HISTOMORPHOMETRIC CHANGES IN THE RABBIT NEURAL RETINA FOLLOWING MONOCULAR DEPRIVATION

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By

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DECLARATION

I hereby confirm that this thesis is my original work and has not been presented elsewhere for examination.

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"In the study of this membrane [the retina] I for the first time felt my faith in Darwinism (hypothesis of natural selection) weakened, being amazed and confounded by the supreme constructive ingenuity revealed not only in the retina and in the dioptric apparatus of the vertebrates but even in the meanest insect eye. ... I felt more profoundly than in any other subject of study the shuddering sensation of the unfathomable mystery of life."

- Santiago Ramón y Cajal

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LIST OF ABBREVIATIONS

ANOVA ..... Analysis of Variance

µm ..... Micrometers

μm^2 ..... Square micrometers

mm^2 ..... Square millimeters

SPSS ..... Statistical Package for Social Sciences

RT ..... Retinal thickness

BDNF ..... Brain Derived Neurotrophic Factor

P14 ..... Postnatal day 14

P21 ..... Postnatal day 21

P28 ..... Postnatal day 28

P35 ..... Postnatal day 35

SUMMARY

Background: Monocular deprivation in experimental animals results in both anatomical and electrophysiological changes in the visual cortex in favor of the non-deprived eye, a phenomenon known as ocular dominance plasticity. Although the retina is considered part of the nervous system based on its embryonic development and its cellular content, there is scarcity of information on the anatomical changes occurring in the retina as a result of monocular deprivation.

Objective: To describe the histomorphometric changes in the neural retina following monocular deprivation.

Study design: Randomized-experimental study using a rabbit model.

Material and methods: 30 rabbits (18 experimental, 12 controls) were studied. Experimental animals were monocularly deprived of light by suturing the lids of one eye at postnatal day 14. At baseline (postnatal day 14), three control rabbits were euthanized, their retina harvested and processed for light microscopy. Thereafter at postnatal days 21, 28, and 35, three control and six experimental animals were euthanized, their retina harvested and processed for light microscopy by paraffin embedding. Retina of both deprived (closed) and non-deprived (open) eyes in experimental animals were studied. Haematoxylin & Eosin stain was used to demonstrate the layers and cellular detail of the retina, while Golgi stain was used to elucidate the processes of ganglion neurons. Photomicrographs of the retina were taken using a Canon® digital camera that was mounted on a Leica® photomicroscope.

Data analysis: The photomicrographs were entered into Fiji-ImageJ® processing and analysis software. The variables measured included: thickness of each layer of the neural retina, cell densities in the nuclear layers, and dendritic features of the ganglion neurons. Data gathered were entered into Statistical Package for Social Sciences software (version 17.0 Chicago, Illinois) for analysis. Analysis of Variance was used to compare the differences in means of each variable with increasing duration of monocular

deprivation. The Student's *t*-test was used to compare the differences in means between the experimental and control animals. A *p value* <0.05 was considered significant at 95% confidence interval.

Results: From the P14 to P35, the neural retina thickness in the deprived eyes, reduced by 40.5% (p=0.001). Compared to controls, statistically significant differences were noted in the ganglion cell layer (p<0.001), inner nuclear layer (p<0.001), rod and cones layer (p=0.001), outer plexiform layer (p=0.008), nerve fiber layer (p=0.010), and inner plexiform layer (p=0.024). There was generalized reduction in the cell densities of the deprived eyes with increasing period of deprivation, with the percent reductions being ganglion cell 60.9% (p<0.001), inner nuclear layer 41.6% (p=0.003), and outer nuclear layer 18.9% (p=0.326). The number of primary and terminal dendrites as well as the dendritic field area reduced with increasing period of deprivation, but the differences were not statistically significant compared to the controls.

Among the non-deprived eyes, the neural retina thickness increased by 9.8% from P14 to P35 (p=0.075). Compared to controls, statistically significant differences were noted in the inner plexiform (p<0.001), inner nuclear (p=0.002), and rods and cones (p=0.007) layers. The cell densities increased with increasing period of deprivation, with the percent increments being ganglion cell 116% (p<0.001), inner nuclear layer 52% (p<0.001), and outer nuclear layer 59.6% (p<0.001). The number of primary and terminal dendrites, and dendritic field area increased with prolonged period of deprivation. Compared to controls, non-deprived eyes had 114.3% more terminal dendrites (p=0.002), while the number of primary dendrites and dendritic field area did not display statistically significant differences.

Conclusion: Monocular deprivation results in retinal thinning, reduction in cell densities and synaptic contacts in the deprived eye, with compensatory changes occurring in the non-deprived eye. These changes in the retina may contribute to the changes seen in the visual cortex in monocularly deprived animals.

1. INTRODUCTION

The retina plays a critical role in visual perception as it contains the initial components of the visual pathway (Masland, 2011). The retina develops from out-pouchings of the neural tube known as optic vesicles (Moore et al., 2013), and is made up of an outer retinal pigment epithelium and inner neural retina. The neural retina is the photosensitive layer, and contains several cell types namely photoreceptors (rods and cones), conducting neurons (bipolar and retinal ganglion cells), interneurons (horizontal, and amacrine cells), and supporting (Muller and microglial) cells (Masland, 2012). These cells are arranged in three histologically distinct ‘‘nuclear’’ layers that contain cell bodies but no synapses, separated by two ‘‘plexiform’’ layers that contain synapses but no cell bodies (Wässle, 2004; Masland, 2012). Axons of ganglion neurons form the optic nerve that synapses with third order neurons at the lateral geniculate body of the thalamus (Kolb et al., 1995). The third order neurons mainly project to the primary visual cortex where processing of the visual information takes place (Masland, 2011, 2012).

Monocular visual deprivation as a result of cataracts occurring in children who previously had normal vision, results in permanent visual deficits if the cataracts are not corrected early (Lewis and Daphne, 2005). However if correction is done early, before the age of ten years, vision is usually restored to normal levels in the affected eye (Vaegan and Taylor, 1979; Ellemberg et al., 2000; Lewis and Maurer, 2005). The basis for these observations has been attributed to the plastic changes in the visual cortex (Ellemberg et al., 2000; Lewis and Maurer, 2005). Studies on monocularly deprived animals through eyelid closure, have demonstrated that the visual cortex undergoes anatomical changes ranging from synaptic modifications to changes in the cell densities of the neurons in favor of the non-deprived eye (Hofer et al., 2006; Lehmann and Löwel, 2008).

Although the retina is considered as part of the nervous system based on its embryonic development and

its cellular content, there is scarcity of information on the anatomical changes occurring in the retina as a result of monocular deprivation. One author reported some changes in the retina following visual deprivation (Fifková, 1972a, 1972b). This author mainly reported changes in the thicknesses of the retinal layer with no report on changes in the cell densities of the ganglion, inner nuclear, and outer nuclear cells. This study therefore aimed at describing the histomorphometric changes in the retina following monocular deprivation using a rabbit model. Rabbits offer a good model for vision research because they are readily available, easier to handle, and their visual capabilities as well as the cell types of its retina have been studied in detail and characterized in a fashion similar to those in humans (Amthor et al., 1989; Takahashi and Oyster, 1989; Strettoi et al., 1994; McGillem and Dacheux, 2001; MacNeil et al., 2004; Muraoka et al., 2012).

2. LITERATURE REVIEW

2.1. STRUCTURAL ORGANIZATION OF RETINA

The retina is morphologically made up of two parts: an outer retinal pigment epithelium and an inner neural retina. The neural retina is the photosensitive layer that contains neurons and photoreceptors (Masland, 2012). The outer pigmented layer is an epithelium that lines the ciliary body and posterior iris. The retinal pigment epithelium has many functions such as being part of the blood-retina barrier, absorbing light passing through the retina, phagocytosing shed components from the adjacent rods and cones, removing free radicals, and isomerizing and regenerating retinoids used by the rods and cones (Palczewski and Baehr, 2001; Sparrow et al., 2010; Strauss, 2005).

The neural retina is a multilayered structure consisting of three nuclear layers separated by two synaptic layers (**Figures 1 and 2**). The nuclear layers from outside inwards are: outer nuclear layer containing cell bodies of the photoreceptors; inner nuclear layer containing cell bodies of bipolar cells, amacrine cells, horizontal cells and Muller cells; and the ganglion cell layer containing retinal ganglion cells (Sanes and Zipursky, 2010). There are two types of photoreceptors in the retina, rods and cones (Masland, 2012). The rods contain visual pigment rhodopsin and are used for vision under dark-dim conditions at night (Peichl, 2005). Cones on the other hand contain cone opsins and are the basis for colour vision (Wikler and Rakic, 1990). Horizontal cells are interconnecting neurons that help integrate and regulate input from photoreceptor cells (Peichl et al., 1998). Bipolar cells transmit signals from the photoreceptors to the ganglion cells (McGillem and Dacheux, 2001; MacNeil et al., 2004). Amacrine cells, like horizontal cells, work laterally affecting the output from bipolar cells (Kolb, 1997). Ganglion cells synapse with bipolar cells at the inner plexiform layer, and have central processes that project to the lateral geniculate body of the thalamus (Masland, 2011, 2012).

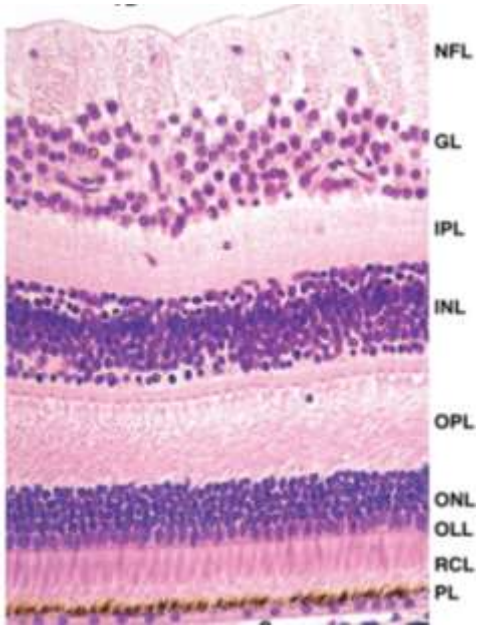

Figure 1: Photomicrograph showing the structural organization of the retina. Note the layers, pigment epithelium layer (PL), rods and cones layer (RCL), outer limiting layer (OLL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion layer (GL), and nerve fibre layer (NFL). *Adapted from Janqueira's Basic Histology Text and Atlas, 12th Edition 2010.*

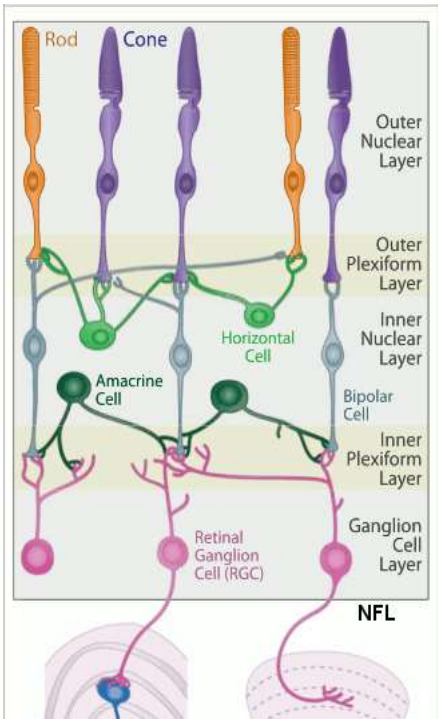

Figure 2: Diagrammatic representation of the composition of the nuclear and plexiform layers of the neural retina. The central processes of the ganglion cells form the nerve fiber layer (NFL) that will eventually form the optic nerve. *Adapted from Sanes and Zipursky, 2010.*

2.2 DEVELOPMENT OF RETINA AND VISUAL PATHWAY

The retina develops from optic vesicles which are outpouchings from two sides of the developing neural tube (Moore and Persaud, 2003). The primordial optic vesicles fold back in upon themselves to form the optic cup, with the inside of the cup becoming the neural retina and the outside remaining a single monolayer of epithelium, known as the retinal pigment epithelium (Agathocleous and Harris, 2009). Initially, both walls of the optic cup are one-cell thick, but the cells of the inner wall divide to form a multilayered neural retina. The neural retina develops in an inside-to-outside manner: ganglion cells are formed first, and photoreceptor cells are last to become fully mature (Agathocleous and Harris, 2009). Additional changes in retinal morphology are accomplished by simultaneous formation of multiple, complex inter-cellular connections (Huberman et al., 2008).

