptors to regenerate their visual pigments (Lem and Fain, 2004).

4.2.1 Light-induced as a potential damage model in the newt

Ultra-intense light is a novel way of inducing retinal damage in red-spotted newts. Light-induced damage models in other animals involved either continually exposing animals (for several days or weeks) to a low intensity of light or exposing the animals to a highly intense light for a shorter period of time. Our study was largely based on studies conducted in albino zebrafish where 30 minutes of ultra intense light from a metal halide lamp was sufficient to damage the photoreceptor cells in the central region of the zebrafish retina, as they swam freely in a beaker (Bernardos et al., 2007; Thummel et al., 2008).

Newts possess retinas that are conserved in function across all vertebrates; however their eyes differ structurally from two animals commonly used in light-induced retinal studies, rodents and zebrafish. Firstly, newt eyes are pigmented whereas the zebrafish used in the Bernardos *et al.* (2007) and Thummel *et al* (2008) studies were albino. Albino animals would be more susceptible to light damage and may require shorter exposure times than pigmented animals because the melanin pigment may absorb some of the light (Lanum, 1978; Organisciak, 2003). In pigmented animals such as the newt, light can only enter through the pupil whereas in albino animals, light can enter through the iris and the pupil, exposing a larger quantity of photoreceptor cells of the retina in a more acute fashion. However, regardless of whether or not pigmentation is present, the way in which light damages the photoreceptor cells is the same (Organisciak, 2003).

Secondly, some retinal cell types found in the newt differ from those found in rodents (Stebbins, 1995). Newts are diurnal, active during the daytime, and their retina consists of rods and cones distributed throughout the entire retina (Hollenberg and Dickson, 1971). Rodents are nocturnal, active during the nighttime, and they possess a rod-dominant retina. The rods, responsible for mediating night vision, are stimulated by dim light while cones, responsible for mediating daylight vision, are stimulated by bright light (Webvision, 2010). Therefore, light sources differing in intensity are required in order to induce retinal damage in newts compared to rodents. While newts require a high intensity of light to stimulate the cones they possess, rodents require a low intensity of light, one- to two-fold greater than ordinary room lighting, to stimulate their rods (Organisciak, 2003).

Since this was the first study to report light-induced photoreceptor cell damage in any species of newt, different light sources, exposure times and experimental conditions were tested.

4.2.2 The use of two different light sources to induce retinal damage in the newt (Phototoxicity 1 vs Phototoxicity 2)

Two different light sources, a 150 W halogen light bulb and a 100 W mercury arc lamp, were tested in order to induce damage to the newt retina. There were no visible apoptotic cells (Figure 18) and no statistically significant effect of light on the ERG amplitudes with the use of the halogen light bulb at 15 000 or 20 3000 lux (data not shown). At ultra-intense light treatment (>100 000 lux) for 30 and 60 minutes, though a reduction of the ERG amplitudes was observed (Figure 19), this did not correlate with the appearance of apoptotic cells.

One reason for the differences observed with the two light sources is that with the 100 W mercury arc lamp, a higher intensity of light actually reached the photoreceptor cells compared to the 150 W halogen bulb. This augmented the chance of increased oxidation in the retina, resulting in apoptosis. The mercury arc lamp was similar in irradiance to the metal halide lamp (Figure 15) used in zebrafish retinal damage studies (Bernardos et al., 2007; Thummel et al., 2008); therefore, it was considered the more-promising light source to induce retinal damage in the newt. Its high irradiance and emission over a broader spectrum of light compared to the halogen bulb would allow for absorption and saturation of a higher number of newt photoreceptor cells, and can potentially induce more damage. Literature suggested that the newt retina contains cones that predominantly absorb light in the yellow-green spectrum and maximally absorbs

light at a wavelength of 580 nm (Stebbins, 1995), which falls near the maximal wavelength of light emitted by the mercury arc lamp. Moreover, Red is not a predominant wavelength of light absorbed by the newt retina which may explain why there was no damaging effect with the halogen bulb (which predominantly emits red light).

The objective of inducing damage to the newt retina using constant ultra-intense light was met with the use of the Super High Pressure mercury arc lamp. Retinal damage was assessed firstly with the loss of retinal function, using the established ERG protocol, and secondly with the appearance of apoptotic cells in the various layers of the newt retina, using TUNEL. The reduction of ERG a- and b-wave amplitudes was successfully correlated with the appearance of TUNEL-positive cells.

4.2.3 Anesthetised versus freely-swimming newts – Method 1 vs Methods 2/3

Newts were exposed to ultra-intense light from the MAL either while anesthetised in an MS222®-soaked dish or swimming around in a beaker or fishbowl (Methods 2.3.1, Figure 10). Both positions were tested for the maximal loss of retinal function with the ERG and the maximal appearance of photoreceptor-cell death with TUNEL.

