Electrophysiological measures of optic nerve function

by

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AUTHOR'S DECLARATION

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

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Abstract

The contribution of retinal ganglion cells (RGCs) to human electroretinograms (ERGs) is known, but that of chicken (*Gallus gallus domesticus*) is not clear. This project seeks to determine the effect of RGC dysfunction on full-field flash ERGs in chickens using established protocols known to test RGC function in humans and other mammals.

Chicks were treated to produce unilateral retinal dysfunction by surgical optic nerve section (ONS group) or by intravitreal injection of tetrodotoxin (TTX group) to block ganglion cell function. Contralateral eyes received sham treatments, consisting of sham surgery or injections of vehicle, phosphate buffered saline (PBS), respectively. For both groups, bilateral, full-field ERGs were recorded in dark-adapted (DA) birds (ONS: n=6; TTX: n=5) or in light-adapted (LA) birds (ONS: n=10; TTX: n=5) prior to the imposed treatment (at one day post-hatch) and on days 3-, 5-, 7-, 14-, and 21- post-treatment on the same birds. In addition, bilateral, full-field, long-flash (150 ms) ERGs were recorded from light-adapted birds (ONS: n=8; TTX: n=5) prior to treatment (at one day post-hatch) and again at 3-, 14- and 21-days post-treatment. Interpolation and curve fitting, including Naka Rushton fitting, were used to report parameters of the ERG stimulus-response series such as maximum amplitudes (Vmax) and sensitivity (k, stimulus producing half Vmax). Cell counts (retinal histology) were conducted of the RGC and inner nuclear layers from histological sections of a separate group of 6 birds sacrificed at 21 days post-ONS.

For both groups, the measures of the DA ERG stimulus-response series (dark-adapted Vmax and k, the oscillatory potential amplitudes, and the interpolated a-wave parameters) did not differ between the treated and sham-treated eyes in either treatment group. In addition, for both treatment groups, the negative waveform of the scotopic threshold response (STR), which reflects RGC function in most mammals, was not apparent in the chick ERGs to dim flashes. No differences between the eyes were

detected for the positive STR/DA b-wave to 0.01 cd.s/m^2 flashes (ONS: p=0.59; TTX: p=0.21). Similarly, the photopic negative response (PhNR) following the light adapted b-wave was small and showed no effect of either treatment (ONS: p=0.92; TTX: p=0.11). However, the offset positivity, the d-wave amplitude, was smaller in the treated eyes in both the ONS and TTX groups (ONS: p=0.008; TTX: p=0.03), but d-wave implicit times did not differ. Cell counts confirmed that RGCs were selectively lost following ONS ($p<0.0001$).

This study suggests that the STR and PhNR do not reflect RGC functions in chickens, as they do in most mammals. Anatomical differences between the chicken and human retinae might underlie differences in the generation of ERG waveforms associated with ganglion cells. In particular, chicken eyes, and avian eyes in general, lack an inner retinal blood supply and associated intra-retinal astroglia which may be necessary for the generation of STR and PhNR waveforms. Finally, this thesis showed that, unlike in humans, the chicken d-wave may reflect the function of cells in the optic nerve including RGCs and cells of the centrifugal vision system in the chicken.

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Dedication

This thesis is dedicated to my lovely kids.

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List of Abbreviations

ADOA	autosomal dorminant optic atrophy
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
CNS	central nervous system
CO_2	carbon dioxide
CVS	centrifugal visual science
DA	dark-adapted
DAPI	4′,6-diamidino-2-phenylindole
DTL	Dawson, Trick, and Litzkow
EEG	electroencephalogram
ERG	electroretinogram
GC	ganglion cell
GCL	ganglion cell layer
IPL	inner plexiform layer
IT	implicit time
ISCEV	International Society for Clinical Electrophysiology Society
LA	light-adapted
LED	light-emitting diode
LWS	long wave sensitive
NIRG	non-astrocytic inner retinal cell
Nav	voltage-gated sodium channel
NK	Naka-Rushton curve
NMDA	N-methyl-D-aspartate
6-OHDA	6-hydroxy dopamine
OKN	optokinetic nystagmus
ON	optic nerve
ONH	optic nerve hypoplasia
ONL	outer nuclear layer
ONS	optic nerve section
OP	oscillatory potential

OPL	outer plexiform layer
PBS	phosphate buffer solution
PERG	pattern reversal electroretinogram
PH	potential of hydrogen
PhNR	photopic negative response
R^2	r squared
RGC	retinal ganglion cell
RH	rod-opsin
RMS	root mean square
RPE	retina pigmented epithelium
SD	standard deviation
SEM	standard error of the mean
STR	Scotopic threshold response
TTX	tetrodotoxin
UV	ultraviolet
VB	vecuronium bromide

Chapter 1
Literature Review

1.1 Retinal anatomy and physiology

This section reviews literature about the vertebrate retina and electroretinogram. This review takes the format of general knowledge across various species first, the human variation, and then the retina of the chicken (*Gallus gallus domesticus*).

1.1.1 Vertebrate Retina

The eye is considered as one of the most complex organs of the body. The eye can be divided into three layers: the outer, middle, and inner layers. The inner layer, the retina, is a complex, layered structure of neurons responsible for detecting light and vision. The middle layer is the vascular component of the eye, which is responsible for providing the majority of materials for the metabolic needs of the structures of the eye. This middle layer has the choroid, the ciliary body and the iris. The pupil size, which is controlled by the iris, is responsible for controlling the amount of light falling on the retina (Remington 2012). The outer layer, which is the protective layer of the eye, consists of the supporting sclera, which is continuous with transparent tissue at the front of the eye, and the cornea, through which most of the light rays coming to the eye are refracted (Remington 2012). The epithelial layer on the sclera around the cornea is the conjunctiva.

The retina contains many types of neurons, including the photoreceptors, cones and rods, bipolar cells and retinal ganglion cells (RGCs) (Remington 2012). Other major classes of neurons are horizontal cells and amacrine cells.

The internal limiting membrane is located at the innermost boundaries of the retina. The membrane is made of the inner end of the principal retinal glial cells, the Müller cells, and are also referred to as footplates of the Müller cells. The inner limiting membrane is smooth, and some modifications occur,

such as astrocytes replacement and inclusion of vitreous fibres at the optic disc and periphery, respectively (Remington 2012). The nerve fibre layer (NFL) runs parallel to the surface of the retina adjacent to the inner limiting membrane. In mammals, the NFL is made of unmyelinated axons of the retinal ganglion cells (RGCs) (Remington 2012), while in avians such as chickens, the RGC axons are myelinated by oligodendrocytes (Fu and Qiu 2001, Fischer et al. 2010). The appendages of Müller cells are found in the NFL where they cover/wrap around retinal vessels (such as the superficial capillary network in vascularized retinae) and nerve fibres (Oyster 1999). The next layer, the RGC layer, is usually one cell thick but might be 8-10 cells thick near the macula in primates (Remington 2012). Some displaced amacrine cells, Müller cell bodies and astroglia cells might be found at this layer (Remington 2012).

The next layer, the inner plexiform layer (IPL), contains the synapses of the RGCs and bipolar cells. Other synapses occur between these cells and amacrine cells. In vascularized retinae, the inner capillary networks of retinal vessels are in this layer (Remington 2012). The next more distal layer, the inner nuclear layer (INL), comprises the neuronal cell bodies of bipolar, amacrine, Müller horizontal and, uncommonly in mammals, displaced RGCs (Remington 2012). In the avian retina, displaced RGCs are common and have their cell bodies and dendrites in the IPL (Mey and Johann 2001). These displaced RGCs have the most extensive dendrites, and their axons project into the accessory optic system of the avian brain (Mey and Johann 2001). Distal to the INL is the outer plexiform layer (OPL), which contains the synapses of the photoreceptors and bipolar cells (Zareen et al. 2011). Also, this layer has horizontal cell synapses. The outer nuclear layer, which consists of the cell bodies of photoreceptor cells, is next to the OPL (Oyster 1999). The external limiting membrane (ELM) is a membrane-like structure made up of the outer processes of the Müller cells surrounding the photoreceptor cells near the junction of their outer and inner segments. It has been suggested by

Panda-Jonas et al. (1996) that the external limiting membrane forms a barrier that prevents the movement of macromolecules from the photoreceptor layer to the inner part of the retina (Zareen et al. 2011). Distal to the outer limiting membrane, the photoreceptor outer segments form the photoreceptor layer of the retina.

The types and composition of retinal cells depend on the species of the animal under study as the retina in most animals has adapted to the visual environment (Bowmaker and Knowles 1977). Light absorption and transduction functions are performed by photoreceptors, which are broadly classified into rods and cones. The photoreceptors absorb light by using visual pigments (chromophores) derived from Vitamin A. Isomerization of a chromophore upon light absorption results in the biochemical cascade of phototransduction, which eventually leads to the halting of glutamate neurotransmitter release.

Rods are designed to capture photons at low light levels and, therefore, are more sensitive than cones and very useful for dim vision. Furthermore, the organization of the cones tends to be concentrated in a specific part of the retina to achieve better visual acuity (Oyster 1999). For humans, many primates and raptors depend on excellent visual acuity for survival, and these cones are concentrated at a specialized depressed (pit) part of the retina called the fovea (Oyster 1999). The chicken retina has a concentration of cones 2 mm dorsal to the optic disc but has no foveal pit (Morris 1982). Primates have three types of cones: long-wavelength sensitive (red light), medium-wavelength sensitive (green light) and short-wavelength sensitive (blue light) (Oyster 1999). However, avian species have additional photoreceptors in the ultraviolet range, and chickens specifically have five cone types, including the double cone (Kram et al. 2010, Wisely et al. 2017).

The photoreceptors synapse with the second-order neuron, the bipolar cells. One feature of the bipolar cells is that they initiate the parallel visual processing pathway in the visual system by dividing the pathway into on- and off-pathways (Famiglietti and Kolb 1976). The on-bipolar cells are depolarized when glutamate release is interrupted by phototransduction in the photoreceptors. Furthermore, because they possess excitatory kainate and α-amino-3hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, they are also known as metabotropic glutamate receptors (mGluRs) (Sasaki and Kaneko 1996).

Contrary to the on-bipolar cells, the off-bipolar cells have inhibitory glutamate receptors and are known to have ionotropic glutamate receptors (iGluRs). Another interesting feature of the bipolar cells is the segregation of the off-bipolar to RGC synapses from the on-bipolar to RGC synapses at the IPL, as shown in Figure 1. Specifically, the off-bipolar cells synapse close to the INL, while the on-bipolar cells connect to the RGCs close to the RGC layer (Nelson and Connaughton 1995). Moreover, this segregation occurs only in mature retinae and is absent if the off-bipolar cells are blocked during early development. The segregation develops independently of the presence or absence of functioning RGCs (Tootle 1993, Chalupa and Gunhan 2004).

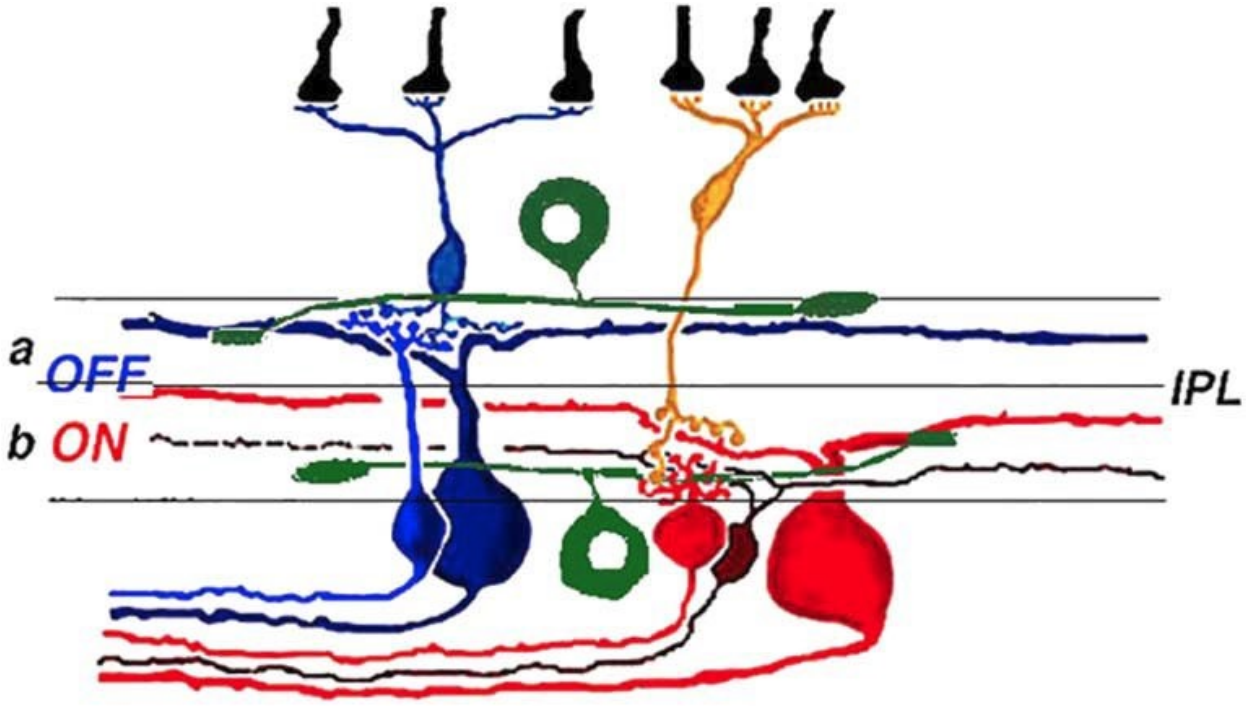

Figure 1: Segregation of off visual pathway (in blue) from the on pathway (in red) at the IPL. From Chalupa and Gunhan (2004) with permission.

The primary retinal cells that transmit highly processed visual information to the brain are the RGCs. These third-order retinal cells are highly diverse. In terms of morphology, some possess small cell bodies but complex dendrites; others have large cell bodies but may have a smaller dendritic spread (Oyster 1999). Functionally, some fire impulses in response to motion, stimuli from specific direction or stimuli from particular orientation. The response to the stimuli can be sustained or transient. Moreover, the RGCs only respond to a specific range of contrast, stimulus size or colour (Oyster 1999).

Embryologically, RGCs are the first to differentiate from primordial retinal stem cells (Cepko, 2014, Rapaport et al., 1996, Tian, 1995). It is known that upon migrating to the ganglion cell layer, RGCs extend their axons before forming extensive dendrites (Maslim et al. 1986, Kirby and Steineke 1991); segregation of the dendrites at the IPL is seen in matured RGCs as shown in Figure 2.

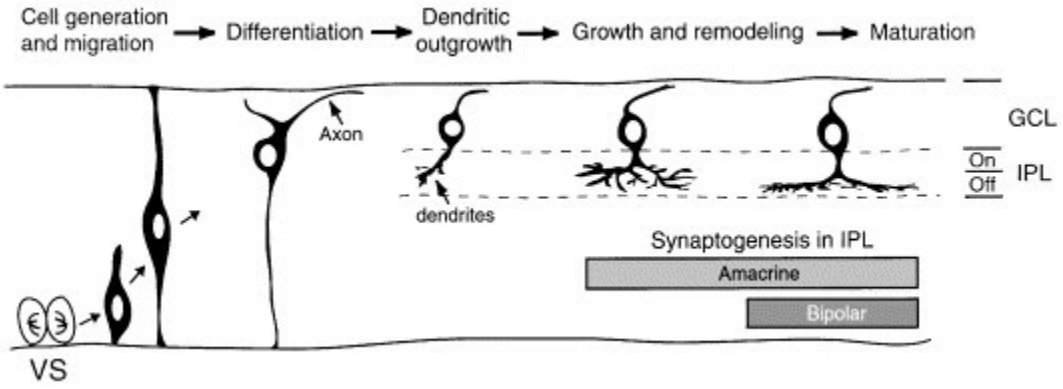

Figure 2: The embryological development of RGCs across species.

VS is the mitotic RGC stem cells. From Sernagor et al. (2001). with permission

Müller cells are the primary glial cells in the retina and span the layers of the retina from the inner to the outer limiting membranes (Figure 3). They have close contact with most retinal cells (Pfrieger and Barres 1996) and serve to control the flow of oxygen, carbon dioxide, nutrients and other metabolites (Reichenbach and Robinson 1995).

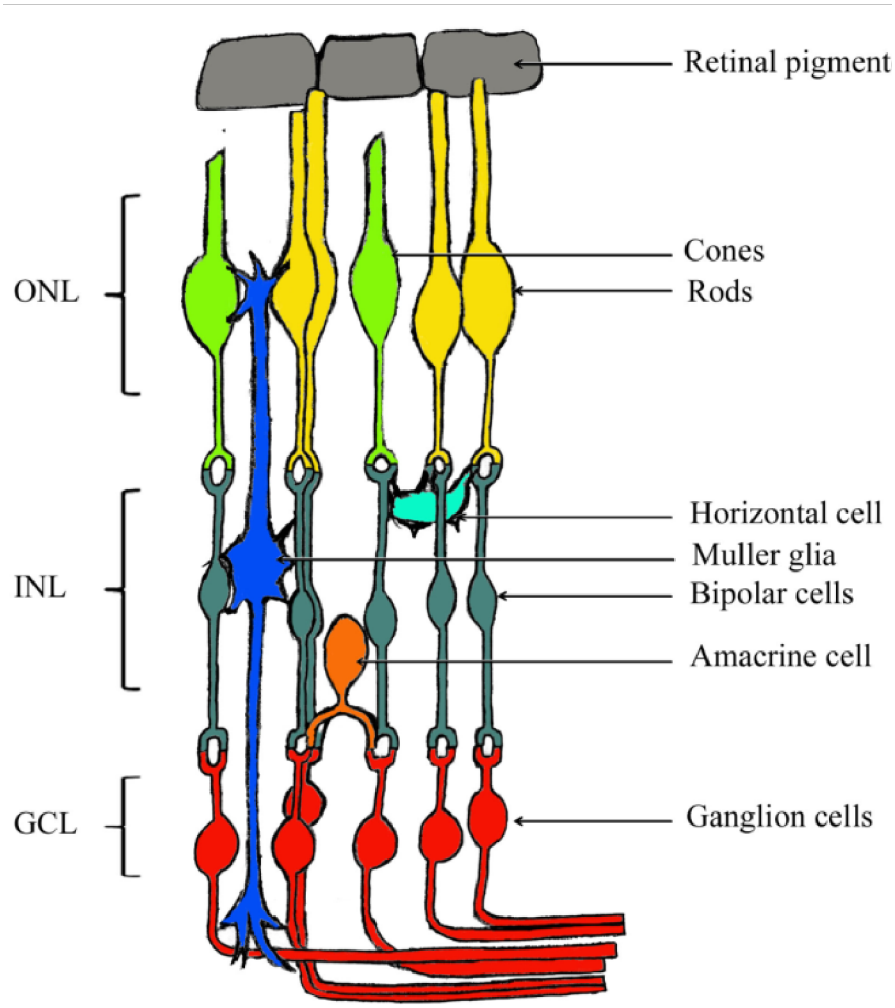

Figure 3: A drawing of the retina cells and its layers, highlighting the role of the Müller cell.

From Belecky-Adams et al. (2013) with permission.

1.1.2 Relationship between astrocytes and Müller cells

In the retina, the astrocytes and Müller cells seem to perform similar functions. Additionally, it has been postulated by Ramírez et al. (1998) and Jammalamadaka et al. (2015) that astrocytes complement the role of Müller cells in performing their functions. These two retinal glial cells store,

and release glycogen, help neurotransmitter metabolism, take up CO_2, regulate potassium ions, and help establish the blood-retinal barriers. Although Müller cells are present and traverse all layers of the retina, the presence or distribution of astrocytes is complex. Whereas elongated astrocytes are found in the NFL of most vertebrates, the intra-retinal stellate (protoplasmic) astrocytes tend to be abundant only in animals with inner retinal blood vessels (Schnitzer 1988, Chojnacki and Weiss 2008). In animals with avascular retinae, intra-retinal astrocytes are rare (Schnitzer 1988, Fischer et al. 2010). In chickens (Fischer et al. 2010), hippopotami and rhinoceri, all of which have avascular inner retina, the astrocytes are restricted to only the optic nerve head (optic disc) (Schnitzer 1987, Schnitzer 1988). In animals with vessels around the disc, the astrocytes are located around the optic disk. Moreover, in the rabbits' partially vascularized retina, the astrocytes are located only around the vascular (medullary artery) part of the retina and are rare in the other parts of the retina (Clark and Mobbs 1992, Trivin et al. 1997, Haddad et al. 2001). Intra-retinal astrocytes are numerous in animals with inner retinal blood supply except in areas of the retina which have no vessels, such as the fovea (Stone and Dreher 1987, Jammalamadaka et al. 2015). The concept of the link between the astrocytes and inner retinal blood vessels has led to the assumption that the intra-retinal astrocytes are necessary for the development of inner retinal vessels, and for most vertebrates, the distribution of astrocytes is based on whether they have inner retinal blood vessels or not. Because of the close functional relationship of the Müller cells and the retinal astrocytes, in animals without inner blood vessels, Müller cells take over the functions of astrocytes (Stone and Dreher 1987, Jammalamadaka et al. 2015).

1.2 Embryology of the human retina

Following the formation of a hollow blastocyst and three germinal layers a neural tube forms by surface invagination the neural plate, which is derived from the primordial ectoderm. At the rostral end of the developing embryo, the primitive vesicles of the developing brain, including the diencephalon (Oyster 1999). An outpouching of the diencephalon gives rise to the earliest stage of ocular development, the optic vesicles. The vesicles fold inwards to form the two-layered cup-shaped optic cups. Although the optic vesicle is initially located beside the surface ectoderm, a layer of derivative neural crest (mesenchymal) cells migrates between the optic cup and the ectoderm. Several eye structures, such as the choroid, the corneal endothelium, and the ciliary body, develop from these mesenchymal cells (Oyster 1999).

The optic vesicle, on contacting the surface ectoderm, causes the surface ectoderm to thicken to form the primordial lens, and the invagination of the optic cup results in a groove called the choroidal fissure (Oyster 1999). The primitive central artery forms and grows into the optic cup through the choroidal fissure. Consequently, the two-layered optic cup forms the retina; the outer layer becomes the retinal pigmented epithelium (RPE) and the inner one results in the neural retina (Oyster 1999). In humans, this process takes place between 25 – 35 days of gestation, and in chicks is complete by about 20 days (Wisely et al. 2017). The Müller cells and the true astrocytes are both of the neuroectoderm origin (Ramírez et al. 1998) and the Müller cells may share the same stem cells as neurons. In some species such as chickens, the Müller cells are progenitor cells for neurons (Ramírez et al. 1998). In humans, the astrocytes originate from the brain and enter the retina through the optic nerve in the 13th week of gestation (Jammalamadaka et al. 2015). Astrocytes follow the pathway of spindle fibres, which form the retinal blood vessels (Ramírez et al. 1998, Jammalamadaka et al. 2015).

1.3 Chicken eye and retina

The basic structure of the chicken retina is similar to that of other vertebrates (Hocking and Guggenheim 2014). In fact, most of the information about the vertebrate retinal structure and function were obtained through the study of the eyes of chickens (Bovolenta and Martinez-Morales 2019). However, there are notable differences, and these differences can be categorized as macro and micro levels. At the macro level, the chicken eye takes about 50% of the cranium space whilst the human eye is 5% of the human cranium (Waldvogel 1990). Differences in the anterior chicken eye compared with most mammals include the presence of ossicles at the limbus, the region between the sclera and cornea, and that the iris and ciliary body contain skeletal muscles, but the most substantial differences are in the retina. The inner retina of the chicken has no inner retinal vessels; instead, it possesses pecten oculi, which provides nourishment to inner retinal structures (Pettigrew et al. 1990).

Moreover, 2 mm dorsal from the chicken optic disc (Morris 1982) is a no "pit" fovea called the area centralis (3mm in diameter), which performs similar functions as a fovea. The photoreceptors of the chicken retina have oil droplets (Figure 4), located between the outer and inner segments of the cone photoreceptors (Bowmaker and Knowles 1977). The oil droplets are pigmented, and the pigments filter light, which except for the double cones, contributes to colour vision (Hart and Vorobyev 2005). The oil droplets' pigmentation is modified by the type of feed (Meyer et al. 1971) given to the chicken (high carotenoid feeds lead to higher pigmentation), and by the light intensity (Hart et al. 2006) of the environment of the chicken (chickens reared in the high intensity of light have more pigmentation). Chickens have single cones with peak sensitivity to short, medium and long wavelengths as in humans but also an additional cone type that is sensitive to ultraviolet (UV) (Osorio et al. 1999). Furthermore, the chicken has double cones, which are sensitive to medium to long wavelengths (Morris and Shorey 1967).