The ganglion cells, when formed in the retina, begin random firing (Graven and Browne, 2008). This in turn stimulates its axon to grow toward the lateral geniculate nucleus of the thalamus. The axons from the retinal ganglion cells form the optic nerve (Reese, 2011). These nerves meet in an X pattern in the optic chiasma. Half of the axons from each eye go to one lateral geniculate nucleus. The neurons in the visual cortex are formed in the germinal matrix in the center of the brain and migrate to the cortex to form six layers (Moore et al., 2013).

2.3 POSTNATAL CHANGES IN THE RETINA AND VISUAL PATHWAY

The retina and visual pathway are fully formed at birth. Visual experience in the early post natal period, the critical period, is important in the maturation of the visual system (Sengpiel and Kind, 2002). In rodents and rabbits, the first two weeks after birth are characterized by maturation of the synapses in the inner plexiform and outer plexiform layers (Reichenbach et al., 1993; Sharma et al., 2003; Coombs et al., 2007). Peak synaptogenesis in the outer plexiform layer occurs within the first seven days after birth (Sharma et al., 2003) while in the inner plexiform layer synaptogenesis is maximal in the second and

third postnatal weeks (McArdle et al., 1977). In addition, during this period, the elements of the outer plexiform layer such as recoverin, calbindin, synaptophysin, beta-tubulin and actin are assembled (Sharma et al., 2003). The thickness of the retina decreases after birth as a result of stretching from the growing eyeball (Reichenbach et al., 1991, 1993). The retina expands more in the peripheral areas more than in the central region (visual streak). Thus, retinal thinning after birth is marked in the peripheral retina, and less pronounced in the visual streak (Reichenbach et al., 1991, 1993; Kuhrt et al., 2012). In all regions of the retina, the thicknesses of the nuclear layers decreases while those of the plexiform layers increase (Reichenbach et al., 1991).

The critical period, also known as the sensitive period, is defined as the period in which normal visual input is required for normal visual development (Hensch, 2004; Levelt and Hübener, 2012). This period corresponds to the time in normal development during which geniculocortical axons attain their mature organization in the form of ocular dominance columns, and is affected by monocular deprivation (Wiesel and Hubel, 1963; Dews and Wiesel, 1970; Huberman et al., 2008). In rodents and cats, plasticity is low at eye opening, peaks around four weeks of age, and declines over several weeks to months (Fagiolini et al., 1994; Levelt and Hübener, 2012). In human beings, the critical period appears to lie within the first 10 years of life (Vaegan and Taylor, 1979; Lewis and Daphne, 2005). Tests on the deprived eye of children who had a normal visual history until they developed a cataract in one eye resulted in abnormal visual acuity if the deprivation began before 8 years of age, but not if it began after 10 years (Maurer and Lewis, 2001).

2.4. EFFECTS OF MONOCULAR DEPRIVATION

2.4.1 Primary visual cortex

Monocular visual deprivation by lid closure leads to a loss of cortical responsiveness of the visually-deprived eye and a concomitant visual impairment (Hensch, 2004). This phenomenon is referred to as

ocular dominance plasticity. Changes occurring in the visual cortex as a result of monocular deprivation were first described by Wiesel and Hubel, (1963). Subsequent studies further reported that monocular deprivation resulted to changes in the neurons in the primary visual cortex in favour of the non-deprived eye (Wiesel and Hubel, 1965; Dews and Wiesel, 1970; Fagiolini et al., 1994). These changes in the visual cortex include modification of the existing synapses, formation of new synapses, and changes in the density of the neurons (Antonini et al., 1999; Trachtenberg and Stryker, 2001; Noppeney, 2007). These changes follow competitive interactions between the two eyes for the control of cortical territory (Daw, 2006). As a direct consequence of shifts in cortical ocular dominance, the weakened input becomes amblyopic, resulting in reduction in visual impairment even when no physical damage to the retina exists (Dews and Wiesel, 1970; Maurer et al., 1999; Daw, 2006; Noppeney, 2007).

2.4.2 Thalamus

Retinal output is relayed to the visual cortex by the dorsal lateral geniculate nucleus of the thalamus (Linden et al., 2009). There are contradicting reports on the effects of monocular deprivation on the thalamus. Some studies have reported that the lateral geniculate body is relatively unchanged by monocular deprivation (Silver and Stryker, 1999; Purves et al., 2001; Linden et al., 2009). Quantitative studies of cell size in the cat's dorsal lateral geniculate nucleus revealed that the cells in the deprived lamina were smaller than those of non-deprived lamina (Hickey et al., 1977; Robertson et al., 1980; Kutcher and Duffy, 2007). Other studies have reported that deprivation leads to synaptic depression on the thalamic region receiving input from the occluded eye (Tieman, 1984; Krahe and Guido, 2011).

2.4.3 Retina

Studies on the retinae of completely dark reared or light reared animals report conflicting results. Initial studies had reported that visual deprivation had no discernible effect on the retina (Wiesel and Hubel,

1965; Hendrickson and Boothe, 1976; Lau et al., 1990). Hendrickson and Boothe (1976) who studied completely dark reared monkey (from postnatal day 14 to 6 months), did not report any obvious changes in the retinal cell number and size compared to light-reared age-matched controls. A study on retinal ganglion cells of hamsters (Lau et al., 1990) did not reveal any difference in the soma size, dendritic field area, and dendritic aborisation pattern between normal and visually deprived (bilateral eye lid closure) animals.

However later studies, mainly electrophysiological, reported that indeed visual deprivation affects the retina (Baro et al., 1990; Shou et al., 1994; Tian and Copenhagen, 2003). For instance, light stimulation has been shown to enhance light-evoked responsiveness of inner retinal neurons in young mice while dark rearing suppresses them (Tian and Copenhagen, 2003). The findings by these authors challenged the commonly held belief that retinal structure and function is immune to visual deprivation. In spite of this, there is no study that gives a detailed account of the anatomical changes in the retinal as a result of monocular deprivation. One author reported some changes in the retina following visual deprivation (Fifková, 1972a, 1972b). This author mainly reported changes in the thicknesses of the retinal layer with no report on changes in the cell densities of the ganglion, inner nuclear, and outer nuclear cells.

2.5 USE OF ANIMALS IN VISION RESEARCH

Several animal models such as the mice, monkeys, rabbits, tree shrews, and cats have been used in vision research (Dews and Wiesel, 1970; Amthor et al., 1989; Baro et al., 1990; MacNeil et al., 2004; Lehmann and Löwel, 2008; Abbott et al., 2011). Rabbits offer a good model for vision research as they are readily available, easier to handle, and their visual capabilities as well as cell types of its retina have been studied in detail and characterized in a fashion similar to those in humans (Amthor et al., 1989; Takahashi and Oyster, 1989; Strettoi et al., 1994; McGillem and Dacheux, 2001; MacNeil et al., 2004; Muraoka et al., 2012).

3. STUDY RATIONALE

Neuroplasticity is the ability of the nervous system to adapt its structural organization to new situations emerging from changes to intrinsic or extrinsic inputs (Antonini and Stryker, 1993; Daw, 2006). Effects of monocular deprivation on the visual cortex and lateral geniculate body of the thalamus have been studied (Dews and Wiesel, 1970; Fagiolini et al., 1994; Purves et al., 2001; Kutcher and Duffy, 2007; Linden et al., 2009). However, the effects of monocular deprivation on the structure of retina, which houses photoreceptors and neurons involved in visual pathway remain largely understudied (Fifková, 1972a, 1972b). To the best of our knowledge, there is no study that gives a detailed account on the anatomical changes in all layers of the retina following visual deprivation. This study therefore aimed at describing the histomorphometric changes in the retina following monocular deprivation using a rabbit model.

Visual deprivation by lid suture has been used as a reliable laboratory model for studying anatomical changes in the visual cortex resulting from visual impairments such as cataracts (Dews and Wiesel, 1970). According to World Health Organization report, cataract is the leading cause of visual impairment, accounting for 50% of blindness in Sub Saharan Africa (World Health Organization, 2012). Monocular visual deprivation as a result of cataracts occurring in children who previously had normal vision results in permanent visual deficits if the cataracts are not corrected before the age of ten years (Vaegan and Taylor, 1979; Ellemberg et al., 2000; Lewis and Maurer, 2005). Although the basis for these observations has largely been attributed to the plastic changes in the visual cortex, recent studies have shown that the retina indeed undergoes some plastic changes (Feller, 2003; Tian and Copenhagen, 2003; Tian, 2004; Krahe and Guido, 2011). Understanding the effect of monocular light deprivation on the neural may therefore further explain the plastic changes seen in the visual system, and may inform eye specialists on the timing of the corrective surgery for pre-retinal causes of blindness such as cataracts

4. STUDY OBJECTIVES

Broad objective: To determine the histomorphometric changes occur in the neural retina following monocular deprivation.

Specific objectives:

1. To determine the changes in the thickness of neural retina following monocular deprivation
2. To determine the changes in the densities of neural retinal cells following monocular deprivation
3. To describe the changes in the dendritic features of ganglion cells following monocular deprivation

5. MATERIALS AND METHODS

5.1. STUDY DESIGN

Randomized trial.

5.2. MATERIALS

Thirty Californian White (*oryctolagus cuniculus*) rabbits obtained from a local private commercial farm were used. The sample size was calculated using the following formula (Sakpal, 2010; Suresh and Chandrashekara, 2012);

$$n = \frac{(r+1)(z_{(\alpha/2)}+z_{(1-\beta)})^2\sigma^2}{r\delta^2}$$

Where: *n = sample size, $Z_{(1-\beta)}$ = desired power (typically 0.84 for 80% power), $Z_{(\alpha/2)}$ = desired level of statistical significance (typically 1.96 for a significance of 0.05), σ = standard deviation, δ = smallest meaningful difference, r = n1/n2 is the ratio of sample size required for 2 groups (typically 1 for equal sample size for 2 groups. If r = 0.5 gives the sample size distribution as 1:2 for 2 groups).*

A previous study by Muraoka et.al (2012) had revealed that the total retinal thickness of a rabbit was 194.3±7.7 μm. Using a statistical significance of 0.05, power of 80%, and δ of 7.7 μm (one standard deviation), and r = 0.5 (ratio of controls to experimental animals of 1:2), the sample size was calculated as follows:

$$n = \frac{(0.5+1)[1.96+0.84]^2*7.7^2}{0.5*7.7^2} = \frac{1.5*7.84}{0.5} = 23.52 = 24 \text{ animals}$$

The sample size was increased to 30 animals to cater for a 25% drop out rate.

Inclusion criteria

Since the peak period for development of ocular dominance plasticity is between the 2nd and 4th postnatal week (Fagiolini et al., 1994; Levelt and Hübener, 2012), the rabbits were recruited into the study on

their 14th postnatal day. The rabbits had opened the eyes between 8th and 9th days after birth.

Exclusion criteria

Rabbits with obvious congenital or acquired eye disorders were excluded from this study.

Sampling technique

The thirty rabbits were obtained from three does each containing 10 kits. The kits in each doe were randomly assigned numbers 1 to 10. The kit bearing number one for each doe was sacrificed at the start of the study (P14), their retina harvested and analyzed. This served as the baseline cluster. For the remaining nine kits, the 4th, 7th and 10th kits were assigned to the control group, while the rest were allocated to the experimental group (**Table 1**). The experimental animals were mixed with the control animals. Rabbits with labels 2, 3, and 4 were sacrificed after 7 days of study (P21), while rabbits 5, 6, and 7 were studied on day 14 of the study (P28). The remaining rabbits had their retina harvested and studied on day 21 of the study (P35).