When newts were anesthetised on a dish and subjected to light exposure, damage was restricted to a focal area of the retina (Method 1, Figure 21). One possible explanation for the focal damage was the newts were placed very close to the condenser (1-2 cm away) with the assumption that a maximal amount of light would enter the pupil. However, the focal point of the light emerging from the mercury arc lamp was not considered at the time. Light was emitted from the light source as a beam of parallel rays

that would eventually converge to one small intense point a certain distance from the light source. Since the newt was positioned very close to the condenser, which was used in the various experiments to converge the scattered light beam, the distance may have been too close for a focal point of the light beam to have been formed. Therefore only a fraction of the scattered light may have actually entered into the newt's pupil. The rest was likely reflected off of the aluminum foil surrounding the anesthetised newt. If the newt was positioned further away from the condenser in a position where the focal point of the rays was right in front of the pupil, this may have allowed for more light to enter the pupil as the rays diverged again beyond the focal point (Figure 41). It was therefore important to consider where the newt pupil was positioned relative to the focal point of the light beam; however, since the newt retina is barely 3mm thick, it was difficult to consistently judge this exact position in front of the pupil.

Since it was possible to visualize where the light beam was most intense, suggestive of the focal point of the light beam, subsequent experiments with the mercury arc lamp involved the beaker or fishbowl positioned in such a way that the light beam illuminated all of the areas within the beaker or fishbowl, and allowed for light to reflect off the surrounding aluminum foil. This was the case when the centre of the fishbowl or beaker was placed approximately 55 cm away from the light source.

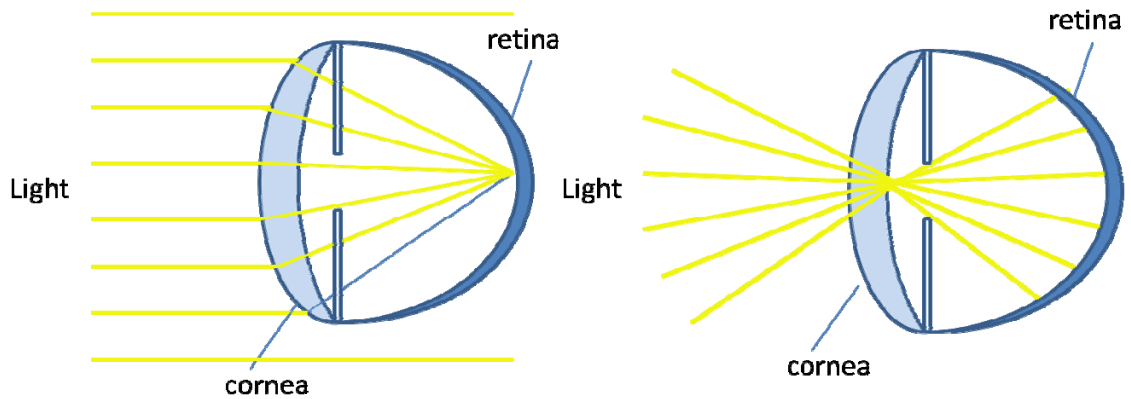


Figure 41. The focal point of a light beam and its effect on maximal illuminance of the retina. When the newt eye is placed close to the light source, only a fraction of the parallel light rays enters the pupil and is focussed to the back of the retina with the cornea (with contribution from the lens, not shown) (Left). If the newt is placed in front of the light source such that the focal point of the light beam is directly in front of the pupil, light entering the pupil will converge to reach more areas of the retina (Right). Since the newt eye is very small (2-3 mm) it is challenging to locate where the light beam focuses in front of the newt pupil, causing variability in the amount of light that reaches the photoreceptor cells to cause damage (personal communication with Dr. Serge LeBlanc).

4.2.4 The effect of exposure time and light intensity

Three different exposure times were tested for each of the two light sources. The 150 W halogen bulb was shone directly into the newt eye at four different intensities: 350 lux, 15 000 lux, 20 300 lux and an ultra-high-intensity greater than 100 000 lux. At each light intensity, three exposure times were tested while the newts remained anaesthetised: 15, 30 and 60 minutes constant light exposure. The only decrease in ERG amplitudes (up to 50%) was observed at 3 days post 30 minutes of constant exposure to the highest intensity of light (>100 000 lux) yet this did not correlate with apoptosis in the retina. These results suggested that at 3 days post-light, the dead cells were either already cleared, leaving no cells for TUNEL analysis, or that the decrease in ERG, in combination with error of recording ERGs on the same animal over time, was simply not significant. The latter was the likely explanation since there was no decrease in ERG amplitudes at the longer exposure time of 60 minutes.

For the 100 W mercury arc lamp, the light was emitted at one maximal intensity of greater than 100 000 lux and newts were exposed for 5, 15 and 30 minutes to determine the photosensitivity of newt photoreceptor cells to this light source. Samples were subjected to ERG and TUNEL analysis at 8 hours, 1 or 2 days post-light. There were very few TUNEL-positive cells observed in any layer of the retina with a 5 minute exposure, sampled at 2 dpl. While some nuclei of the inner nuclear layer and photoreceptor cell layer were TUNEL-positive at an exposure of 15 minutes, it was difficult to assess whether the staining was nuclear (Figure 21 K,L).

The most successful conditions with the use of the MAL were achieved from Experiments 2 and 3, which involved the exposure of the dark-adapted, dilated newt eye to 30 minutes of ultra-intense light reaching the retina at various angles, while the newt swam alone in a fishbowl located 55 cm away from the light source. The filtration of red or UV light did not appear to impact on the overall effect of light damage from the mercury arc lamp. The damage observed correlated to a reduction of retinal function, measured by the ERG.

