Sex-Related Differences in Retinal Structure and Function of Normal Rats And The Role of Estrogen in Rodent Models of Oxidative Retinopathies

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Dedicated To

My beloved parents for helping to make me the person that I am today,

My dear husband, for accompanying me all the way &

My bundle of joy, who I can hardly wait to meet, I look forward to one day sharing this special accomplishment with.

"This is not the end.

It is not even the beginning of the end.

But, it is, perhaps, the end of the beginning."

Speech given at the Lord Mayor's Luncheon, Manision House, London, November 10, 1942.

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PREFACE: PART I

1. ABSTRACT

In human, the function and structure of the retina can be influenced by the biological sex, possibly due to the different sexual hormone profiles. Among the sexual hormones, the neuroprotective effects of estrogen, the major female sexual hormone, has been well documented. As a neuroprotective agent, estrogen also has some antioxidant properties and consequently it can protect the tissues against environmentally-induced oxidative damage. The discovery of estrogen receptors within the retinal layers suggests that this sexual hormone can also play an important role in the normal functioning of this neural tissue. Additionally, it might exert protection against oxidative damage in the retina, a tissue that is extremely vulnerable to oxidative stress. The purpose of this study was to clarify the role of biological sex on the retinal structure and function of male and female Sprague-Dawley (SD) rats, and also to investigate the neuroprotective effects of estrogen on the retina of SD rat pups exposed to two oxidative conditions (i.e. hyperoxia or bright light).

To our knowledge, no study has reported the sex-related differences in the retinal function and structure of normal male and female rodents as they age. Aging is considered as a biological process leading to accumulative oxidative stress. The purpose of the first study was to elucidate the role of biological sex and age on the retinal function and structure of adult male and female SD rats. In this study, the retinal function and structure of normal male and female SD rats were investigated at specific age points with the electroretinogram (ERG) and histology, respectively. Also, ERG recorded from premenopausal and menopausal female SD rats were compared in order to clarify the role of the estrus cycle on the retinal function. Findings showed that biological sex could influence the retinal function and structure of SD rats. Of interest, between the age of P60 and P200, an increase in ERG responses was found in female rats but not

in male rats. We also showed that the ERG responses obtained from premenopausal rats were of larger amplitudes compared to age-matched male rats, while the amplitudes of those recorded from menopausal rats were smaller compared to age-matched male rats. The latter results suggest that estrogen modulates the retinal function of female rats as witnessed with the ERG.

Oxygen-induced retinopathy (OIR) and light-induced retinopathy (LIR) are two well-known animal models of oxidative retinopathies that are induced by exposing rat pups to either hyperoxia (80 ± 5 % O_2) or intense light (10,000 Lux), respectively. Given that estrogen has some antioxidant properties, the aim of the second study was to determine whether it could prevent the functional and structural consequences of OIR and LIR. Our results showed that while 17β-estradiol could protect the retina in rat model of LIR, it had a detrimental effect in the OIR model.

Taken together, our results indicate that biological sex influences the retina of normal rodents. Age-related retinal changes were more pronounced in female rats, possibly due to fluctuations in the plasmatic level of estrogen. We also suggest that estrogen can efficiently protect the retina against oxidative damage targeted at the outer retina (as in LIR), whereas estrogen potentiates oxidative damages aimed at the inner retina (as in OIR).

2. RÉSUMÉ

Chez les humains, la fonction et la structure de la rétine peuvent être influencées par le sexe biologique. Ceci est possiblement dû à la différence de profil des hormones sexuelles. Parmi ces hormones sexuelles, les effets neuroprotecteurs de l'estrogène, la principale hormone sexuelle féminine, ont été bien documentés. En tant qu'agent neuroprotecteur, l'estrogène possède également des propriétés antioxydantes et c'est pour cette raison qu'elle peut protéger les tissus contre les dommages oxydatifs environnementaux. La découverte des récepteurs d'estrogène dans les couches rétiniennes suggère que cette hormone sexuelle pourrait jouer un rôle très important dans le fonctionnement normal de ce tissu. De plus, elle pourrait avoir un effet protecteur contre les dommages oxydatifs rétiniens, un tissu qui est extrêmement vulnérable au stress oxydatif. L'objectif de cette étude était de déterminer l'impact du sexe biologique dans de la structure et la fonction rétinienne des rats Sprague-Dawley (SD), ainsi que d'investiguer les effets neuroprotecteurs de l'estrogène sur la rétine des ratons SD mâles exposés à deux conditions oxydatives (i.e hyperoxie où lumière intense).