In the inner retina, the AII amacrine cell, which mediate the rod pathway in humans and many other mammals, is reported to be lacking in the chicken retina (Shi and Stell 2013). Possibly, the AII amacrine cells is lacking because chickens are diurnal, and their retina is dominated by cones.

Additionally, chickens have an enhanced centrifugal vision system (CVS), efferent fibres from the brain to the retina (Cowan et al. 1961, Gutierrez-Ibanez et al. 2012, Miles 1972), while such efferent fibres are rare in the primate retina (Gastinger et al. 2006). Most of the CVS fibres terminate close to the off-bipolar cells and excite RGCs (Lindstrom et al. 2010). This CVS is implicated in the feedback system, which plays an important role in the feeding habit of chickens. Since the synapses of the CVS cells are closer to the RGC layer of the chicken retina, it is expected that off-pathway may play an important role in influencing the ERGs of RGCs of chickens. For instance, in toads and salamanders, also animals with enhanced CVS, dysfunctional RGCs influenced the electroretinogram of their off-pathway. In chickens, sectioning of the CVS only has the same effect on refractive error as full optic nerve section. (Dillingham et al., 2017).

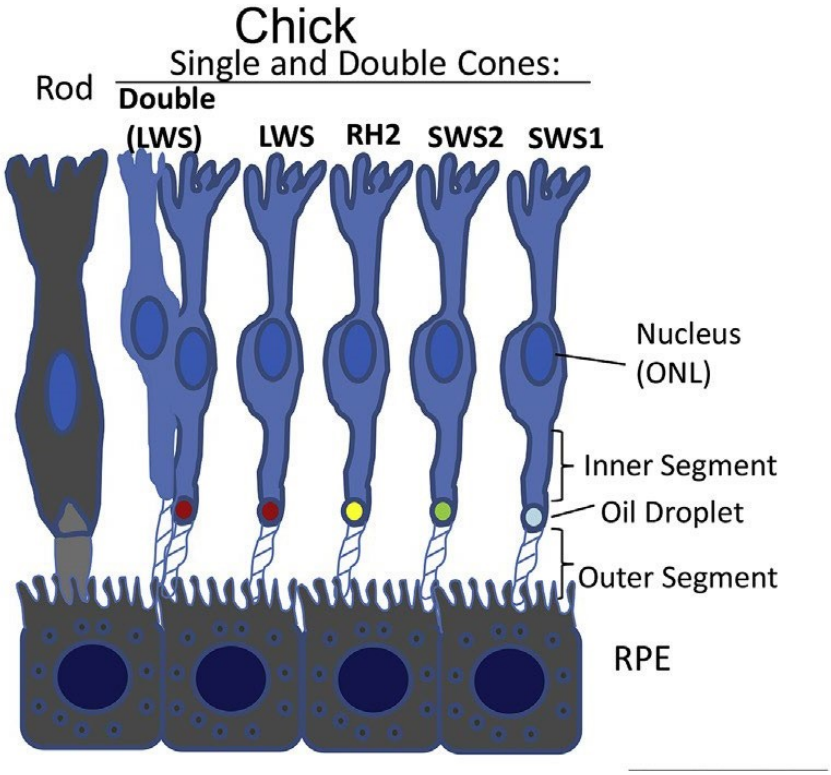

Figure 4: Chick photoreceptor cells showing the oil droplet.

RPE is retinal pigmented epithelium, ONL (outer nuclear layer), LWS (long wave sensitive), RH2 (rod-opsin-like cone opsin), short wavelength sensitive (SWS). Adapted from Wisely et al. (2017), with permission.

At the microlevel, chickens, like other vertebrates with avascular inner retinae, such as guinea pigs and other birds, have no or very few intra-retinal astrocytes. Unlike other vertebrates, chickens have oligodendrocytes surrounding the nerve fibres (Fischer et al. 2010). Additionally, the chicken possesses non-neuronal intra-retinal non-astrocytic inner retinal cells (NIRGs) (Zelinka et al. 2012). It has been suggested by Fischer et al. (2010) that these NIRG cells play a role in Müller cell and neuronal retina cell survival.

1.4 Models of RGC damage

Surgical excision of the optic nerve (optic nerve sectioning; ONS) or intravitreal injection of tetrodotoxin are employed as methods to destroy or block RGC function, respectively (Wildsoet and Wallman 1995, Chong 2013). Chong et al. (2013) and Choh et al. (2004) showed that ONS of chicken eyes causes a significant and progressive loss of the number of RGC cells starting from days three post-surgery. This finding makes ONS a particularly excellent model choice for studying RGC deficits as it allows longitudinal observation of the effect of progressive reduction of RGC numbers. The RGC reduction is mediated by apoptotic processes (Choh et al. 2004, Chong et al. 2013).

Tetrodotoxin is a neurotoxin that blocks action potentials in neurons with axons by blocking the voltage-gated sodium channel (Nav) (Narahashi 1974). TTX is, therefore, effective in inhibiting the RGCs and spiking amacrine cells. Because the affinity of the TTX to a particular Nav depends on the composition of the channels' amino acid isoforms, and different species have different amino acid isoforms in their Navs, some species are resistant to the effect of TTX (Catterall et al. 2005). Out of the 10 types of Navs, TTX has a high affinity for Nav 1.4, and Nav 1.6, which frequently are associated with the neurons in the CNS, including the chicken retina (Lee and Ruben 2008). The TTX effect in the retina is reversible. Intravitreal injection of 0.8 µg in 8 µl citrate buffer is known to effectively stop the function of the RGC in domestic chicken eyes (Wildsoet and Wallman 1995) for up to 72 hours (McBrien et al. 1995).

1.5 Electroretinograms (ERGs)

The activity of retinal neurons in the form of the electrical response of the retina to light flashes can be recorded from the surface of the cornea. This non-invasive method of assessing retinal cell function has become an essential tool for studying the retina in clinical and laboratory settings (McCulloch et al. 2015).

Generally, it is widely accepted that retinal responses such as ERGs occur due to voltage changes generated by inward and outward ion flow from the changes to localized conductance across the active retinal cell membranes (Brown 1968, Frishman 2006). These voltage changes cause currents to flow through the extracellular space, following the potential gradients. Part of the active cell membrane serves as a generating source of these currents or as a sink, and these currents are directed towards a less active part of the membrane. Therefore, the aggregate effect of many cells generating the extracellular current flowing in the same vector direction synchronously results in potential changes referred to as field potentials (Brown 1968, Frishman 2006, Perlman 2015). The measurement of the field potential can be recorded on the eye surface if the field potential is radiating outward around the eye. Most retinal neurons are involved in the generation of ERGs (Frishman 2006). Based on the influence of specific factors, such as the position and orientation of the cell, and the relative strength of the cellular responses, the ERG waveforms generated can be attributed to specific types of retinal cells. Mainly, radially oriented retinal cells generate most ERG signals (Brown 1968, Frishman 2006, Perlman 2015). Therefore, photoreceptor cells, bipolar cells and Müller glial cells are the dominant generators of ERGs. Conversely, the small to undetectable signals originating from specific retinal cells that are not radially oriented, such as amacrine and horizontal cells are associated with much smaller field potentials such as the oscillatory potentials that are superimposed on the leading edge of b-waves. The current dipole, created as a result of the

movement of current from the generating area to the receiving area (current sink) of radially oriented cells, flows mostly intraretinally. A small amount of the current generated by the dipoles flows through tissue external to the retina, i.e., through the vitreous and the highly resistant RPE, and returns to the neural retina (Brown 1968, Frishman 2006, Perlman 2015). From the above explanation, it stands to reason that placement of the active electrode has a considerable influence on the magnitude of ERGs. Other factors, such as the background illumination, the adaptation of the retinal cells to the light stimulus, the strength of the flash stimulus, and the stimulus wavelength, affect the relative contribution of the cells to the ERGs (Frishman 2006). For instance, in light-adapted ERGs, most rods are suppressed and hence have little contribution from rod photoreceptor cells (Frishman 2006).

Using drug neuro-active drugs, research studies have made significant contributions to understand the ERG waveform. Ragnar Granit won the Nobel prize for medicine for his research in sensory physiology, including his studies of ERGs. His classic work, published in 1933, showed that ERGs have three components, which he named the PI, PII, and PIII, in order of their loss when exposed to anesthesia (Granit 1933), as shown in Figure 5.

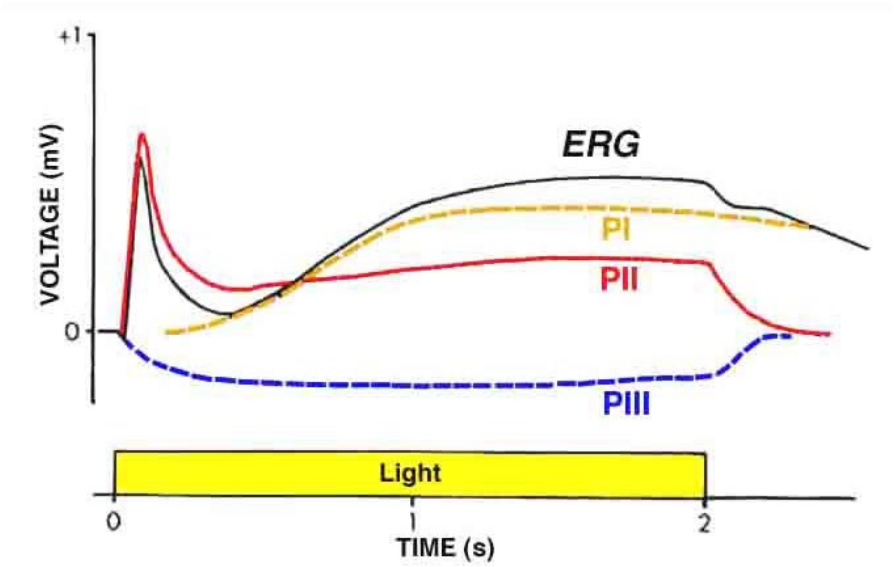

Figure 5: A representation of Granit's view of the components of the long-flash ERGs. Adapted from (Perlman 2015) with permission.

Before Granit's dissection of the ERG components, several researchers, including Holmgren and Armington, made major contributions to the detection and our understanding of ERGs (Perlman 2015). Einthoven and Jolly discovered the a-wave and b-wave components of ERGs (Einthoven and Jolly 1908). The use of drugs to inhibit specific cell types of the retina, thus studying the uninhibited part, is termed pharmacological dissection, and this method has contributed greatly to understanding the sources of ERG waveforms. Additionally, the use of intra-retinal electrophysiological recordings also contributed immensely to our understanding of the source of the ERGs (Perlman 2015). Since the discovery of the pharmacological agent, L-aspartate, which can isolate the function of the photoreceptors, it has been found that photoreceptors are the source of a-wave (Perlman 2015). Because of the proximity of the potassium sink of the Müller cells and the bipolar cells, the studies to determine the source of b-wave were quite controversial. The Müller cells were initially thought to be the source of b-wave (Miller and Dowling 1970). However, intra-retinal recording and

pharmacological dissection of the retina revealed that the ON bipolar cells are the major generators of b-waves. In definitive studies, Lei and Perlman (1999) used barium ion salt, which specifically blocks the function of the Müller cell, to show that b-waves originate from bipolar cells. In their study, although Müller cell function was blocked, there was an enhancement of the b-wave.

1.5.1 Brief-flash ERGs

In humans, brief flash ERGs are widely used as retinal function tests (McCulloch et al. 2015), possibly because testing is easily performed clinically and has been standardized by the International Society for Clinical Electrophysiology of Vision (ISCEV). The ISCEV standard for full-field ERG testing includes the DA ERG amplitudes in response to 0.01 cd.s/m^2 stimuli (DA b-wave) to assess rod pathway function, dark-adapted 3 cd.s/m^2 flashes for both rod and cone driven retinal function, dark-adapted 10 cd.s/m^2 flashes for enhanced a-waves to evaluate photoreceptor function, dark-adapted oscillatory potentials (OPs) to reflect middle retinal cells, light-adapted 3 cd.s/m^2 flashes and light-adapted 30 Hz flicker ERG to study the cone-driven retinal pathway (McCulloch et al. 2015). For light flashes below the human psychophysical threshold, negative and positive ERG waveforms are observed on the human dark-adapted ERG (Frishman et al. 1996). This waveform is called the scotopic threshold response (STR), the negative and positive STR (nSTR and pSTR), respectively. These STRs are distinguished from a- or b-waves because they are removed with pharmacological agents that block inner retinal cells, and because the STR is elicited with very weak flashes below the perceptual threshold. In macaque monkeys, pSTRs are small because part of their waveforms are negated by nSTR (Frishman et al. 1996). The STR waveforms are easily elicited in rod-dominant mammals such as rodents and in non-human primates. However, these STRs are not universally present in all vertebrate dark-adapted retinae.

1.5.2 Diagnostic potential of brief ERGs to measure optic nerve or RGC deficit

Whereas it is relatively easy to test for the function of radially oriented retinal neurons such as photoreceptor cells (a-waves) and bipolar cells (b-waves), non-invasive testing of the RGC function is more challenging (Frishman 2006, Perlman 2015). However, in humans, the STR, photopic negative response (PhNR), and pattern reversal ERG (PERG) protocols reflect RGC function (Viswanathan et al. 1999, Frishman 2006, Perlman 2015). As described above, the STRs are dark-adapted ERGs elicited by very weak flashing (-6 to -3 log cd.s/m^2) stimuli; the PhNR is the negative potential that follows the b-wave in light-adapted ERGs, whilst the PERG is generated through contrast changes such as alternating checkerboard stimuli.

While standard ERGs are useful to test retinal function, they provide limited usefulness in the detection of RGC deficits. The PERG is the most studied ERG known to objectively assess the central vision of primates. Standard PERGs are elicited using checkerboard stimuli and require clear ocular media and refraction. The PERG is made of two main waveforms, P50 and N95. The P50 waveform reflects macular function, and the N95 waveform originates from RGCs. In several mammals, PERG waveforms are reduced on intravitreal injection of TTX (Viswanathan et al. 2000) or ONS surgery (Harrison et al. 1987).

In humans (Morny et al. 2015, Joshi et al. 2017), monkeys (Wilsey et al. 2017) and dogs (Takada et al. 2017), PhNRs hold great potential for diagnosis of RGC deficits. First described by Viswanathan et al. (1999), the PhNR has been useful for the detection of RGC deficits in that it has become a useful substitute to PERG in the study of RGC function. Additionally, the PhNR has the added advantage of not requiring clear and optimal correction of refractive error of subjects. Clinically, studies indicate that PhNR amplitudes are sensitive to RGC cell functions (Morny et al. 2015, Wilsey and Fortune 2016, Frishman et al. 2018); however, other studies point to the fact that the amplitude is

sensitive to the integrity of glial cells around the RGC layer of the retina (Machida et al. 2008, Raz-Prag et al. 2010). Animals with inner retinal vascular supplies tend to have many glial cells, notably intra-retinal astrocytes and Müller cells, and these animals are also reported to have PhNRs reflecting RGC functions; it stands to reason that the PhNR may indirectly reflect RGC function but be generated by glial cells. Animals with avascular inner retina could help to differentiate the RGC from the glial origins of the PhNR.

1.5.3 Long-flash ERGs

ERGs elicited by long-duration (150-200 ms) stimuli in the presence of rod suppressing backgrounds are useful for obtaining ERGs from on- and off- retinal pathways. D-waves are positive responses to the flash offset, while onset responses are indicated by a- and b-waves. The stimulus strength and duration affect the amplitudes obtained in long-flash ERGs. Sustar et al. (2006), showed that the optimal offset d-wave is obtained with flashes 150 to 200 ms; shorter flash durations result in incomplete separation of the on- and off responses, showing an i-wave, superimposed on the onset b-wave rather than a separated d-wave at off-set. Also, the longer the duration of the stimuli, the larger the amplitude of the d-wave. The amplitudes of the b- and d-waves also depend on the stimulus strength in a non-linear manner (Al Abdlseaed et al. 2010).

ISCEV recently published an extended protocol for long-flash ERG recordings with a white stimulus of 150-350 cd/m^2 for eliciting the onset ERG and off-set d-wave (Sustar et al. 2018). However, several studies point to the use of other wavelengths such as orange on a green background to selectively stimulate the L and M-cones (Sustar et al. 2006). Evers and Gouras (1986) and Sustar et al. (2006) report that a long-wavelength flash stimulus generates significantly smaller d-wave amplitudes; hence long wavelength stimuli should not be used to elicit the off-set responses. However, published work still makes use of long-wavelength flashes to successfully elicit d-waves

(Morny et al. 2015, Morny et al. 2019). Furthermore, due to significant interspecies differences in retinal constituents, the long-flash ERG protocol is expected to be specific to the species.

Seiving et al. 1993 pioneered work in the long-flash ERGs in primates, describing the on-response of the ERG as being a typical biphasic waveform, as seen in short flash ERGs. The second component, the d-wave, is typically a positive off-response. It has subsequently been suggested that the positive off-response is not universal and is found selectively in vertebrates with significant cone photoreceptors (Lei, 2003). Therefore, vertebrates with retinae containing a substantial population of cones such as primates, birds and guinea pigs tend to have a positive polarity of the d-wave, while retinae with fewer number of cones, such as those in rats, typically show a negative-going waveform after offset. Furthermore, there seems to be a positive relationship between the number of cones and the magnitude of the d-wave. It has been suggested that the on a-wave reflects the functions of photoreceptors with some contribution from hyperpolarizing bipolar cells (Bush and Sieving 1994) while the on b-wave reflects functions of the depolarizing on-bipolar cells with some contribution from the horizontal cells (Sieving et al. 1994). In contrast, the d-wave appears to reflect the off-pathway with a major contribution from the hyperpolarizing off-bipolar cells, which are cone-specific with no direct connection to rod photoreceptors (Sieving et al. 1994). Additionally, Seiving et al. (1994), in proposing the 'push and pull" model of brief-flash b-wave amplitude, showed that the amplitude of the brief-flash b-wave is influenced by increased ("push") of the flash onset b-wave from depolarizing on-bipolar cells and decreased ("pull") amplitude of the offset d-wave from the hyperpolarizing (off) bipolar cells.

1.5.4 Development of chick electroretinogram

The a-wave and b-wave onset are known to be recordable in chickens from pre-hatch day 18 of incubation (Ookawa 1971). Ookawa (1971), also noted that the a- and b-wave amplitudes increase up

to day 14 post-hatch. However, in the post-hatch chick, there is a notable drop in amplitude of the a- and b-wave on day seven post-hatch (Ookawa 1971).

Moreover, the b-wave is most affected by a decrease in chicken body temperature far more than the a-wave. This phenomenon is contrary to what is expected, as the a-wave, having its source from photoreceptors, should be more affected because it is closer to the systemic blood supply through the choroid (Ookawa and Tateishi 1970). Chick b-wave latencies are the same as the evoked potential of the optic tectum in Rhode Island chickens (Crampton and Boggs 1959).

Furthermore, chicken oscillatory potentials (OPs) are thought to originate from inhibitory feedback pathways in the inner retina (Wachtmeister 1998) and are similar to human OPs (Yonemura et al. 1963). However, Li et al. (1992) showed that, unlike human OPs, 6-hydroxy dopamine (6-OHDA), causes an increase in chicken OP amplitudes but reduced a- and b-wave amplitudes.

Interocular differences in ERGs are common. Inter-ocular differences in ERG parameters have been explored in humans and primates with normal differences noted to be ±13% (Rotenstreich et al. 2003), ±30% (Viswanathanm et al. 1999), respectively. McGoogan et al. 2000, demonstrated that chickens' ERGs are prone to volume conduction of the field potentials such that ERGs could be recorded at the untested contralateral eye (ERG crosstalk). To account for inter-ocular differences, Armstrong (2013) suggested that in a situation where both eyes (treated and fellow control eyes) are to be analyzed for differences, paired t-test or Analysis of Variance (ANOVA) should be employed.

Chapter 2
Introduction

2.1 Retinal Ganglion Cells (RGCs) in disease

Retinal cells, like all neural cells in the central nervous system, do not regenerate. Most human blinding diseases are results of photoreceptor or RGC dysfunction. Moreover, conditions associated with RGC dysfunctional conditions in humans are not reversible. Of these conditions, glaucoma is most prevalent globally (Pascolini et al. 2004). Also, other conditions with RGC dysfunction, such as optic nerve hypoplasia (ONH), are important for childhood blindness ((Rahi et al. 2003, Garcia-Filion and Borchert 2013). Therefore, the contribution of RGC functional measurement is important. Moreover, since these conditions cannot be cured, studies to inform the prevention of RGC dysfunctions are very relevant. More importantly, early diagnosis of these conditions is vital to management, prevention from further deterioration and/or cure. One aspect of early detection of RGCs dysfunction is the in vivo electroretinogram (ERG). ERG tests are non-invasive and are known to detect retinal dysfunction before structural damage is detectible.

2.2 Animal models of the retina

Several animal models exist for the study of RGC functions. Mostly these models use mammals such as cats, rodents, and non-human primates. The macaque monkey is frequently used because its retina is closest to humans (Viswanathan et al. 1999). However, primates are very expensive to maintain, and usually, few are available for research, thereby reducing the power needed for statistical analysis. Additionally, they are not easily amenable to genetic manipulation. Rodents are the most abundant animal model for RGC function studies. However, rodents have fewer cones and, in general, have retinae that are more suited for nocturnal vision. Chickens are inexpensive to acquire and maintain, have good visual acuity, and are diurnal. Also, the chicken has an avascular inner retina, which

eliminates ischemia as a confounding factor in RGC retinal research. Although there are anatomical differences in the retina of chickens and humans (Section 1.3), the spectral sensitivity of the chicken retina and the origin of ERG waveforms associated with glial cells might be expected to differ from that of humans. However, surprisingly, but for the UV cones, the spectral sensitivity of chick vision closely matches that of humans. ERG protocols with no UV stimulation (i.e. wavelengths above 420 nm) are well-matched to the human relative spectral sensitivity (Li et al., 1992, Schnapf et al., 1987)

Several studies of the structure (retinal histology) and function (PERG, PhNR and STR) of RGCs exist for non-human primates and rodents (Frishman et al. 2000, Bui and Fortune 2004, Porciatti 2015), but similar studies in chickens are rare. Ostrin et al. (2016) showed that it is possible to record PERGs in chickens but conclusively demonstrated that the PERG waveforms do not reflect RGC function. Petersen-Jones et al. (2010) suggested that d-waves reflect the non-spiking RGC function in white leghorn chicks.

Although a chicken's retina is cone-dominated and therefore perhaps ideal for isolating PhNRs and d-waves, a literature search showed a paucity of data relating flash ERGs to RGC function in chickens.

2.3 Objectives

This project ultimately seeks to determine the effects of ganglion cell death and dysfunction, using ONS and pharmacologic blockade with TTX, respectively, on the flash ERGs in chickens using PhNR, STR and photopic long-flash ERG protocols; specifically, it is hypothesized that ONS will cause selective reduction of PhNR and STR waveforms in the ERGs of maturing chicks and that intravitreal injection of TTX will cause selective reduction of the PhNR and the STR in the ERG waveforms recorded from the retinae of maturing chicks. In addition, it is expected that there will be a selective reduction of RGC in retinal histological cell count in ONS eyes.

2.3.1 Specific aims:

These studies aim to develop the chick model for RGC development and function.

Specifically:

1. To monitor the time course of development of light- and dark-adapted ERG luminance-response functions in the chick from hatching to 21 days post-treatment.

2. To determine the effect of ONS on light- and dark-adapted ERG waveforms in the maturing chick retina.

3. To determine the effect of TTX on light- and dark-adapted ERG waveforms in the maturing chick retina.

4. To determine the effect of ONS on long-flash ERG waveforms in the maturing chick

5. To determine the effect of TTX on long-flash ERG waveforms in the maturing chick.

Chapter 3
Materials and Methods

3.1 Introduction

This chapter describes the procedures used in the studies reported in this thesis, including procedures for inducing RGC degeneration (ONS) and pharmacological blockade of the RGCs. In addition, the procedures and protocols for the evaluation of retinal function (ERGs) are included. The procedures which are common to all study subjects are described in this chapter.