Table 1: Study allocation of kits per doe

Study number	Allocated group	Age at time of tissue harvesting
1	**Baseline**	**Postnatal day 14 (P14)**
2	**Experimental**	**Postnatal day 21 (P21)**
3	**Experimental**	**Postnatal day 21 (P21)**
4	**Control**	**Postnatal day 21 (P21)**
5	Experimental	Postnatal day 28 (P28)
6	Experimental	Postnatal day 28 (P28)
7	Control	Postnatal day 28 (P28)
8	**Experimental**	**Postnatal day 35 (P35)**
9	**Experimental**	**Postnatal day 35 (P35)**
10	**Control**	**Postnatal day 35 (P35)**

5.3. ETHICAL CONSIDERATIONS

The approval to carry out the study was granted by the Biosafety, Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nairobi.

5.4. HANDLING OF STUDY ANIMALS

All the study animals were obtained from a local private farm that breeds rabbits for commercial as well as research use. Since the study involved use of young rabbits that were still breastfeeding, and so as to minimize stress related to the rabbits adapting to a new environment, the initial stages of the study from acquisition of the animals up to tissue harvesting were conducted at the farm. The later stages of the study involving histology and data analysis were conducted at the Department of Human Anatomy, University of Nairobi. The rabbits were kept in wire cages measuring 4 feet by 4 feet, floored with saw dust. Each cage housed one doe and its litter, and contained a nest box where the litter stayed. Since a nursing female and its litter require a minimum floor space of 7.5 square feet (for a doe more than 5kg body weight), and one doe would have 6-12 kits per litter (average 9), then a 16 square foot cage was be spacious enough for each doe and its kits. For this study, three does each containing 10 kits were used. The kits were weaned on their 28^{th} postnatal day, by being fed on commercial rabbit pellets, half a cup of pellets per 5 kilogram body weight daily. They were also offered water ad libitum through sipper bottles with nozzles. The rabbit cages were cleaned on a daily basis. The rabbits were raised on 12:12-hour light-dark cycle with fluorescent light (500 lux intensity) onset at 6am.

Restraint techniques

During procedures such as weighing, injections and tarsorrhaphy, the rabbits were restrained as follows:

a) Rabbits were removed from their cage by grasping the excess skin over the dorsal cervical region with one hand while the other supports its hind quarters.
b) Polycarbonate Rabbit Restrainer (from Harvard Apparatus Inc.) was used when measuring the weights of the rabbits as well as during administration of drugs
c) During tarsorrhaphy, the rabbits were restrained by wrapping a clean towel on the animals' body.

5.5. MONOCULAR DEPRIVATION

Eighteen rabbits (experimental animals) were recruited on their 14th postnatal day. These rabbits were then clustered into two groups each containing eight rabbits. One group had their right eye lids sutured together while the other group had their left eye lids stitched up. These animals were anesthetized with intramuscular ketamine (50mg/kg) and also given intramuscular analgesic (Flunixin meglumine 1.1mg/kg). Two drops of gentamycin (antibiotic) eye drops were applied on the eye to be deprived. The margins of the upper and lower lids of one eye were trimmed and sutured together using Nylon 5.0 single vertical mattress stitch in aseptic technique.

Following tarsorrhaphy, the rabbits were returned to their home cages and observed daily for suture breakdown or infection. Only rabbit developed suture dehiscence, and was subsequently disqualified from the study. The experimental and control kits for each doe were put in the same cage. Post-operative pain was managed by intramuscular Flunixin meglumine (1.1mg/kg) every 24 hours for 4 days. In addition, the animals were clinically assessed for signs and symptoms of pain such as poor feeding, facing the back of the cage (hiding posture), vocalization by means of a piercing squeal, kicking and scratching, and teeth grinding. Rabbits that continued experiencing pain despite being on the regular analgesic received a further dose of Butorphanol 0.5 mg/kg IM 12 hourly until they were pain free.

5.6. TISSUE HARVESTING

Three control animals were sacrificed at the start of the study (P14). This was on the same day the experimental animals had their eyelids sutured together. Thereafter nine rabbits, 3 controls and 6 experimental, were sacrificed each successive week (P21, P28, and P35). Following weight determination, the rabbits were euthanized using intravenous Euthasol® (Sodium pentobarbital 390 mg/ml + sodium phenytoin 50 mg/ml) at a dose of 1mL per 4.5Kg body weight (86.7mg/kg pentobarbital and 11.1mg/kg phenytoin). Once death was confirmed by loss of pupillary light reflex and corneal reflex,

the thoracic cavity was opened then intra cardiac perfusion with normal saline commenced. Following perfusion both eyes were enucleated. Each eye was bisected horizontally into two hemispheres oriented along the optic nerve. This was followed by removal of the vitreous humor from the eyecup so as to facilitate penetration of the fixing medium. The carcasses were incinerated after the tissues were harvested.

5.7. TISSUE PROCESSING

Both hemispheres obtained from each eye were processed for light microscopic examination by paraffin wax embedding. Within the wax blocks, each hemisphere was oriented along its horizontal meridian. One hemisphere was stained with Haematoxylin & Eosin stain to demonstrate cellular detail of the different layers of the retina, while the other was stained with Golgi stain to elucidate the neuronal processes (axons and dendrites) of the ganglion neurons. Sectioning of the blocked tissues started from the central retina (retina near the optic nerve). In each block, every fourth serially cut section was picked in a glass slide and dried. Only the first four picked sections were stained and used for analysis.

a) Haematoxylin and Eosin method

The harvested retinae were fixed in formaldehyde by immersion technique for at least 24 hours followed by dehydration in increasing strengths of alcohol (70%, 95%, and 100%) each 30 minutes. The tissues were then cleared in xylene before being infiltrated with wax for 24 hrs. This was followed by embedding in paraffin wax. The embedded tissues were then serially cut into 5 micrometer thick sections using a microtome, starting with the central retina (as oriented by the optic nerve). Every fourth section was picked on a glass slide then dried in an oven. The dry sections were dipped in xylene to remove the paraffin wax, followed by rehydration in descending grades of ethanol (100%, 95% and 70%). After rehydration the sections were dipped in a jar containing Haematoxylin for 15 minutes then washed in

running water for 2 minutes to remove excess stain. This was followed by staining the sections in 1% eosin solution for 3 minutes, followed by dehydration in ascending grades of ethanol from 70% to absolute alcohol. The sections were then cleared in xylene before being covered with cover slides.

b) Golgi method

Once the vitreous humor was removed from the eye cup, the retinae were fixed in 3% potassium dichromate and 0.25% osmium tetroxide (pH 5.8) at 25°C for 16 hours. Following fixation, the retina and choroid were detached from the sclera and rinsed several times with silver nitrate solution (0.75%) until the formation of red-brown precipitate stopped. The tissues were then impregnated with silver by being placed in 50mls of fresh solution of silver nitrate for periods of 24 hours at room temperature.

After silver impregnation, the retinae were rinsed in double-distilled water overnight then dehydrated in graded series of alcohols (10 minutes each), followed by absolute ethanol (changed every 30 minutes for 2 hours). The dehydrated retinae were cleared in cedarwood oil for a 36 hour period, followed by clearing in xylene for 30 minutes and mounting in paraffin wax for 2 hours. The mounted tissues were serially sectioned at 80 micrometers using a microtome, starting with the central retina (as oriented by the optic nerve). Every fourth section was picked for the next step. These sections were dried in an oven then deparaffinised in xylene followed by rehydration in decreasing concentration of alcohol (100%, 95%, 70%, and 50%). The sections were counterstained with cresyl violet then dehydrated in increasing concentration of alcohol (50%, 70%, 95%, and 100%). The sections were then cleared in xylene before being covered with cover slides.

5.8. MORPHOMETRIC ANALYSIS

Photomicrographs of the sections were taken using Canon® digital camera (12 megapixels). These photographs were transferred to a computer installed with ImageJ-Fiji software (Schindelin et al., 2012) for morphometric analysis. ImageJ is open source software developed by United States National Institute of Health for processing and analyzing images. It has inbuilt morphometric and stereological tools. The following data were collected: vertical thickness of each retinal layer; cell densities in inner nuclear, outer nuclear and ganglion cell years; and dendritic features of ganglion neurons (number of primary dendrites, number of terminal dendrites, dendritic ramification ratio and dendritic field area).

a) Vertical measurements

The following vertical measurements were taken in Haematoxylin and Eosin stained sections:

- outer nuclear layer thickness, taken as an index of photoreceptor number (Michon et al., 1991)
- outer plexiform layer thickness, taken as synaptic index of the photoreceptors and bipolar cells
- inner nuclear layer thickness, taken as index of bipolar, amacrine and horizontal cells
- inner plexiform layer thickness, taken as synaptic index of the bipolar and ganglion cells
- Ganglion cell layer thickness, taken as index of ganglion neurons.

To minimize intra-observer error, these measurements in each section were taken from four random locations of the central retina, and then averaged. Each measurement was expressed in micrometers.

b) Estimation of cell densities

A grid generated by the FIJI software, containing boxes 20 × 20 µm in size, was superimposed on photomicrographs of Haematoxylin and Eosin stained sections. These photomicrographs had been taken at x200 magnification. Cell bodies in the outer nuclear, inner nuclear and ganglion cell layers within the central retina (visual streak) were counted. For each of the three layers, cells from four boxes obtained

by picking the first, third, fifth, and seventh boxes within the row overlying the layer were counted then averaged. Each box had green inclusion lines and red exclusion lines (**Figure 3**). To avoid oversampling, only the cell bodies located in the box or crossed the green inclusion lines were counted. Nuclei touching or crossing the red exclusion lines were not counted.

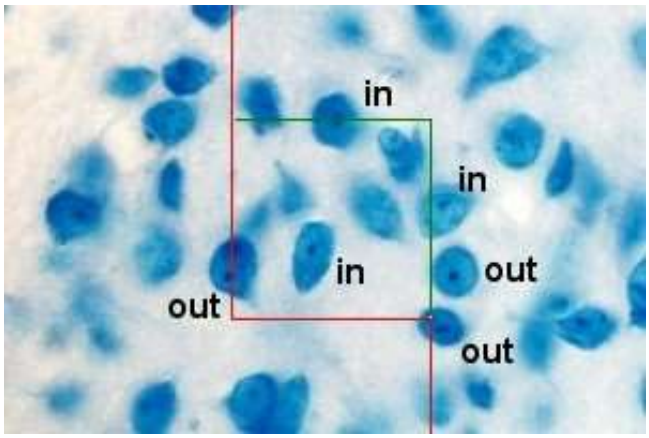

Figure 3: Cell counting protocol. *The green lines are the inclusion lines while the red lines are the exclusion lines.*

The cell densities were determined using the following formula:

Cell density *(n/mm^2)* = number of cell bodies counted *(n)* / grid area *(mm^2)*

c) Dendritic features

Photomicrographs from Golgi stained sections were used to determine the dendritic features of the ganglion neurons. Using the Simple Neurite tracer plugin in Fiji software, the dendrites of each ganglion neuron was followed to the inner plexiform layer. Simple Neurite Tracer is open source software that is designed to allow for reconstruction, visualization and analysis of neuronal processes (Longair et al., 2011). The following parameters were determined as described by previous studies (Coombs et al., 2007; Germain et al., 2010; Milatovic et al., 2010):

- ***Number of primary dendrites:*** *the number of dendrites emerging from the soma. (**Figure 4**)*

- ***Number of terminal dendrites:*** *the number of outermost tips of the dendrites from a single-cell*
- ***Dendritic ramification index****: number of terminal dendrites / number of primary dendrites*
- ***Dendritic field size:*** *area bounded by a line connecting the tips of the terminal dendrites*

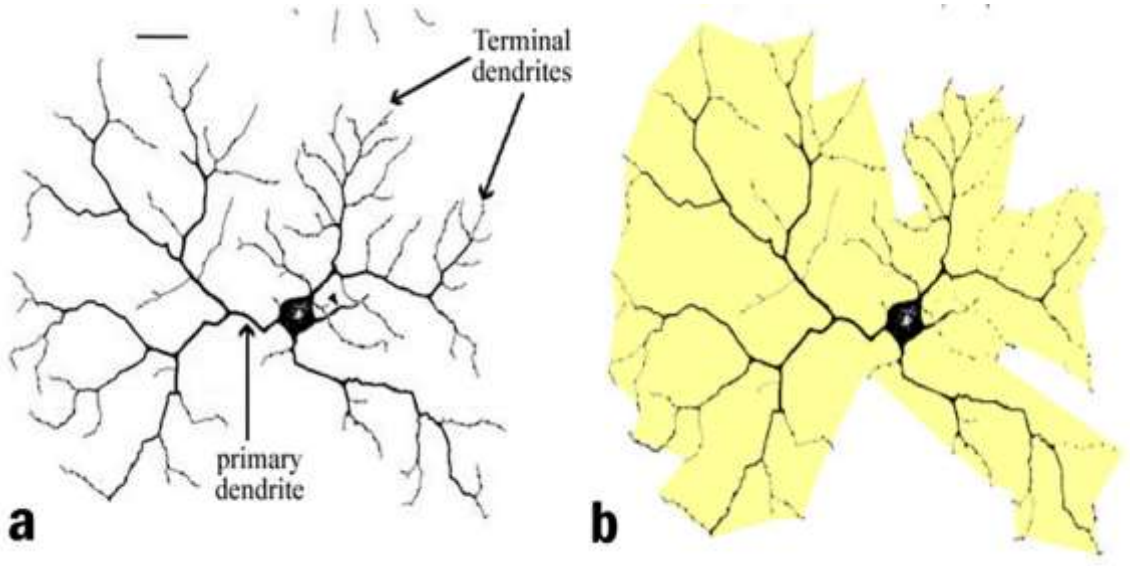

Figure 4: Dendritic features of a neuron. a) Primary dendrites emerge directly from the cell body (soma), then divides into secondary branches that finally end as terminal branches. **b)** The dendritic field area is the yellow shaded area.