À notre connaissance, aucune étude n'a signalé des différences reliées au sexe biologique sur la fonction et la structure rétinienne durant le vieillissement normal des rongeurs. Le vieillissement est considéré comme un procès biologique menant à l'accumulation de stress oxydatifs. Le but de la première étude était d'élucider le rôle du sexe biologique et de l'âge sur la fonction et la structure rétinienne mâle et femelle de rats SD adultes. Dans cette étude, la fonction et la structure rétinienne des rats SD a été étudiées à l'aide l'électrorétinogramme (ERG) et de l'histologie. De plus, l'ERG a aussi été enregistré chez des rats femelles SD en préménopause et en ménopause afin de clarifier le rôle du cycle ovarien sur la fonction rétinienne. Les résultats montrent que le sexe biologique peut influencer la fonction et la

structure rétinienne des rats SD. Par exemple, entre l'âge de P60 et de P200, un accroissement des réponses ERG a été observé uniquement chez les rats femelles. Nous avons aussi montré que les réponses d'ERG obtenues de rats femelles en prémenopause étaient d'une amplitude plus large comparés à ceux des rats mâles du même âge, tandis que les amplitudes de ceux obtenus à partir des rats femelles en ménopause étaient moins larges comparés à ceux des rats mâles du même âge. Ce dernier résultat suggère que l'estrogène contribue largement à la fonction rétinienne des rats femelles.

La rétinopathie induite par l'oxygène (RIO) ainsi que la rétinopathie induite par la lumière (RIL) représentent deux modèles animaux connus de rétinopathies oxydatives qui sont induites en exposant des ratons soit à une hyperoxie (80 ± 5 % O_2) ou à une luminosité intense (10,000 Lux). Étant donné que l'estrogène possède des effets antioxydants, le but de la deuxième étude était de déterminer si l'estrogène peut aussi prévenir les anomalies fonctionnelles et structurelles de la RIO et de la RIL. Nos résultats montrent que l'injection de 17β-estradiol peut protéger la rétine des rats exposés à la lumière tandis qu'il accélère la détérioration dans le modèle de la RIO.

En résumé, nos résultats montrent que le sexe biologique influence la structure et la fonction rétine normale des rongeurs. Les changements rétiniens liés à l'âge étaient plus prononcés parmi les rats femelles et ceci est possiblement dû aux fluctuations plasmatiques de l'estrogène. Nos résultats suggèrent aussi que l'estrogène pourrait protéger la rétine contre les dommages oxydatifs dirigés contre la rétine externe (comme dans la RIL), alors que les dommages oxydatifs affectant la rétine interne sont (comme dans la RIO) amplifiés par l'estrogène.

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4. LIST OF ABBREVIATIONS

AC	Amacrine cell
AMD	Age-related macular degeneration
BC	Bipolar cell
βE_2	17β-estradiol
ERG	Electroretinogram
ERs	Estrogen receptors
GCL	Ganglion cell layer
GDP	Guanosine diphosphate
Gpx	Glutathione peroxidase
GTP	Guanosine triphosphate
HC	Horizontal cells
ILM	Inner limiting membrane
INL	Inner nuclear layer
IOP	Intraocular pressure
IPL	Inner plexiform layer
IS	Inner segment
L	Long wavelength
LGN	Lateral geniculate nucleus
LIR	Light-induced retinopathy
M	Medium wavelength
MC	Müller cell
NFL	Nerve fiber layer
NO	Nitric oxide
NOS	Nitric oxide synthase

OCT	Optical coherence tomography
OIR	Oxygen-induced retinopathy
OLM	Outer limiting membrane
ONH	Optic nerve head
OPL	Outer plexiform layer
OPs	Oscillatory Potentials
OS	Outer segment
P	Postnatal day
PL	Photoreceptor layer
POAG	Primary open-angle glaucoma
RGCL/FL	Retinal ganglion cell layer/fiber layer
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
RPE	Retinal pigmented epithelium
S	Short wavelength
SD	Sprague-Dawley
SOD	Superoxide dismutase
Tα	Transducin α- subunit
VEGF	Vascular endothelial growth factor
VS	Vaginal smear

PREFACE: PART II

1. CONTRIBUTION OF AUTHORS ON CO-AUTHORED PAPERS

1.1 Manuscript 1 (Chapter II)

Chaychi S, Lachapelle P. *Differences in retinal structure and function between aging male and females Sprague-Dawley rats are strongly influenced by the estrus cycle.*

For this manuscript, the study was designed by Dr. Pierre Lachapelle and myself. I recorded the ERG from all the experimental animals. The retinal histology was also performed by me. Data analysis (ERG and histology) along with the correction and rewriting of the several iterations of this manuscript were always done in constant collaboration with Dr. Pierre Lachapelle.

1.2 Manuscript 2 (Chapter III)

Chaychi S, Lachapelle P. *Evaluating the neuroprotective effect of 17β-estradiol on the structure and function of the retina exposed to an intense oxidative stress.*

For this second manuscript, the study was designed by Dr. Pierre Lachapelle and myself. Injections (17β-estradiol and vehicle) to experimental groups were done by me. The exposure to oxygen and light were also carried out by me. I did the recording of the ERGs from the all the experimental groups and also performed the retinal histology. Data analysis (ERG and histology) along with the correction and rewriting of the several iterations of this manuscript were always done in constant collaboration with Dr. Pierre Lachapelle.