3.2 Animal protocols

All research undertaken in this thesis adhered to the standards of the Canadian Council on Animal Care. Ethical approval for the study was obtained from the Animal Care Committee of the University of Waterloo. *Gallus gallus domesticus* (White Leghorn chicken) mixed, unsexed hatchlings were purchased from Maple Leaf Poultry Foods Inc, New Hamburg, ON, Canada, for the study. The chickens were fed ad libitum with feed obtained from Jones Feed Mills Ltd, Mitchell, ON, Canada. All chicks that were 0 to 13 days old were housed in 1 meter by 1 meter heated stainless steel brooders, and floor housing was provided for 14 to 21 day-old chicks. The housing for the chicks was kept at room temperature of the building. The chicks were kept on a 12h light and 12h dark cycle throughout all studies. Both retinal dysfunction studies were initiated when chicks were 1-day old (day 0 of the experiment).

3.3 Protocol to induce RGC dysfunction: optic nerve section (ONS)

Each chick (n=18) was anesthetized with 2% isoflurane prior to and during optic nerve section (ONS) surgery. For each eye, the skin lateral to the lateral canthus was wiped with sterile alcohol pads. At the anterio-lateral orbital bone of the chick, a 4 mm vertical cut was made on the skin. Another

incision was made to the underlying fascial sheath. The eyeball was held away from the temporal orbital wall by the aid of forceps to expose the optic nerve. The exposed sheath covering the nerve was poked into using a small (3 mm) knife, and the forceps were used to enlarge the hole to expose the nerve fibers. Forceps were used to break the nerve fibers by pulling them upwards. To ensure all the fibers had been cut off, the hole was visualized, and any fiber left uncut was excised.

The contralateral eye was treated to the same procedures as above, but the nerve fibers were not cut (sham surgery). The eye designated as the treated or control eye alternated between groups, such that if the right eyes of all chicks in a group for surgery were chosen as the treated eyes, the left eyes of the next group of chicks for surgery would be chosen as the treated eyes.

3.4 Protocols to induce RGC dysfunction: intravitreal injection of tetrodotoxin

A different set of chicks (n= 14) were used for the tetrodotoxin (TTX) intravitreal injection procedure. Each chick was anesthetized as described above. Then, using a Hamilton syringe, 10 µl of phosphate buffer solution (PBS) containing 1.0 µg of TTX was injected into the vitreous of the treated eye. A preliminary experiment showed that this concentration (0.1 µg/ul) of TTX causes the desired blockade in each age group of the chicks. The contralateral sham eye was intravitreally injected with 10 µl of the PBS vehicle only. The injections were repeated 1 hour before every ERG procedure.

3.5 Verification of optic nerve / RGC dysfunction

To ascertain whether the optic nerve section procedure was successful, each bird's pupillary reaction were tested (one eye at a time) a day before ERG testing for the ONS group. Pupils were tested one hour after injection for birds in the TTX group. The bird was restrained, and its beak placed on a head holder whilst a custom-made lid retractor was used to open the eyelid. The left eye was patched with

a black electrical tape. A pen torch was then switched on to elicit the pupillary test on the right eye. The light was directed to the center of the pupil. The pupillary response was then noted. The pupillary reaction of the left eye was similarly tested with the right eye patched. The pupillary test is known to be an effective indicator of whether ONS treatments (McBrien et al., 1995, Wildsoet and Wallman, 1995, Wong-Riley et al., 1989a) or TTX treatments on the eye (Wildsoet and Wallman 1995) were successful, indicated by fixed dilated pupils when stimulating the treated eyes, and light-induced pupillary constriction in the control eyes.

To further confirm the success the optic nerve dysfunction procedure, each chicken for this study went through visual tracking testing. Each bird was restrained with their heads allowed to move freely and placed on a stage with the untested eye occluded. The chicken was then presented at the central view with the OKN Strips IOS application on an Iphone 6 (Apple, Cupertino, USA), which produced a moving 0.2 cpd (cycle per degree) white and 100% black square wave grating placed 5 cm away from the chicken eyes. The stripes moved nasal to temporal first and from temporal to nasal. The success of the treatment procedure is indicated by the absence of visual tracking of the chicken in the treated (dysfunction) eye and the presence of the tracking movement in the sham (control) eye. In all cases, the ONS/TTX injections were successful.

3.6 Chicken retinal histology procedure

Six chickens were used for histological analyses (6 optic nerve-sectioned eyes and their contralateral sham eyes). The ONS and sham procedure was as described in Section 3.3. The enucleated eyes were cut at the ora serrata. The vitreous and the anterior portion were removed, leaving the posterior eye cup. The eye cup was fixed in 4% paraformaldehyde (w/v) with 3% sucrose (w/v) in 0.1M Sorensen's sodium buffer (23.996 g/L NaH_2PO_4, 23.392 g/L Na_2HPO_4 in deionised water; pH of 7.5) for 20 mins. The tissue was then washed in 0.1M Sorensen's buffer for 5 min. The washing was

repeated for 3x and the eyecup was then cryoprotected in 30% sucrose (w/v) in 0.1M Sorensen's buffer for 12 hrs. Optimal cutting medium (VWR CA27900-2460) was used to embed the eyecups in 22 mm by 22 mm molds, before freezing. The eye cups were sectioned at 12 µm using a cryotome (Leica CM 1900 UV). The sections were mounted on Superfrost™ Plus glass slides (VWR CA CA48311-703) and air-dried before being stored in a -20°C freezer.

Only tissue from the central retina was used for the histological staining. After washing with PBS (3x in 5 minutes), slides were stained with 4',6-diamidino-2-phenylindole (DAPI; 1M, 5 min; Invitrogen, Walthon, MA, USA) to label the retinal cell nuclei. Slides were mounted with antifading mountant and a cover slip before viewing under a microscope.

3.6.1 Microscopic Imaging, and histology analyses.

An upright fluorescence deconvolution microscope (Zeiss Axio Image.Z2) was used to capture the microscope images. The image of the chicken central region of the retina was taken at 20x magnification. The total number of cells at GCL layers was counted across the retinal image length. Cells within one cell diameter away from the GCL were counted. For the INL,100 µm wide rectangular boxes containing the entire INL height were superimposed on each image, and all cells located in or touching the box were counted.

The cells were counted three times and averaged. The averaged cell count from the treated and control eyes were statistically analyzed with paired t-test. $P \leq 0.05$ was considered statistically significant.

3.7 Measurement of visual function: Electroretinograms (ERGs)

For both the ONS and TTX groups, ERGs were recorded on day 0, prior to any treatment, then again 3, 5, 7, 10 and 21 days after the day 0 recording. To anesthetize the chick cornea, one drop of proparacaine (Alcon Inc., Mississauga, ON, Canada) was applied to each cornea. After 1 minute, one

drop of vecuronium bromide mixture (VB) was applied to the cornea to dilate the chick's pupil. The contents of the VB were 3 mg/ml of vecuronium bromide (Sigma-Aldrich, Oakville, ON, Canada), 1% methylcellulose (Sigma-Aldrich, Oakville, ON, Canada), and 0.13% benzalkonium chloride (Sigma-Aldrich, Oakville, ON, Canada). One drop of VB was applied every minute for a total of four drops in each eye. The chick was then injected with a combination of ketamine/xylazine (53.3 mg/kg and 5.3 mg/kg, respectively).

After anesthesia induction (no movement upon prodding), the chick's head was placed in a head-holder that was attached to a heated platform. A sterile lubricant, Cellulvisc (Allergan Inc., Irvine, CA, USA), was applied to a temperature probe which was placed in the bird's rectum to monitor the well-being of the bird. The back of the head was wiped with a sterile alcohol pad. Two platinum needle reference electrodes (Diagnosys LLC., Lowell, MA, USA), one for each eye, were inserted under the skin of swabbed areas 3 mm from the lateral eye canthus of each eye. Eyelids were kept open with custom-made lid retractors. To keep the cornea moist, one lubricating drop of Celluvisc was placed on each cornea before placing a custom-made loop electrode on the cornea.

The light stimulation and ERG recording system used in this study was an Espion E2 with AC amplifier (Diagnosys LLC., Lowell, MA, USA). The light was delivered through two mini-ganzfeld stimulators (Espion ColorBurst units, Diagnosys LLC., Lowell, MA, USA). The ganzfeld stimulators were placed such that eyes were equally illuminated. For each eye, the ganzfeld was consistently placed such that the eye of the chick was at the center, and the plane of the opening was parallel to the surface of the chick's eye. ERGs for the treated eyes and the control eyes were tested simultaneously in each bird to avoid confounding effects, such as anesthesia (Choh et al., 2017) on ERG variability from sequential testing.

3.8 Stimulus protocols for ERG studies

3.8.1 Dark-adapted ERG stimulus-response series

Full-field, dark-adapted ERGs were recorded bilaterally. All dark-adapted ERGs were recorded in the night-time to activate the chicken rods (Schaeffel et al. 1991). Before the ERG recordings, the chick was dark-adapted for an additional 20 min to negate any effect of the dim red illumination. Each dark-adapted ERG was recorded for 500 ms after the flash, with a 50 ms pre-trigger, and a sampling frequency of 1000 Hz with a band pass of 0.1 to 1000 Hz. The stimulus was a 4 ms full-field white flash on dark background. White flash was generated using the three sets of narrow band light-emitting diodes (LEDs) incorporated in Colorburst® mini ganzfeld stimulators. The central wavelengths of the LEDs were 650 nm (red), 588 nm (Green), 524 nm (amber) and 470 nm (blue), respectively, and the equivalent colour temperature of the white stimulus was 6500°K.

Following a no-stimulus baseline measure, flash stimuli were incremented in 0.25 log unit steps from -5.25 log cd.s/m^2 to the maximum available stimulus of +1.50 log cd.s/m^2 (Table 1). All measurements, regardless of adaptation state, are expressed in photopic units as is conventional in clinical electrophysiology (McCulloch and Hamilton, 2010, McCulloch et al. 2015).

The dark-adapted ERG parameters measured were the amplitudes and implicit times of nSTR, pSTR, a- and b-waves. Implicit times were measured to the maximum or minimum of the peaks or troughs, and amplitudes were either from baseline or peak to peak described in Table 1. Oscillatory potentials were simultaneously isolated from the dark-adapted ERGs to 3.0 cd.s/m^2 stimuli. A separate channel with a band pass frequency filter of 50-300 Hz was used to isolate the OPs, as done in a similar study in chicken flash ERGs (Ostrin et al. 2016).

Table 1: The stimulus parameters and ERG waveform measurements for dark-adapted ERG luminance-response series.

Step range	Type of ERG	Log steps	Range of flash strength cd.s/m^2	ERG parameters recorded	No. of sweeps	Measurement of Amplitude
1	No ERG	NA	No flash	No parameters	20-30	
2-11	Scotopic Threshold Responses	0.25	-5.25 to -3.0 log	nSTR	20-40	From the baseline to STR trough.
18-21	Scotopic ERGs	0.25	-2.25 to -1.75 log	DA standard b-wave	10-20	From the to the peak of the DA standard b-wave.
22-31	Scotopic ERGs	0.5	-1.25 to +1.5 log	a-, b-waves and OPs	3-6	a-wave: from the trough to baseline b-wave: from the trough of a-wave to the peak of b-wave OPs: RMS between 5 to 55 ms

3.8.2 Light-adapted ERG stimulus-response series

For the light-adapted ERG protocols, the dilated eyes were light-adapted to 30 cd/m^2 white light for 10 minutes using the mini ganzfeld stimulators. Full-field, light-adapted, ERGs were recorded simultaneously from both eyes to 4 ms long-wavelength (red) flashes (LED with peak λ = 650 nm) in increasing flash luminance from 0.1 to 8 cd.s/m^2 on a rod suppressing short-wavelength (blue) background (LED peak λ = 462 nm) of 30 cd/m^2. Table 2 shows the stimulus parameters. Each ERG

was recorded from a pre-triggered baseline of 50 ms to 500 ms after the flash, with a sampling frequency of 1000 Hz. An in-built analogue 60 Hz notch filter from the ERG system was used to reduce line frequency noise. Each step of the ERG protocol had at least 4 flash stimulus sweeps, which were then averaged as one result. For weak stimuli, up to 10 flashes were averaged to determine whether a detectable ERG was present.

Table 2: The stimulus parameters and ERG waveform measures for the light-adapted ERG luminance-response series.

Step range	Type of ERG	No. of sweeps	Range of flash strength (log cd.s/m^2)	ERG parameters recorded	Measurement of Amplitude
1	No ERG	≥5	No flash	-	-
2-9	Light adapted ERGs	≥5	-0.1 to 0.75 in log steps of 0.25	a-wave b-wave PhNR	a-wave: from trough to baseline b-wave: from a-wave trough to peak the of b-wave PhNR: from the trough of PhNR to the peak of b-wave

The parameters measured for this light-adapted ERG protocol were a-wave, b-wave, and PhNR. The Espion E2 software (Diagnosys LLC., Lowell, MA, USA) was set to autodetect the trough of the a-wave, PhNR, and the peak of the b-wave based on maximum and minimum points in pre-selected post-stimulus time ranges. These were manually adjusted after reviewing the ERG waveforms and deleting any sweep contaminated by artifact. The amplitude of the a-wave was measured from the baseline to trough of a-wave, the b-wave from the trough of the a-wave to the b-wave peak and the

PhNR was measured from the peak of the b-wave to the trough of the PhNR. The implicit time of all the parameters was measured from the mid-point of the stimulus flash to the peak of the ERG wave. The amplitude and implicit time of the ERG parameters were then exported as Microsoft Excel files (Richmond, Washington, USA) for statistical analysis.

3.8.3 Long-flash ERGs

Full-field, long-flash (150 ms) ERGs were recorded bilaterally. The filters, sampling frequency and the pre-trigger and post-trigger times were matched to the protocol for recording the light-adapted ERG stimulus-response function (section 3.8.2). However, the long-flash duration and luminance matched those of the ISCEV extended protocol (250 cd/m^2 flashes on 30 cd/m^2 background), but the wavelengths determined in pilot study 2 (section 4.2) were used. The parameters measured were onset a- and b-waves, and offset d-waves. The responses to at least 5-10 long-flash stimuli were averaged and analyzed. The bilateral ERGs were recorded on day 0 (baseline, no treatment) and again on days 3, 5, 7, 14 and 21 post-baselines for the ONS (8 birds) and TTX (5 birds) groups on day 0 and again on days 3, 14 and 21 post-baselines.

The amplitude and implicit time of the onset a- and b-waves were analyzed as above (section 3.8.2, Table 2), and the offset d-wave amplitude was measured relative from the offset point (at 150 ms) to the peak of the d-wave onset as shown in Figure 6.

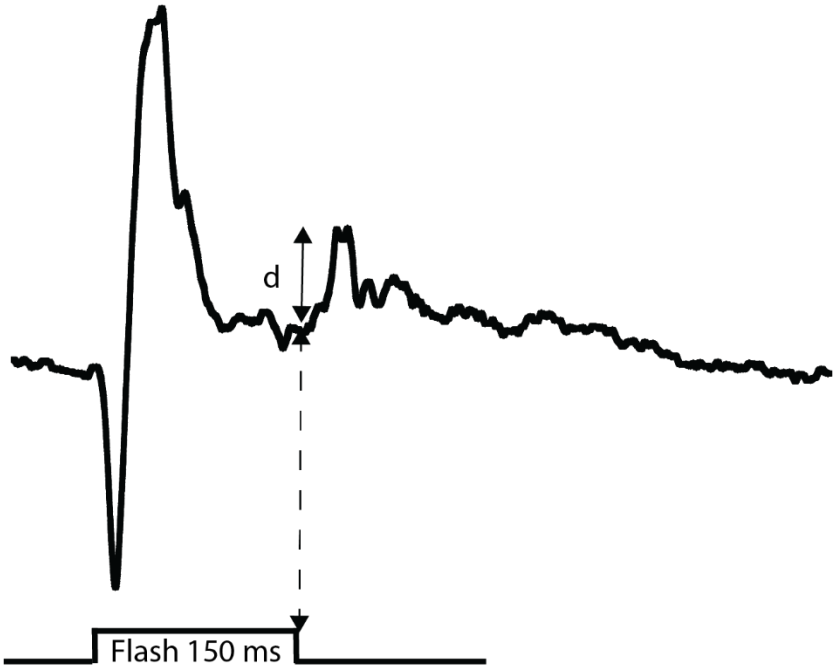

Figure 6: Chicken long-flash ERGs, showing how the d-wave amplitude parameters were measured. d, denotes the measurement of the amplitude from the off-set point to the first peak of d-wave.

3.9 Data analysis of ERGs.

Dark- and light-adapted a-, b-wave amplitudes and implicit times, light- and dark-adapted OPs, light-adapted d-wave amplitudes and implicit times were measured and analyzed. For each luminance-response series, some overall parameters were derived using the appropriate curve-fitting methods.

Curve fitting for the stimulus-response series for the amplitudes of the dark-adapted a- and b-waves, the light-adapted b-wave and the PhNR were fitted using least-squares curve fitting based on the ERG measurements for each subject at each time point. For these measurements, parameters were obtained through curve fitting using the Naka-Rushton curve:

$$= \frac{(\mathrm{Vmax}^{n} * \mathrm{I}^{n})}{(\mathrm{I}^{n} + \mathrm{K}^{n})}$$

where V is the amplitude in response to a flash strength of I, Vmax is the maximum (saturated) amplitude, K is the sensitivity (flash strength at half Vmax) and the slope parameter (n) is constrained to 1 (Hamilton et al. 2007).

R-studio set codes (Appendix E) was used for the least-squares fitting of Naka Rushton curves. In all cases of fitting $R^2 \geq 0.88$ or better was used. The set of codes was written by Vivian Choh, PhD and was modified by the candidate with permission. The Naka-Rushton equation of the dark-adapted a- and b-waves, as well as the light-adapted b-wave and PhNR, was constrained to a slope (n) of one (1) as suggested by Hamilton et al (2007) and as used in some chick stimulus-response fittings by Montiani-Ferreira et al. (2007). Following the suggested protocols of Severns and Johnson (1993) and Joshi et al. (2017), the Naka Rushton curve was used to fit all points of the ERG stimulus-response function, unless the last point of the function showed a decline following saturation or a sharp rise of the secondary rising function (Figure 7).

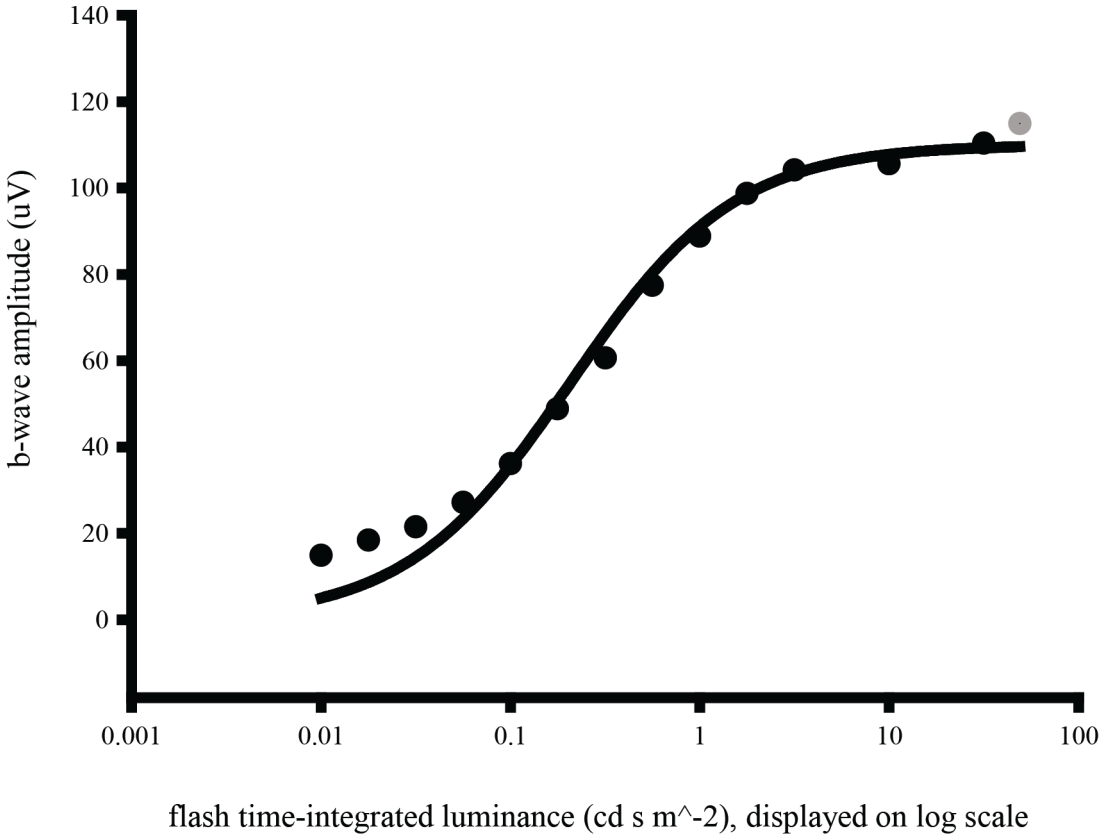

Figure 7: A representative fitting of the Naka-Rushton curve to a dark-adapted b-wave amplitude stimulus-response curve. The dark line indicates the part of the curve that was fitted, and grey point was excluded as part of the secondary rising phase (Severns & Johnston 1993).

All light-adapted a-waves did not saturate at higher light intensities, and therefore were fitted with a power function:

$$V = a * i^b$$

where V is the light-adapted a-wave amplitude in response to the stimulus intensity, i, and a and b are parameters of the curve fit. In all cases, $R^2 \geq 0.88$ was considered a good fit. The light-adapted

amplitude used for the analysis was interpolated from the individually fitted power functions for a stimulus of 3 cd.s/m^2.

The extracted OPs amplitudes were quantified by calculating the root mean square (RMS) of signals within 5 to 55 ms post-flash. A similar method was used in the study of OPs by Wang et al. (2015) and Gur et al. (1987).

The data were analyzed using GraphPad Prism version 9 software (GraphPad Prism Inc, San Diego, USA). The differences between eyes at baseline (day 0) for the chicken ERG data were analyzed a priori using paired t-tests.

Two-way repeated-measures analysis of variance (ANOVA) was used to analyze the main effects of treatment (sham vs treatment) and age (maturation period of 3,5,7,14 and 21 days post-treatment), and the interaction (treatment x age) on NK-Rushton parameters (Vmax, K) of DA a- and b-waves and LA b-waves and PhNR), interpolated a-wave (for light-adapted a-wave) or long-flash ERG parameters as well as implicit times. In the event of missing data (as was the case in all the ONS studies), the two-way repeated ANOVA method was replaced by mixed-effects modelling. If the assumption of sphericity was violated, Greenhouse-Geisser corrections were used.

Bonferroni test was used for post-hoc comparison. $P \leq 0.05$ was considered statistically significant for all statistical tests in this study.

Cohen's d, d, was used for post-hoc power analysis to estimate the effect size of the main effect of treatment:

$$d = \frac{\mu_1 - \mu_2}{\sqrt{[(\sigma_1^2 + \sigma_2^2)/2]}}$$

Where μ is the pooled mean and σ is the pooled standard deviation of the main effects in sham, 1, and treated, 2, eyes. As used by Ahmadieh et al. (2021) on ERG parameters, the Cohen's d can be grouped into "negligible" (d<0.2), small (0.2<d<0.5), medium (0.5<d<0.8), large (d>0.8).

3.9.1 Sample size calculations

Due to the paucity of data on the effect of ONS on ERGs in birds, the sample size arrived at for this study was based on the rat model (L Alarcon-Martinez et al. 2009) and non-human primate model (Viswanathan et al., 1999) where at least 40% of STRs or PhNR were lost after RGC loss respectively. However, since there are notable differences in chicken and rodents' and primates' retinae, power calculations were based on a more conservative value for the expected difference of 25%. Two-tailed analysis with an expected change of 25%, a standard deviation of 10%, alpha of 0.05 and power goal of 0.80 gave the sample size of 4 (minimum), calculated using Statistica V8 software (Statsoft Inc, Tulsa, OK). Hence, this study uses a minimum of n=4 birds for statistical analysis with 80% power to detect amplitude differences of 25%.

The sample size calculation for the histology work is based on work done in our lab (Chong et al. 2013) with chicks which shows n=3 for statistical analysis with 80% power to detected differences.

3.9.2 One-tailed or two-tailed analysis.

For this thesis, two-tailed statistical analysis was employed throughout. Currently, several studies point to the fact that stress on RGC could lead to either reduction of ERG amplitudes (Bui and Fortune, 2004, Viswanathan et al., 1999) or its enhancement (Choh et al., 2016, Tan et al., 2018). Since the effect of RGCs dysfunction could go either way, a two-tailed statistical analysis was performed throughout this study.