5.9. STATISTICAL ANALYSIS

Data collected were entered into the Statistical Package for Social Sciences software (Version 17.0, Chicago, Illinois) for coding, tabulation and statistical analysis. After confirming that the data was normally distributed using histograms and box plots, parametric tests were used to compare the means of the variables measured. Analysis of Variance (ANOVA) test was used to compare the means of each variable studied from baseline to the end of third week of study. The Student's t-test was used to compare the differences in means between the non-deprived *and* deprived eyes, non-deprived *and* control eyes, and deprived *and* control eyes. A *p value* <0.05 was considered significant at 95% confidence interval.

6. RESULTS

Of the thirty rabbits recruited into the study, one rabbit in the experimental group was excluded from the study as it developed suture dehiscence (dropout rate 3.3%). Thus, fifty eight retina (from 29 rabbits) were studied. Retinae from all the study animals (experimental and control) had the classic layers of the neural retinae (**Figure 5**). The ganglion cell layer in all the retinae studied was one cell thick. All variables measured did not reveal any statistically significant differences between the right and left eyes.

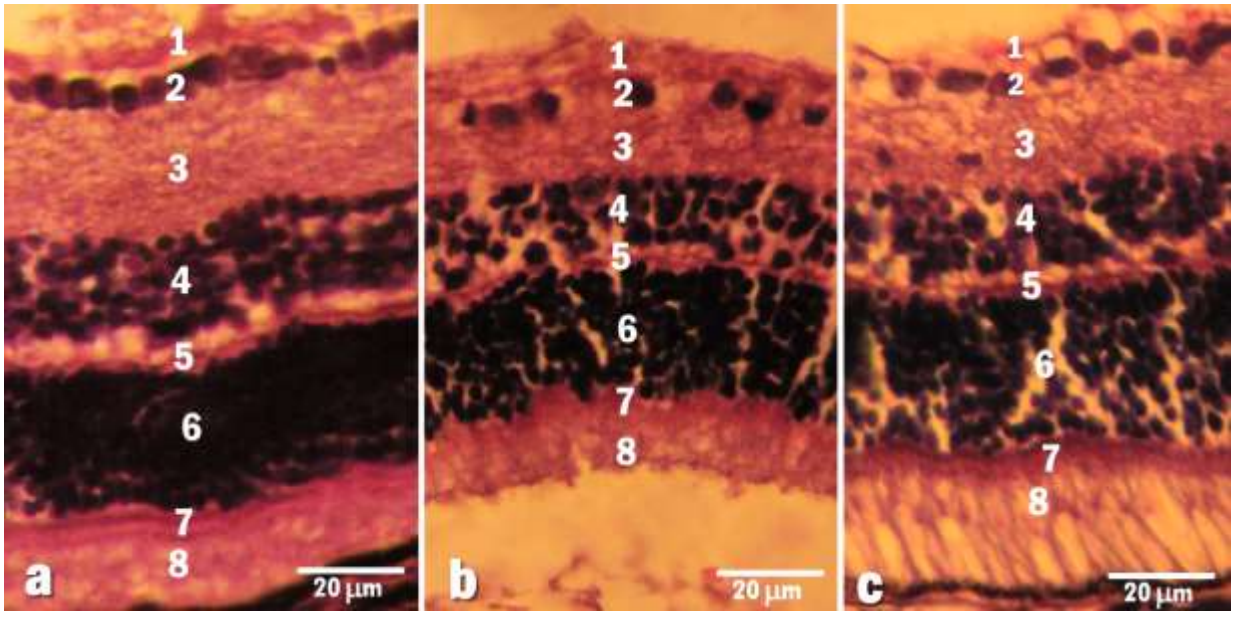

Figure 5: Photomicrograph displaying layers of neural retina in five week old rabbits. a) Non deprived eye, **b)** Deprived eye, **c)** Control eye. Note the layers are numbered from the innermost to the outermost. *1- Nerve fiber layer, 2-ganglion cell layer, 3-inner plexiform layer, 4-inner nuclear layer, 5-outer plexiform layer, 6-outer nuclear layer, 7-outer limiting membrane, 8-rods and cones layer (Haematoxylin and Eosin stain, x92).*

6.1 RETINAL LAYER THICKNESS

6.1.1 Deprived eyes

The total thickness of the neural retina reduced with increasing duration of deprivation (**Figure 6**). The thickness of the neural retina at the start of the study (P14), after 7 days (P21), 14 days (P28) and 21 days (P35) of deprivation was 118.66±12.28µm, 105.84±13.04µm, 94.37±25.06µm, and 70.66±18.07µm respectively (p=0.001). Thus, the total neural retina thickness reduced by 40.5% from P14 to P35. There was generalized reduction in the thicknesses of all individual layers of the retina with increasing duration of deprivation (**Table 2**). The ANOVA test revealed statistically significant reduction in the thickness of the inner plexiform (58.4%, p<0.001), inner nuclear (50.3%, p<0.001), rods and cones (51.4%, p=0.005) and ganglion cell (39.7%, p=0.008) layers only (**Table 2**).

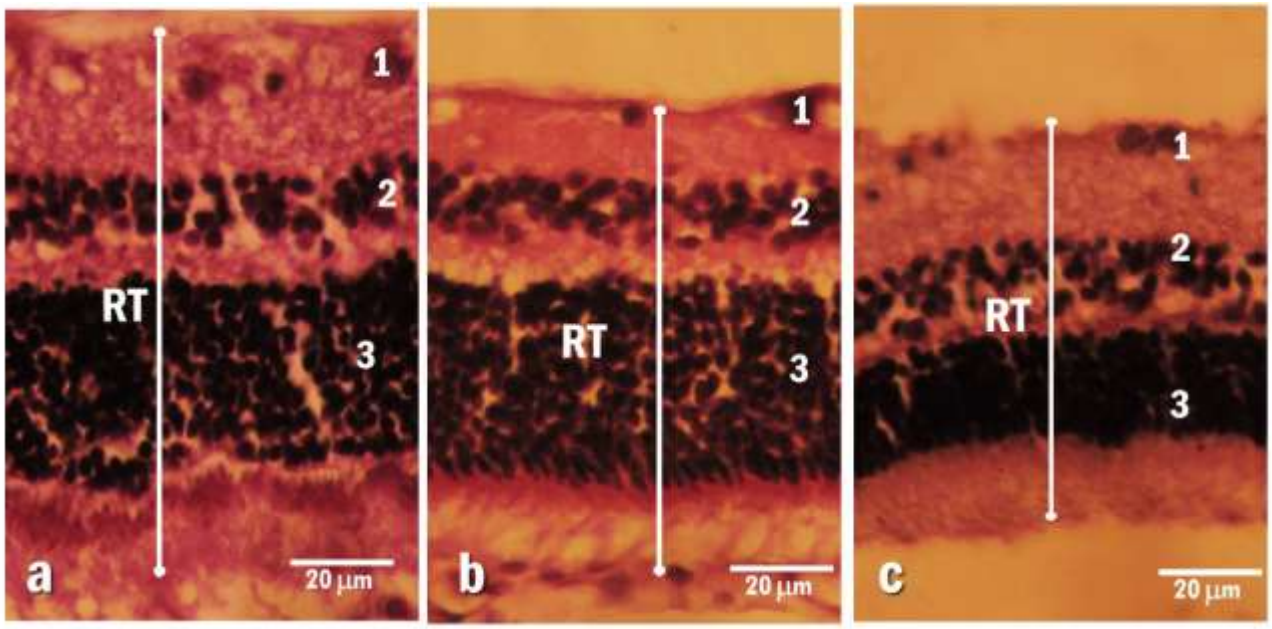

Figure 6: Photomicrograph of the neural retinal layer thickness in the deprived eyes. **a)** After 7 days of monocular deprivation (P21), **b)** After 14 days of monocular deprivation (P28), **c)** After 21 days of monocular deprivation (P35). Note that the neural retinal thickness (RT) decreases with increasing duration of deprivation. *For orientation, the cell body layers are marked 1-3. 1-Ganglion cell layer, 2-Inner nuclear layer, 3-outer nuclear layer.* (*Haematoxylin and Eosin stain, x92*).

6.1.2 Non-deprived eyes

There was no marked change in the thickness of the neural retina with increasing duration of deprivation (**Figure 7**). The total neural retinal thickness in the non-deprived eye at the start of the study, after 7 days, 14 days and 21 days of deprivation was 119.36±12.75µm, 120.26±14.18µm, 125.96±13.38µm, 132.4±5.11 µm respectively (p=0.075). Thus, the total neural retina thickness increased by 9% from P14 to P35. Although the thickness of the layers of the retina in the non-deprived eyes increased with increasing period of monocular deprivation, ANOVA test revealed statistically significant differences in the inner plexiform and nerve fibre layers only (**Table 2**).

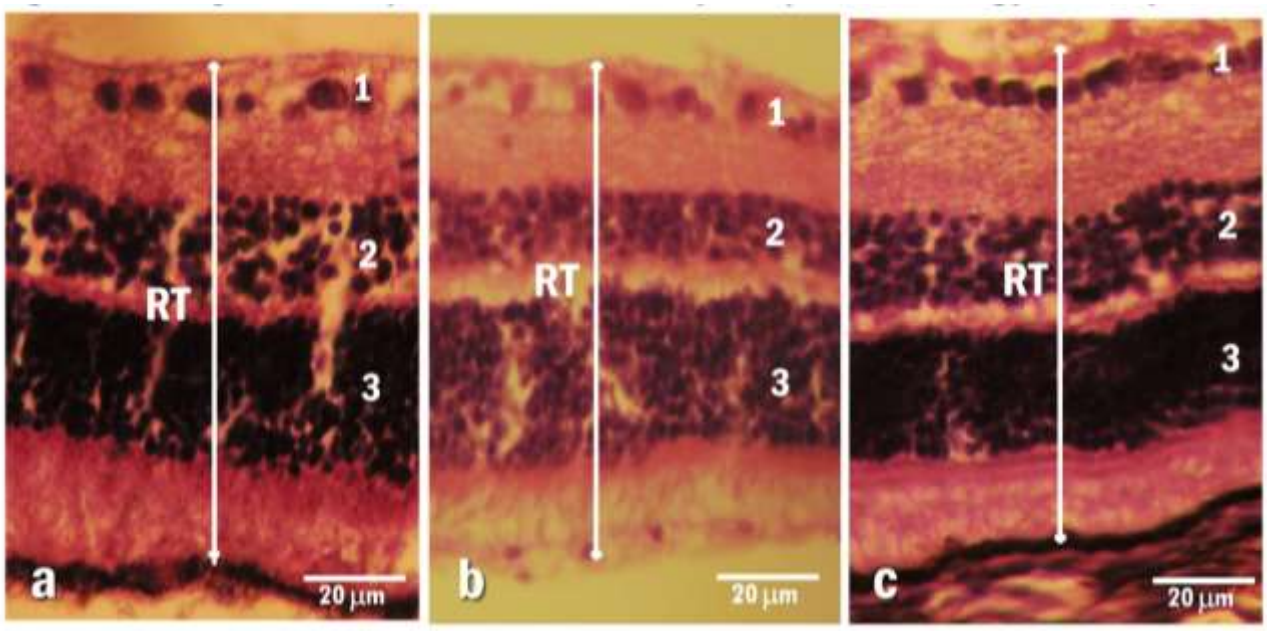

Figure 7: Photomicrograph of the neural retinal layer thickness in non-deprived eyes. a) After 7 days of monocular deprivation (P21), **b)** After 14 days of monocular deprivation (P28), **c)** After 21 days of monocular deprivation (P35). Note that there is no marked difference in the neural retinal thickness (RT) with increasing duration of deprivation. *For orientation, the cell body layers are marked 1-3. 1-Ganglion cell layer, 2-Inner nuclear layer, 3-outer nuclear layer. (Haematoxylin and Eosin stain, x92).*

6.1.3 Control eyes

There were no marked age related changes in the thickness of the neural retina among the control eyes (**Figure 8**). The neural retina thickness at the start of the study, after 7 days, 14 days and 21 days of study were 119.36±12.75µm, 114.85±15.82µm, 108.11±27.01µm, 109.10±9.48µm respectively (p = 0.722). ANOVA test did not reveal any statistically significant differences in the thickness of the individual layers of the retina among the control eyes (**Table 2**).