4.2.5 Examining the onset and extent of damage in the newt retina due to light

It was hypothesized that the damage due to light would be acute and photoreceptor-cell specific. The results demonstrated that light from the MAL induced damage to several cell-types of the retina, as early as 8 hours post-light treatment of 30 minutes exposure time (Figure 23). Therefore, while the light-induced damage appeared in a short period of time, it was not photoreceptor-cell specific. A possible factor considered was that at thirty minutes, the mercury arc lamp was potentially producing heat by the emitted wavelengths of light, even though an IR filter was in place. However, the temperature of the water was measured throughout the various tests and after thirty minutes of light exposure, the water temperature elevated by a maximum of 1.5°C, which was not a substantial increase.

Another factor considered to contribute to non-specific retinal damage was UV light. Ultraviolet emission accounts for about half of the output of the mercury arc lamp (Davidson, 2010). The light sources were equipped with a UV filter; nevertheless, some residual UV light (2%) was emitted by the Super High Pressure mercury arc lamp, as

detected by the Ocean Optics spectrometer (Figure 17). However, it was determined that the residual UV light emitted from the machine did not impact on the non-specific damage observed (Experiment 3, Figures 33 and 34).

It has been suggested that a longer duration of light exposure at such high light levels can lead to damage in the inner retinal layers (Organisciak, 2003), yet the mechanism is not known. Light damage does not typically affect the cone cells and inner retinal layers of rodents at low light levels (LaVail et al., 1987; Organisciak, 2003). As well, 30 minutes of constant light exposure of the zebrafish to the mercury halide lamp, which irradiates light at a higher power than the mercury arc lamp, resulted in photoreceptor-cell specific damage. When newt eyes were exposed to light for 30 minutes and sampled only 8 hours later, in Experiment 2, TUNEL-positive nuclei were observed in both photoreceptor cells and ganglion cells, further indicating that damage was acute but not photoreceptor-cell specific (Figure 23). While the photosensitivity of newt neurons is relatively unknown, it is possible that newts possess neurons that are more light-sensitive compared to those of zebrafish.

To further assess the timing of the onset of retinal damage, experiments were conducted at 1 day post-light treatment. In anesthetised newts, a funnel-like damage effect was observed at 1 day post-light, with the most TUNEL-positive cells in the photoreceptor layer compared to significantly fewer TUNEL-positive ganglion cells. At 2 days post-light, an increase in TUNEL-positive cells was observed in the inner nuclear layer compared to 1 day post-light. This could be due to the fact that once the photoreceptor cells were damaged, the phototransduction cascade was no longer functioning properly, the neurotransmitter glutamate was no longer being released from

the synaptic terminal of the photoreceptors, and the light signal was not further processed to second order neurons in the retina such as bipolar and horizontal cells (Webvision, 2010). It was possible that without adequate stimulation with neurotransmitters, the second order neurons underwent apoptosis. An interesting extension to this study could measure the initial loss of neurotransmitter activity and the electroretinogram after light treatment, and the subsequent appearance and maturation of the ERG can be monitored and correlated with the synthesis of the neurotransmitters in the retina during regeneration.

4.2.6 Variability and limitations of the light damage model

Different phototoxicity methods resulted in variable degrees of retinal damage. Experiments with the 150 W halogen bulb were largely unsuccessful. Even though different intensities of light, durations of exposure and distances from the source were attempted, very little damage was seen in the retinas by ERG or TUNEL analysis. Phototoxicity with the MAL was more effective at inducing damage compared to the halogen bulb. Various experimental conditions were tested. Newts were either anaesthetised or freely swimming in a beaker or fishbowl. The greatest phototoxicity was achieved with newts freely swimming in a fishbowl. Under these conditions, reductions in ERG amplitudes were seen and these correlated with the appearance of TUNEL-positive cells. Even so, degree of phototoxicity was extremely variable even between animals treated under similar conditions, with some animals showing greater than 50% reductions of ERG amplitudes compared to baseline and others showing no effects at all (Figure 33).

One possible explanation for such variability is the activity of the newts. Some were less mobile than others. Thus, light would damage smaller regions of the retinas of underactive newts and considerably extensive regions in the retinas of active newts. One relevant experiment to address the lack of mobility in certain newts would be to have the beaker rotate during the experiment, or the light source itself rotate. The former setup may force the newt to continually move, resulting in light reaching the retina at various angles. Furthermore, the maximal amount of damage could be limited by the small pupil size of the newt, which may be variable from one newt to the next. In addition, dilation may also have different effects on different newts. A newt pupil that would dilate more would allow for more light to reach the retina, resulting in more damage when exposed to constant light. Newts were acquired from the wild at unknown age and different genders, and this may also have contributed to experimental variability.

Apart from the variability observed with the various experiments, some limitations of establishing a light-damage model in the newt exist. Newts have pigmented eyes whose melanin may absorb some of the ultra-intense light. There is a prominent band of pigmented cells found in the iris that may also reflect off some of the light. In addition, unlike zebrafish, newts may blink during the assay, preventing their retinas from being continually exposed to ultra-intense light. It would be of interest to expose newt retinas to a period longer than 30 minutes, to see whether or not increased time would allow for a greater number of animals to be affected. Moreover, newts may exert overall less motility compared to the zebrafish who are constantly swimming around; thus, light may not reach some regions of the newt retina. Furthermore, the newt retinal

cells of the inner nuclear layer and ganglion cell layer may be more sensitive to light compared to those of other animals, causing non-specific damage.