Chapter 4
Pilot studies

4.1 Introduction

Because of the paucity of ERG data in chicks using coloured LED stimuli, preliminary experiments were carried out to inform the stimulus selection for the main studies.

4.1 Pilot study 1: Effect of intra-orbital bleeding on subsequent light-adapted ERGs in chicks

Purpose: It has been documented that the blood supply to the retina affects the ERGs of some species (Block and Schwarz, 1998). Little is known of the effect of the blood supply in chicken ERGs. Moreover, some surgeries in this study resulted in intra-orbital bleeding; therefore, a pilot study was undertaken to determine if chickens with intra-orbital bleeding should be excluded from the study.

Methods: Light-adapted ERGs were recorded on day three post-ONS, using eyes of chicks with sham surgery performed at 1-day after hatch that bled during the procedure (n=5) or that did not bleed (n=5) during the procedure. The setup for the surgery and ERG is as described in section 3.3, and the setup for the ERGs was as described for light-adapted ERGs (section 3.8.2), except that stimuli were white flashes (3.0 cd.s/m^2) on a white (30 cd/m^2) background.

Results and discussion: Three days after surgery, there were no differences in the amplitudes of the a-waves ($p = 0.88$, t-test) or b-waves ($p=0.980$, t-test) or implicit times (a-wave: $p = 0.73$; b-wave: $p = 0.69$) between the ERGs of sham-treated eyes in chicks that had intra-orbital bleeding and those that did not.

Conclusion: No further exclusion criteria relating to surgical bleeding were added to the study. Each eye of chicks with and without intra-orbital bleeding was included in all cohorts.

4.2 Pilot study 2: Comparison of broad spectrum and long wavelength stimuli for chick dark-adapted ERG luminance-response series

Purpose: To determine the normal chick scotopic threshold response (STR) and dark-adapted ERG luminance-response series. Since the long-wavelength light activates mostly chicken retinal cones, a comparison of the ERGs from white light and red light could be used to indicate the differences of the cone ERG waveform from the rod ERG waveform.

Method: The same procedure (section 3.8.1) for recording bilateral dark-adapted ERGs was used for 3 normal (not treated) birds day 15 post-hatch. An additional red flash ERG luminance-response series using the long-wavelength LED flash stimulus (peak output of 650 nm) matched to the white stimuli for photopic time-integrated luminance up to the maximum of log -1.75 cd.s/m^2 was conducted a day after the white flash dark-adapted ERGs series but within the same time (midnight to 3 am). A description of the ERG waveforms of the left eye was reported.

Results: For all chicks, there was no detectable dark-adapted ERG for very dim white or red flashes (-6 to -4 log cd.s/m^2). For red and white flashes from -3.5 to -2.25 log cd.s/m^2, ERGs tended to be small, below 4 μV. For both red and white light stimuli of -2.75 log cd.s/m^2 and stronger, chicks show no negative going ERGs but a positive going ERGs. With increasing stimulus strength, the positive ERG waveform showed increasing amplitudes and shorter implicit times. The response from the white light showed larger amplitudes at lower thresholds as compared responses to red stimuli, which appeared smaller and double peaked. A typical dark-adapted luminance-response series for red and white flash stimuli is shown in Figure 8.

Conclusion: Although the ERGs recorded between -3 and -2.25 log cd.s/m^2 were small and most were below the 4 μV, it could be observed that such responses were real because they had low noise levels with averaging and the amplitude increased with stronger stimuli.

The positive waveforms of these near threshold ERGs to flash stimuli of -2.5 log cd.s/m^2, -2.25 log cd.s/m^2 and -2 log cd.s/m^2 were considered the dark-adapted b-wave and were averaged for this analysis. White light stimuli were used for the dark-adapted ERGs in the main studies because white light produced larger amplitudes and single peaks, facilitating analysis.

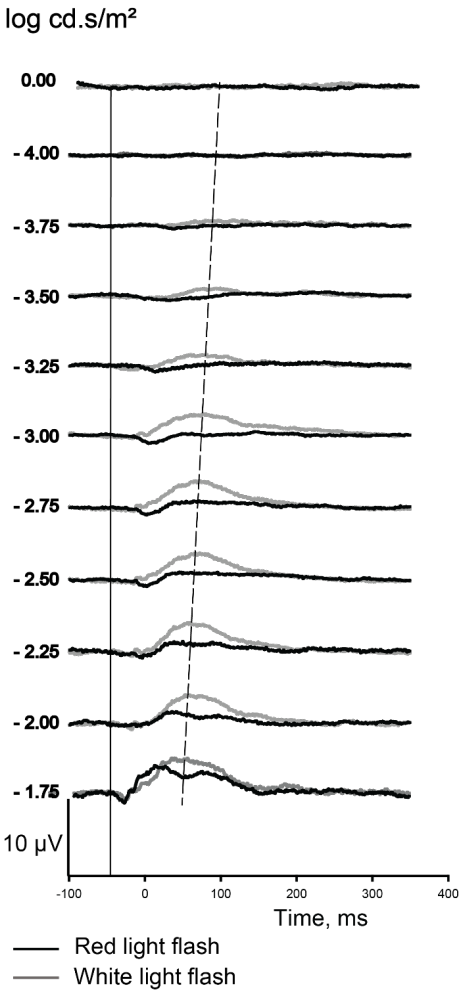

Figure 8: A representative dark-adapted ERG luminance-response series to weak white- and red-flash stimuli.

Dark-adapted ERGs to red (black line) and white (grey line) stimuli from a chick at day 15 (white flash) and day 16 (for red flash series). The dotted line shows decreasing implicit time of the DA standard b-wave to white flash, while the vertical line depicts the midpoint of the 4 ms flash stimuli.

4.3 Pilot study 3: Effects of spectral characteristics and duration on light-adapted ERGs in chicks

Purpose: To inform the spectral characteristics and flash duration for stimulus selection for the light-adapted ERG studies.

Methods: Bilateral ERGs were recorded using the standard preparation and anesthesia (see section 3.7) for four hatchlings with no other experimental intervention (normal) when they were 14- and 21-days post-hatch. Following 10 minutes of light-adaptation, different stimulus combinations were used to determine suitable spectral characteristics for the flash stimulus and the 30 cd/m^2, background illumination for brief flash ERGs. Additional studies were conducted to determine a suitable duration for long-flash stimuli using the same normal chicks. The parameters for the flashes used for the light-adapted brief and long-flash ERG pilot studies are presented in Table 3.

The mini-ganzfeld stimulators were supplied with three sets of narrow band-width LEDs, with peak outputs at a long wavelength (650 nm, 'red'), a medium wavelength (510 nm. 'green') and a short wavelength (462 nm 'blue'). All stimuli were calibrated using photopic spectral sensitivity. The three LED sources could be combined. Balanced output from the three sets of LED simulated broad spectrum (white) light. Long and medium wavelength outputs were combined for yellow stimuli. The strength of brief flashes was controlled by a combination of power to the LEDs and flash duration, as shown in the Table 3.

Table 3: Stimuli and background illumination for light-adapted ERGs.

Comparison	Type of ERG	Stimulus strength	Stimulus/ Background*
Spectral combination	Brief-flash	1 cd.s/m2	White on White Red on Blue Green on Blue Yellow on Blue
Spectral combination	Long-flash 150 ms	250 cd/m2	White on White Red on Blue Green on Blue
Stimulus duration	Long-flash 5, 10, 30, 50, 100, 150 and 200 ms	250 cd/m2	Red on blue
Stimulus luminance	Brief-flash	0.3, 1, 3, 5 & 8 cd.s/m2	Red on blue

* All light adapting backgrounds were 30 cd/m^2 for 10 minutes.

Results: The light-adapted, 1 cd.s/m^2, brief flash ERG waveforms using the different spectral combinations in Table 3 (top row) are shown in Figure 9. The series shows that ERGs to a red flash stimulus on blue rod-suppressing background elicit the largest amplitudes for a- and b-waves. This was also found for the oscillatory potentials and i-waves.

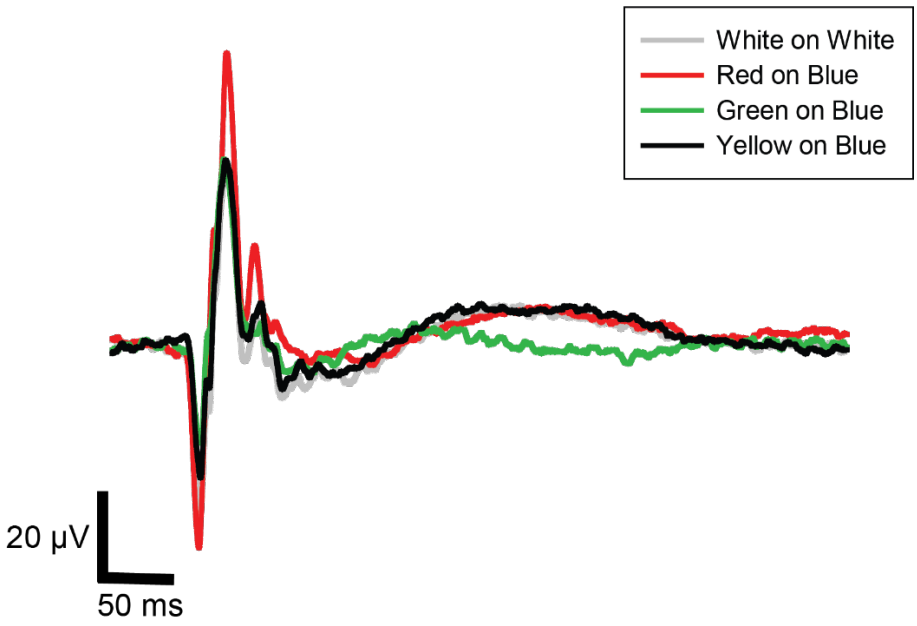

Figure 9: Representative ERGs from an untreated eye of a 14-day-old chick are shown for brief 1.0 cd.s/m² flashes with 30 cd/m² backgrounds using different spectral combinations (Table 3).

ERGs for the long-flash stimuli of 250 cd.s/m^2, using different spectral combinations, are shown in Figure 10. The series shows that the ERGs in response to the red, long-flash stimulus on blue background provide the largest amplitudes and a well-defined offset d-wave. To determine the stimulus strength to use for the long-flash ERG studies, the 250 cd/m^2 gave a larger d-wave compared with those for 25 and 700 cd/m^2; the difference reached significance ($p = 0.04$) compared to the 25 cd/m^2 stimulus (Figure 11).

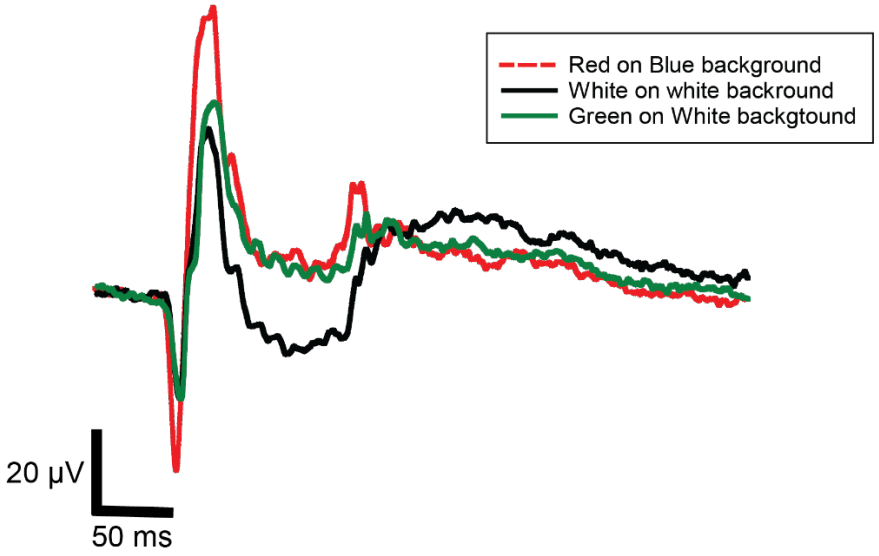

Figure 10: Representative ERGs for the long-flash stimuli of 150 ms at 250 cd/m^2, using the different spectral combinations in Table 3.

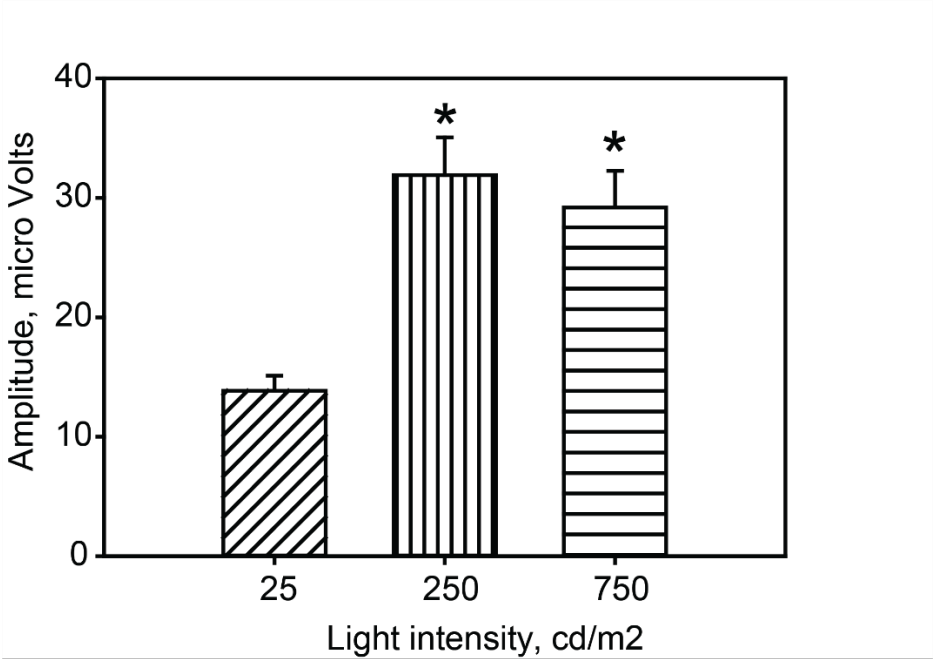

Figure 11: The d-wave amplitude is shown for 4 normal chicks to different luminance levels for a long-red flash stimulus.

The ERGs were recorded on 4 chicks who were 14-day post-hatch. * amplitudes (p = 0.04) were higher for stronger stimuli, (250 cd/m^2 and 750 cd/m^2) than those for the long-flash stimulus of 25 cd/m^2 stimulus.

For flashes from 5 to 20 ms duration the onset and offset ERG waveforms overlapped and could not be independently distinguished (Figure 12). For red-on-blue long-flashes, the onset and offset ERGs were clearly separated for long-flashes exceeding 20 ms (Figure 12).

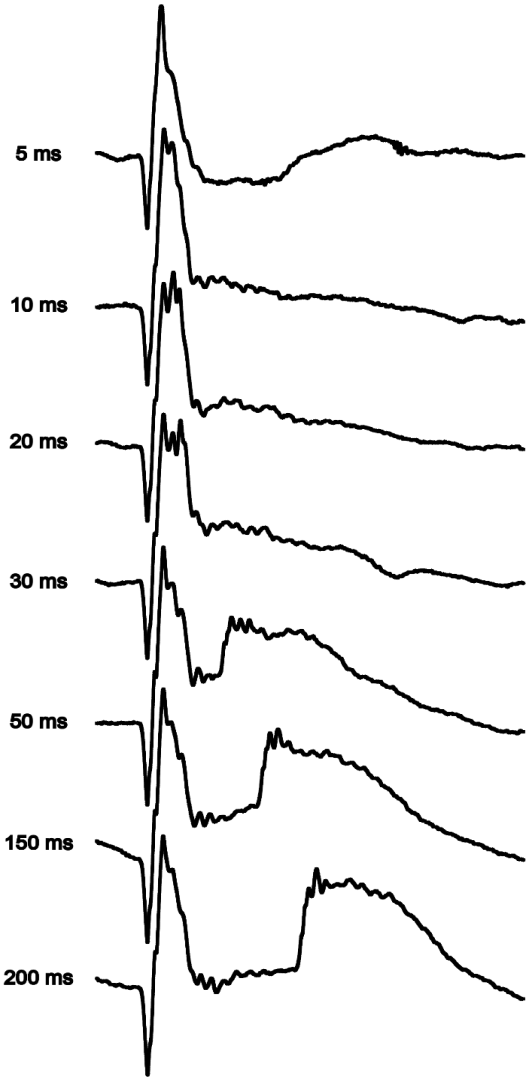

Figure 12: A representative long-flash ERG series from a normal chick recorded 14 days post- for stimuli with different stimulus durations (5 - 200 ms) using 250 cd/m^2 blue flash on a 30 cd/ m^2 blue background.

Conclusions: Red flash (1 cd.s/m^2) on a blue (30 cd/m^2) background was chosen as the stimulus combination to measure the PhNR in the main light-adapted ERG study. For the long-flash ERGs studies, the red on blue stimulus was chosen with the red flash luminance of 250 cd/m^2. The duration

of 150 ms was chosen to match the ISCEV extended protocol, although only the very short durations (< 30 ms) caused overlap between the on- and off-responses.

4.4 Summary of final method

Following the pilot studies, the protocols for the main were fixed. These are summarized below along with a synopsis of the methods from Chapter 3.

4.4.1 The effect of ONS on chicken flash ERGs and retinal histology

Histology

For the histology, six chickens with ONS treated (n=6) and contralateral sham (n=6) eyes were used. The total number of cells at GCL layers and INL layers were counted across the Z-stack images of the chicken central region of the retina.

ONS and ERGs

The optic nerve sectioning (ONS) and electroretinograms (ERG) procedures were performed as described in sections 3.2 to 3.5, 3.7 and 3.8**.** Separate ONS birds were used to collect the dark-adapted and light-adapted ERGs (ONS total: n=22). The ERGs done on the chickens were grouped into dark-adapted (DA) ERGs (DA standard b-wave, dark-adapted a- and b-waves, n=6), light-adapted (LA) ERGs (a-, b-waves, PhNR, n=10), long-flash ERGs (a-, b-, and d-waves, n=8). From the pilot 2 study, data from 0.01 cd.s/m^2 stimuli were analyzed as the DA standard (0.01) b-wave, and DA b-wave curve fitting used responses to 0.01 cd.s/m^2 and stronger because responses from below 0.01 cd.s/m^2 stimuli were below noise level in some cases. The sample size of each group is as shown in Figure 13. The minimum sample size of 4 was calculated as described in section 3.9.1. Each study was carried out on separate birds except for the light-adapted ERGs groups, for which two birds in the ONS group (Figure 13) were used for both brief and long-flash light-adapted ERGs. For the optic nerve-sectioned

(ONS) group of birds, ERGs were collected when the bird were one-day old, just prior to ONS surgery (refers to 0 post-treatment), and again at days 3, 5, 7, 14 and 21 post-treatment.

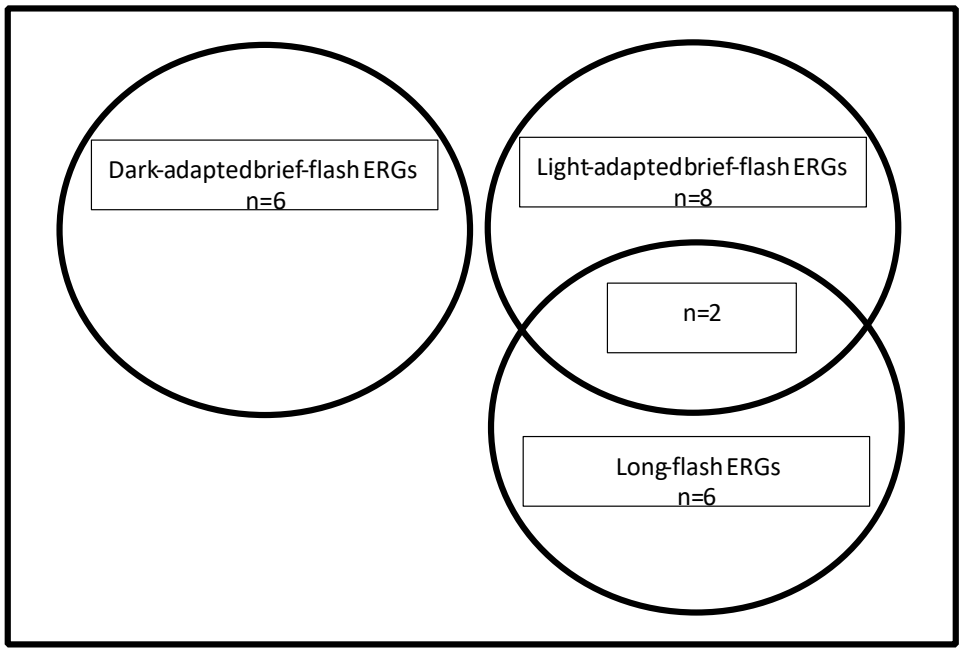

Figure 13: A Venn diagram outlining the number and distribution of birds in the ONS experimental groups.

4.4.2 The effect of TTX on chicken flash ERGs

Similar to 4.5.1, the intravitreal injections of TTX and ERG procedures were performed as described in 3.2 to 3.5, 3.7 and 3.8**.** Separate TTX birds were used to collect the dark-adapted and light-adapted ERGs (total TTX: n=14). The ERGs done on the chickens were grouped into DA ERGs (n=5), LA ERGs (n=5), long-flash ERGs (n=6). From the pilot 2 study, only data from 0.01 cd.s/m^2 stimuli was analyzed for DA standard (0.01) b-wave because the response from below 0.01 cd.s/m^2 stimuli was below noise level. Each study was carried out on separate birds except for the light-adapted ERGs groups, for which two birds in the TTX group were used for both brief and long-flash light-adapted

ERGs. The sample size of each group is as shown in Figure 14. The minimum sample size of 4 was calculated as described in section 3.6.1. For the intravitreal injection of TTX groups of birds, ERGs were collected when the birds were one day old, prior to the first intravitreal injection of TTX (refers to 0 post-treatment), and again at days 3, 5, 7, 14 and 21 after the first injection (post-baseline). The injection was repeated before each ERGs as described in section 3.4.

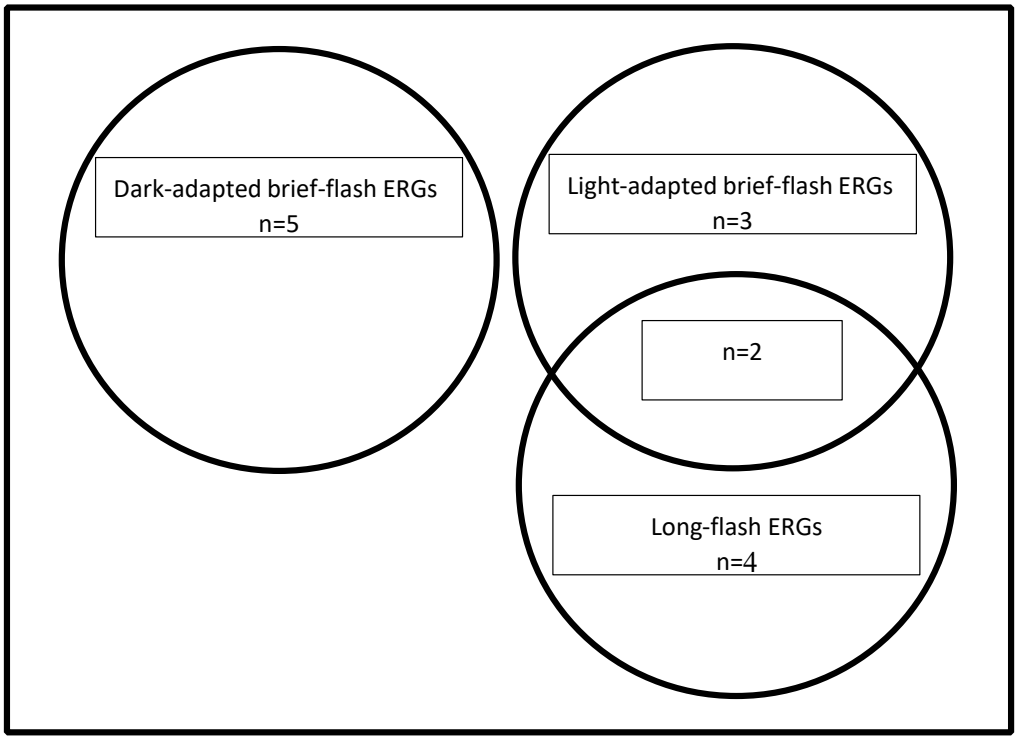

Figure 14: A Venn diagram outlining the intravitreal injection of TTX experimental groups and the distribution of birds.