CHAPTER I

GENERAL INTRODUCTION

Preface to chapter I

This chapter is divided into three sections. The first section focuses on the normal retinal structure and its cellular organization and also describes the electroretinogram (ERG) components by which the retinal function can be properly evaluated. The second section provides an overview about the role of biological sex on the retinal structure and function and briefly highlights the role of estrogen, the major female sexual hormone, which also has some protective effects on the neural tissues, including retina. The third section describes two rodent models of oxidative retinopathies, oxygen-induced retinopathy (OIR) and light-induced retinopathy (LIR), which were used in the present research project in order to evaluate the neuroprotective effects of estrogen on the retinal structure and function. Finally, the last section explains the general objectives of the present research project.

SECTION I

1. The retina

The retina is a thin layer of tissue lining the inside of the eye. It develops from a pouch of the embryonic forebrain; therefore, it is considered as a part of the brain (Kolb, 2003). It contains light sensitive neurons to detect the photons as well as the neural pathways that are involved in the initial processing of the visual world which will be transmitted to the brain (Kolb, 2003).

1.1 Retinal cytoarchitecture

The rays of the light entering the eye through the pupil must cross the entire retina before reaching the photoreceptors, which are the light-sensitive cells of the retina. In human, as in all vertebrates, the retina is composed of three layers of nerve cell bodies and two layers of synapses (Kolb, 1995). The outermost layer of the retina, named the outer nuclear layer, contains the cell bodies of photoreceptors. The photoreceptors are classified into two different types: rods and cones. Added to its cell body, the photoreceptor is composed of an inner and an outer segments by which the photoreceptor attaches to the underlying pigmented layer named the retinal pigmented epithelium. The second nuclear layer, the inner nuclear layer, consists of the cell bodies of bipolar, horizontal, amacrine, and Müller cells. Finally, the last layer contains the retinal ganglion cells whose axons form the optic nerve that brings the retinal output to the visual centers of the brain. The three neuronal layers are separated by two plexiform layers where the synaptic interactions occur to transmit the visual signal between different nuclear layers. The outer plexiform layer connects the outer and inner nuclear layers, while the junction between inner nuclear layer and ganglion cell layer is achieved with the inner plexiform layer. A schematic representation of the retina is shown in Figure 1.