While the light damage model was reproducible and effective in other animal models, such as rodents and zebrafish, light damage was not as conclusive and reproducible a method in newts and the results were not as trivial to generate, compared to other species. Light penetration to the newt retina was also limited by the size of the newt pupil. Because of the extreme variability in damage, even among newts treated under similar experimental conditions, the light damage model is not the best choice for inducing damage and regeneration in the newt.

4.3 Monitoring the restoration of retinal function after surgical removal of the retina

Since the light damage model has not proven to be a trivial and reliable model for studying retinal damage and regeneration in the newt, another well-documented method of inducing retinal regeneration was attempted – retinectomy (surgical removal of the retina). Previous studies have used biochemical and histological methods to monitor the regenerative process of the newt retina after surgical removal (Araki, 2007; Cheon et al., 1998; Del Rio-Tsonis and Tsonis, 2003; Kaneko et al., 1999; Mitashov et al., 1995). As previously mentioned, there are no other publications to date reporting the use of ERGs as an *in vivo* method to monitor the functional restoration of surgically-removed retina in adult red-spotted newts. One goal of this study was to correlate the initial loss and subsequent recovery of the ERG amplitudes following retinectomy, to an initial loss and subsequent regeneration of functional retina.

4.3.1 Electrophysiological and histological assessment post-retinectomy

We established an *in vivo* newt ERG protocol and used it to measure the electrical activity produced by the regenerated newt retinas at various stages following retinectomy. Hematoxylin and eosin staining was used to assess the morphology of the partial and full retinal regenerates, comparing them to functional, untreated retinal controls. After two separate retinectomy experiments in a total of 15 newts, we observed the successful complete restoration of retinal function in 6 viable newts, with the amplitudes of ERG a- and b-waves reaching baseline levels. This data took into account the potential variability of recording ERGs from the same animal on a different day (21.2% for the a-wave and 25.1% for the b-wave). The appearance of plexiform layers was used to mark the end stages of retinal regeneration (Figure 5). Hematoxylin and eosin staining of sampled retinal cryosections indicated that laminations in the regenerated retinas are clearly present from mid to final stages of regeneration in most sections (Figures 36, 38- 40).

The first appearance of the ERG waveform was seen at 4 wpr in some animals, for both experiments (Figure 35 and Figure 37). In total, 6/15 animals showed initial loss and subsequent recovery of the ERG a- and b-waves to baseline. Of the 9 animals that did not show full recovery of the ERG, 6 showed partial recovery; two of which were sampled early to assess structural restoration over time. The histological assessment of these two animals showed regions that had incomplete lamination and reduced compaction of retinal layers. Overall, retinas that had incomplete restoration of the ERG were also more difficult to section. This was likely due to the loosely packed tissue that resulted in tearing of the cryosections. Histological assessment of these newts showed proper plexiform formation and retinas appearing structurally similar to the controls by

the time they were sampled, suggesting that structure may be formed before functional restoration or that the regions which underwent substantial retinal removal in these newts had not fully restored all their neurons or neuronal connections.

4.3.2 Variability of ERG recovery

After two separate experiments, variability was observed in the amount of time it takes for retinal function to be restored and in the extent to which the ERGs recovered compared to baseline levels.

Two main factors were considered that could contribute to variable recovery of the ERG, as it was expected that variability may arise. The first factor was the intactness of the retinal pigment epithelium (RPE) during retinectomy. Due to the fact that the cells of the RPE are essential for retinal regeneration, care was taken to ensure the RPE was not damaged or removed during surgery. The removal of RPE results in a variable amount of regenerated retina; thus contributes to variable recovery of the ERG over time. To control for this factor, it was important to gently apply water to the retina during surgery. However, force from the water could potentially have lead to detachment of the RPE. In the event that RPE was removed, which was evident from its dark pigmentation, it was necessary to disregard this animal. For this reason, newts in which no RPE was visibly removed were studied; however, based on histology no conclusion can be made on the amount of RPE that was damaged during the surgery.

The second factor was the amount of retina removed during surgery. We could not confidently report the amount of retina removed, due in part to its transparency and due to our inability to see clearly to the entire back of the eye during the surgery. While it was

possible to visualize removed retina, it was not possible to quantify the amount remaining in the retina. Therefore, the change in ERG amplitude from baseline to 2 dpr in Retinectomy 1 and from baseline to 2 wpr in Retinectomy 2 was the major determinant of retinal removal. The newt eye measures only 2-3 mm in diameter and even with the use of powerful microscopes, peripheral regions of the retina could not be seen through the tiny newt pupil. We were therefore restricted to observing the general location of our tools through the pupil and the peripheral regions of the retina through the site of incision. A larger incision in the corneal/scleral junction would likely result in significant loss of vitreal fluid (fluid found behind the lens and in front of the retina) as the pressure of the eye would drastically decrease. Vitreal fluid has not been reported to regenerate; therefore, the best approach was to carefully remove retina from various regions in the back of the newt eye through a small incision site.

In summary, the incomplete recovery of the ERG in these newts can thus be potentially explained by one or more of the following reasons: retinal structure was restored prior to retinal function, there was incomplete regeneration due to RPE damage, resulting in an overall loss of neurons compared to baseline levels, or that there was variability in the regeneration process due to the degree of retina removed during surgery.