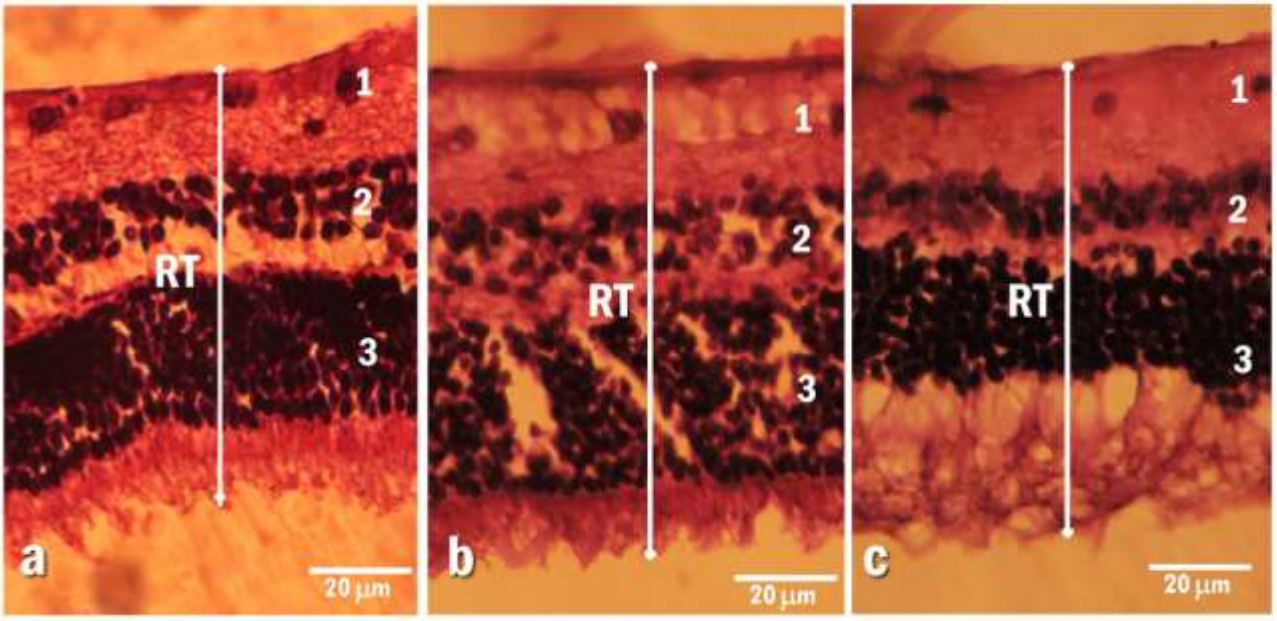

Figure 8: Photomicrograph of the neural retinal layer thickness among control eyes. a) After 7 days of study (P21), **b)** after 14 days of study (P28)**, c)** after 21 days of study (P35). Note that there is no marked difference in the overall neural retinal thickness (RT) with increasing age. *For orientation, the cell body layers are marked 1-3. 1-Ganglion cell layer, 2-Inner nuclear layer, 3-outer nuclear layer*. (*Haematoxylin and Eosin stain, x92*).

Table 2: Changes in the retinal layer thicknesses with increasing period of deprivation

Retinal layer	Duration of deprivation (Days)	Postnatal Age (days)	Deprived eyes		Non deprived eyes		Control eyes	
			Mean± SD (µm)	p-value [c]	Mean± SD (µm)	p-value [c]	Mean± SD (µm)	p-value [c]
Rod and cones layer	0[a]	14	23.53±7.66	0.005 [b]	23.53±7.66	0.977	23.53±7.66	0.162
	7	21	18.99±4.35		23.81±2.12		20.57±3.39	
	14	28	16.30±5.99		24.10±3.00		18.41±4.28	
	21	35	11.44±3.69		24.45±5.47		17.83±1.41	
Outer limiting Membrane	0[a]	14	4.51±0.52	0.941	4.51±0.52	0.676	4.51±0.52	0.952
	7	21	4.51±0.97		4.83±1.86		4.66±0.95	
	14	28	4.49±1.66		5.07±1.28		4.73±1.21	
	21	35	4.37±1.95		5.37±1.24		4.81±0.59	
Outer nuclear layer	0[a]	14	36.43±5.26	0.460	36.43±5.26	0.938	36.43±5.26	0.978
	7	21	33.06±9.07		35.15±6.56		36.45±5.77	
	14	28	31.53±11.12		34.99±9.18		36.51±13.34	
	21	35	26.58±14.62		34.16±3.71		36.60±7.86	
Outer plexiform layer	0[1]	14	7.07±2.03	0.245	7.07±1.58	0.828	7.07±1.58	0.997
	7	21	7.05±3.70		7.18±3.12		7.07±0.09	
	14	28	5.73±2.38		7.29±0.69		7.30±3.89	
	21	35	4.41±1.70		7.90±1.83		7.34±1.07	
Inner nuclear layer	0[a]	14	16.49±3.16	<0.001 [b]	16.49±3.16	0.109	16.49±3.16	0.413
	7	21	15.09±3.24		16.95±2.61		17.39±3.20	
	14	28	12.66±1.95		18.12±2.19		16.07±4.87	
	21	35	8.29±2.07		19.81±4.15		13.89±1.32	
Inner plexiform layer	0[a]	14	20.51±5.24	<0.001 [b]	20.51±5.24	0.012 [b]	20.51±5.24	0.195
	7	21	17.67±5.39		20.41±4.35		20.18±3.86	
	14	28	14.20±3.82		22.73±4.38		14.79±7.86	
	21	35	8.53±2.45		26.53±4.41		14.17±5.21	
Ganglion cell layer	0[a]	14	5.84±0.83	0.008 [b]	5.84±0.83	0.285	5.84±0.83	0.425
	7	21	5.88±1.18		6.93±1.80		7.06±0.88	
	14	28	5.49±1.80		7.13±1.29		7.44±2.46	
	21	35	3.59±0.65		7.19±0.84		7.50±1.81	
Nerve fibre layer	0[a]	14	4.98±2.17	0.200	4.98±2.17	0.033 [b]	4.98±2.17	0.214
	7	21	4.46±1.21		4.99±1.39		4.72±2.23	
	14	28	3.97±1.69		6.55±2.21		4.97±0.73	
	21	35	3.46±0.59		6.99±1.69		6.96±2.97	

[a] The values on postnatal day 14 are repeated in the three groups as they are the baseline values.

[b] p-value < 0.05. [c] p-value obtained after ANOVA test

6.1.4 Comparison between deprived and non-deprived eyes

The deprived eyes retinal layers were thinner than the non-deprived counterparts, with the differences in thickness being marked with increasing duration of deprivation (**Figures 6 and 7**). Comparison of the differences in means in the retinal layer measurements between the non-deprived and deprived eyes, using Student's *t*-test, revealed statistically significant differences in all layers of the retina apart from the outer nuclear layer and outer limiting membrane (**Table 3**).

6.1.5 Comparison between deprived and control eyes

The deprived eyes retinae had thinner retinae compared to the control eyes (**Figures 6 and 8**). The differences between the deprived and control eyes were more marked with increasing duration of deprivation. Comparison of the differences in means in the retinal layer measurements after 21 days of deprivation, using Student's *t*-test, revealed statistically significant differences in all layers apart from the outer limiting membrane and outer nuclear layer (**Table 3**).

6.1.6 Comparison between non-deprived and control eyes

The non-deprived eyes had thicker retina compared to the controls (**Figures 7 and 8**). Comparison of the means between the non-deprived and control eyes using Student's *t*-test revealed statistically significant differences in the rod and cones, inner nuclear, and inner plexiform layers after 21 days of deprivation (**Table 3**).

Table 3: Comparison of retinal layer thicknesses between deprived, non-deprived eyes and controls

Retinal layer	Eye	Duration of monocular deprivation		
		7 days (p value[a])	14 days(p value[a])	21 days(p value[a])
Retinal thickness	*Deprived vs. Non deprived*	0.034 [b]	0.002[b]	<0.001[b]
	Non deprived vs Control	0.525	0.070	<0.001[b]
	Deprived vs. Control	0.348	0.279	<0.001[b]
Rod and cones layer	*Deprived vs. Non deprived*	0.002[b]	0.001[b]	<0.001[b]
	Non deprived vs Control	0.033[b]	0.003[b]	0.007[b]
	Deprived vs. Control	0.537	0.387	0.001 [b]
Outer limiting Membrane	*Deprived vs. Non deprived*	0.651	0.368	0.197
	Non deprived vs Control	0.867	0.556	0.282
	Deprived vs. Control	0.809	0.716	0.575
Outer nuclear layer	*Deprived vs. Non deprived*	0.536	0.436	0.116
	Non deprived vs Control	0.727	0.765	0.384
	Deprived vs. Control	0.510	0.373	0.136
Outer plexiform layer	*Deprived vs. Non deprived*	0.930	0.058	0.003[b]
	Non deprived vs Control	0.946	0.993	0.477
	Deprived vs. Control	0.992	0.296	0.008 [b]
Inner nuclear layer	*Deprived vs. Non deprived*	0.151	0.003[b]	<0.001[b]
	Non deprived vs Control	0.781	0.225	0.002[b]
	Deprived vs. Control	0.260	0.213	<0.001 [b]
Inner plexiform layer	*Deprived vs. Non deprived*	0.203	<0.001[b]	<0.001[b]
	Non deprived vs Control	0.926	0.010[b]	<0.001[b]
	Deprived vs. Control	0.423	0.830	0.024 [b]
Ganglion cell layer	*Deprived vs. Non deprived*	0.139	0.023[b]	<0.001[b]
	Non deprived vs Control	0.895	0.718	0.623
	Deprived vs. Control	0.103	0.055	<0.001 [b]
Nerve fibre layer	*Deprived vs. Non deprived*	0.360	0.006[b]	<0.001[b]
	Non deprived vs Control	0.771	0.056	0.977
	Deprived vs. Control	0.781	0.116	0.010 [b]

[a] *p-value obtained after Student t-test analysis.*

[b] *p-value < 0.05.*

6.2 CELL DENSITIES

6.2.1 Non-deprived eyes

The cell densities in the non-deprived eyes increased with increasing duration of monocular deprivation (**Figure 9**). From the baseline, the percent increase was as follows: ganglion cells by 116%, inner nuclear layer cells by 52%, and outer nuclear layer cells by 59.6% (**Table 4**). All the differences noted in all the nuclear layers were statistically significant (ANOVA, $p<0.05$).