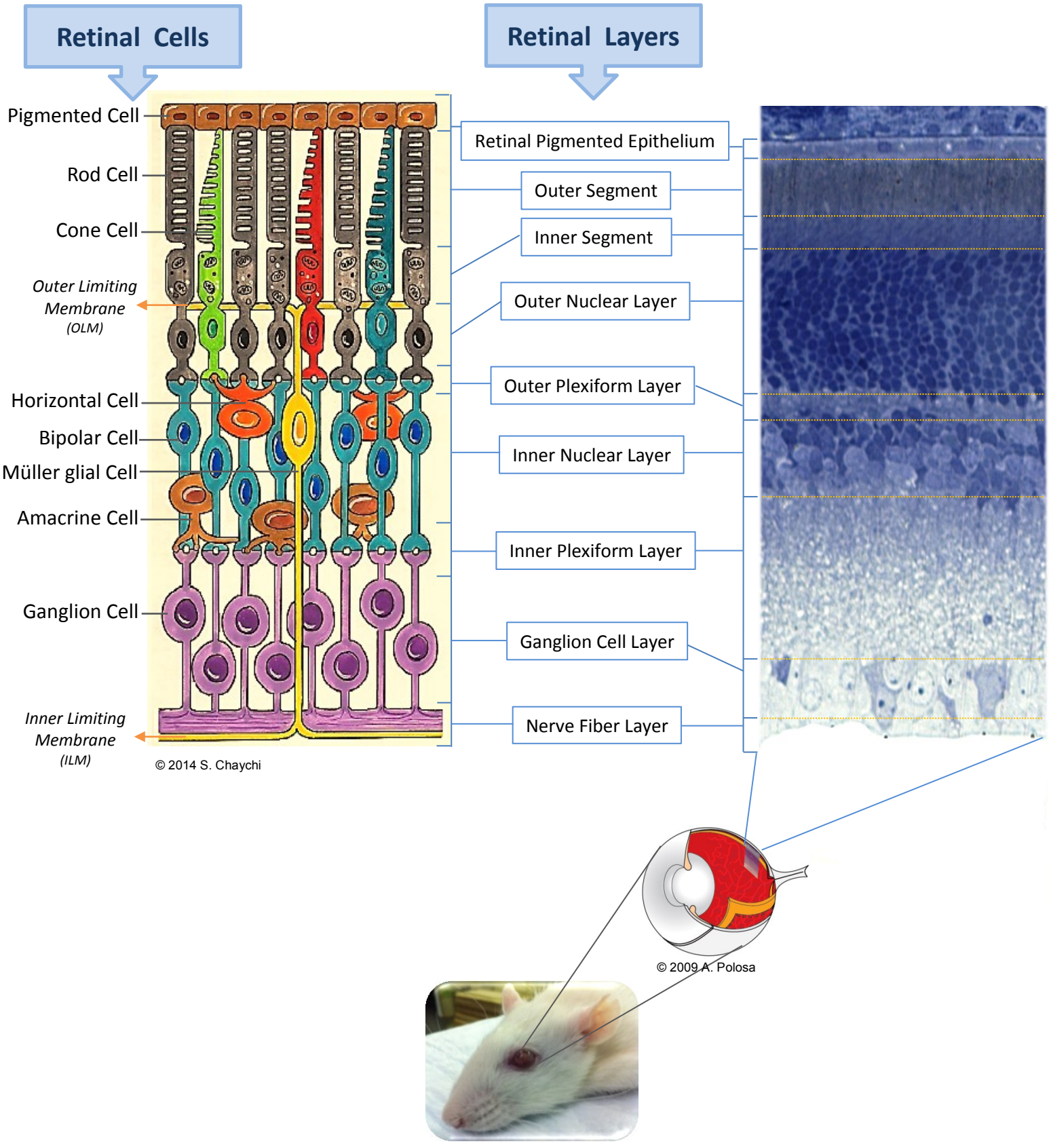

Figure 1: Schematic Representation of retinal structure.

Briefly, the retina is made up of 10 distinctive layers, from the outermost to the innermost: the retinal pigmented epithelium (RPE), the photoreceptor layer (PR) with outer and inner segments, the outer limiting membrane (OLM) the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL), the ganglion cell layer (GCL) and the nerve fiber layer (NFL) and the inner limiting membrane (ILM). These layers are described in further details below.

1.1.1 Retinal pigmented epithelium (RPE)

The RPE is composed of a monolayer of cuboidal/columnar epithelial cells and is located outside the neurosensory retina (Forrester et al., 2002). This pigmented layer, extending from the optic nerve head to the ora serrata, is completely attached to the underlying choroid on one side and to the light sensitive outer segments of the photoreceptors on the other side (Strauss, 2005). The RPE plays specific roles including phagocytizing of the shed outer segments of the photoreceptors, absorbing the light and reducing the light diffusion, providing a permeable barrier between the retinal cells and the underlying choroid, transferring and storing of water, ions, metabolites and vitamins (Steinberg, 1985; Bok, 1993).

1.1.2 Photoreceptor layer

The photoreceptors are the most abundant cell types in the mammalian retina (Brzezinski et al., 2010) and act as the main retinal sensory neurons involved in absorbing the light and converting it into electrical signals through an intracellular process named phototransduction. There are two types of photoreceptors: the rods which are responsible for dim light (scotopic) vision and the cones which are responsible for bright light (photopic) vision as well as color discrimination (Peichl et al., 2000). The human retina contains approximately 100-120 million rods and 7-8 million cones (Ferwerda, 1998). The photoreceptor distribution varies throughout

the retina with eccentricity. The most central part of the retina, the fovea, is densely packed with the cones whose density decreases significantly towards the peripheral part of the retina. In contrast, the rods are absent in the central fovea and a few millimeters outside the fovea the rods rapidly outnumber the cones. Towards the periphery of the retina, the photoreceptors become larger and more widely spaced (Farber et al., 1985).

Rod and cone photoreceptors have a similar structure that is made up of four functional regions (Kolb et al., 2012), namely: 1) The outer segment (OS), located at the distal extremity of the neural retina, contacts with the apical surface of RPE cells. It contains the light-absorbing visual pigments such as rhodopsin, which are responsible for light absorption; 2) The inner segment (IS), located more proximally, contains cellular organelles such as mitochondria, Golgi apparatus and smooth endoplasmic reticulum that are required for cell metabolism (Kawamura and Tachibanaki, 2008) ; 3) The cell body contains the nucleus and forms the outer nuclear layer (ONL) and 4) The synaptic terminal, namely pedicle in cone cells and spherule in rod cells, where the visual signals are transmitted to second order neurons. In spite of structural similarity, the morphology of rod and cone's outer segment is different. While rods have a long, cylindrical outer segment within which the stack of disk membrane containing visual pigment rhodopsin are separated from the plasma membrane, cones have a shorter, conical outer segment, and the disks containing visual pigment molecules, known as opsins are continuous with the outer membrane (Figure 2).