4.4 Controls and considerations for future experiments

In this study, the sham control was an important control as it verified that the surgery itself did not have an impact on the ERG a-and b-waves. There were no statistically significant changes in the ERG amplitudes of this control, measured at the

same time point and under the same conditions as the newts that underwent retinectomies (Figure 37 C, D).

Furthermore, we considered that nicking or displacement of the lens during surgery may have impacted the ERG response. If the lens was nicked, opacification (cloudiness of the lens) would have occurred, causing the light to improperly focus on the retina and the subsequent variable ERG responses pre- and post-retinectomy. We did not observe any opacification in any of the sampled newt eyes.

It is important to note that while observation of cryosections from various areas of each newt retina was attempted; some sites in which the retina was removed may have been missed. Serial sectioning of the newt eyes was done carefully; however a few samples were lost either during the sectioning process or during the H and E staining if they had not fully adhered to the microslides. These may represent sections that had a marked loss of retinal cells and therefore could not completely adhere to the microslides.

Moreover, since the eyes were not scored prior to fixation, it was difficult to locate the exact incision site and conclude which areas of the retina were confidently damaged or removed. In the future, the area adjacent to the surgery site should be scored to mark the location of the incision and to allow for embedment of each sample in a consistent orientation. This would facilitate the identification of retinal cryosections to stain for comparison purposes.

In conclusion, this current study contributes to our understanding of the global functionality of regenerated retinal cells. We have shown that surgical removal of the retina of six newts in two different experiments resulted in the full recovery of the ERG a- and b-waves as early as 11 weeks post-retinectomy, with proper lamination observed

with histology. While four other eyes were sampled at time points prior to their full recovery, these newts showed the potential of attaining full recovery of the ERG. Future experiments should include sampling retinectomized eyes at early time points, as early as 1 day post-retinectomy, to confirm proper surgical technique and to observe areas in which retina was freshly removed.

4.5 Final Conclusion

In conclusion, the aim of this study was to develop a novel way of inducing retinal damage and to monitor the recovery of function in regenerated retinal cells of the adult red-spotted newt. A novel *in vivo* method to monitor retinal function in this species of newt was optimized and developed, using a modern full-field electroretinography system used in the clinic (the Espion™ Electrophysiology software). Future studies can apply this non-invasive tool to further elucidate the molecular mechanisms that occur during retinal regeneration, since these are poorly understood.

We hypothesized that ultra-intense light treatment and surgical removal of the newt retina will result in loss of the ERG response, while a subsequent recovery of the ERG will occur as a result of retinal regeneration. While light treatment did not prove to be a relevant and conclusive model to induce retinal damage in the newt, our hypothesis was confirmed with surgical retinectomy. Since full-field ERGs were used to monitor the overall electrical signals emanating from the different retinal layers, we proposed that after surgical removal of the adult newt retina, function was restored to the photoreceptor cells and the cells of the inner nuclear layer. These results significantly enhance our appreciation of retinal regeneration in the adult newt. Future studies will explore the

differentiation of these retinal subtypes during regeneration, shedding light onto the key factors required for proper differentiation of damaged neurons. This would have tremendous application to models of retinal degeneration and would enhance our understanding of the regenerative limitations in humans.

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Appendices

Appendix 1. Buffers/Reagents

Antifade/1xPBS (50mLs)

25 mls PBS

25 mls glycerol (Fisher Scientific)

0.5 g N-propyl gallate (Sigma)

- Heat antifade to 65°C and vortex to dissolve

MS222 (0.05%)

Dissolve 0.5 g 3-aminobenzoic acid ethyl ester (Sigma) and 0.6 g sodium hydrogen carbonate (BDH) in 1L dechlorinated water. Store at 4°C for up to one week or freeze at -20°C.

4% Paraformaldehyde (PFA)

1. Warm 800 mL 1x PBS to 65°C.
2. Dissolve 40 g paraformaldehyde (Acros) into 800 mL 1xPBS.
3. Transfer to the fume hood, place flask on top of the hotplate/stirrer and stir gently while heating to no more than 65°C.
4. Add 2-3 NaOH tablets (J.T. Baker), 1 at a time, to help dissolve the PFA.
5. The solution will turn from being cloudy to clear when dissolved.
6. Filter. Adjust pH to 7.4. Top up to 1L with 1xPBS
7. Allow to cool. Store at 4°C.

Phosphate Buffered Saline (10X)

To make 1L of 10X PBS solution:

1. In 800 mL ddH₂O, dissolve:

4.26 g Na₂HPO₄·7H₂O (EMD)

1.44 g KH₂PO₄ (Fisher Scientific)

90g NaCl (Sigma)

2. Adjust pH to 7.4 with NaOH or HCl.

3. Fill to a final volume of 1000 mL with ddH₂O

Appendix II. Basic protocols.

1. Electroretinography

Materials

Electrodes: 2 x 48" Platinum subdermal needle electrodes (Grass Product Group of
Astro-Med, Inc, #F-E2)

1 main silver electrode with a small loop at tip to place on cornea

Biological Amplifier (Espion)

Ganzfeld (Manfrotto)

MS222® (1L) (0.05%):

0.05% tricaine methanesulphonate (MS222) Store at 4°C; warm to RT prior to use

1 petri dish

0.5 g 3-aminobenzoic acid ethyl ester

Cheesecloths

0.6 g sodium hydrogen carbonate

newts

1L dechlorinated water

Program: Espion™ by Diagnosys

Protocol:

Before starting an ERG:

1. Access Espion™ Program and connect to the appropriate Database from the Database Centre (for example, MaggieV4.GDB).
2. Access a created file or create a new one. Select the protocol Newt ERGS v2.
3. Take a dark reading of the oscilloscope before preparing the newt. When the appropriate protocol is selected, select Run. A pop-up will appear on the screen to begin dark reading.
4. Cover the ganzfeld completely with a towel or hand to calibrate a dark measurement.