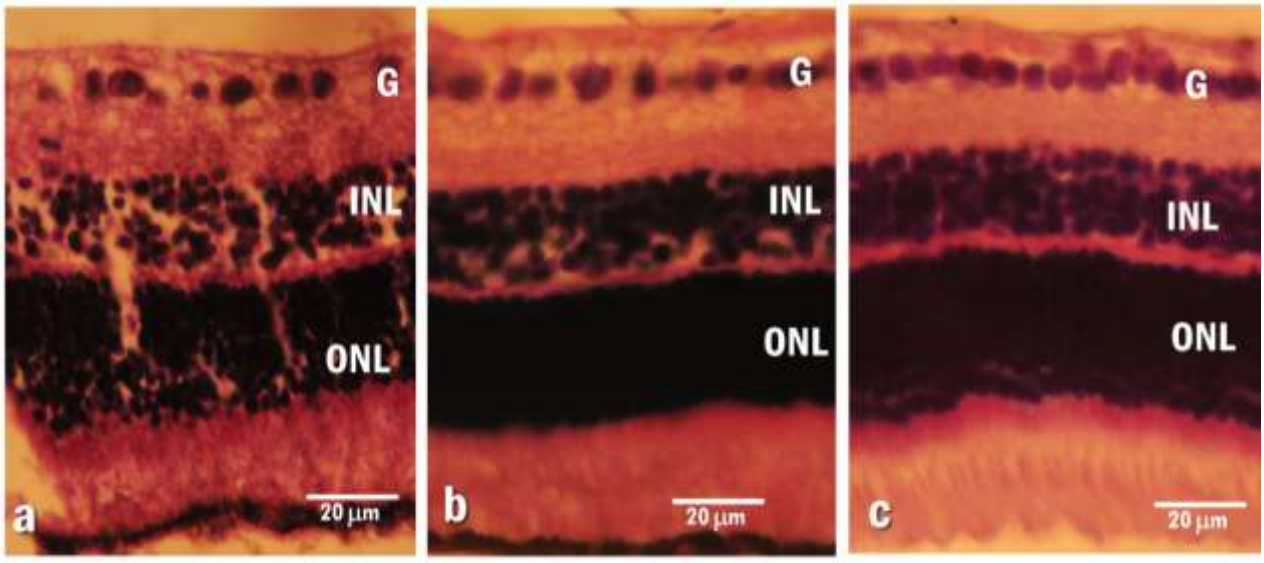

Figure 9: Photomicrograph of cell densities in the non-deprived eyes. . a) After 7 days of monocular deprivation (P21), **b)** After 14 days of monocular deprivation (P28), **c)** After 21 days of monocular deprivation (P35). Note the cells become more densely packed with increasing duration of monocular deprivation. This is clearly depicted in the ganglion cell layer (G). *G-Ganglion cell layer, INL-Inner Nuclear Layer, ONL-Outer Nuclear Layer. (Haematoxylin & eosin stain, x92).*

6.2.2 Deprived eyes

There was generalized reduction in the cell densities in the deprived eyes with increasing duration of monocular deprivation (**Figure 10**). The percent reductions of the densities from the baseline were 60.9%, 41.6%, and 18.9% in the ganglion cells, inner nuclear cells and outer nuclear cells respectively (**Table 4**). Statistically significant reduction in the densities were noted in the ganglion and inner nuclear cell layers (ANOVA test, $p<0.05$).

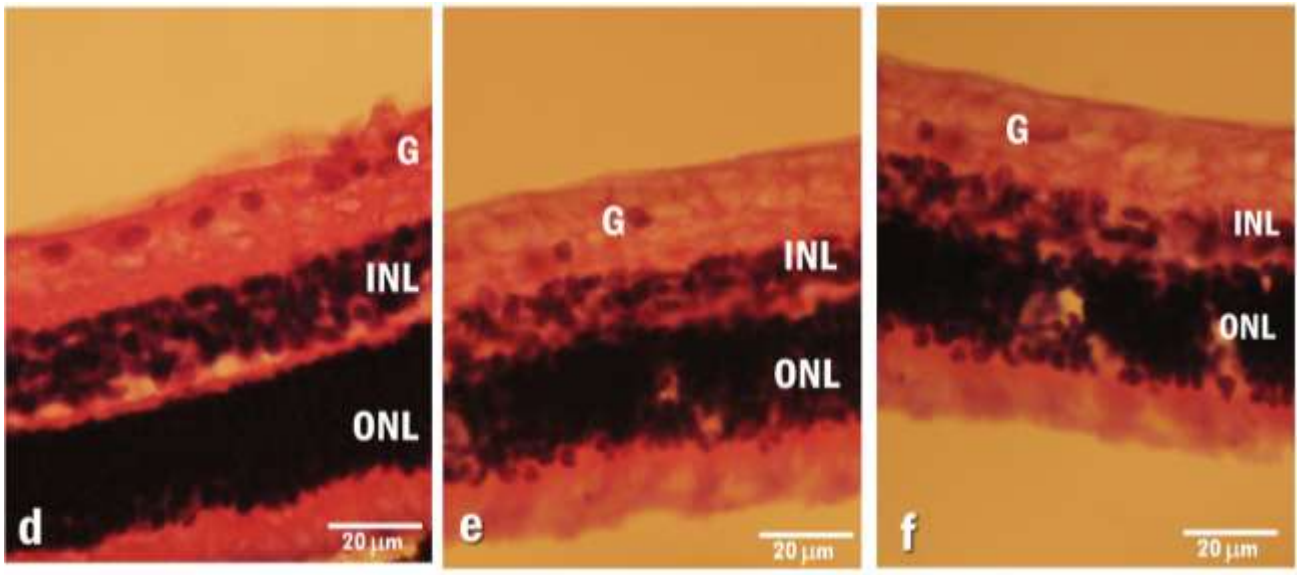

Figure 10: Photomicrograph of cell densities in the deprived eyes. . a) After 7 days of monocular deprivation (P21), **b)** After 14 days of monocular deprivation (P28), **c)** After 21 days of monocular deprivation (P35). Note that the population of the ganglion cells (G) significantly reduces with increasing monocular deprivation. *INL-Inner Nuclear Layer, ONL-Outer Nuclear Layer (Haematoxylin & eosin stain, x92).*

6.2.3 Control eyes

The retinae of the control eyes did not display any statistically significant age related changes in their cell densities (**Figure 11 and Table 4**).

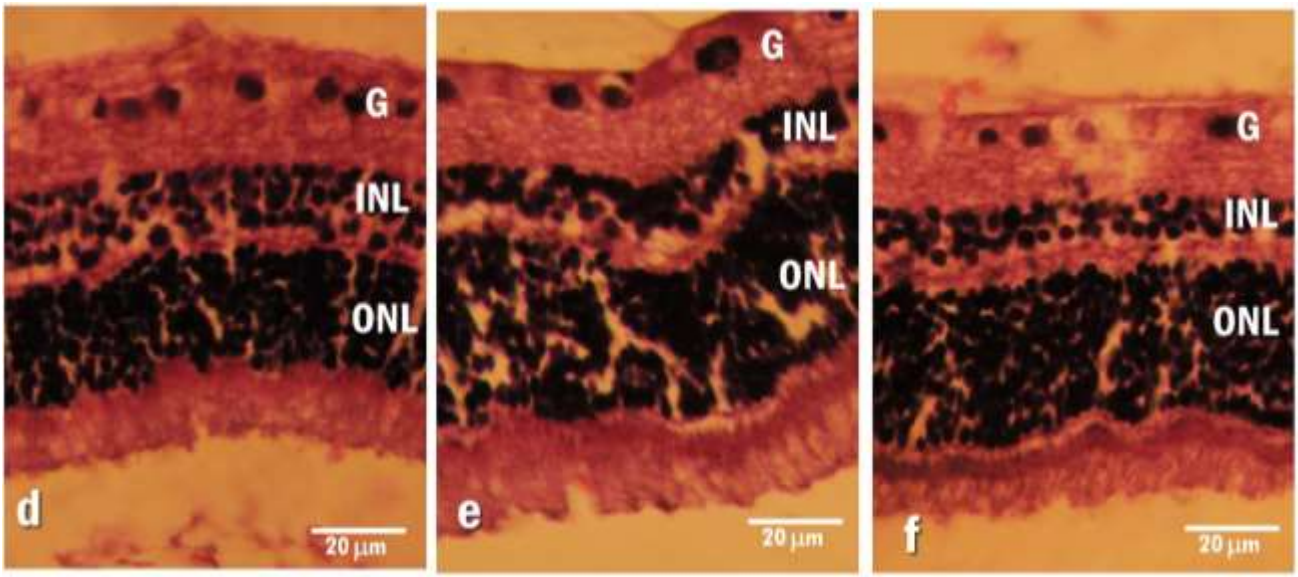

Figure 11: Photomicrograph of cell densities in the control eyes. a) *After 7 days* of study (P21), **b)** *after 14 days of* study (P28)**, c)** *after 21 days* of study (P35). *G-Ganglion cell layer, INL-Inner Nuclear Layer, ONL-Outer Nuclear Layer. (Haematoxylin & eosin stain, x92 magnification).*

Table 4: Changes in the retinal cell densities with increasing period of deprivation.

	Deprivation time (days)	Deprived		Non-deprived		Controls	
		Mean± SD	p value[b]	Mean± SD	p value[b]	Mean± SD	p value[b]
Ganglion cell density (cells/mm²)	0[a]	7775.0±1831.9	<0.001[c]	7775.0±1831.9	<0.001[c]	7775.0±1831.9	0.679
	7	4787.6±774.6		8456.0±3807.3		7049.8±2658.2	
	14	4135.9±1087.0		11589.5±2401.2		6775.7±1787.7	
	21	3040.7±1086.9		16803.2±5158.5		6575.3±821.1	
Inner nuclear Cell Density (cells/mm²)	0[a]	38488.8±1834.5	0.003[c]	38488.8±1834.5	<0.001[c]	38488.8±1834.5	0.995
	7	35579.1±8322.8		37218.6±8029.3		38325.3±1490.6	
	14	34147.7±5372.0		52894.8±11016.4		38432.0±8879.4	
	21	22490.8±8872.8		58845.3±9177.6		39163.4±4201.9	
Outer nuclear Cell density (cells/mm²)	0[a]	59669.2±961.1	0.326	59669.2±961.1	<0.001[c]	59669.2±961.1	0.996
	7	54006.8±11721.5		65480.3±11414.0		61731.0±1281.0	
	14	53105.8±9736.7		87952.9±20311.5		61361.3±22132.4	
	21	48406.8±10814.6		95240.5±17834.6		60824.6±2982.3	

[a] The values on postnatal day 14 are repeated in the three groups as they are the baseline values
[b] p-value obtained after ANOVA analysis. [c] p-value < 0.05.
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6.2.4 Comparison of cell densities between deprived and non-deprived eyes

Monocular deprivation resulted in increase in the cell populations of the non-deprived eyes while the cell densities of the deprived eyes reduced (**Figures 9** and **10**). Comparison of cell densities between deprived and non-deprived eyes using Student's *t*-test revealed statistically significant differences, which were more prominent with increasing duration of monocular deprivation (**Table 5**).

6.2.5 Comparison of cell densities between non-deprived and control eyes

The non-deprived eyes had higher cell densities compared to the control eyes in all the three study weeks (**Figures 9** and **11**). Statistically significant differences were however noted after 14 and 21 days of deprivation (**Table 5**). Compared to the control retinae, after 21 days of deprivation, the non-deprived retinae had 115.6%, 50.3%, and 56.6% increments in the ganglion cell, inner nuclear cell, and outer nuclear cell densities respectively. All these differences were statistically significant ($p<0.05$).

6.2.6 Comparison of cell densities between deprived and control eyes

The deprived eyes had less cell densities compared to the control eyes, with the differences being more marked with increasing period of deprivation (**Figures 10** and **11**). There were more marked changes in the ganglion cell densities (**Table 5**). Compared to the controls, the ganglion cells in the deprived eyes suffered a 32%, 39%, and 54% reduction in its cell densities after 7, 14, and 21 days of monocular deprivation respectively.

Table 5: Comparison of cell densities between deprived, non-deprived and control eyes.