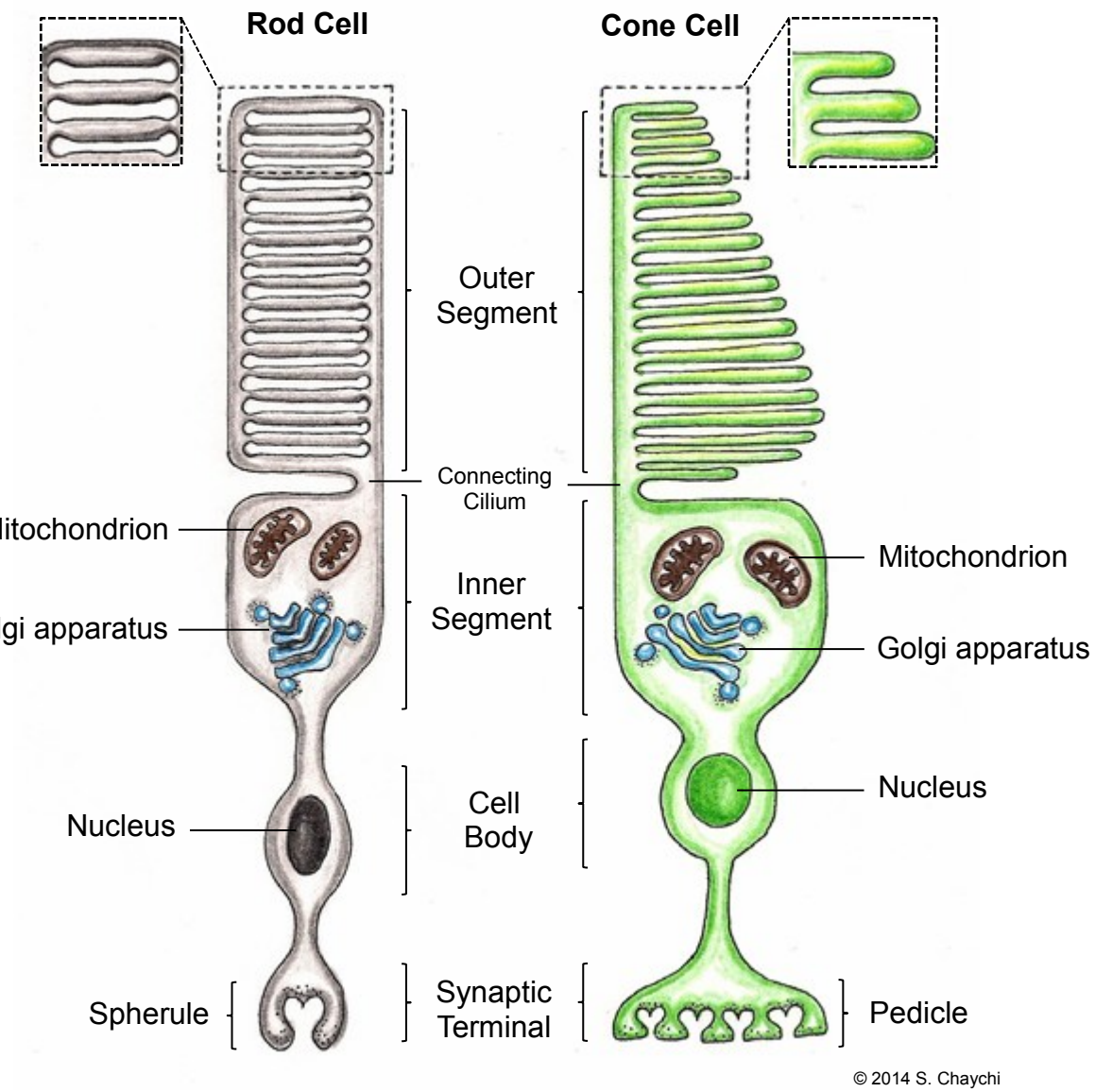

Figure 2: Structural features of rod and cone photoreceptors.

The visual pigment is an essential molecule that is required to absorb the photons and initiate the phototransduction cascade which is at the onset of the phenomenon of vision. The cone photoreceptors contain three classes of photopigments encoded by different opsin gene families (Bowmaker, 2008). These cone pigments have different absorption spectra. Cone opsins are maximally sensitive to either long wavelengths (L) of light (red light), medium wavelengths

(M) of light (green light) or short wavelengths (S) of light (blue light), depending on their respective molecular structures. The maximal absorption wavelength sensitivity of L-, M- and S-cones peaks at 564nm, at 533nm and at 437nm, respectively (Kolb, 2003). In contrast, the rod photoreceptors only have one type of visual pigment, namely rhodopsin, (Peichl, 2005) which is sensitive to the blue-green wavelength of light with peak sensitivity at approximately 500 nm (Kolb, 2003).

1.1.2.1 Phototransduction

Phototransduction is the process by which the light photons captured by the photoreceptors are converted to electrical signals. Analogous to other neurons, the membrane potential of a photoreceptor relies on the distribution of ions, particularly sodium (Na^+) and potassium (K^+), on both sides of the cell membrane. The concentration of K^+ is higher within the cell, whereas that of Na^+ is higher outside the cell. This ionic equilibrium is maintained by the activity of ATP-dependent Na^+-K^+ pumps located in the IS of the photoreceptor.

In darkness, the photoreceptors are in a depolarized state due to Na^+-cGMP-gated channels which are located in the OS of the photoreceptors. At the level of IS, there is another type of ionic channel, the K^+-selective non gated channel. These two types of channels are open in dark condition. Consequently, the Na^+ ions flow into the photoreceptors and K^+ ions leak out of the photoreceptors according to their electrochemical gradients. This ionic relocation across the cell membranes of the photoreceptors is known as the "dark current". In dark condition, depolarized photoreceptors incessantly release the neurotransmitter glutamate.