5. Anaesthetize the newts in 0.05% MS222® for **15 minutes**.
6. Setup appropriate electrodes:
 - 1 Ground Subdermal Electrode is plugged in the Amplifier in the 'REF' Channel. This is to be inserted into the newt's tail, under the skin.
 - 1 Subdermal Electrode is plugged into the Amplifier in the Channel labeled 1 (-). This is to be inserted under the skin of the newt's head.
 - 1 main test silver electrode that will be contacting the cornea is plugged into Channel 1 (+), above the reference electrode. Since the needle electrodes are small, ensure that they are first plugged into a lead that is itself connected into the amplifier.
7. Place cheesecloth along the bottom of a 10-25 cm dish. Soak gauze with MS 222® and place under the oscilloscope.
8. For measurements taken of the right eye, set up anaesthetized newt on the cheesecloth-soaked dish with tail facing the left and rotate the newt so its eye is facing up towards the ganzfeld.
9. Dilate the pupils with one drop of Mydfrin® (2.5%) (Alcon) for **40 minutes**.
10. Insert all the appropriate electrodes into the animal (head, tail).
 - a) Place the electrode so that it is just touching the central cornea and not the skin surrounding the eye. It helps to protrude the eye from the inside of the mouth with an old silver electrode that is covered with the tip of a transfer pipet. Gently apply pressure.
 - b) Check impedance. Aim for low impedance, which is 10 k or less.

- Factors that affect impedance include not having the proper reference and ground electrodes in place and not having the main electrode contacting the cornea. Movement displaces voltage around in the room which also affects ERG recordings. Do not approach the newt while recording is happening.
- c) If impedance is high, place one drop of GenTeal® Artificial Tears (Novartis) onto the newt eye.
- d) Lower the ganzfeld and position it so that it is directly over the newt's head.
- e) Keep lights on during the test.

11. Running an ERG:

- a. Once all the correct parameters are in place, hit run. 50 pulses will be measured.
- b. To repeat the experiment, click Run again. Repeat 2-3 times.
- c. When done with test, click Edit and Save the test.
- d. ERG notes can be added (anesthetic time, dilation time etc, by clicking Notes.

Note: If flatlining is observed, click Pause until the machine resets itself and traces are observed again.

2. TUNEL Protocol on Newt Eye Cryosections

The following protocol is adapted from the Apoptag® Kit Red In Situ TUNEL kit. The kit supplies plastic coverslips, which are cut in half. A 5cm² area is usually sufficient to cover one Fisherbrand *Superfrost/Plus* microscope slide (Fisher Scientific) containing 5 cryosections.

Materials/Solutions to Prepare:

4% Paraformaldehyde (1L).

Dilute to 1% using 1xPBS

DN Buffer components (for DNase I positive control) (2 mls).

MgCl₂ (1 M):

MgCl ₂ • 6 H ₂ O (BDH)	20.3 g
ddH ₂ O -----	100 ml (final)

Tris base, pH 7 (1 M):

Tris base (Fisher Scientific)	12.1 g
ddH ₂ O -----	80 ml

Adjust pH to 7 with concentrated HCl and add ddH₂O to final 100 mL volume.

DTT (100 mM) (New England Biolabs)

DNase (Sigma):

Dilute to 25 µg/ml with DN buffer

Ethanol: acetic acid (300mL):

200 mL ethanol

100 mL acetic acid

TdT Buffer Stock Solution (125mM Tris-HCl, 1M Sodium Cacodylate, 1.25mg/ml BSA, pH 6.6):

Tris-HCl (MW 157.6)	1.97 g
Sodium cacodylate, Trihydrate (MW 214.0)	21.4 g
BSA	0.125 g
ddH ₂ O-----	100 ml

Adjust pH to 6.6 and aliquot and store at -20 °C.

Cobalt Chloride Stock Solution (25mM Cobalt Chloride in Distilled Water):

Cobalt chloride, Hexahydrate (MW 237.9)	0.6 g
ddH ₂ O-----	100 ml

Mix to dissolve. Aliquot and store at –20 °C.

TdT Reaction Buffer (25mM Tris-HCl, 200 mM Sodium Cacodylate, 0.25 mg/ml BSA, 1mM Cobalt Chloride):

TdT Buffer Stock Solution	40 µl
Cobalt Chloride Stock Solution	8 µl
ddH ₂ O-----	160 µl

Mix well. Store at –20 °C

TdT Storage Buffer (60mM K-phosphate, pH 7.2, 150mM KCl, 1mM 2-Mercaptoethanol, 0.5% Triton X-100, 50% glycerol):

K ₂ HPO ₄ (MW 174.18)	1.05 g
KCl (FW 74.55)	1.12 g
ddH ₂ O-----	50 ml

Stir to dissolve and adjust pH 7.2 using concentrated HCl. Add 50 ml of glycerin (100% glycerol), 0.5 ml of Triton X-100, and 8 µl of 2-Mercaptoethanol (99% Solution, FW 78.13). Store at –20 °C

*Enzyme Reagent:

Terminal Transferase (TdT) (Roche Diagnostic) ----	4 µl
TdT Storage Buffer -----	100 µl

Mix well and store at –20 °C

**Label Reagent:

DIG-11-dUTP (Roche Diagnostic) -----	4 µl
TdT Reaction Buffer -----	1 ml

Mix well and store at –20 °C

* + ** = TdT Reaction Mixture:

Enzyme Reagent -----	100 µl
Label Reagent -----	900 µl

Mix just before use.