4.4.3 Effect of ONS or TTX on long-flash light-adapted chicken ERGs

For the long-flash ERGs studies, the red on blue stimulus was chosen with the red flash luminance of 250 cd/m^2. The duration of 150 ms was chosen to match the ISCEV extended protocol, although only

the very short durations (< 30 ms) caused overlap between the on- and off-responses. A total of 8 birds for ONS and 5 birds for TTX groups were used for this study.

Chapter 5

Effect of Optic Nerve Section (ONS) on the chicken ERGs and retinal histology.

5.1 Introduction

The chapter covers the results of the experiments to determine the effect of disrupting retinal function using optic nerve-section surgery (ONS). The focus of this thesis is to find out whether ERG waveforms known to detect RGC deficits in humans can also detect RGC deficits in chickens.

5.2 Effect of the ONS on chicken retinal histology

To determine the effect the ONS on the chicken retinal GCL (ganglion cell layer) and INL (inner nuclear layer), the retina from 6 ONS chicks sacrificed at 21 days post-treatment were analyzed. Retinal tissues were stained with DAPI, and the retinal images were analyzed (see section 3.6 and 3.6.1). Images of the retina of the treated (ONS) and sham control eyes are shown in Figure 15, where these representative images show the depleted RGC cells from the ONS eye**.** The cell count across an average length of 464 µm of the GCL layers (Figure 16**)** of the ONS treated eyes were fewer than those of the sham treated eyes (52.2 ± 18.9 vs 245.3 ± 34.9 cells/mm, respectively; $p < 0.0001$, N=6, paired t-test).

For the INL (Figure 17), the cell count for 6 pairs of eyes sampled in 100 µm wide boxes that span the depth of INL (average of three counts) were similar for the treated and sham ONS eyes (numbers, $p=0.41$, N=6, paired t-test). ONS selectively induced reduction of cells in the chicken GCL with a mean loss of 78.7% of the nuclei in the GCL. However, the cell counts in the INL were not different, and showed no effect of ONS versus sham surgery on the INL thickness or cell density.

A

B

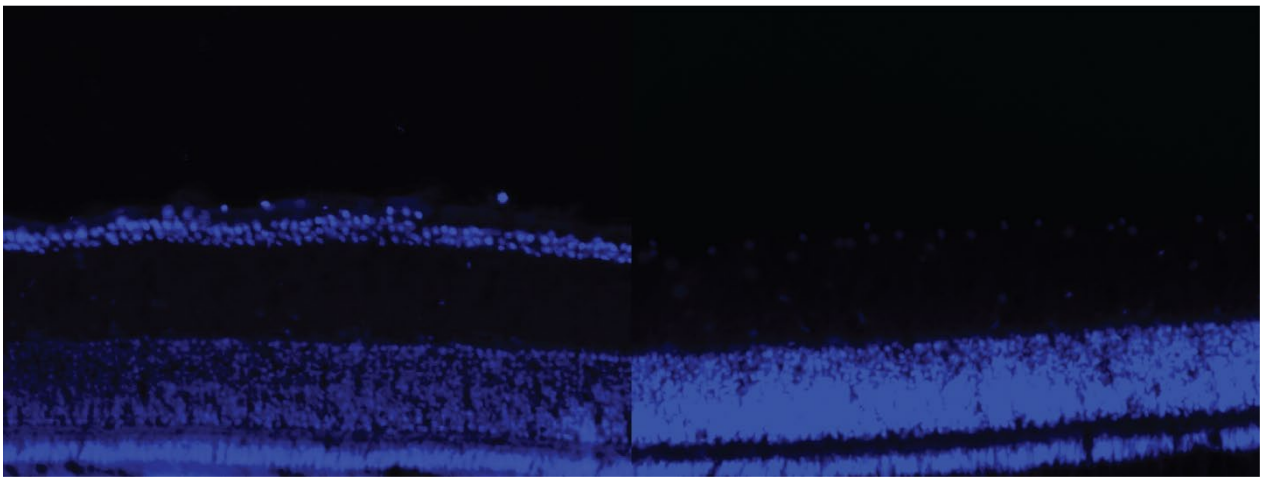

Figure 15: Representative retinal sections labelled with DAPI (blue nuclear stain) of sham-ONS (A) chicken retina and ONS (B) chicken retina.

The figure shows depletion of cells in the GCL of the ONS eye and a clear GCL in the sham eye.

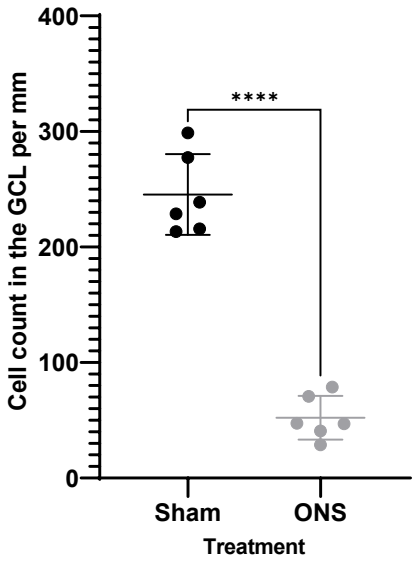

Figure 16: Total number of cells in the ganglion cell layer (GCL) across a 464 µm image of the optic nerve sectioned (ONS) and sham treated chicken eyes.

Mean, error bars showing standard deviation and dots showing the average counts from individual eyes. The **** denotes p-value of ≤ 0.0001 (n=6).
The figure shows that there were fewer cells in the GC layer of the ONS eye than in the sham treated eyes of the same chicks sacrificed on day 21 post-ONS and sham treatments.

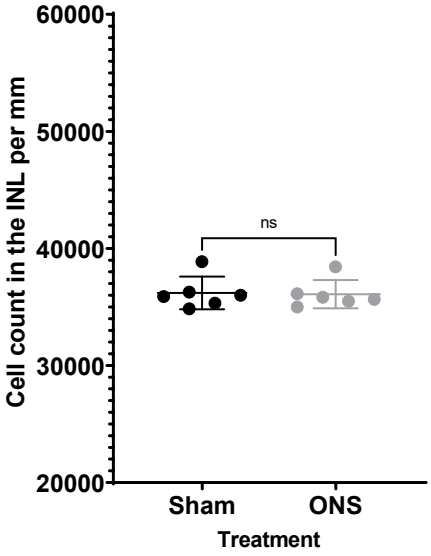

Figure 17: Total number of cells in the inner nuclear layer (INL) per mm of the optic nerve sectioned (ONS) and of sham operated chicken eyes with the mean, error bars showing standard deviation and dots shows the averaged counts from individual eyes.

The ns denotes no statistical difference (n=6). The figure shows that there were similar number (p=41) of cells in the INL in the ONS and sham eyes.

5.3 Survival for the ONS chicks for ERGs.

Three birds (1 for dark-adapted group and 2 light-adapted group) were lost on day 10 post-ONS. Therefore, complete dark-adapted (DA) ERG results are reported for 5 birds along with results up to 7 days for the one additional bird. For light-adapted (LA) ERGs, complete results are reported for 8 birds along with results up to 7 days for the two additional birds.

5.4 Dark-adapted ERGs of ONS chicken eyes

5.4.1 Effect of ONS on chicken dark-adapted b-wave amplitudes in response to 0.01 cd.s/m^2 (DA 0.01 b-wave).

The DA b-wave ERG amplitudes in response to 0.01 cd.s/m^2 stimuli (DA 0.01 b-wave) was similar (paired t-test, $p = 0.21$, N=6) between the pre-treated eyes at day 0 (baseline). The ONS and sham treated fellow eyes (treatment) were not different (main effect of treatment: $p=0.59$, Cohen's d, $d=0.36$, N=6), suggesting that ONS does not affect the DA 0.01 b-wave ERG amplitudes. A main effect of age was detected ($p=0.003$). Bonferroni's post-hoc showed increasing amplitudes between day 3 post-treatment to 7 ($p=0.02$) and decreasing amplitudes between days 14 to 21 post-treatment ($p=0.04$; Figure 18). There was no significant interaction of age and treated eyes on the DA 0.01 b-wave ($p=0.78$).

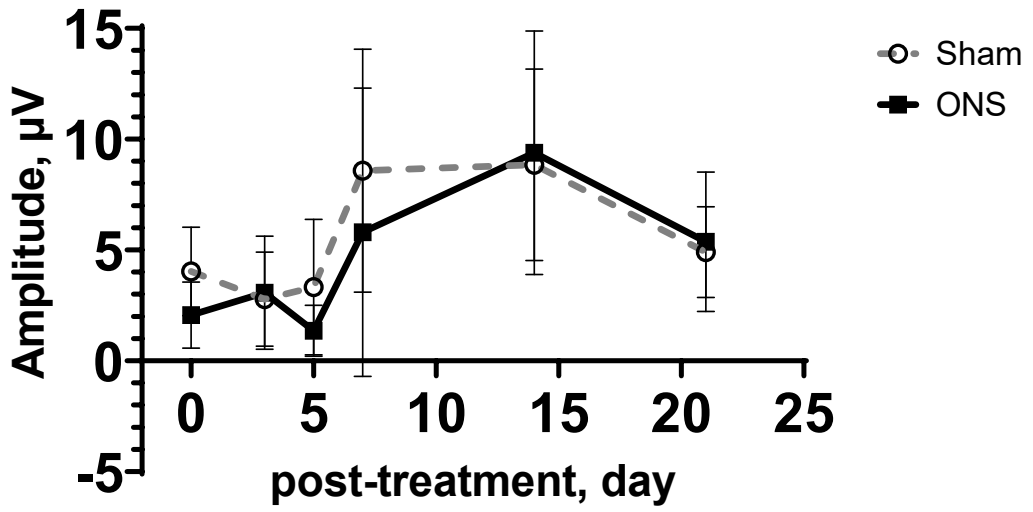

Figure 18: Mean dark-adapted Standard b-wave amplitudes ± SD of ONS-treated eyes and sham fellow eyes at 0, 3, 5, 7, 14- and 21-days post-treatment (n=6 chicks)

Stimuli were 4ms DA flashes of 0.01 cd.s/m^2. Note that on day 0 neither eye had undergone treatment. A main effect of maturation (age) was detected (p=0.003).

5.4.2 Dark-adapted oscillatory potentials of ONS chicken eyes

OPs were clearly recorded from all chick eyes, as shown in Figure 19. The RMS amplitudes between 5 and 55 ms for the 3 cd.s/m^2 white flash stimulus are shown in Table 4. The pre-treatment eyes (day 0) were not different (p=0.34, N=6) in OP amplitude. There was no significant difference (p=0.51) between the sham and ONS eyes (Table 4), as demonstrated by the inconsistent trend of the lower RMS amplitudes for the ONS eyes on days 3, 7 and 21 but higher RMS amplitudes for days 5 and 21 post-treatments compared with the sham eyes (Table 4). There was no significant difference of the OP RMS amplitudes across the days post-treatment, age (p=0.51), nor the interaction of treatment and age (p=0.35).

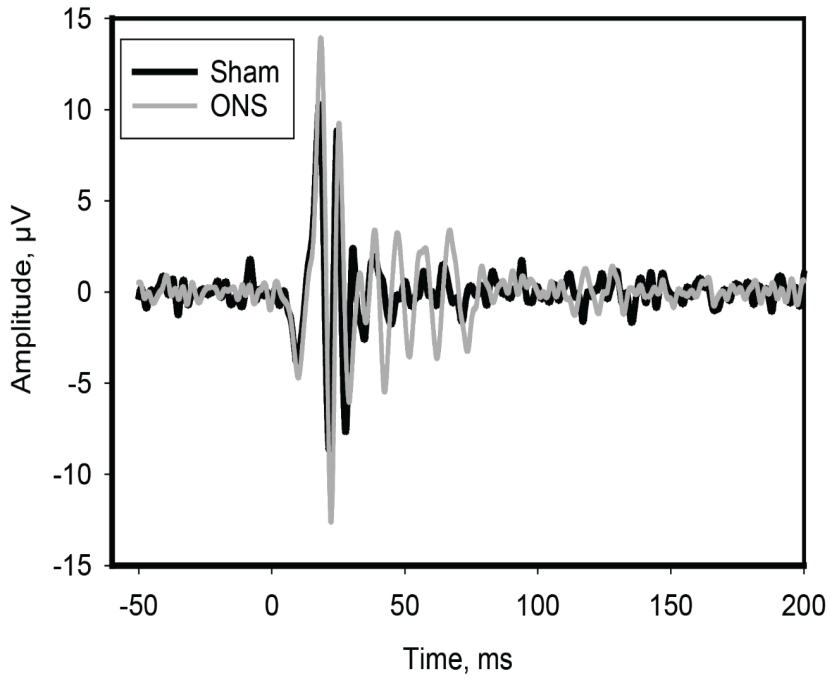

Figure 19: Representative DA oscillatory potentials of optic nerve-sectioned and sham eyes of the same chick.

The chick was 14 days post-ONS. Stimuli were brief flashes of 3.0 cd.s/m^2.

Table 4: Table of RMS amplitude of dark-adapted OPs to 3.cd.s/m^2 flash in the ONS groups of chickens.

Days post-treatment N=6	Treatment, Mean RMS amplitude ± SD, µV	
	Sham	ONS
0 (pre-treatment)	31.60 ± 16.68	31.21 ± 6.08
3	37.21 ± 10.42	36.84 ± 6.16
5	24.14 ± 3.55	28.38 ± 6.54
7	33.95 ± 14.88	28.78 ± 12.54
14	35.76 ± 9.88	32.10 ± 12.50
21	31.19 ± 9.76	33.04 ± 9.80

For the DA OPs, this study of 6 pairs of chicks' eyes had the power (80%) to detect a difference in RMS amplitude of 10.0 µV ($p<0.05$).

5.5 Dark-adapted a- and b-waves of ONS chicken eyes

The DA saturated amplitudes (Vmax of the amplitudes fit with the NK equation) for the DA-ERG a-waves are illustrated in Figure 20. The pre-treated eyes (day 0) ERG data showed no significant differences in DA a-wave Vmax ($p=0.34$, $N=6$) and IT ($p=0.74$, $N=6$). The ONS did not affect the Vmax or the interpolated IT at 3 cd.s/m^2 of the DA a-waves (Vmax: $p=0.38$, $d=0.32$; IT: $p=0.52$, $d=0.32$) post-treatment (3 to 21 days). The a-wave amplitudes of both eyes generally grew (not significant) till day 14 and dipped (not significant) on day 5 and 21 post-treatment, while the IT of the eyes generally reduced across the period, but there was no main effect of age between 3- and 21-days post-treatment (a-wave: $p=0.051$, $d=0.48$, IT: $p=0.11$) and no interaction (a-wave: $p=0.11$, IT: $p=34$)

The Vmax of DA b-wave amplitudes and interpolated ITs showed no significant difference between the untreated eyes at day 0 (b-wave Vmax: $p=0.49$, IT: $p=0.10$) and no significant change of between the post-treated eyes (amplitude: $p=0.98$, $d=0.03$; IT: $p=0.22$, $d=42$). There was no significant difference in both eyes with age ($p=0.12$), as demonstrated by the inconsistent trend of the Vmax amplitudes of both eyes dipping on days 5, and 21 but increasing on days 3, 7 and 14 post-treatments (Figure 21). Similarly, the IT of the interpolated b-wave of both eyes did not change significantly across over the period of the experiment (IT: $p=0.90$). There were no interactions of age and treatment in either the Vmax or IT (b-wave: $p=0.21$, IT: $p=0.27$).

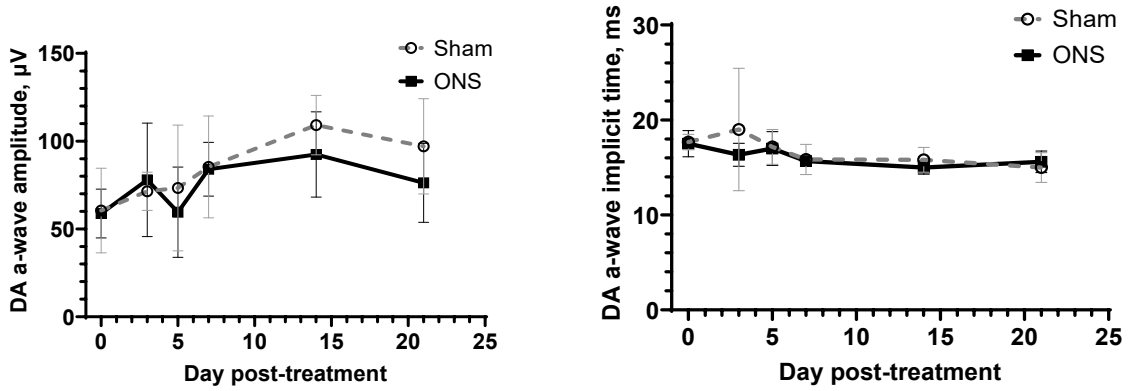

Figure 20: The time course of dark-adapted a-wave saturated amplitudes (Vmax), and implicit time interpolated at 3 cd.s/m^2 without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-treatment in treated chicks (n=6), for DA ERG luminance series from 0.0562to 31.6 cd.s/m^2.

The point is the mean and error bars standard deviation (SD). The figure shows no statistical significance in Vmax or IT for DA a-waves.

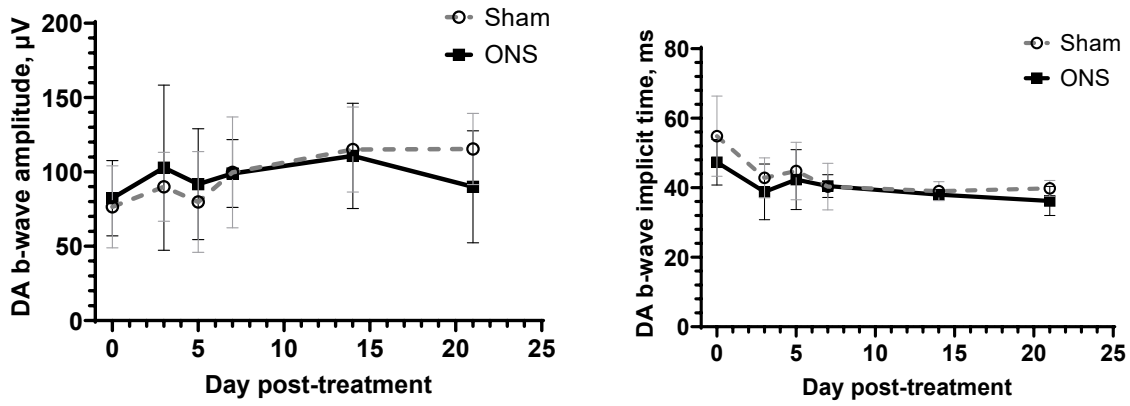

Figure 21: The time course of dark-adapted b-wave saturated amplitudes (Vmax), and implicit time interpolated at 3 cd.s/m^2 without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-treatment in treated chicks (n=6), for luminance 0.0562 to 31.6 cd.s/m^2.

The point is the mean and error bars standard deviation (SD). The figure shows no statistical significance in Vmax or IT for DA b-waves.

For the NK Rushton sensitivity parameter, K, the dark-adapted a-waves reached half of Vmax for stimuli below the strength of the standard flash (mean (from log values) K = 1.74 cd.s/m^2). B-waves were more sensitive, with K values for half Vmax at an average of 0.360 cd.s/m^2 (converted from the mean of log values). Sensitivity, K values, for DA a- and b-waves showed no differences between eyes on baseline day 0 (a-wave: p=0.45, b-wave p=0.13), showed no effect of the ONS on K for either a-waves (a-wave: p=0.17, d=0.60) or b-waves (p=0.25, d=0.35) between the treated and the sham eyes as illustrated in Table 5. In addition, sensitivity did not change significantly with age for a-wave: p=0.51 but changed for b-waves (p=0.02) between 3- and 21-days post-ONS. No interaction of treatment and age was detected for both a-wave (p=0.44) and b-wave (p=0.52).

Table 5: Naka-Rushton equation sensitivity, K (flash strength at half Vmax) of dark-adapted a- and b-waves of chickens in the ONS group.

Post-ONS Time, Days N=6	**Mean DA a-wave sensitivity, K (cd.s/m^2) ± SD, µV**	
	Sham	**ONS**
0 (pre-treatment)	2.05 ± 1.07	1.69 ± 0.37
3	1.17 ± 0.21	1.26 ± 0.57
5	1.57 ± 0.64	1.25 ± 0.64
7	2.34 ± 1.38	1.52 ± 0.76
14	2.39 ± 0.51	2.47 ± 0.78
21	2.47 ± 0.75	1.64 ± 0.72
Post-ONS Time, Days n=6	**Mean DA b-wave sensitivity, K (cd.s/m^2) ± SD, µV ***	
	Sham	**ONS**
0 (pre-treatment)	0.49 ± 0.23	0.42 ± 0.12
3	0.21 ± 0.10	0.32 ± 0.05
5	0.41 ± 0.21	0.39 ± 0.09
7	0.43 ± 0.16	0.42 ± 0.09
14	0.31 ± 0.08	0.19 ± 0.08
21	0.41 ± 0.09	0.49 ± 0.09

* A main effect of maturation (age) was detected (p=0.003) for b-wave but not a-wave.

5.6 Effect of ONS on chick light-adapted ERG

Over the 21-days for all the light-adapted (LA) ERGs, the a- and b-waves were clearly recordable in all eyes for flashed from 0.3 to 5.6 cd.s/m^2 (Table 2). Figure 22 shows representative Standard LA 3.0 ERGs. For the LA standard ERGs, were no differences in the amplitudes between the treatments (p=0.30, d=0.49 and p=0.33, d=0.22 for a-and b-wave amplitudes, respectively), nor were there differences with maturation (p=0.16 and p=0.19, for a-and b-wave amplitudes, respectively) nor were there any significant differences in interactions (p=0.82 and p=0.60, for a-and b-wave amplitudes, respectively)

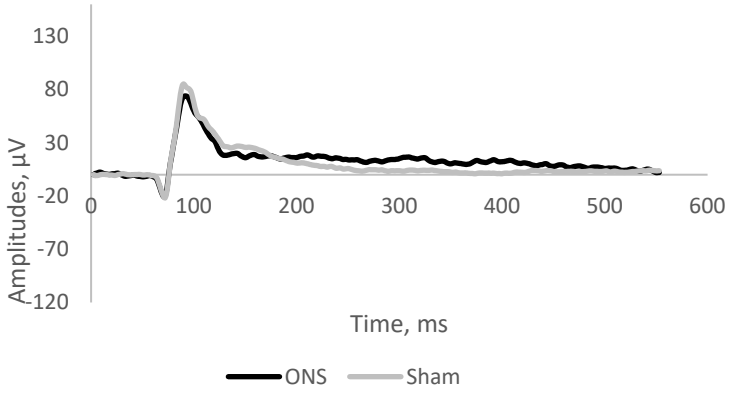

Figure 22: Representative LA Standard ERGs from Sham and ONS eyes of a five-day post-ONS chicken.

ERGs stimulus was a 3.0 cd.s/m^2 white flash on a 30 cd/m^2 background.

At pre-treatment day 0 (1-day post-hatch), the retinal function of the chickens' right and left eyes were similar for all parameters measured (p>0.05). The ONS did not affect the LA implicit times (interpolated at 3 cd.s/m^2) of the a- or b-waves (p=0.39, d=0.21 and p=0.63, d= 0.12, for a-and b-

wave interpolated ITs, respectively). Figure 23 shows the LA ERG results for power function interpolation of a-waves at 3 cd.s/m^2 and for Vmax for LA b-waves in ONS and sham treated eyes across the age range. The LA a-wave interpolated amplitude and b-wave Vmax of both eyes appears increased (not significant) with maturation except for a transient decrease (not significant) at day 7 post-treatment, but these changes in the amplitude of both eyes were not significant over age (p=0.24 and p=0.30, for a- and b-wave ITs, respectively). There were no interactions of the two (p=0.61 and p=0.91, for a- and b-wave ITs, respectively). The sensitivity NK Rushton equation parameter, K, (flash luminance resulting in half Vmax) of light-adapted b-waves (Table 6) were not statistically different between the treated and the sham eyes (ONS: p = 0.50, d=0.13), nor was there an effect of age (p=0.08), nor the interaction between eye and time (p=0.46).