Retinal layer	Eye	Duration of monocular deprivation		
		7 days (p value [a])	14 days (p value [a])	21 days (p value [a])
ganglion density (cells/mm²)	*Deprived vs Non deprived*	0.011[b]	<0.001[b]	<0.001[b]
	Deprived vs Control	0.032[b]	0.001[b]	<0.001[b]
	Non deprived vs Control	0.509	<0.001[b]	<0.001[b]
Inner nuclear Cell Density (cells/mm²)	*Deprived vs Non deprived*	0.654	<0.001[b]	<0.001[b]
	Deprived vs Control	0.535	0.199	0.001[b]
	Non deprived vs Control	0.793	0.005[b]	<0.001[b]
Outer nuclear Cell density (cells/mm²)	*Deprived vs Non deprived*	0.039[b]	<0.001[b]	<0.001[b]
	Deprived vs Control	0.228	0.303	0.036[b]
	Non deprived vs Control	0.837	0.015[b]	0.001[b]

[a] *p-value obtained after Student t-test analysis.*

[b] *p-value < 0.05.*

6.3 GANGLION CELL DENDRITIC FEATURES

Using the Simple Neurite tracer plugin in Fiji software, the dendrites of each ganglion neuron was followed to the inner plexiform layer. The following parameters were determined: number of primary branches, number of terminal branches, dendritic ramification index (number of primary branches/number of terminal branches), and dendritic field area (**Figure 12**).

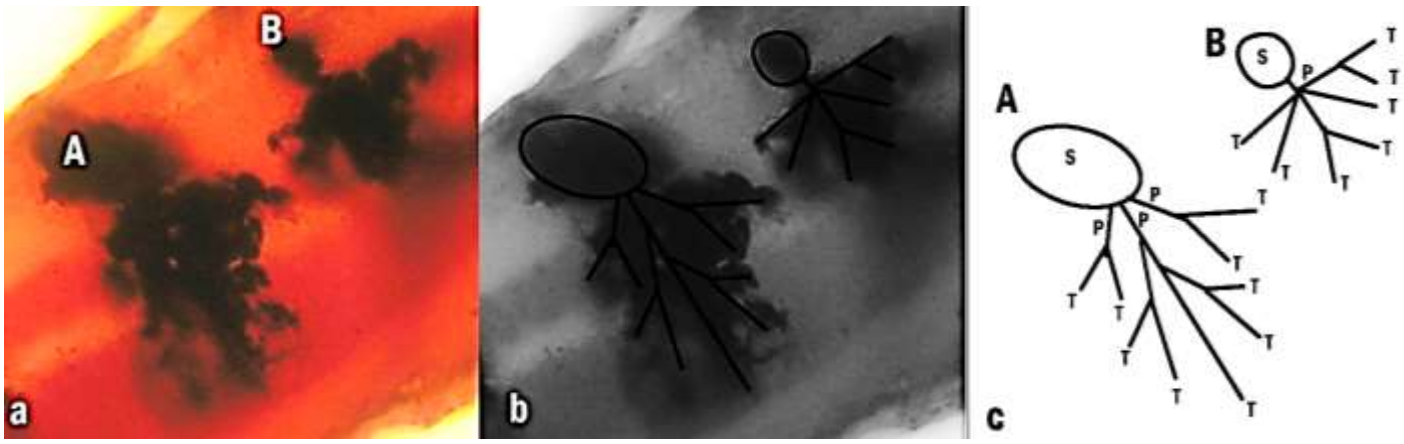

Figure 12: Photomicrograph of the neural retina of a non-deprived eye after two week of monocular deprivation (P28). a) Displaying two ganglion neurons A and B (Golgi stain with cresyl violet counter stain x92). **b**) Displaying two ganglion neurons A and B with overlaid tracings (gray scale image of [a]). **c)** Tracing of two ganglion neurons A and B. *P-Primary dendrite, T-terminal dendrite, S-Soma.*

6.3.1 Deprived eyes

The number of the primary and terminal dendrites initially increased until the 14th day of deprivation thereafter decreased as duration of deprivation increased (**Table 6**). The dendritic field area peaked at seven days of deprivation then rapidly reduced with further deprivation. However, ANOVA analysis of these differences did not reveal any statistically significant changes.

6.3.2 Non-deprived eyes

The number of both the primary and terminal dendrites increased with increasing deprivation (**Table 6**), resulting in statistically significant change in the dendritic ramification index *(p=0.040).* From the

baseline (P14), the number of primary dendrites increased by 66.7% *(p=0.385)*, while the terminal dendrites increased by 400% *(p=0.002)*. The dendritic field area increased rapidly in the first seven days of deprivation then plateaued. However, there were no statistically significant changes in the dendritic field area with increase in deprivation time.

6.3.3 Control eyes

There were no statistically significant age-related changes in the number of primary dendrites, terminal dendrites, dendritic ramification index and the dendritic field area among the control rabbits (**Table 6**).

Table 6: Dendritic variables in deprived, non-deprived, and control eyes

	Duration of deprivation (days)	Deprived		Non-deprived		Controls	
		Mean ± SD	p-value [b]	Mean ± SD	p-value [b]	Mean ± SD	p-value [b]
No. of primary branches	**0** [a]	1.5±0.7	**0.679**	1.5±0.7	**0.385**	1.5±0.7	**0.560**
	7	1.9±0.2		2.3±0.5		2.0±0.7	
	14	2.3±0.4		2.3±0.6		2.2±0.8	
	21	2.3±1.0		2.5±0.7		2.4±0.5	
No. of terminal branches	**0** [a]	1.5±0.7	**0.792**	1.5±0.7	**0.002** [c]	1.5±0.7	**0.065**
	7	2.5±0.7		4.3±1.3		2.5±0.7	
	14	3.0±1.4		5.3±0.6		2.8±0.8	
	21	2.8±2.1		7.5±0.7		3.5±0.6	
Dendritic Ramification Index	**0** [a]	1.0±0.0	**0.834**	1.0±0.0	**0.040** [c]	1.0±0.0	**0.727**
	7	1.4±0.5		1.9±0.3		1.4±0.8	
	14	1.3±0.4		2.4±0.7		1.4±0.5	
	21	1.1±0.5		3.2±1.2		1.6±0.5	
Dendritic field area (μm^2)	**0** [a]	990.4±134	**0.878**	990.4±134	**0.523**	990.4±134.0	**0.999**
	7	1213.8±253		1903.0±774.0		1053.1±37.7	
	14	1020.4±120		1914.1±935.9		1059.4±319.0	
	21	840.6±350		1866.6±546.9		1049.6±853.9	

[a] *The values on postnatal day 14 are repeated in the three groups as they are the baseline values*

[b] *p-value obtained after ANOVA test.* [c] *p-value < 0.05.*

6.3.4 Comparison of the dendritic variables between non-deprived and deprived eyes

The non-deprived retinae had more primary dendrites, terminal dendrites, dendritic field area, and dendritic ramification indices compared to the deprived eyes (**Table 6**). These were more marked with increasing period of deprivation. Student's *t*-test revealed statistically significant differences in the terminal dendrites after 21 days of deprivation *(p=0.039).* This lead to statistically significant differences in the dendritic ramification indices (**Table 7**).

6.3.5 Comparison of the dendritic variables between deprived and control eyes

While the dendritic features of the ganglion cells of the deprived eyes decreased with increasing deprivation, those of the control group either increased or plateaued (**Table 6**). The Student *t*-test analysis did not reveal any statistically significant differences in the variables between the deprived and control animals (**Table 7**).

6.3.6 Comparison of the dendritic variables between non-deprived and control eyes

Non-deprived eyes had more primary dendrites, terminal dendrites, dendritic ramification index and dendritic field area compared to the controls (**Table 6**). However, statistically significant differences were noted in the terminal dendrites after 14 days of deprivation (**Table 7**). The terminal dendrites in the non-deprived eyes were 90.5% *(p=0.004)* and 114.3% *(p=0.002)* more compared to controls after 14 and 21 days of deprivation respectively.

Table 7: Comparison of dendritic variables between non-deprived, deprived and control eyes.

Retinal layer	Eye	Duration of monocular deprivation		
		7 days (p value [a])	14 days (p value [a])	21 days (p value [a])
Primary branches	*Deprived vs Non deprived*	0.383	0.900	0.765
	Deprived vs Control	0.831	0.941	0.823
	Non deprived vs Control	0.633	0.818	0.804
Terminal branches	*Deprived vs Non deprived*	0.153	0.073	0.039[b]
	Deprived vs Control	1.000	0.817	0.510
	Non deprived vs Control	0.153	0.004[b]	0.002[b]
Ramification Index	*Deprived vs Non deprived*	0.147	0.142	0.032[b]
	Deprived vs Control	0.957	0.829	0.285
	Non deprived vs Control	0.314	0.062	0.066
Dendritic Field area (μm^2)	*Deprived vs Non deprived*	0.373	0.290	0.105
	Deprived vs Control	0.815	0.879	0.699
	Non deprived vs Control	0.217	0.141	0.298

[a] *p-value obtained after Student t-test analysis.*

[b] p-value < 0.05.

7. DISCUSSION

The present study has revealed that monocular deprivation leads to significant reduction in the neural retinal thickness of the deprived eyes compared to controls and non-deprived eyes. This is in agreement with previous studies on monocularly deprived mice (Zhou et al., 2010) and tree shrews (Abbott et al., 2011). Similar findings have been reported in other stimulus deprived receptor organs such as the olfactory mucosa after unilateral naris occlusion (Coppola, 2012; Huart et al., 2013), and the organ of Corti after unilateral hearing loss (Terayama et al., 1977; Syka, 2002). For instance, in the olfactory mucosa, the occluded side becomes significantly thinner compared to the open side (Coppola, 2012; Huart et al., 2013). These findings have been attributed to under-expression of pro-mitotic genes and increased expression of apoptotic genes in the deprived side leading to reduced cellular proliferation (Firszt et al., 2013; Zhao et al., 2013). In the retina, growth factors such as Brain Derived Neurotrophic Factor (BDNF) have been shown to influence its cellular proliferation (Seki et al., 2003; Mandolesi et al., 2005). In monocularly deprived eyes, BDNF expression is reduced in the deprived eyes and increased in the non-deprived eyes (Seki et al., 2003). Consequently, the reduction in the retinal thickness in the deprived eyes in the current study could be as a result of reduced proliferation of the retinal cells due to reduced expression of promitotic factors such as BDNF or increased expression of apoptotic factors.

The deprived eyes in the present study had statistically significant thinner rods and cones layer compared to controls. The non-deprived eyes on the other hand had thicker rods and cones layer compared to controls. These findings are hitherto undescribed. The rods and cones layer is made up of the outer segments of the photoreceptors which contain visual pigments such as rhodopsin (Mescher, 2005). Biosynthesis of visual pigments especially rhodopsin is a light dependent process (Schwemer, 1984). It is therefore plausible that lack of light as in the case with the deprived eyes in the current study may

have resulted into reduced synthesis of the visual pigments leading to reduction in the thickness of the rods and cones layer. On the other hand, presence of light may have caused increased synthesis of the photo pigments leading to increase in the thickness of this layer among the non-deprived eyes.

The outer nuclear layer contains the cell bodies of photoreceptors (Masland, 2012). In the present study, this layer was relatively not affected by visual deprivation. There were no statistically significant differences in the thickness of this layer between the deprived and control eyes. Abbott et al. 2011 who studied three tree shrews that had been monocularly deprived of light for 19 months, reported that the deprived eyes had significantly thinner outer nuclear layer compared to the non-deprived eye. The differences noted between our study and this previous study could be due to the differences in duration of deprivation, species of animal, or the sample size used. In the same study however the authors noted that the outer nuclear layer contributed least in the total thinning of the retina among the deprived eyes (Abbott et al., 2011). Our findings on the thickness of the outer nuclear layer concur with the findings on the outer nuclear layer cell density whereby we observed no statistically significant differences in the cell density with increasing duration of deprivation in the deprived eyes. Thus, visual deprivation has little effect on the outer nuclear layer.

In the present study, the non-deprived eyes had thinner outer nuclear layer compared to controls, with the differences being more marked with increasing period of deprivation. This concurs with the findings by Fifkova 1972a, who reported that increasing duration of deprivation resulted to greater decrease in the thickness of outer nuclear layer of non-deprived eyes compared to the controls. This author postulated that the animal with one eye keeps it more open compared to the one with both eyes open, leading to damage to the photoreceptors as a result of over stimulation (Fifková, 1972a). Subsequent studies have demonstrated that prolonged exposure to light leads to damage to the photoreceptors (Organisciak and Vaughan, 2010; Marchiafava, 2012; Okano et al., 2012). It is therefore plausible that

the experimental animals kept open their non-deprived eyes for a longer duration leading to damage to the photoreceptors which in turn lead to reduction in the thickness of the outer nuclear layer compared to the controls.