Note: The remaining 100 µl of Label Solution can be used for negative control.

Stop Wash Buffer (300mM NaCl, 30mM Sodium Citrate):

NaCl (MW 58.44) -----	1.75 g
Sodium citrate, Trihydrate (MW294.11) -----	0.88 g

ddH₂O ----- 100 ml
Mix to dissolve and store at room temperature.

PROTOCOL

1. Fix and Pre-incubate Specimens.

- a. Fix slides in **1% PFA/1x PBS (pH 7)**, for 10 mins at room temperature (RT). Drain off excess liquid.
- b. Wash in 2 changes of PBS for 5 mins each wash.
- c. Post fix in precooled **ethanol:acetic acid (2:1)** for 5 mins at – 20°C in a coplin jar.
- d. Drain but do not allow to dry (this solvent permeabilizes cells).
- e. Wash in 2 changes of PBS for 5 mins each wash. Leave in PBS.
 - For positive control, proceed to end of protocol (**DNase treatment**).
- f. **Pre-incubation.** To all slides (including controls), add **TdT Reaction Buffer** and incubate for 10 minutes at RT.

2. Apply TdT Enzyme

- a. Gently tap off excess liquid and carefully blot or aspirate around the sections.
- b. Prepare **TdT Reaction Mixture** by adding 200 µl enzyme to 1800 µl label solution. **NOTE:** DO NOT include enzyme for negative control, substitute with ddH₂O.
- c. Incubate in a humidified chamber at 37°C for 1 hour.

3. Apply Stop/Wash Buffer

- a. Add working strength **Stop/Wash Buffer** directly onto the slides, agitate if possible for 15 seconds and incubate for 5 min at room temperature.
Possible stopping point: Place in 70% ethanol for up to 2 days at at -20°C.

4. Apply Working Strength Anti-Digoxigenin Conjugate

- a. Wash the specimen in 3 changes of PBS for 3 mins each wash at RT.

- b. Add blocking solution to each slide (**0.3% TritonX-100 in 1%BSA /1x PBS**) and incubate for 30 mins, at RT. (**BSA: 100 mg/10mls**).
- c. Remove an aliquot of **anti-dig-rhodamine** from the stock vial sufficient to process the desired number of specimens. Warm the aliquot to RT while avoiding exposure to light.
- d. Gently tap off excess liquid and blot or aspirate around each section.
- e. Dilute warmed working strength **anti-dig-rhodamine** conjugate 1:8 in blocking solution.
- f. Incubate in a humidified chamber by first soaking paper towels in PBS and placing them in a coverslip chamber. Add slides to the chamber, cover and incubate for 30 mins, at RT. Avoid exposure to light!

5. Wash in PBS

- a. Wash the specimen in 2 changes of 1x PBS in a coplin jar for 3 mins per wash, at RT.

6. Counterstain with DAPI

- a. Dilute **DAPI** (Sigma) in a coplin jar 1:8500 - 1:10 000 (3.5µl in 35 mls).
- b. Incubate specimens in DAPI for 3mins, while avoiding exposure to light
- c. Wash the specimens in 2 changes of PBS in a coplin jar for 5 mins per wash, at RT.
- d. Mount with **Antifade** under a glass coverslip.
- e. Seal with nail polish and examine with a Zeiss Axiovert light microscope. TUNEL-positive cells can be detected at 488 nm (red).
- f. Store at 4°C in the dark.

Positive Control (DNase treatment):

Treat tissue with DNase I as follows:

- 1. Pretreat the section with **DN buffer** for 5 minutes, at RT.
 - a. DN buffer (2 mls):
 - 1930 µl ddH₂O
 - 8 µl MgCl₂
 - 60 µl Tris
 - 2 µl 100 mM DTT

2. Dissolve **DNase I** in DN buffer to a final concentration 25 µg/ml DNase. Do not vortex. Mix end-on-end.
3. Apply DNase solution and incubate for 10 mins, RT.
4. Rinse with 4 changes of ddH₂O, 3 mins each change.

3. Phototoxicity Assay (Mercury Arc lamp)

Materials:

Nikon super high pressure mercury arc lamp
 500 mL MS222 (0.05%)
 petri dishes embedded with wax
 Cheesecloths
 5" x 5" carbon paper with 1" diameter hole cut in middle
 Condenser
 Aluminum foil
 Recovery tanks for newts

Protocol:

Safety First: NEVER look directly into the light. Wear UV protected glasses at all times.