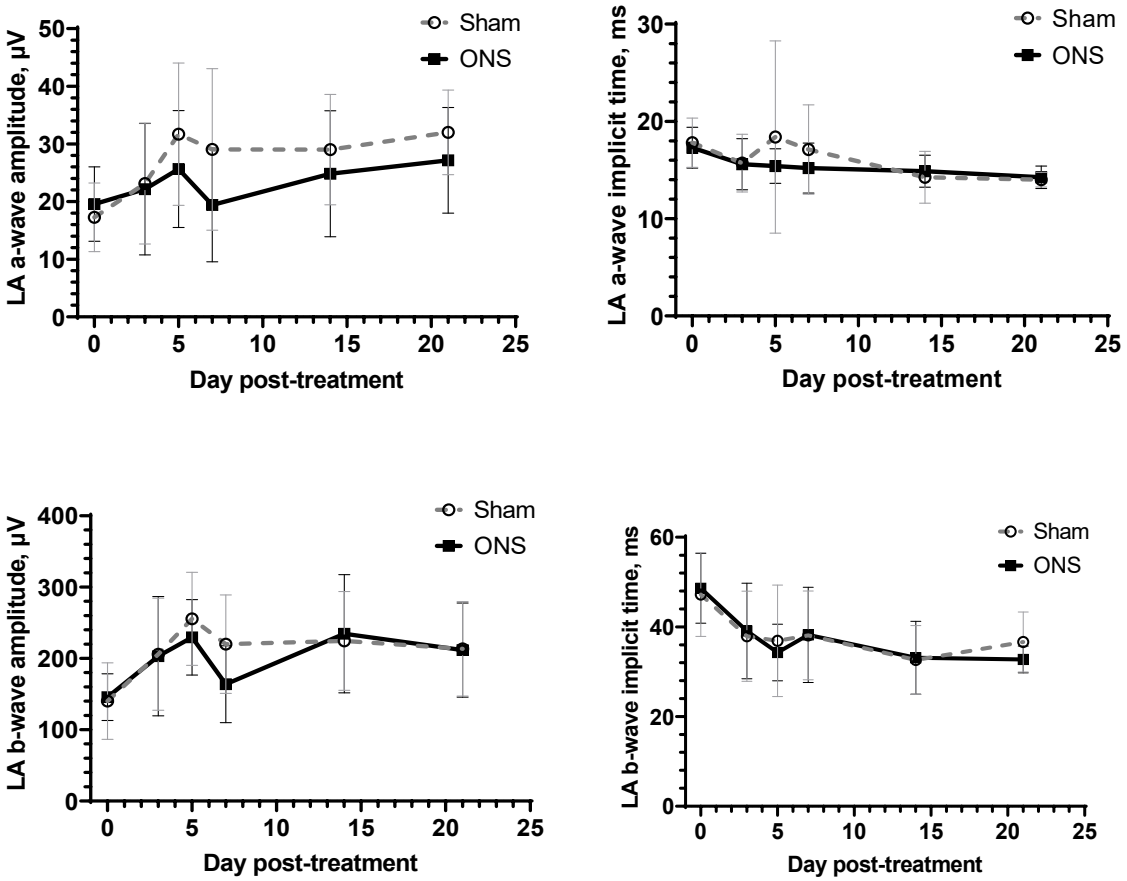

Figure 23: The time course of light-adapted a-wave amplitude interpolated at 3.0 cd.s/m^2, saturated b-wave amplitudes (Vmax), and implicit times for a- and b-waves at 3 cd.s/m^2 without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-treatment in ONS treated chicks (n=10), for an ERG luminance series with stimuli ranging from 0.0562 to 5.6 cd.s/m^2.

The point is the mean and error bars standard deviation (SD). The figure shows no statistical significance in LA a-wave amplitude interpolated at 3.0 cd.s/m^2 and a-wave IT and LA b-wave Vmax or IT

Prior to treatment, there was no difference in the Vmax of the PhNR (Figure 23), (p=0.90) nor in the PhNR IT (p=0.32) between sham treated and ONS eyes prior to treatment (day 0). There were no significant differences in Vmax or implicit time, IT, between sham and ONS treated eyes (Vmax: p=0.92, d=0.18; IT: p=0.13, d= 0.31). Figure 23 shows the saturated amplitudes, Vmax, of the PhNR measured to the b-wave and ITs to the PhNR trough. While Vmax of the ONS eyes increased till day 5 post-ONS and decreased marginally from 7 to 21, the control sham eyes dropped by 15% at day 7 and 21 post-treatment. However, these changes were not significant by age (Vmax: p=0.42, IT: p=0.10) and there were no interactions between age and treatment (Vmax: p=0.23, IT: p=0.71). No differences in PhNR sensitivity (Table 6), pre-treated eyes (p=0.09), in the post treated eyes (p=0.17), by age (p=0.44), nor was there an interaction between age and treatment (p=0.70) for sensitivity (K value). The results, therefore, suggest that chicken PhNR does not reflect RGC functions.

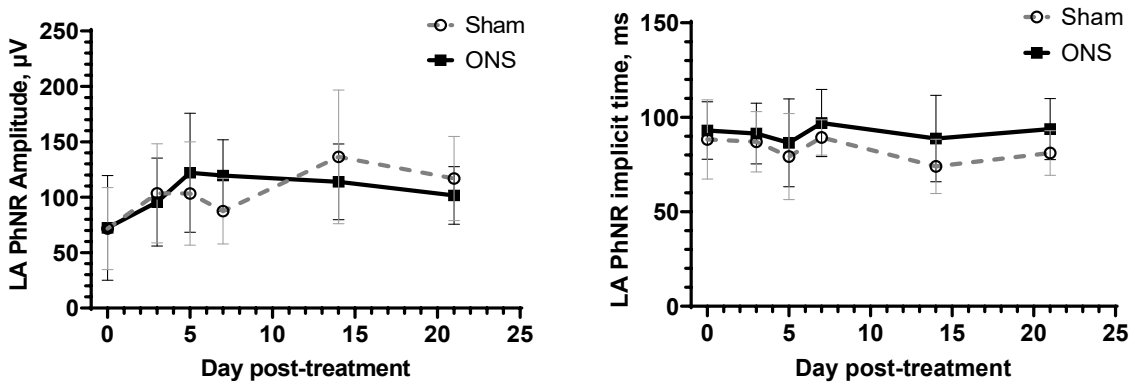

Figure 24: The time course of light-adapted PhNR saturated amplitudes (Vmax), and implicit time at interpolated at 3 cd.s/m^2 without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-treatment in treated chicks (n=10), for luminance 0.0562 to 5.6 cd.s/m^2.

The point is the mean and error bars, standard deviation (SD). The figure shows no statistical significance in Vmax or IT for LA PhNR.

Table 6: Sensitivity, K (luminance at half Vmax), of light-adapted b-wave and PhNR of ONS group chickens.

Post-ONS Time, Days n=10	**LA b-wave sensitivity, K (cd.s/m^2) ± SD**	
	Sham	**ONS**
0 (pre-treatment)	1.97 ± 0.05	1.92 ± 1.26
3	1.87 ± 0.43	1.80 ± 0.99
5	1.37 ± 0.92	1.50 ± 0.89
7	1.37 ± 0.79	1.47 ± 0.98
14	1.22 ± 0.56	2.38 ± 0.92
21	0.92 ± 0.37	1.23 ± 0.82
Post-ONS Time, Days n=10	**LA PhNR sensitivity, K (cd.s/m^2) ± SD**	
	Sham	**ONS**
0 (-pre-treatment)	1.52 ± 0.13	1.64 ± 0.19
3	1.24 ± 0.20	1.16 ± 0.17
5	1.03 ± 0.12	1.06 ± 0.20
7	1.00 ± 0.13	0.90 ± 0.18
14	0.72 ± 0.19	1.90 ± 0.90
21	0.40 ± 0.05	0.97 ± 0.39

5.7 Effect of ONS on chick long-flash ERG amplitudes and implicit time

The long-flash (250 cd/m^2) ERG onset waveforms were clearly recordable in all eyes at all ages. Neither amplitudes nor implicit times of the onset a-waves differed between the ONS and sham treated eyes, and there was no effect of age nor an interaction between age and treatment (pre-treatment p=0.56, treatment; p=0.38, age: p=0.69 or interaction: p=0.67). Similarly, no differences in the onset b-wave amplitudes were found by pre-treatment (p=0.46), by treatment (p=0.48), by age (p=0.07), nor was there an interaction between treatment and age (p=0.93). Indicating that the ONS did not affect the photoreceptors and bipolar cells between the eyes and over the period of studies.

The offset d-waves measured from the treatment at offset were clearly recordable in all eyes. D-wave amplitude (mean 64.29 μV ±14.35 μV) was approximately half of the amplitude of the onset b-waves for the same stimulus. Figure 25 shows there was steady rise of d-wave amplitude to day 7, which then plateaued. The ONS eye dropped below the treatment at day 7, increased in day 14 but marginally dropped at day 21. For the offset d-wave amplitude, there was an overall a main effect of smaller amplitudes in the ONS eyes compared with the sham eyes (p=0.008), but there were no differences in amplitude by age (p=0.67) or interaction between age and treatment (p=0.45). On Bonferroni's multiple comparisons post-hoc testing for differences at specific ages, the d-wave amplitude for the ONS treated eyes was decreased significantly (p=0.04) at day 7. No difference in d-wave implicit times (mean ±SD) were found between eyes pre-treatment (p=0.34), by treatment (p=0.17), with age (p=0.12) or by interaction (p=0.22).

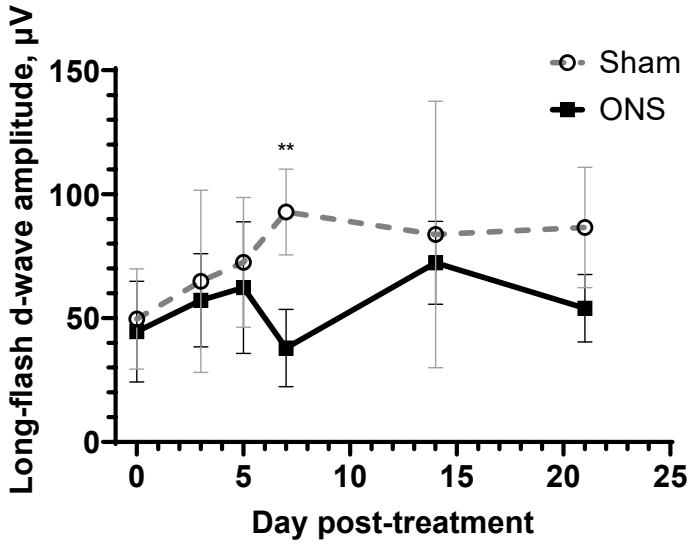

Figure 25: The time course of long-flash (250 cd/m2) offset d-wave amplitudes before ONS and 3-, 5-, 7-, 14- and 21-days post-ONS (n=8).

There is a main effect of the ONS treatment and the significant difference (p=0.04) of eye treatment on day 7 post-treatment is indicated (**).

5.8 Summary

In summary, the ONS study shows no effect of treatment on the DA standard b-wave amplitude, LA PhNR but showed treatment effect of the d-wave. Furthermore, this study also detected main effect of age (maturation) on DA standard b-wave amplitudes and Naka-Rushton equation sensitivity, K (half Vmax intensity) of the DA b-wave.

Chapter 6

Effect of intravitreal injection of tetrodotoxin, TTX, on the chicken ERGs.

6.1 Survival for the TTX chicks for ERGs.

There was no loss of birds in the TTX experiment, and repeated measures ANOVA was used to analyze the ERG data from the post-treated chickens.

6.2 Effect of intravitreal injection of TTX on chicken DA b-wave to 0.01 cd.s/m^2 stimuli.

The b-wave amplitudes of the sham and TTX treated eyes for luminance level at 0.01 cd.s/m^2 (DA 0.01 b-wave) at each of the time points, as illustrated in Figure 25. The sham b-waves amplitudes increased from day 5 post-treatment to day 14 and plateaued at 21 days. A similar trend was observed for the TTX treated eyes except for a dip (not significant) in the amplitude on day 5 and marginally at 14. At day 0 (baseline), there were no differences (p=0.21) in the DA standard b-wave amplitudes between the eyes (pre-treatment). The amplitude difference between TTX and sham injected, fellow eyes (treatment) was not significant (p=0.96, post-hoc power analysis, d=0.05, n=5). The DA 0.01 b-wave amplitudes did increase significantly with age of both eyes (p=0.009). The interactions of treatment and age of both eyes were not significant (p=0.57).

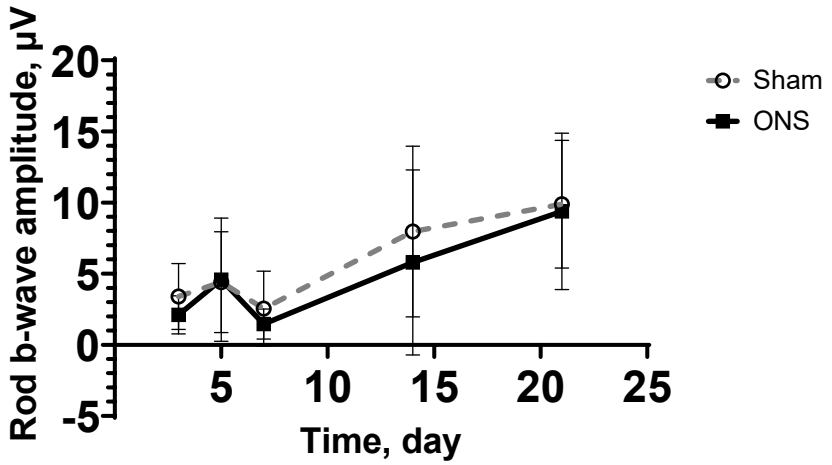

Figure 26: Dark-adapted DA 0.01 b-wave amplitudes at 0, 3, 5, 7, 14 and 21-days post-baseline is shown for five chicks with TTX treated eyes and sham fellow eyes.

Stimuli were 4ms flashes 0.01 cd.s/m2. Note that on day 0 neither eye had undergone baseline. The point is the mean and error bars standard deviation (SD). The DA 0.01 b-wave amplitudes did increase significantly with age of both eyes (p=0.009)

6.3 The effect of TTX on chicken oscillatory potential

The root mean square (RMS) of the OP amplitudes between 5 and 55 ms for the 3 cd.s/m^2 flash stimulus are shown in (Table 7). At baseline day 0 (pre-treatment), there were no differences (p=0.77) in OP amplitudes between the eyes, and there were no differences (p=0.18, d=0.24, n=5) between the treated eyes and sham eyes (treatment) across the period, 3- to 21 days post-baseline. Table 7 shows the chicken DA OPs RMS increased from day 5 to day 7 before saturating to day 21, but the increases by age were not significant (p=0.06), and there were no interactions of treatment and age (p=0.90).

Table 7: Table of RMS amplitude of dark-adapted OPs to 3.cd.s/m^2 flash in the TTX groups of chickens.

Days post-baseline	**Treatment, RMS amplitude (μV)**	
N=5	**PBS (sham)**	**TTX**
0	2.28 ± 1.11	1.89 ± 0.84
3	2.02 ± 1.43	2.05 ± 1.46
5	1.56 ± 0.59	1.92 ± 0.93
7	3.86 ± 2.62	4.11 ± 2.21
14	2.94 ± 0.41	3.03 ± 0.39
21	2.75 ± 0.43	3.08 ± 0.29

6.4 Effect of intravitreal injection of TTX on chicken dark-adapted, DA, ERG

The TTX DA-ERG a-wave saturated amplitude (Vmax) and interpolated implicit time (IT) at 3 cd.s/m^2 are illustrated in Figure 27. From Figure 27, at baseline (day 0), there were no differences in a-wave Vmax amplitudes and IT between the pre-treatment eyes (a-wave: p=0.86, IT=p=0.56, n=5). Additionally, no differences (a-waves: p=0.17, d=0.24; IT: p=0.52, d=0.31, n=5) in the post-baseline eyes across the treatment period. The a-wave amplitudes of both the sham and treated eyes appear to decrease marginally from day 0 to day 5 post-baseline but grew (observed) at day 7 before saturating at 14- and 21- days post-baseline but these changes over time were not significant (a-wave: p=0.27). The a-wave IT of both eyes generally appear to decrease (observed, not statistically significant, p=0.11) with age from post-baseline day 5 to 21. There were no interactions (Vmax: p=0.77, IT: p=0.46) between the effects of the treatment and age.

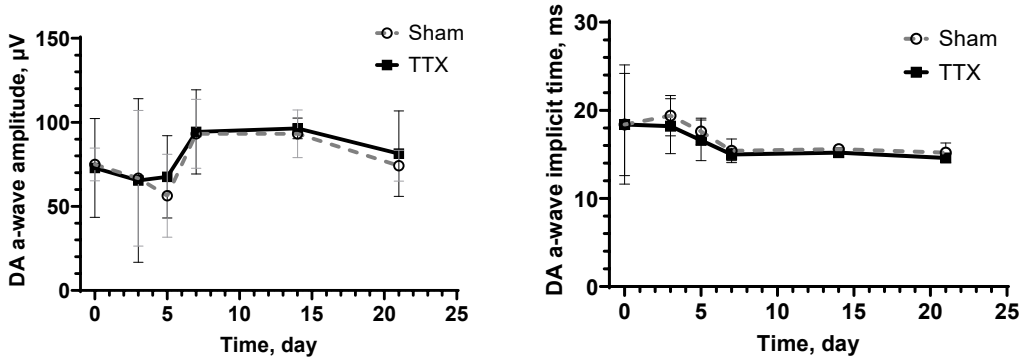

Figure 27: The time course of dark-adapted a-wave saturated amplitudes (Vmax) and IT without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-baseline in treated chicks (5 TTX), for luminance 0.0562 to 31.6 cd.s/m^2.

The point is the mean and error bars standard deviation (SD). The figure shows no statistical significance in Vmax or IT for DA a-waves of the treated eyes.

The DA b-wave Vmax amplitude of untreated baseline eyes (p=0.03) differed between the eyes that was subsequently treated with TTX and those that became sham eyes, although the b-wave IT was same (p=0.52), but Vmax or implicit times (IT) of the b-waves did not change as a function of treatment (DA b-wave Vmax: p=0.69, d=0.06, IT: p=0.36, n=5). The pattern of b-wave ERGs (saturated amplitude and implicit time) changes with age followed a similar observed trend as the a-wave amplitudes, except for decreased saturated amplitudes of the treated (TTX) eyes at day 5 and a dip (not significant) in the IT at day 3 post-baseline (Figure 28) but the observed changes with age were not significant (DA b-wave: p=0.28, IT: p=0.31) as well, there was no interaction of age and treatment (amplitude: p=0.50, IT: p=0.37).

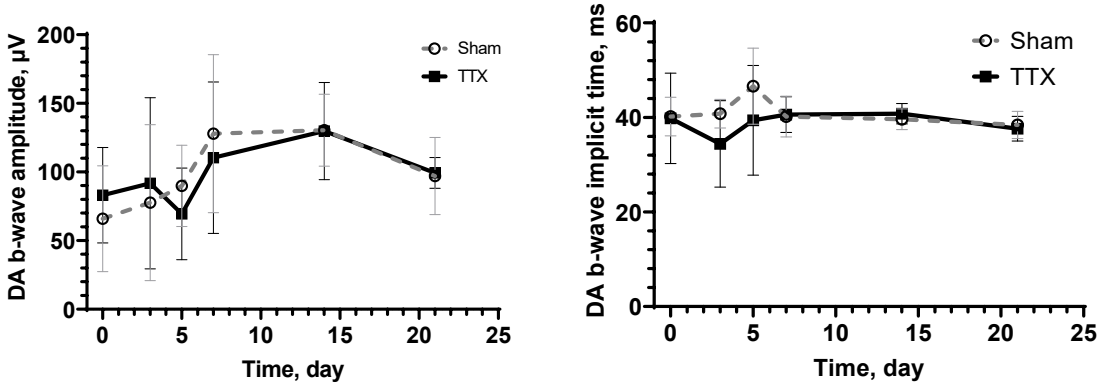

Figure 28: The time course of dark-adapted b-wave saturated amplitudes (Vmax) and IT without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-baseline in treated chicks (5 TTX), for luminance 0.0562 to 31.6 cd.s/m^2.

The point is the mean and error bars standard deviation (SD).

The NK Rushton sensitivity parameter, K, of the dark-adapted a-waves and b-waves baseline ERG data shows no differences between the untreated eyes at day 0 (p>0.50), and no differences in the K

values for the treated and sham eyes (a-wave: p=0.59, d=0.11; b-wave: p=0.64, d=0.07, n=5). From the Table 8, the sensitivity, K, appears to decrease with age except for day 3- and 5- post-baseline for a- and b-waves of the two eyes, respectively, but the changes with age were not significant (a-wave: p=0.34; b-wave: p=0.16). The interactions of baseline and age (a-wave: p=0.94; b-wave: p=0.90) of both eyes were also not significant.

Table 8: Sensitivity (K) (flash strength at half Vmax) of dark-adapted a- and b-waves of chickens in the TTX group.

Post-baseline Time, Days. n=5	**DA a-wave sensitivity, K (cd.s/m^2)**	
	PBS (sham)	**TTX**
0 (Pre-treatment)	2.06 ± 0.93	1.76 ± 0.80
3	1.93 ± 0.88	1.56 ± 0.49
5	2.06 ± 0.84	1.95 ± 1.01
7	1.37 ± 0.34	1.42 ± 0.50
14	1.65 ± 0.72	1.47 ± 0.66
21	1.56 ± 0.29	1.66 ± 0.64
Post-baseline Time, Days. n=5	**DA b-wave sensitivity, K (cd.s/m^2)**	
	PBS (sham)	**TTX**
0 (Pre-treatment)	0.48 ± 0.28	0.65 ± 0.23
3	1.99 ± 3.42	1.17 ± 0.93
5	0.79 ± 0.71	0.73 ± 0.42
7	0.41 ± 0.39	0.31 ± 0.07
14	0.32 ± 0.04	0.29 ± 0.09
21	0.29 ± 0.10	0.40 ± 0.28

6.5 Effect of TTX on chick light-adapted ERG amplitudes

The pre-treatment (day 0) LA a-wave amplitude interpolated at 3 cd.s/m^2 were asymmetric, with smaller a-waves in the eyes that were subsequently treated (p=0.003), but the ERGs from the TTX treated eyes were not significantly smaller than those of the sham eyes (p=0.06, d=0.58, n=5). The LA a-wave amplitude interpolated at 3 cd.s/m^2 from both eyes decreased at day 5 post-baseline as shown in Figure 28. Both amplitudes increased (p=0.01) with age, and the interaction of treatment and age was not significant (p=0.09). The LA a-wave IT at 3 cd.s/m^2 of between eyes were not different pre-treatment (p=0.70), or with treatment (p=0.06, d=0.48) but ITs in both eyes decreased (p<0.001) with age. The interaction of the LA a-wave IT with treatment and age was not significant (p=0.56).

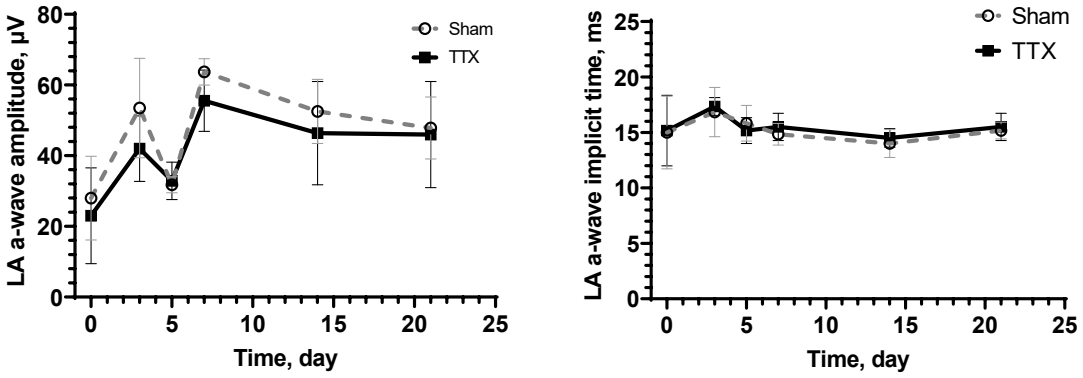

Figure 29: The time course of for the maturation of the light adapted a-wave amplitude (interpolated at 3 cd.s/m^2) and IT, before at day 0 (baseline, no treatment) and at 3-, 5-, 7-, 14- and 21- post-baseline in the TTX group.

LA a-wave amplitude interpolated at 3 cd.s/m^2 increased (p=0.01). The ITs in both eyes decreased (p<0.001) with age between 3 and 21 after treatment.