Compared to controls, the deprived eyes had statistically significant reduction in the thickness of the inner nuclear layer with increasing duration of deprivation. On the other hand, the non-deprived eyes had generally thicker inner nuclear layer compared with the controls. Our findings are in accord with those of a study by Abbott et al., 2011, who demonstrated that monocular deprivation resulted into statistically significant thinning of the inner nuclear layer in the deprived eyes compared to non-deprived eyes. The inner nuclear layer contains the somata of the bipolar, amacrine and horizontal cells (Kolb et al., 1995; Masland, 2012). In the present study, the deprived eyes had statistically significant reduction in the inner nuclear cell density while their non-deprived counterparts had their cell density increase. Proliferation of these cells is dependent on the interplay between extrinsic factors such as presence of light stimulus, and intrinsic factors such as growth factors (Tropepe et al., 2000; Fischer et al., 2002; Li et al., 2002). For instance, Brain-Derived Neurotrophic Factor (BDNF) which is a nerve growth factor, has been localized in the inner nuclear cells (Perez and Caminos, 1995; Vecino et al., 1998), and has been shown to influence retinal cell proliferation. Monocular deprivation has been shown to result in reduced expression of BDNF in deprived eyes compared to the non-deprived counterparts (Seki et al., 2003). Consequently, absence of light stimulus could have caused reduced expression of BDNF in the deprived eyes leading to reduced cellular proliferation and subsequent thinning of the inner nuclear layer.

The ganglion cell layer contains the cell bodies of the ganglion neurons while the nerve fibre layer contains the axons of these neurons (Masland, 2012). In the current study, the ganglion cell and nerve fibre layers in the deprived eyes were significantly thinner compared the controls and the non-deprived

eyes. Thinning of the ganglion cell as well as the nerve fibre layers resulting from deprivation has been reported in previous studies (Abbott et al., 2011; Szumiński and Bakunowicz-Łazarczyk, 2012; Zhao and Jiang, 2013). Proliferation of the ganglion cells has also been shown to be influenced by growth factors such as retinal BDNF (Seki et al., 2003; Mandolesi et al., 2005). BDNF has also been localized in the retinal ganglion cells (Perez and Caminos, 1995; Vecino et al., 1998), and monocular deprivation has been shown to result in reduced expression of BDNF in deprived eyes compared to the non-deprived counterparts (Seki et al., 2003). In the current study the ganglion cell density significantly increased in the non-deprived eyes, and reduced in the deprived eyes. Thus, the reduction in the thickness of the ganglion cell and nerve fibre layers seen in the deprived eyes could have resulted from reduced proliferation of the ganglion cells as a result of reduced expression of growth factors such as BDNF following light deprivation.

Although all the cells demonstrated changes in their densities with monocular deprivation, it was the ganglion cells that displayed the most marked changes. Among the non-deprived eyes, the ganglion cell density increased by 116%, while in the deprived eyes it reduced by 62% from baseline. The retinal ganglion cells are key in the visual pathway as they are the output neurons from the retina (Berson, 2008; Masland, 2011, 2012). Our findings are in agreement with previous studies on the Rhesus monkeys (Von Noorden et al., 1977), rats (Hsiao and Fukuda, 1984), and tree shrews (Abbott et al., 2011) which reported a decrease in the ganglion cell density in the deprived eyes compared to non-deprived eyes. A study on three cats raised with monocular deprivation for 5.2–7.2 years however did not reveal any differences in ganglion cell densities (Spear and Hou, 1990). The findings by the latter study could be due to small sample size used, species of animal studied or the longer period of deprivation in this study.

In the present study, the thickness of the inner plexiform layer among the deprived eyes significantly

reduced with increasing time of deprivation. Abbott et al. 2011, also reported that the inner plexiform layer of monocularly deprived eyes of tree shrews was significantly thinner compared to control eyes. The inner plexiform layer contains synapses between the bipolar and ganglion cells, as well as between the amacrine and ganglion cells (Kolb, 1997; Masland, 2012). Synaptic plasticity involves strengthening or weakening of synapses over time in response to increase or decrease in the synaptic activity (Antonini et al., 1999; Trachtenberg and Stryker, 2001; Ho et al., 2011). Monocular deprivation has been shown to lead to shrinkage of the primary visual cortex and the lateral geniculate nucleus of the deprived eye as a result of reduction in the synaptic activity in these areas (Daw, 2006; Hofer et al., 2006; Hayano and Yamamoto, 2008; Linden et al., 2009). Consequently, the reduction in the thickness of the inner plexiform layer observed among deprived eyes could be as a result of reduced synaptic activity in this layer due to visual deprivation. Indeed in the present study, prolonged deprivation resulted in reduction in the number of terminal branches as well as dendritic field area among the deprived eyes.

The ganglion cells in the non-deprived eyes in the present study had a steady increase in the number of primary and terminal branches as well as the dendritic field area with increasing duration of deprivation. Our findings concur with reports by other workers who demonstrated increased synapses in the inner plexiform layer of non-deprived eyes (Fifková, 1972b; 1973). Usually, the ganglion cell dendritogenesis begins with the extension of several primary dendrites that project out from the soma, toward the inner plexiform layer (Huberman, 2007). After initiation from the soma, the primary dendrites then branch actively, adding and retracting branches to remodel the dendritic arbor (Coombs et al., 2007). The morphology of ganglion cell dendrites continues to be modified by pruning mechanisms that persist long after initial synaptic contacts are formed in the visual system (Coombs et al., 2007; Huberman, 2007). The extent and form of the dendritic arbor is modulated within the retina by afferent input mediated through neurotransmitters produced by bipolar and amacrine cells (Cohen-Cory and Lom, 2004). As

was observed in the present study, the cell densities of the inner nuclear layer that contains bipolar and amacrine cells, had significantly increased in the non-deprived eye with increasing duration of deprivation. It is therefore plausible that the increased number of the inner nuclear cells produced more dendritogenic factors in the non-deprived eyes, leading to the increased dendritic aborisations of the ganglion cells and subsequent increase in the dendritic field area.

The deprived eyes in the present study had initial increase in the number of both primary and terminal dendrites as well as the dendritic field area before reducing after the first week of deprivation. This implies that the ganglion cells initially attempted to compensate the lack of light by increasing its synaptic contacts however with prolonged deprivation, these synapses could not be sustained therefore they were pruned. Studies have shown visual stimulation is required for refinement of the retinal ganglion synaptic contacts (Tian and Copenhagen, 2003; Tian, 2004). Dark rearing results in initial increase in the inner plexiform layer synapses in rodents (Fisher, 1979), and turtles (Sernagor and Grzywacz, 1996) in an attempt to compensate for the lack of light, and has been associated with increased spontaneous expression of acetylcholine by the immature ganglion cells (Sernagor and Grzywacz, 1996). Visual experience controls the later stages of retinal ganglion cell dendritogenesis, and has been shown to abolish the spontaneous acetylcholine expression in the immature ganglion cells (Tian and Copenhagen, 2003; Tian, 2004; Cohen-Cory and Lom, 2004). Visual experience modulates the expression of neurotrophic factors such as retinal BDNF, which is produced by the inner nuclear cells (Cohen-Cory and Lom, 2004; Wong and Ghosh, 2002). Thus, reduction in the number of the dendrites and dendritic field area seen with further deprivation in the present study could be as a result of reduced production of neurotrophic factors from the degenerating inner nuclear layer cells in the deprived eyes.

8. CONCLUSION

The present study has demonstrated that monocular deprivation results in activity-dependent changes in the neural retina. These changes include retinal thinning, reduction in all cell densities and reduction in the dendrites of ganglion neurons in the deprived eyes. On the other hand, the non-deprived eyes experience compensatory increase in neural retinal thickness, cell densities and the dendrites of the ganglion cells. In both the deprived and non-deprived eyes, most changes were seen in the layers associated with the neurons namely, nerve fibre layer, ganglion cell layer, inner plexiform, and inner nuclear layer. These changes in the retina may contribute to the changes seen in the visual cortex in monocularly deprived animals. However, there is need for further studies to determine whether these changes in the retina are reversible, and if they are, then the maximum period of deprivation beyond which these changes cannot occur should be ascertained.

9. LIMITATIONS

1. Structural changes occurring to the supporting cells of the retina such as the Muller cells may have been missed out because demonstration of the cellular details of these cells requires special stains.
2. Lack of three dimensional visualization of the dendritic arbor of the ganglion cells in light microscopy may have affected the counting of the primary and terminal dendrites.
3. The structural changes occurring to the cell types of the inner nuclear layer namely bipolar, amacrine and horizontal cells could not be ascertained from our study because identification of these cells require use of special stains.

10. SUGGESTIONS FOR FUTURE STUDIES

1. Electron microscopic studies on the effect of monocular deprivation on the structure of:
    a. Supporting cells of the retina.
    b. Photoreceptors (rods and cones)
    c. Inner nuclear cells (Bipolar, amacrine and horizontal neurons)
    d. Ganglion cells
2. Immunohistochemistry study on the effects of monocular deprivation on the synaptic contacts in:
    a. Inner plexiform layer (amacrine-ganglion, bipolar-ganglion cell synapses)
    b. Outer plexiform layer (photoreceptor-bipolar, bipolar-horizontal cell synapses)
3. Electron microscopic and immunohistochemistry study on the effect of reversal of monocular deprivation on the structure of the retina

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12. APPENDIX I: DATA SHEET

DATA SHEET

Study Number: _____ Age: _____ (days)

1. Is the animal in the experimental or Control group? *Experimental* ☐ *Control* ☐
2. If animal is in experimental group,
    a. Which eye was sutured (deprived)? *Right* ☐ *Left* ☐
    b. How long was the duration of deprivation? _____(days)
3. Retinal thickness measurements

Right eye	Left eye
a) Rods and cones layer: _____µm	a) Rods and cones layer: _____µm
b) Outer limiting membrane:_____µm	b) Outer limiting membrane:_____µm
c) Outer nuclear layer: _____µm	c) Outer nuclear layer: _____µm
d) Outer plexiform layer: _____µm	d) Outer plexiform layer: _____µm
e) Inner nuclear layer: _____µm	e) Inner nuclear layer: _____µm
f) Inner plexiform layer: _____µm	f) Inner plexiform layer: _____µm
g) Ganglion cell layer: _____µm	g) Ganglion cell layer: _____µm
h) Nerve fibre layer: _____µm	h) Nerve fibre layer: _____µm

4. Cell densities

Right eye	Left eye
a) Outer nuclear cells: _____cells/mm^2	a) Outer nuclear cells: _____cells/mm^2
b) Inner nuclear cells: _____cells/mm^2	b) Inner nuclear cells: _____cells/mm^2
c) Ganglion cells: _____cells/mm^2	c) Ganglion cells: _____cells/mm^2

5. Dendritic features of the ganglion cell

Right eye	Left eye
a) Number of primary dendrites: _____	a) Number of primary dendrites: _____
b) Number of terminal dendrites: _____	b) Number of terminal dendrites: _____
c) Dendritic ramification index: _____	c) Dendritic ramification index: _____
d) Dendritic field area: _____(µm^2)	d) Dendritic field area: _____(µm^2)

13. APPENDIX II: ETHICAL APPROVAL LETTER

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

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Dr Philip M. Mwachaka
Dept of Human Anatomy

22/10/2013

Dear Dr Mwachaka,

RE: Approval of proposal by Biosafety, Animal use and Ethics committee

Structural changes in the rabbit neural retina following mononuclear deprivation
By P.M. Mwachaka H56/82271/2012

We refer to the above proposal that you resubmitted on 26th September 2013. We have noted that you have addressed the concerns regarding animal husbandry and anaesthesia, analgesia following tarsorraphy and finally euthanasia. We trust that all these treatments will actively involve Veterinary supervision.

We hereby approve your study on the rabbits as per your final revised proposal.

Yours sincerely

Rodi O. Ojoo BVM M.Sc Ph.D
Chairman,
Biosafety, Animal Use and Ethics Committee
Faculty of Veterinary Medicine.