Absorption of a photon by the photopigment contained in the OS triggers the phototransduction cascade through which the captured photon is transformed into an electrical signal. This phototransduction cascade has been well described for the rod photoreceptors, but

similar mechanisms are also observed for the cones as well. Rhodopsin, the visual pigment of the rod cells, is made up of an opsin and a chromophore (retinal). The capture of a photon will cause a structural change in the retinal molecule from an 11-cis to an all-trans form. The change in the shape of the retinal molecule creates a conformational change in the opsin to an activated form namely, metarhodopsin II. It interacts with an intracellular protein binding to a molecule of guanosine diphosphate (GDP), transducin, and promotes the exchange of GDP with guanosine triphosphate (GTP). The transducine has three subunits: alpha, beta, and gamma. The alpha-subunit GTP complex will dissociate from the other two subunits to activate the phosphodiesterase enzyme that will hydrolyze cGMP to 5'-GMP. The reduction in cGMP concentration will cause the closure of Na^+-cGMP-gated channels. Consequently, the inward flow of Na^+ ions will decrease, while the outward flow of K^+ will still continue. Finally, this change in ionic flow will lead to the hyperpolarization of the photoreceptors and cessation of the glutamate release (Arshavsky et al., 2002).

The photoreceptor response to a single photon terminates when metarhodopsin II is inactivated by a specific rhodopsin kinase. Subsequently, GTPase protein bound to the transducin α-subunit (Tα) converts GTP to GDP and then Tα-GDP will recombine with the other two subunits (beta and gamma). In order to regenerate the visual pigment, the all-trans retinal is transported from rod cells to the pigment epithelial cells in which it will be converted to 11-cis retinal and then moved back into the rod cells.

1.1.3 Outer plexiform layer (OPL)

The first step of visual information processing takes place in the OPL, where the cone pedicles and rod spherules make synaptic contacts upon bipolar and horizontal cells. The bipolar cells transmit the visual signals vertically from the OPL to the IPL, while the horizontal cells

provide lateral interactions with adjacent photoreceptors through their wide spread processes (Dowling, 1970). Interestingly, the cone pedicles transfer some electrical signals between each other and between rod spherules through specific gap junctions resulting in the integration of photoreceptor signals at this layer (Raviola and Gilula, 1975).

1.1.4 Inner nuclear layer (INL)

This layer is composed of four distinct cell groups including bipolar cells, horizontal cells, amacrine cells, and Müller cells that are closely attached to each other. The cell bodies of these cells are located in different stratifications. The outermost margin of the INL is occupied by the horizontal cells, while the innermost part of this layer is made up of amacrine cells (Dowling, 1970; Kolb, 2003). The cell bodies of bipolar cells lie in the distal half of the INL and those of Müller cells are found at the centralmost row of the INL (Strettoi and Masland, 1995).

1.1.4.1 Bipolar cells (BC)

Structurally, the bipolar cells are made up of dendritic terminals, a cell body containing the intracellular organs such as nucleus, and the axon terminals. Bipolar cells, the second neurons involved in signal transduction, connect on one side to the photoreceptors through their single or multiple dendrites, and on the other side they synapse to the ganglion cells through their single axons. Hereby, they transfer the visual information from the photoreceptors to the ganglion cells. They also make connection with horizontal cells.

Golgi staining has identified eleven types of bipolar cells in the human retina. One type among them is identified as the rod bipolar cells and the other types are classified as cone bipolar cells (Wässle and Boycott, 1991). The dendritic terminals of a bipolar cell synapse exclusively to rod or cone photoreceptors. The cone bipolar cells, depending on their shape and arborization, are subdivided into two different categories including the diffuse cone BCs and midget BCs that

receive information from several cones or just one cone, respectively. Interestingly, different types of BCs express various types of receptors for neurotransmitter glutamate, as a result they react differently to photoreceptor signals. Accordingly, ON BCs have inhibitory glutamate receptors resulting in being inactive in darkness and active in brightness. These cells detect light stimuli against a darker background. In contrast, OFF BCs possess excitatory glutamate receptors leading to being fired in the absence of light when the photoreceptors release glutamate. These cells detect dark stimuli against a lighter background (Kolb, 2003).

1.1.4.2 Horizontal Cells (HC)

Horizontal Cells are the laterally interconnecting neurons in the outer plexiform layer and synapse with the photoreceptors through their terminal processes. They also have connections with the bipolar cells. Three morphologically different HCs: HI, HII and HIII, have been identified in the mammalian retina (Kolb, 1994). HI cells synapse with both cones and rods, while HII cells only connect to the cones. The interactions of HIII cells are not well known. Although, they synapse with the cones through their dendritic terminals, their axons have not been precisely followed to a terminal yet. However, it is proposed that they probably receive mix connections from both rods and cones (Kolb et al., 2007b).

At the level of the OPL, HCs provide lateral inhibition to improve visual sharpness and recognize simultaneous contrast. HCs release γ-aminobutyric acid (GABA) with which they modulate the neuronal signals by providing inhibitory feedback to the photoreceptors or inhibitory feedforward to the BCs (Kolb, 2003).

1.1.4.3 Amacrine Cells (AC)

A wide variety of ACs has been identified based on their morphologies and functions. Amacrine cells can be classified either as diffuse or stratified. Diffused ACs expand their

processes throughout the thickness of the IPL, while the other type, stratified ACs, elongate their processes to one or a few stratum of the IPL (Kolb et al., 1992).