1. If using the red-cut-off filter (Method 1), Place a 5" x 5" carbon paper around the red cut-off filter to ensure any diffuse light is blocked from reaching the newt's body.
2. Turn on mercury arc lamp and allow the lamp to heat for 30 minutes before use.
3. Meanwhile, anesthetize newt(s) for 15 minutes in MS222 (0.05%) if running test for 15 or 20 minutes, and anesthetize for 20 minutes if running test for 30 minutes.
4. If keeping newt anesthetized during test, setup petri dish so that aluminum foil is taped to the side that faces the mercury arc lamp. This will shield the newt's body from any diffused light. Place this in front of the mercury arc lamp and mark where the centre of the light beam focuses on the aluminum foil, using a fine permanent marker. Cut out a hole in this area ~2mm in diameter, the approximate diameter of a newt's eye.

5. After anesthesia, place newts on a Petri dish that contains MS222-soaked cheesecloth. Dilate pupils with 1 drop Mydrin® for 10 minutes (Method 1) or 20 minutes (Method 2)
6. **Method 1:** Position the newt on the Petri dish so that its eye is pressed against the aluminum foil, directly behind the hole. This will ensure that the light beam will enter only the newt eye and any heat will be dissipated out with the aluminum foil.
7. **Method 1:** Position the petri dish so that the light's focal point is right in front of the newt's pupil. This location seems to be 50 cm away from the light source. Ensure the stage is set to 130.9 and that the edge of the Petri dish is on the edge of the stage in front of the condenser. This positioning is essential for ensuring the light maximally illuminates the retina.
8. **Method 2:** Position the fishbowl or beaker so that the light's focal point is at the centre of the fishbowl or beaker (about 52 cm away from the light source).
9. Expose newts to 30 minutes of light. After ~13 mins, anesthetize the next newt.
10. Allow animals to recover in darkness for at least 15 minutes.
11. Control newts are exposed to normal light (~280 lux).
12. Shut off the mercury arc lamp and allow to cool before covering.

Tissues fixation and cryosectioning of retinal cryosections

1. Using forceps to hold the eye in place, enucleate eye from the surrounding tissue in the eyecup by cutting around it with fine microscissors, severing the eye from the optic nerve.
2. Place dissected eye into 4% PFA/1X PBS pre-cooled to 4°C.
3. Shake end-on-end at 4°C O/N for 30 minutes
4. Wash 3x 10 mins in 1X PBS
5. Add 30% sucrose/1X PBS. Store upright at 4°C O/N until the eye sinks to the bottom. Sucrose is a partial dehydrant that prevents the formation of ice crystals in frozen tissue sections.
6. Aspirate 30% sucrose/1X PBS and replace with 50:50 / 30% sucrose/1X PBS: embedding medium (OCT).
7. Rock at RT for at least 2 hours at 4°C
8. Fill a plastic flat-bottom freezing container with embedding medium in 50:50 / 30% sucrose/1xPBS. Place eye inside the container and orient eye so that it faces the corner of the dish.
9. Freeze eye on top of a chilled cryo-safe dish. Test temperature by dropping in a piece of dry ice in the cryo-safe dish, until it freezes (turns white).
10. Do not leave sample on dry ice for more than 1 minute.
11. Store blocks at -70/80°C until use.

Sectioning of retina cryosections:

12. Thaw section to -23°C. Section newt eyes at 10 µm thickness (note change) on a cryostat and transfer sections to Superfrost slides (VWR 48311-703). Air dry for at least 2 hours.
13. Store slides desiccated at -20°C. Warm slides at room temperature for around 1 hour before opening box.

Haematoxylin – Eosin (frozen tissue)

- | | |
|---|---------------|
| 1. Let frozen sections come to room temp for around 1 hour | |
| 2. Dip slides into dH ₂ O, wipe bottom of slide, leave slides lying flat for a few minutes | |
| 3. Post-fix slides in 4% PFA (or acetone, or some other fix) | 3 mins |
| 4. Wash out fixative in running tap water | 5 mins |
| 5. Rinse briefly in dH ₂ O | quick dip |
| 6. Stain in filtered haematoxylin (length of time depends on age, If haematoxylin is fresh, 1 minute is maximal time) | 30sec – 1 min |
| 7. Rinse in tap water until water is no longer blueish in colour | 1-3 min |
| 8. Add 5 drops of concentrated hydrochloric acid to 250 ml of 70% ETOH. Make fresh just before using. | 3 quick dips |
| 9. Rinse in tap water. | 3-5 dips |
| 10. Filter saturated Lithium Carbonate, dilute 1:1 with dH ₂ O (Lithum carbonate: 3g in 300 mls, dilute by half) | 7-10 dips |
| 11. Wash/Rinse in tap water at least for | 10 mins |
| 12. Counterstain in aqueous Eosin Y | 30s-1 min |
| 13. 50% ETOH | 1 min |
| 14. 70% ETOH | 1 min |
| 15. 95% ETOH | 1 min |
| 16. 100% ETOH | 1 min |
| 17. 100% ETOH | 2 min |
| 18. 100% ETOH | 2 min |
| 19. Xylene | 2 min |
| 20. Xylene | 2 min |
| 21. Xylene **leave in last xylene bath while mounting slides** | |
| 22. Use Permount to mount slides | |

Eosin Y

Working solution:

1. 1 gram eosin Y in 200 mls ddH₂O (stock solution, Sigma)
 2. Prior to use, add 0.5 ml of glacial acetic acid /100mls solution
- Note: Do not add glacial acetic acid to stock solution.