Similar to the DA b-wave amplitude, the LA b-wave saturated amplitude, Vmax, differed between the eyes that were subsequently treated with TTX and those that became sham eyes (p=0.003) at pre-treatment day 0. The Vmax for the treatment sessions was not significantly different (p=0.86, d=0.09, n=5) between the TTX treated eye and the fellow eye but was different with age (p=0.003). The Vmax of both eyes appears to decrease at day 5, increase day 7 and decrease marginally by day 21 post-baseline (Figure 29), but these differences are not significant. There was no interaction of treatment and age (p=0.72). The LA b-wave IT at 3 cd.s/m^2 shows no significant differences between pre-treated eyes (p=0.77), between treated and sham eyes (p=0.26, d=0.17, n=5) but generally decreased (p=0.01) over the period of the experiment. There was no interaction (p=0.17) of treatment and age.

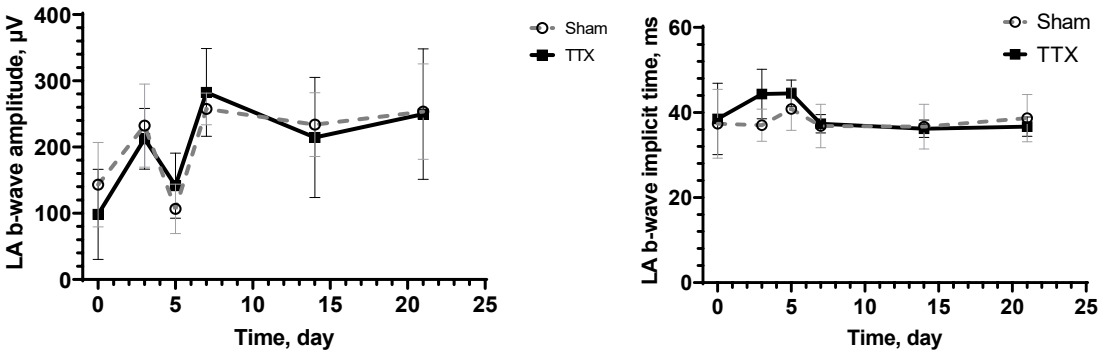

Figure 30: The time course of for the maturation of the light adapted Vmax b-wave amplitude and IT, before at day 0 (baseline, no treatment) and at 3-, 5-, 7-, 14- and 21- post-baseline in the TTX group.

The point is the mean and error bars standard deviation (SD).
LA Vmax of b-wave increased (p=0.003) and b-wave ITs decreased (0.01) with age.

For the sensitivity parameter K, (flash luminance resulting in half Vmax) of the light-adapted b-waves, there were no differences (Table 9) between pretreated eyes ($p=0.09$), the treated and the sham eyes ($p=0.051$, $d=0.61$, $n=5$), nor did K change as a function of age ($p=0.55$). There was no significant interaction of treatment and age ($p=0.10$).

Table 9: Sensitivity, K (luminance at half Vmax) of light-adapted b-wave and PhNR of TTX group chickens.

Post-TTX Time, Days n=5	**LA b-wave sensitivity, K (cd.s/m^2) ± SD**	
	Sham	**TTX**
0 (pre-treatment)	1.49 ± 0.81	1.44 ± 1.19
3	1.41 ± 0.70	1.39 ± 0.92
5	1.24 ± 0.14	1.93 ± 0.77
7	1.21 ± 0.33	1.61 ± 0.51
14	1.45 ± 0.50	1.56 ± 0.27
21	1.01 ± 0.29	1.31 ± 0.37
Post-TTX Time, Days n=5	**PhNR sensitivity, K (cd.s/m^2) ± SD**	
	Sham	**TTX**
0 (pre-treatment)	1.55 ± 0.19	1.61 ± 1.89
3	1.29 ± 0.43	1.29 ± 0.77
5	1.62 ± 0.60	2.64 ± 1.51
7	2.65 ± 0.70	2.06 ± 1.08
14	1.95 ± 0.66	2.13 ± 0.74
21	1.15 ± 0.37	1.26 ± 0.38

The day 0 baseline PhNR Vmax was different between the pretreated eyes (p=0.02) with the eye subsequently treated with TTX being smaller during the baseline sessions, but there was no difference in the PhNR Vmax values between the TTX eyes and the sham eyes (p=0.11, d=0.36, n=5). PhNR Vmax values did not change over time (p=0.06) despite a dip in the values between day 3 and 5 and an apparent increase between day 5 and 7 (Figure 30). There was interaction of treatment and age (p=0.02)

The day 0 treatment IT was not different between the pretreated eyes (p=0.25), and there was no difference in the IT values between the eyes during the treatment sessions (p=0.09, d=0.36, n=5). The IT from both eyes generally decreased over time, but change was not significant (p=0.19), and the interactions were also not significant (p=0.15), as shown in Figure 30.

No differences in PhNR sensitivity, K, of between the eyes with pre-treatment (p=0.08), during the treatment sessions (p=0.17), with age (p=0.17), nor was there an interaction between treatment and age (p=0.18).

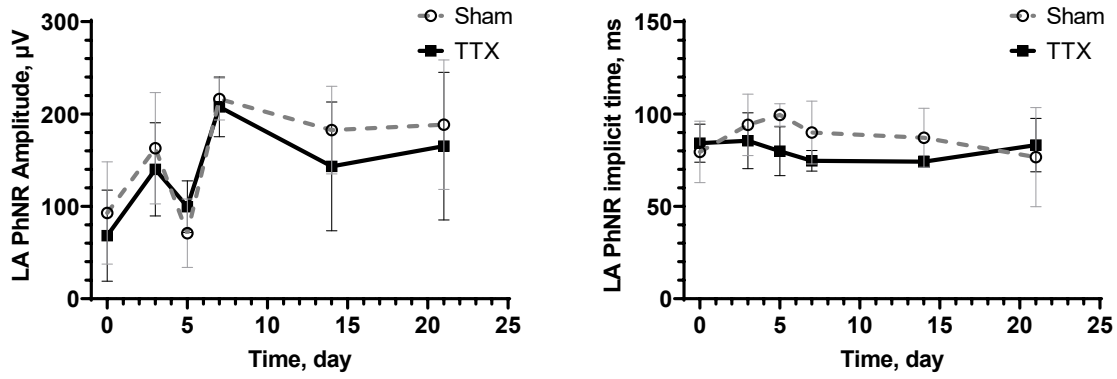

Figure 31: The time course of light-adapted PhNR saturated amplitudes (Vmax), and implicit time at interpolated at 3 cd.s/m^2 without treatment at day 0 and at 3-, 5-, 7-, 14- and 21-days post-baseline (TTX) in treated chicks (n=5), for luminance 0.0562 to 5.6 cd.s/m^2.

The point is the mean and error bars standard deviation (SD).
LA Vmax of PhNR increased (p=0.02) with age.

6.6 Effect of TTX on chick long-flash ERG amplitudes and implicit time

TTX group waveforms did not differ significantly between pre-treated eyes at day 0 (p>0.05). There were no differences between the intravitreally TTX injected eyes and the sham injected control eyes in onset a-wave and b-wave (Table 10) amplitude (a-wave: p=0.40, b-wave: p=0.38, n=6) nor by age

(a-wave: p=0.13, b-wave: p=0.09) nor by interaction of age and treatment (a-wave: p=0.45, b-wave: p=0.51). Similarly, there were no differences in the implicit times between the treatment eyes for the onset a- and b-waves (p = 0.71; p= 0.54 for a- and b-waves, respectively, n=6), and no differences by age (a-wave: p=0.37, b-wave: p=0.20). The interactions for both long-flash a- and b-waves ITs were also not significant (p=0.83; p=0.61 for a- and b-waves, respectively).

Table 10: Amplitudes of the long-flash onset ERG of TTX treated eyes and sham injected eyes.

Treatment, n=6	Time post-baseline (days)	Long-flash b-wave Amplitude ± SD (µV)	Long-flash a-wave Amplitude ± SD (µV)
Pre-treated eye	0	130.10 ± 9.16	39.3± 4.34
Vehicle and TTX (treated)	3	149.10 ± 9.56	48.90 ± 3.92
	14	122.03 ± 24.16	50.70 ± 7.30
	21	152.75 ± 27.83	48.47 ± 7.83
Pre-treated eye	0	132.13 ± 10.35	40.54 ±5.89
Vehicle (sham)	3	155.28 ± 13.62	53.44 ± 4.54
	14	132.67 ± 16.46	57.14 ± 4.20
	21	154.73 ± 23.25	49.04 ± 5.76

At pre-treatment, the d-waves were not different between the eyes (p=0.10). For the light-adapted long-flash offset d-wave, although there were no differences in the implicit time (p=0.10), there was a statistically significant difference (p=0.03, n=6) with the amplitude (Figure 33). The sham eyes' d-waves amplitudes were bigger than those of the treated eyes (Figure 32). There were no differences in both eyes by age (d-wave amplitude: p=0.08, IT: p=0.20) nor interaction of age and treatment (d-wave amplitude: p=0.12, IT: p=0.32)

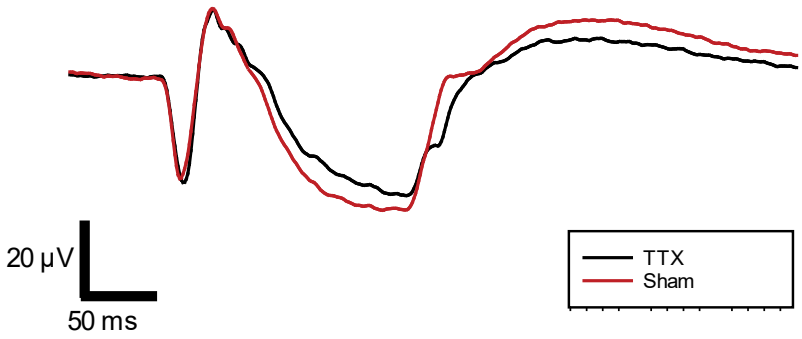

Figure 32: Representative of long-flash ERGs from the sham and TTX eyes of a 14-day post-treatment chicken, demonstrating the reduced d-wave in the TTX eye.

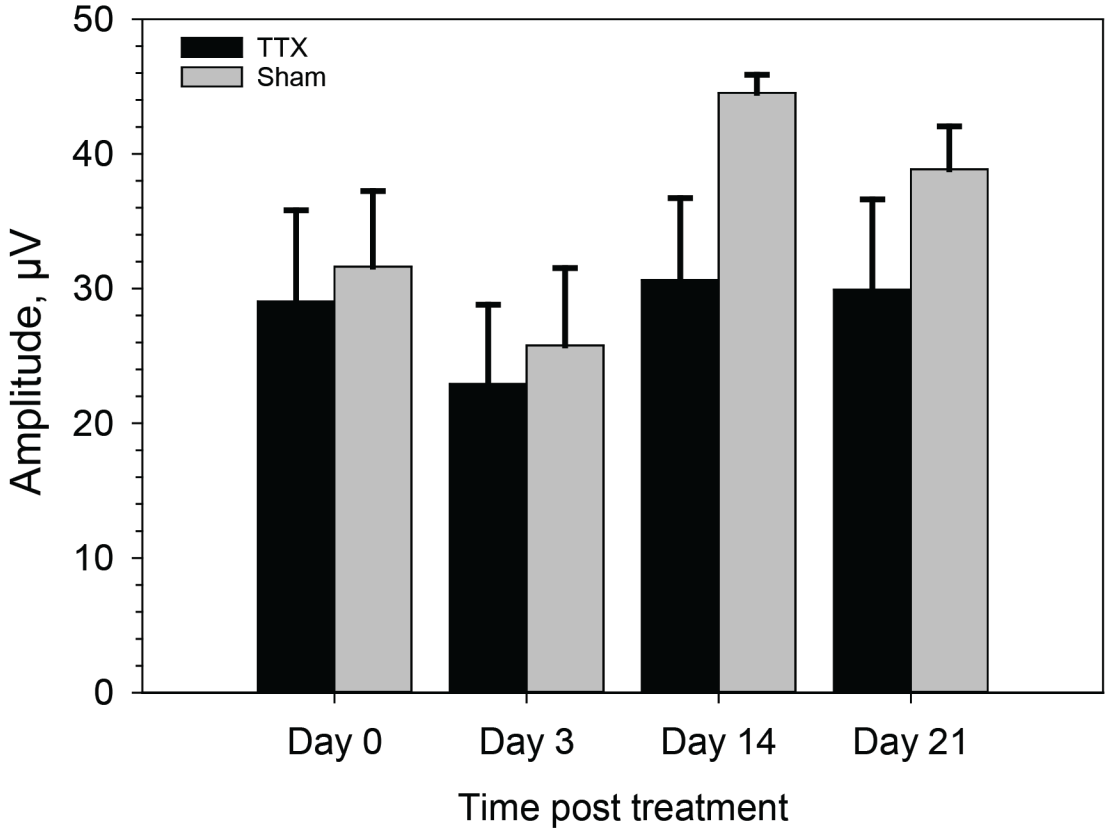

Figure 33: The time course of long-flash (250 cd/m^2) offset d-wave amplitudes before TTX treatment and 3-, 14- and 21-days after baseline with TTX treatment each time (N=6).

The sham eyes' d-waves amplitudes were bigger than those of the treated eyes

Chapter 7
Discussions

7.1 Introduction

This project ultimately seeks to determine the contribution of chicken RGCs to flash ERGs using PhNR, STR and photopic long-flash ERGs protocols with optic nerve section (ONS) and tetrodotoxin (TTX) which destroy or block RGC function, respectively.

7.2 Optic nerve dysfunction

This study shows that ONS- or TTX-induced optic nerve dysfunction results in delinking of the eye to the brain. This was demonstrated by the lack of pupillary constriction on direct pupillary tests on all the chickens used in this study. It has been reported by several authors that pupillary reaction test is a reliable method of testing the efficacy of the ONS/TTX (McBrien et al., 1995, Wong-Riley et al., 1989b). Furthermore, the optokinetic method was employed in this study to further demonstrate the loss of retinal ganglion cells functions. The observed absence of the optokinetic response in the treated eyes further confirms the success of the RGC dysfunction methods employed in this study, and this work is consistent with study by Ostrin et al. (2016).

ONS Histology

The histology experiment shows selective reduction of retinal ganglion cells in the treated eye. Chong et al. (2013) also demonstrated that ONS is associated with loss of chicken RGCs. However, this study further demonstrated that the cells INL were not affected in ONS treated chicken eyes. Since the INL has both amacrine, on- and off-bipolar cells, it is likely that these cells were not affected by ONS in chicken.

7.3 Dark-adapted ERGs and the STR

The absence of a negative-going waveform in the scotopic threshold responses (STRs) in very dim flashes in chicks was reported in this study. Given that STRs are elicited from the rod pathway, it is

not surprising that chickens lack STRs. Unlike humans who have 1:20 rod:cone ratio (Jonas et al. 1992), chicks have 2:3 ratio (Morris 1970, Wisely et al. 2017) as well as a circadian suppression of rod function during the daytime (Schaeffel et al. 1991). For very dim flashes, the chicken ERG, was relatively small as compared with human ERGs. It was suggested by Montiani-Ferreira et al. (2007) that perhaps the relatively fewer rods may account for the lower amplitude of the entire ERG waveform generated in across the scotopic range.

The observed ERGs are like those described by Montiani-Ferreira et al (2007), who reported similar positive-going waves from dark-adapted ERGs from -2.4 log to -2 log cd/m^2. Although a previous study by Schaeffel et al. (1991) shows that, in the dark-adapted state at the appropriate time in the circadian cycle, the rod pathway dominates the chickens' vision, no dark-adapted ERGs were detected from stimuli of -4.32 log cd.s/m^2. In fact, the dark-adapted ERGs in the present study supports the work of Shi and Stell (2013), who found chickens threshold luminance is about – 4 log cd.s/m^2 in 5- to 13-days old chicks. The slight difference in the threshold of -0.32 log cd.s/m^2 found in the same age range as in the present study might be explained by the strain of chick used by the different studies. Shi and Stell (2013) used bovan chickens, a chicken strain reported to have higher night sensitivities, and Shi and Stell (2013) speculated that this strain might have higher rod:cone ratio than the domestic chick (used in this present study).

Some mammals, such as guinea pigs that have a non-vascularized inner retina (Cringle et al. 1996), and also lack STR waveforms in ERGs elicited through the stimulus range that is sub-threshold for ERG b-waves (Lei 2003). Although the paucity of data on chick ERGs to subthreshold luminance levels makes it difficult to compare this result with other studies, it could be suggested that domestic chicks do not have negative STR waveforms and, therefore, the observed positive-going waveforms to dim stimuli is associated with b-waves (Figure 35). Furthermore, it can be observed from Figure 8

that the positive-going wave peak for dim stimuli has similar implicit times to those of b-waves, suggesting that the waves reflect rod bipolar activity and are not positive STRs.

The absence of the negative STR waveform in all chicks gives rise to questions about the protocol and recording techniques, particularly as the STR is eliminated by incomplete dark adaptation or small amounts of light exposure. We, therefore, reproduced our chick STR protocol using a human participant for comparison (Appendix A); given the same protocol/stimulus parameters, STRs with both a negative and positive waveform are recordable in humans. The human negative STR was apparent for stimuli between -5.25 and -4.25 log cd.s/m^2 (Figure 35). The use of similar instruments, recording parameters and light stimulus levels elicited STRs in the human subject but not in the chicks (Figure 35) gives credence to the suggestion by this study that chicks might not have STRs.

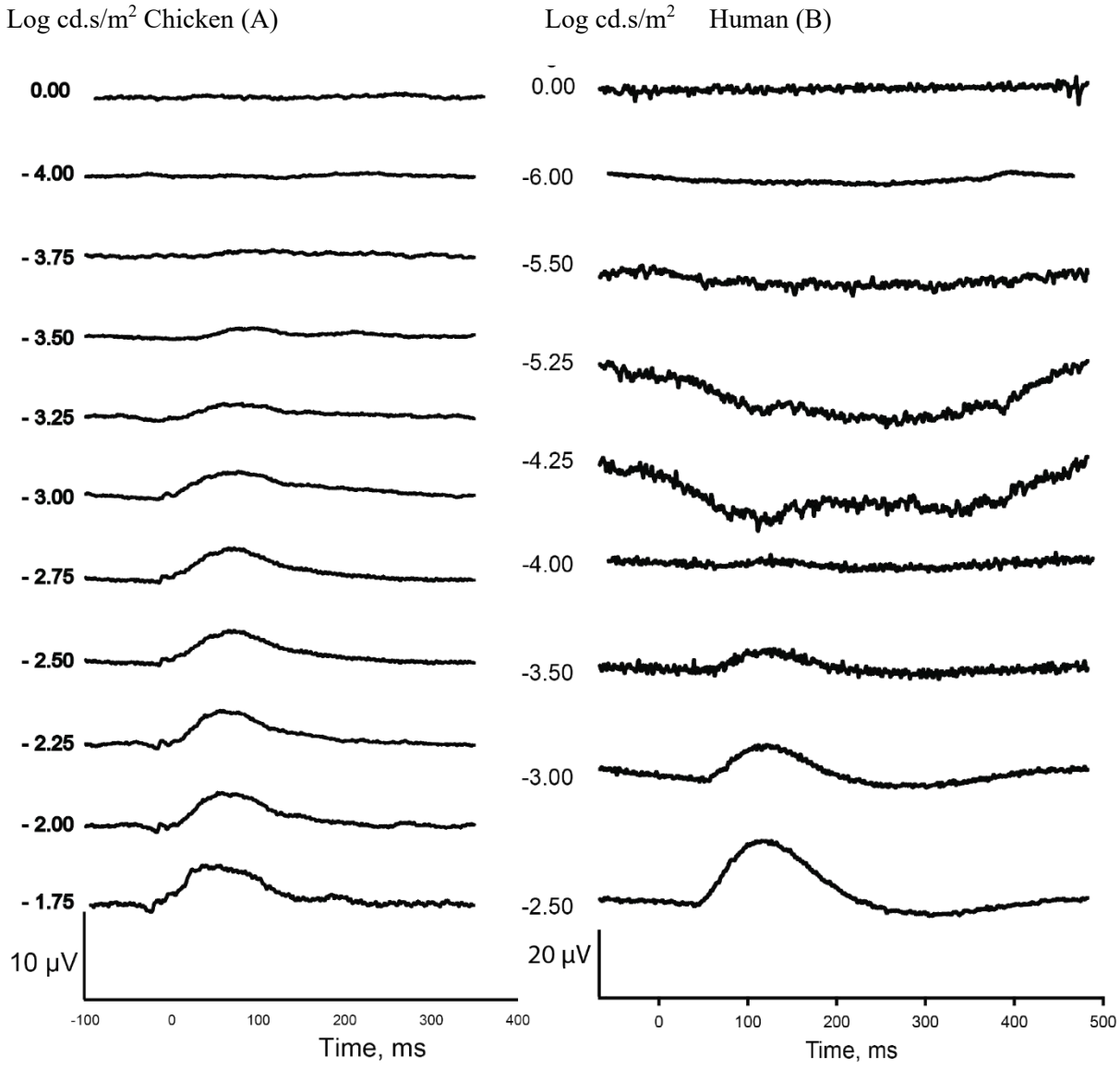

Figure 34: A chicken (A) and human (B) representative dark-adapted ERG response to very dim white flash stimuli.

The chick was 14 days old post-hatch. The human was an adult with brown iris.

7.4 Spectral characteristics and light-adapted long-flash ERGs

From pilot study 3, red on blue long-flash ERGs had larger amplitudes than the other combinations of wavelengths used. This is different as compared to human long-flash where ERGs with red light on a blue background elicited the smallest long-flash ERGs (Sustar et al. 2006); Although the relative threshold sensitivity of the chick retina for medium and long-wavelength light is similar to that of humans, chicks have cone types with red/orange oil droplets (Bowmaker & Knowles, 1977; Kram et al. 2010). It is, therefore, possible that for suprathreshold stimuli, more photoreceptors in the chick respond to the red flash, resulting in a larger a-waves, and therefore also generating a larger b-wave. More importantly, the chick red on blue long-flash ERG showed a more distinct off response d-wave compared with those for the white-on-white stimulation. The d-waves of the chick to light-adapted long-flash ERGs show double peaks as in humans (Sustar et al. 2009, Horn et al. 2011, Morny et al. 2019) and in non-human primates (Sieving et al. 1994, Ueno et al. 2006). Although limited studies have been done to conclusively determine which peaks of the d-wave reflect off bipolar cells in chicks, it is likely that the first peak reflects off-bipolar cell function as it does in the human. This assumption is based only on the fact that the chick and human long-flash ERGs have similar waveforms. Further investigation into the double peak of chick ERGs is indicated.

7.5 Chicken photopic negative response (PhNR)

As for the STRs, the light-adapted short-flash ERGs did not show remarkable PhNR waveforms regardless of the spectral characteristics of the stimulus. For brief flashes (pilot study 2), red on blue elicited ERGs with a clearer PhNR as well as prominent i-wave, as compared to the white-on-white ERGs. As above, the protocol was tested on a human participant to verify that that the PhNR could be recorded using these stimuli. A typical light-adapted luminance-response function from a 2-week-old chick is compared with that of the adult human participant is shown in Figure 35. The

rudimentary PhNR in the chick is consistent with ERGs shown by Montiani-Ferreira et al. (2007) and by Ostrin et al. (2016). Nonetheless, this result is in contrast to the large negative PhNR from primates (Viswanathan et al. 1999, Frishman et al. 2018). It has been shown by Raz-Prag et al. (2010) that these negative going waves come from potassium rectifying channels in the glial cells in the ganglion cell layer that are activated upon retinal ganglion cells function. Perhaps the unremarkable PhNR in chicks is linked to the lack of intra-retinal astrocytes (Fischer et al. 2010). Furthermore, the K^+ channels of glial cells (mostly Müller and intra-retinal astrocytes) draw into the cells the excess K^+ in the extracellular space upon excitation of the RGC cells, Raz-Prag et al. (2010) suggesting that K^+ ions are released in response to RGCs function. Since the chicks lack intra-retinal astrocytes, and therefore the associated rectifying channels, their PhNR amplitude is expectedly, small.