Amacrine cells are located in the inner part of the INL. They receive inputs from bipolar cells and other amacrine cells and send their outputs to bipolar cells, other amacrine cells and ganglion cells. In fact, amacrine cells integrate and modulate the visual impulses that ultimately reach the ganglion cells (Kolb, 2003).

1.1.4.4 Müller Cells (MC)

Three fundamental types of glial cells, including Müller Cells, astrocytes, and microglia, are identified in the human retina (Kolb et al., 2007a). Müller Cell, the principal glial cell of the retina, stretches radially across the entire retinal thickness. The cell bodies of MCs lie in the inner nuclear layer and project processes towards the outer and inner surface of the retina in order to form the limiting membranes. The outer limiting membrane is formed by the projections of the MCs and is located at the base of the photoreceptors thus providing a junction between the Müller cells and the photoreceptor cells. The inner limiting membrane is formed by the expanded conical endfeet of the Müller cells at the inner surface of the retina (Kolb et al., 2007a). It is situated between the retina and the vitreous body.

The MCs have numerous functions such as supplying end-products of anaerobic metabolism (breakdown of glycogen) to fuel aerobic metabolism in the nerve cells, scavenging the neural waste products, recycling the glutamate in order to protect retinal neurons from exposure to extra glutamate (Kolb et al., 2007a), synthesizing of retinoic acid from retinol as well as forming a structural support for retinal neurons (Kolb et al., 1995). They also play an important role in maintaining the ionic environment of neurons by taking up extracellular potassium and redistributing it within the retina (Newman and Reichenbach, 1996). Interestingly,

following exposure to environmental stresses such as intense light, MCs are also known to upregulate neurotrophic factors including fibroblast growth factor-2 (FGF-2) and ciliary neurotrophic factor (CNTF) (Wen et al., 1995; Valter et al., 2005).

1.1.5 Inner plexiform layer (IPL)

Inner plexiform layer, the second interconnection layer, contains the axons of the retinal cells that are located in the INL such as BCs, ACs and MCs as well as the dendritic branches of ganglion cells. BCs and ACs extend their axons towards the ganglion cells in order to transfer visual impulses to these cells. Interestingly, at this level some ACs connect to the BCs through electrical synapses known as gap junctions (Kolb, 1994).

1.1.6 Ganglion cell layer (GCL)

The innermost layer of the retina is made up of the cell bodies of ganglion cells. Ganglion cells modulate the visual information and transfer it to the specialized regions of the brain. Various types of GCs have been recognized with respect to their morphologies and functions. Two types of GCs include the midget GCs and diffuse GCs. Midget ganglion cells, known as P-cells, project to the parvocellular layer of the lateral geniculate nucleus (LGN) and are specifically used for color perception and shape, while diffuse ganglion cells, referred to as M-cells, project to the magnocellular layer of the LGN and are used for information about movement (Kolb, 1991). The P-cells have smaller size and make connections with ACs and a midget BC, whereas the M-cells are larger in size and receive synapses from different types of BCs. Interestingly, some studies showed that a specific type of GCs (1-3% of GCs) synthesize a photopigment, known as melanopsin, by which they could directly absorb the light (Berson et al., 2002; Fu et al., 2005). Indeed, these intrinsically photosensitive GCs are considered as the third class of photoreceptors in the mammalian retina and are claimed to be involved in adjusting the

circadian rhythm, regulating the pupillary light reflex as well as modulating the release of melatonin by the pineal gland (Berson, 2007).

1.1.7 Nerve fiber layer (NFL)

The axons of ganglion cells form the optic nerve, which terminates in visual centers located in different regions of the brain including the lateral geniculate nucleus and the superior colliculus.

1.2 Retinal function

Undoubtedly, perfect visual perception of the surrounding environment requires proper functioning of the neural cells of the retina, which can be evaluated by electroretinography (ERG), a non-invasive method to record the electrical activity of retinal neurons.

1.2.1 The electroretinogram (ERG)

The ERG is an evoked potential representing the summed electrical activity of the retinal cells in response to a light stimulus. Following light stimulation, a graphic representation of potential changes across the eye is recorded (Cameron et al., 2008). Briefly, the full-field ERG can be recorded with an active electrode placed on the corneal surface and a reference electrode placed on the external canthus in humans or the mouth in rodents. In addition, other electrode placed on the forehead in human or inserted into the tail in rodent will act as a ground electrode.

The ERG plays an important role both as a clinical tool to evaluate the retinal function and detect a variety of retinal disorders that may lead to serious consequences such as blindness as well as a research tool in humans and animals in order to study the retinal function.