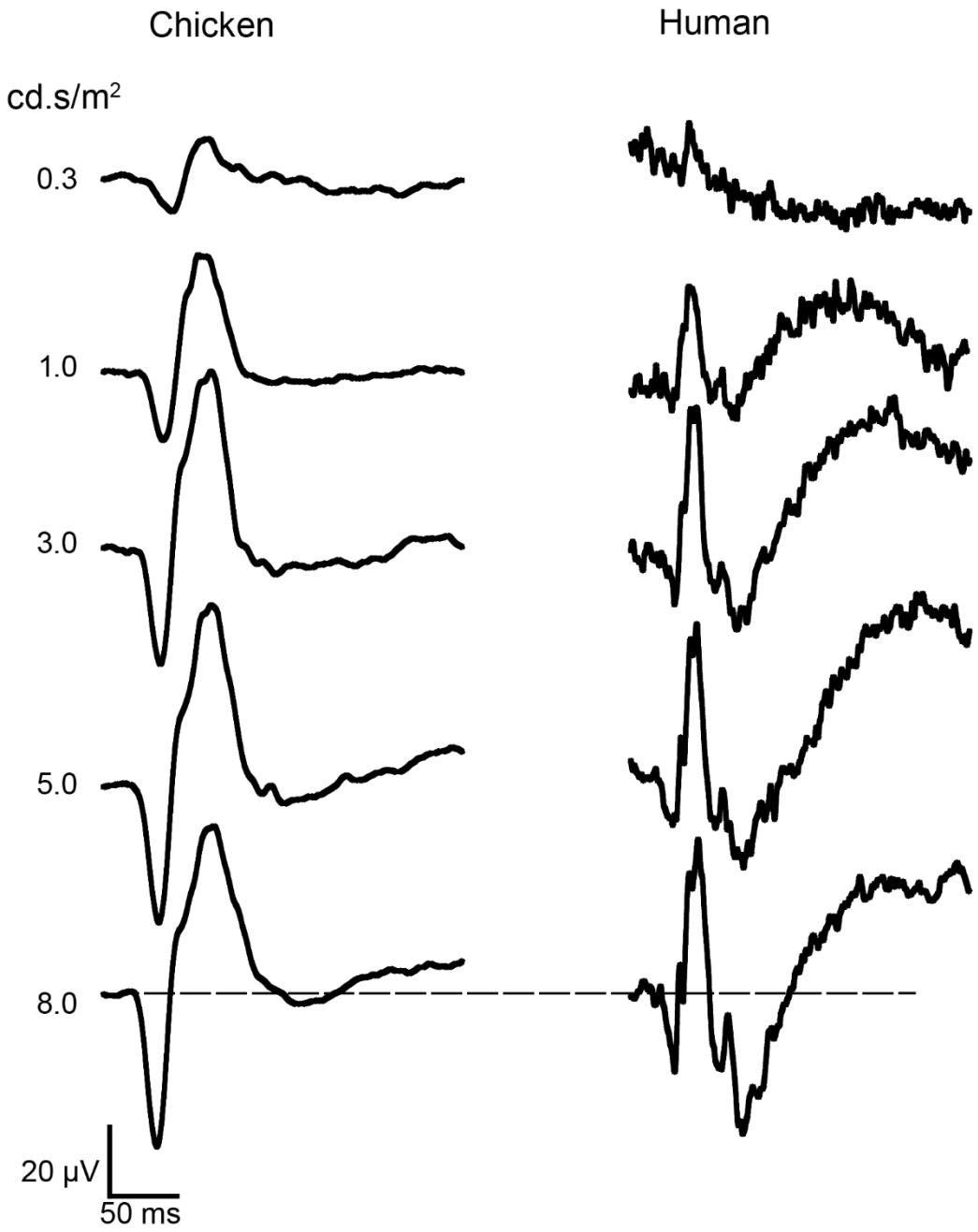

Figure 35: A representative ERG luminance series of a 14-day old light-adapted chicken (left) and human (right) light-adapted ERGs to a luminance series of 4ms red flashes on a blue 30 cd.s/m^2 background.

7.6 Effect of optic nerve-section (ONS) and intravitreal of tetrodotoxin (TTX) on the dark-adapted ERGs.

The present study shows that ONS at one day post-hatch does not affect the rod bipolar of chick ERG throughout the 21 days after treatment (1 day post-hatch). A possible explanation relates to the absence of AII amacrine cells in the chicken retina (Quesada et al. 1988, Shi and Stell 2013). AII amacrine cells mediate dark-adapted vision in rod-dominant mammals as they connect rod bipolar cells to ganglion cells.

Because in some species, RGC damage models affect b-waves and oscillatory potentials of dark-adapted flash ERGs, analysis of the dark-adapted chick ERGs was carried out to determine if ONS or TTX affects flash dark-adapted chick ERGs. Not surprisingly, none of the waveforms of the dark-adapted ERGs was affected by the RGC attenuation. The limited report of the effect of the ONS or TTX on chick dark-adapted ERGs makes it difficult to compare this result with others. In this study, the dark-adapted chicks' a- and b-waves were not affected by RGC deficits. Other studies have suggested that some vertebrate bipolar cells have TTX sensitive channels (Saszik and DeVries 2012) so that b-waves may be affected by TTX if the chick bipolar cells had such properties. However, in the present study, the reduction of the b-wave in the TTX group did not reach significance ($p = 0.311$).

In some species such as rats and mice, it has been documented that attenuation of RGCs results in loss of dark-adapted OP amplitudes (Raviola and Gilula 1973). However, in this report of chicken ERGs, the amplitude of dark-adapted OPs was not affected by RGC deficits caused by either ONS or TTX. This study also differs from studies in humans (Raviola and Gilula 1973), where RGC deficit conditions resulted in the loss of OP amplitude. Although TTX blocks the chicken spiking amacrine cells (Wildsoet and Wallman 1995), TTX did not reduce the amplitudes of the dark-adapted OPs. The results suggest that amacrine cells may contribute to OP generation in humans but not in chicks, perhaps accounting, at least in part, for the interspecies differences.

7.7 Effect of ONS and TTX on chick light-adapted ERG luminance-response series

The PhNR was not significantly reduced in the treated eyes of either the ONS or TTX studies. However, the sham eyes PhNR amplitudes in the ONS study showed a trend towards being larger ($p = 0.09$). Similarly, in the TTX study, the PhNR tended to be larger in the sham (PBS injected) eyes but not significantly. The present results contrast with similar work done by Viswanathan et al. (1999) in primates, showing a substantial diminution of the PhNR amplitude with TTX injection. It could be suggested that since the PhNR was not reduced significantly in the treated chick, perhaps spiking RGCs do not contribute substantially to chick PhNR waveforms. This author did not find published data on chick PhNR; hence comparison within species is not possible.

The ONS surgery and the subsequent loss of RGCs did not affect the a- and b-wave of the chick light-adapted ERGs. Similarly, no differences in the light-adapted a- or b-waves was found for eyes injected with TTX. These data match previous work done in our lab (Choh et al. 2004, Chong et al. 2013) that shows that ONS selectively affects RGCs, hence no effect was demonstrated in the ERG waveforms generated by more distal cells. This work is also consistent with similar work done by Ostrin et al. (2016). The present study includes additional ERG protocols to probe cone function as

compared with Ostrin et al. (2016), such as the use of red on blue stimuli and a light-adapted luminance series as against white on white to one 3 cd.s/m^2 short flash. Both studies showed that the b-wave amplitude reduction was not significant for any stimulus protocol. B-waves primarily originate from the bipolar cells and thus are not expected to be affected by ONS or TTX. There is limited literature pointing to the loss of bipolar cells in any species after ONS. However, we cannot discard the fact that some components of the b-wave might be affected. It has been noted by Sieving et al. (1994) that short flash b-wave amplitude reflects the function or contributions of both on- and off bipolar cells. Also, RGCs might contribute to the pull factor of the push and pull model of b-wave as postulated by Sieving et al. (1994). However, because the push factor (on-bipolar cell activated by the brief-flash stimuli) has more input and hence any reduction associated with RGC input to the pull factor (off bipolar cells) was not detectable.

For the TTX experiment, DA b-wave, LA a-wave, LA b-wave, and LA-PhNR amplitudes had significant inter-ocular differences (ID) in pre-treatment eyes. However, the results post-baseline is still valid due to the robust method used in the analysis. Firstly, the method of analysis repeated-measures ANOVA was performed as suggested by Armstrong (2013) and as used in chicken ERG by Ostrin et al. (2016) on only the post-baseline data, and any significant differences in the treated and control eyes will have been captured. Secondly, although interocular differences were observed in the pre-treatment, it is worth noting that, in post-baseline, these differences became narrower, suggesting that the lack of significant differences in post-baseline could not be attributed to the pre-treatment ID. Thirdly, to account for the pre-existing difference between the eyes prior to treatment, the inter-ocular differences (calculated as treated eyes minus fellow eye changes, were compared as a function time) in LA ERG parameters at the various ages were analyzed (repeated measures ANOVA) (not

reported), where any significant changes from day 0 represent additional differences than the pre-existing day 0 inter-ocular difference, but the result was not different from the reported results.

7.8 Effect of TTX and ONS on chick light-adapted long-flash ERGs

The investigation into which component of the short flash b-wave reflects chick RGC function resulted in a study of the effect of ONS on the on b-wave and off d-wave of the chick long-flash ERGs. Here, the results suggested that both TTX and ONS affect the retinal off-pathway. This result is consistent with work done by Petersen-Jones et al. (2010), which found that another RGC pharmacological blocking agent, NMDA, reduces the d-wave of chicks. The drug employed by Petersen-Jones et al. (2010) targets non-spiking RGCs, TTX targets spiking cells and ONS blocks both spiking and non-spiking RGC. Since all of these treatments reduce d-wave amplitudes, it could be postulated that both spiking and non-spiking RGCs contribute to chickens' d-waves. Moreover, Awatramani et al. (2001) also noted this generalized trend and stated that in blocking the inner retinal functions, d-waves tend to be affected more than the on b-waves. Given that d-wave amplitude and implicit time seems to be preserved in RGC destructive conditions such as glaucoma (Horn et al. 2011) and autosomal dominant optic atrophy (Morny et al. 2015), it could be suggested that an anatomical reason might account for the difference. Chicks have an enhanced centrifugal vision system (CVS), efferent fibres from the brain to the retina, while such efferent fibres are rare in the primate retina (Gastinger et al. 2006). Most of the CVS fibres terminate close to off-bipolar cells and excite RGC cells (Lindstrom et al. 2010). It could be suggested that dysfunction of the CVS fibres by ONS or by blockage of RGCs with TTX in the treated eye could lead to decreased activity of the off-bipolar cells and hence the reduced d-wave.

Both ONS and TTX studies show growth in the ERG waveform over time. This is consistent with studies done by Ookawa, T. (1971), which shows that the chicken ERGs grow over time. Ostrin et la. (2016), also demonstrated this maturation in ONS and sham-treated eyes.

7.9 Summary and limitations

This thesis explored the use of chicken ERGs to detect RGC functions. The ERGs protocols employed in this study were known to detect RGC functions in human subjects. However, this study suggests that chicken does not have a sub-threshold response, which implies that STRs do not reflect RGC functions in chicks. Moreover, another negative going ERG waveform, which is known to reflect RGCs function, the photopic negative response (PhNR), is small in growing white leghorn chickens (compared to humans). Finally, this thesis showed that offset d-wave possibly reflects RGCs functions.

Furthermore, the pre-treatment day 0 (baseline) data shows bigger ERG values for the TTX group than the ERGs from the birds in the ONS group. This may be due to different batches of chickens used for each study. We noticed the ERG amplitudes from the batch of chicken for the TTX group were bigger than for the batch for the ONS group, although the experiment was carried out in the same experimental conditions. For instance, the mean of the DA a-wave amplitude from the TTX group of birds was 15 μV, bigger than the ONS group at day 0. These differences in the treatment ERG data and the generally increased amplitude of ERG data from the TTX group make it difficult to compare the ERGs from the two groups. Also, both study groups were not carried out concurrently. It is suggested that future studies into chicken ERG studies be carried concurrently using the same batch of chickens.

Moreover, the main effects of treated eye of flash ERG parameters measured in the thesis showed no significant difference. However, since the effect size analysis with Cohen's d, shows none of these main effects of treatment had d> 60, it could be suggested the effect sizes were also not large enough. It is suggested that future studies, especially PhNR in chicks, should consider the low observed power analysis from this thesis and factor it into the sample size calculations.

Although this study clearly demonstrated that the inner nuclear layer cells were not affected by the ONS, the d-wave from the chick retina was affected, suggesting future investigations into the effect of ONS or TTX on off-bipolar cells should be investigated in the future using immunohistology. Additionally, ERGs for the treated eyes and the control eyes were tested simultaneously in each bird to avoid testing effects, such as anesthesia (Choh et al., 2017) on ERG variability from sequential testing in this thesis. Although this type of design is the most used in ERG studies in chickens, it is possible that the ERGs could cross to the contralateral eye through volume conduction (ERG crosstalk) because chickens have small heads and have eyes close together. However, the possibility of this ERG cross talk affecting the results was minimized in this study since ERGs crosstalk from each eye could cancel out. In the future, chicken ERG interocular differences should be explored. The sham controls instead of naïve controls were used in this thesis because sham controls had the advantage of cancelling out the effect of the surgery/injection in both chicken eyes.

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Appendices

Appendix A: Human ERG Studies

Purpose: To record STR waveforms in a human participant using the protocols designed for chick ERG testing with similar levels of light control and dark adaptation

Method: ERGs were recorded from one human subject (male black, 38yrs) after ethical approval (ORE 22678) for the study was obtained from Human Research Ethics Committee of University of Waterloo. After informed consent, the visual acuity, intraocular pressure, and anterior chamber assessments were recorded, then eyes were dilated with 1% tropicamide (Alcon Inc., Mississauga, ON, Canada). After achieving pupil dilation of > 6 mm diameter, the ERGs were recorded bilaterally from the cornea using standard DTL® fiber electrodes (Diagnosys LCC, Lowell, MD, USA). References were standard EEG skin electrodes (Fenton Tech. Inc., Markham, ON, Canada) placed on the lateral canthus and a ground placed on the right wrist. Two micro-manipulators (one for each eye) were used to hold and position the ganzfeld such that each eye was were looking at center of its ganzfeld. Protocols completed included those used for the main studies of the chick. Specifically, the dark- and light-adapted protocols (section 3.8.1 and 3.8.2). For dark-adapted ERGs, eyes were dark adapted for 20 min, and for light adapted ERGs, eyes were light adapted to the background (see Table 1 and Table 2) for 10 min.

Results: This study revealed that given the same protocol/stimulus parameters, STRs with both a negative and positive waveform are recordable in humans. The human negative STR was apparent for stimuli between -5.25 and -4.25 log cd.s/m^2. As the use of the same instruments, recording parameters and light stimulus levels elicited STRs in the human subject but not in the chicks (Figure 34), also

gives credence to the suggestion by this study that chicks might not have STRs. The LA-ERG results also show unlike chickens humans have enhanced PhNR.

Appendix B: Human Ethics

Dear Researcher:

A University of Waterloo Research Ethics Committee is pleased to inform you the study named below has been reviewed and given ethics clearance.

Title: Electrophysiological Measure of Retinal Ganglion Cells Function in humans ORE #: 22678 Faculty Supervisor: Daphne McCulloch (daphne.mcculloch@uwaterloo.ca) Faculty Supervisor: Vivian Choh (vchoh@uwaterloo.ca) Student Investigator: Clement Afari (clement.afari@uwaterloo.ca)

A signed copy of the notification of ethics clearance will be sent to the Principal Investigator (or Faculty Supervisor in the case of student research). Ethics approval to start this research is effective as of the date of this email. The above named study is to be conducted in accordance with the submitted application (Form 101/101A) and the most recent approved versions of all supporting materials.

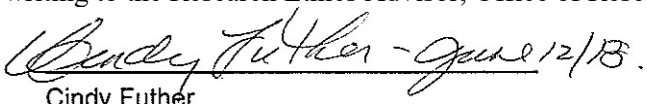
University of Waterloo Research Ethics Committees operate in compliance with the institution's guidelines for research with human participants, the Tri-Council Policy Statement for the Ethical Conduct for Research Involving Humans (TCPS, 2nd edition), Internalization Conference on Harmonization: Good Clinical Practice (ICH-GCP), the Ontario Personal Health Information Protection Act (PHIPA), and the applicable laws and regulations of the province of Ontario. Both Committees are registered with the U.S. Department of Health and Human Services under the Federal Wide Assurance, FWA00021410, and IRB registration number IRB00002419 (Human Research Ethics Committee) and IRB00007409 (Clinical Research Ethics Committee).

Appendix C: Animal Ethics

UNIVERSITY OF WATERLOO
OFFICE OF RESEARCH ETHICS
ANIMAL CARE COMMITTEE
CERTIFICATE OF FULL ETHICS APPROVAL:
RENEWAL OF ANIMAL UTILIZATION PROJECT PROPOSAL (LAST RENEWAL)
All research and teaching activities at the University of Waterloo which use live, non-human vertebrate animals must be conducted in compliance with the Animals for Research Act of Ontario (Revised Statutes of Ontario), the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care and the University of VVateloo's Guidelines for the Care and Use of Animals in Research and Teaching.

Principal Investigator(s):	Vivian Choh;
Department or School:	Optometry;
Co-Investigator(s):	Daphne McCulloch;
Student Investigator(s):	Clement Afari; Ziqing Li;
Projoct Title •	Comparison of three tests that measure retinal ganglion cell function in chickens
AUPP # :	15-11 Approval Date: May 29, 2018
Number of Animals Approved:	66 Chickens Invasiveness Category: c

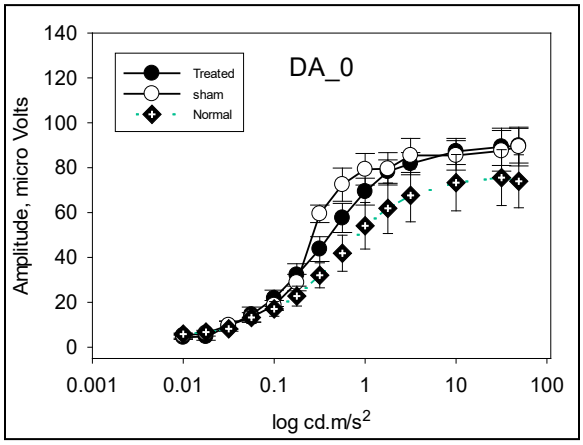
The above Animal Utilization Project Proposal (AUPP) Renewal Form has been reviewed by members of the Animal Care Committee at the University of Waterloo in compliance with the requirements of the Animals for Research Act, the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals, and the University's Guidelines for the Care and Use of Animals in Research and Teaching.
Approval of the original AUPP is extended for an additional twelve month period from the date shown.
This year's renewal represents the last of the 4 year renewal cycle under Animal Care Committee guidelines since an AUPP representing continuing research may be renewed three times only after original ethics approval. In the event you wish to continue this project beyond May 2019, you will need to submit a full AUPP not later than the end of April 2019 in order to avoid any break in ethics approval status.
Note: the project covered by this AUPP must be conducted according to the procedures described in the application. Requests for subsequent modifications to approved AUPPs must be communicated in writing to the Research Ethics Advisor, Office of Research Ethics, using the Modification Form.

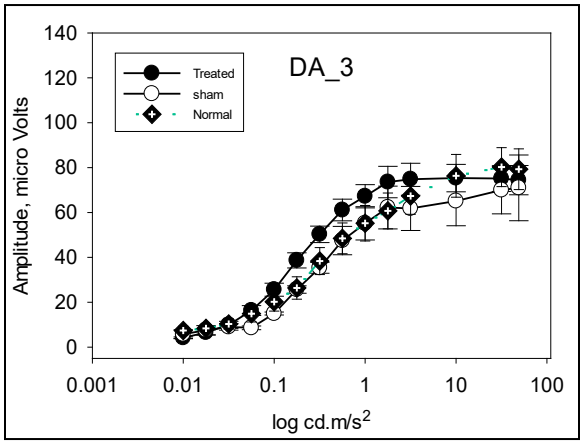


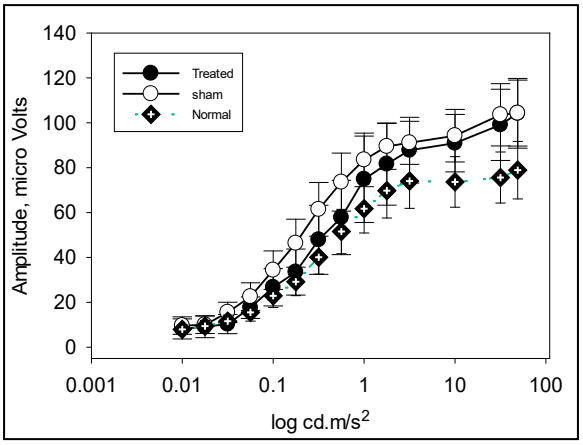
Cindy Futher
Research Ethics Advisor
Office of Research Ethics
https://oreprod.private.uwaterloo.ca/ethics/animals/aupp/ad/AUPPrenewal certificate.asp?i...

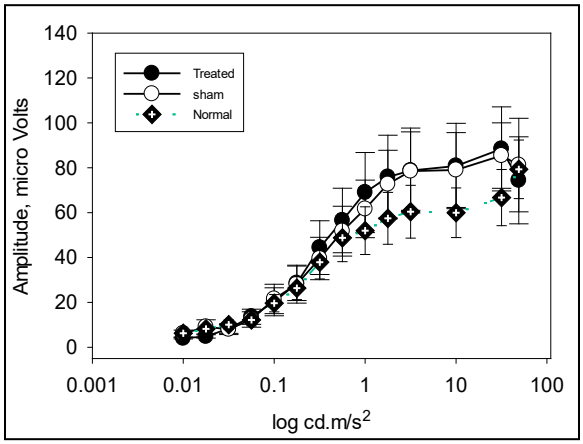
Appendix D: DA Stimulus Response Curves

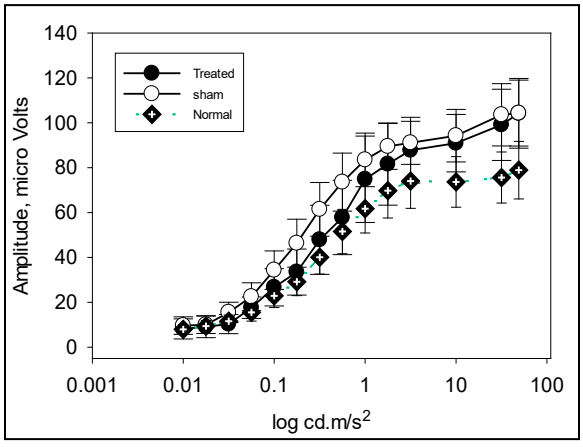
Stimulus response curves form the DA chickens from the ONS group. The order of graph is day 0, 3, 4,5,7 post-baseline.

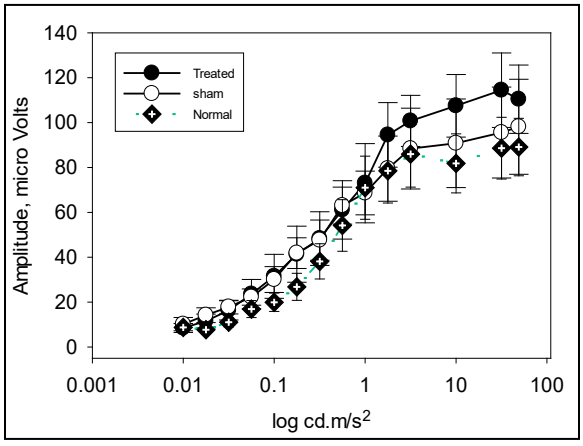












Appendix E: Naka-Rushton Curve Fitting R Codes

Naka-Rushton curve fitting

Coded by Vivian Choh, vchoh@uwaterloo.ca

Modified with permission by Clement Afari, a5clemen@uwaterloo.ca

```
######  Set up function to fit  #####
library(tcltk) require(nlmrt) library(nls2)
library(ggplot2)
log.fit <- "y ~ sqrt(a^2)/(1+(x/sqrt(x0^2))^-1)"


#####          Initialise      files               #####
if(exists("NRdata.a"))              {rm(NRdata.a)}
if(exists("NRdata.b")) {rm(NRdata.b)}

#####  Read in data and set out.path  ##### df <- df[,
colSums(is.na(df)) != nrow(df)] my.out.path <-
tclvalue(tkchooseDirectory()

#####  Find expected variables in data file  #####
birdName <- unique(df$Bird) whichWeek <-
unique(df$week) fit.a <- unique(df$a.amp) fit.b <-
unique(df$b.amp)

#####  Assuming multiple weeks and multiple birds  #####  weekNum =
whichWeek[weekLoop]
```



```
#####  Assuming multiple weeks and multiple birds  #####
weekNum = whichWeek[weekLoop]
```


```
#####  Assuming multiple number of birds   #####
whichBird = birdName[birdLoop]

 #####  For the individual bird at a certain time point   #####
```