1.2.1.1 History of the ERG

In 1865, the first ERG was recorded by Holmgren who showed that a light stimulus creates a change in the electrical potential of the amphibian eye (Perlman, 2011). Afterward, in 1877, Dewar recorded the first human ERG from the surface of the cornea (Dewar, 1877). Gotch in 1903 defined the components of the ERG which included two main waveforms: a cornea-negative deflection which was followed by a larger cornea-positive waveform (Gotch, 1903). Interestingly, five years later, in 1908, a supplementary slower positive wave was recognized by Einthoven and Jolly (Einthoven and Jolly, 1908) who named the three ERG waveforms as the a, b and c waves, respectively. This appellation is still used to define the ERG components today. In addition to these waves, Cobb and Morton in 1954 (Cobb and Morton, 1954) reported some fast oscillations on the ascending limb of the b-wave which were later named as oscillatory potentials (OPs) by Yonemura et al. (Yonemura et al., 1962).

The above mentioned ERG components are recorded in response to the onset of the light stimulus and, accordingly, are considered as components which require the activation of the ON-retinal pathway. Of interest, by increasing the flash duration (>100ms) an additional corneal-positive wave, the d-wave, is obtained at the offset of the light stimulus, in particular under photopic conditions, reflecting the activation of the OFF-pathway (Ueno et al., 2006).

Nowadays, the clinical diagnosis of retinal disorders is usually based on the amplitude and implicit time measurements of the two principal waves of the ERG, the a- and b-wave, as well as the OPs. These components are described in further details below.

1.2.1.2 Types and components of the ERG

The recording of the scotopic and photopic ERGs depends on the background light used during the ERG recording. Dark-adapted ERGs, referred to as scotopic ERGs, evaluate the

function of the rod pathway, while light-adapted ERGs, referred to as photopic ERGs, provide information about the function of the cone pathway.

Regardless of the recording techniques used to obtain scotopic and photopic ERGs, the ERG waveform is composed of different components (Figure 3), each of which reflecting a specific step of the visual information processing that occurs within a particular layer of the retina and demonstrate the activation of a special group of retinal neural cells.

1.2.1.2.1 a-wave

The first negative deflection observed in the ERG, the a-wave (Figure 3), is believed to reflect the hyperpolarization of the photoreceptors in response to a light stimulus. Using an intra-retinal microelectrodes approach, Brown and Wiesel showed that the a-wave of the ERG resulted from an electrical activity originating at a more distal level than the b-wave (Brown and Wiesel, 1961). They claimed that the outer segment of the photoreceptor was the most likely origin of the a-wave. A recent study showed that in addition to the voltage produced by extracellular flow of photocurrent from outer to inner segments, a considerable part of the a-wave is generated by current that flows in the outer nuclear layer (ONL) towards the synaptic terminal (Robson et al., 2014).

1.2.1.2.2 b-wave

The positive deflection following the a-wave, the b-wave (Figure 3) is thought to reflect the postsynaptic activity of the inner retina. It has been shown that blocking synaptic transmission from the photoreceptors to the inner retina, by saturating the post-synaptic receptors with L-aspartate or L-glutamate, abolishes the ERG b-wave, while the ERG a-wave remains intact (Perlman, 2011). Furthermore, blockage of the central retinal artery, which supplies the neural retina, eliminates the electrical activity of the inner retinal cells, thus causing a

considerable reduction in the b-wave amplitude (Noell, 1954). These findings confirm the role of postreceptoral neurons as the main origin of the b-wave.

Among the inner retinal cells, the BCs and the MCs are the most likely generators of the b-wave. Several studies have shown the role of these cells in the generation of the b-wave. For example, Miller and Dowling, using intracellular recording techniques, showed that the intracellular light-induced activation of MCs was very analogous to the b-wave component of ERG (Miller and Dowling, 1970). In addition, Slaughter and Miller reported a reduction of the b-wave following the administration of 2-amino-4-phosphonobutyric acid (APB), a specific agonist of glutamate metabotropic receptors which selectively blocks the ON-DBC activity (Slaughter and Miller, 1981). The "Müller cell hypothesis" highlights the role of MCs in generation of the ERG b-wave. According to this hypothesis, following light stimulation the extracellular K^+ concentration will change that modulate the membrane potential of the MCs, and this modification will be reflected as the corneal ERG b-wave (Perlman, 2011). In fact, the cell membrane of MCs is highly permeable to K^+ and, as a result, following the light-evoked depolarization of second order neurons such as bipolar cells that will lead to an increase in extracellular K^+ concentration, the MCs will depolarize (Newman, 1980) and presumably take part in generating the b-wave. To confirm the role of MCs, some studies reported that administration of DL-α-amino-adipic acid (DL-αaaa), a gliotoxic agent, led to the b-wave disappearance, while the a-wave remained intact (Bonaventure et al., 1981; Bruce et al., 1981).

1.2.1.2.3 Oscillatory potentials (OPs)

A series of low-voltage, high frequency wavelets observed on the ascending limb of the b-wave are named as oscillatory potentials (OPs) (Figure 3). These wavelets are much faster than the complex of the a- and b-wave in the ERG and their frequency is in the range of 100-150Hz

(Perlman, 2011). Hence, the best method to isolate them is to apply a band-pass filter on the ERG waveform in order to remove the slow, high amplitude a- and b-wave and to increase the amplification in order to selectively enhance them (Asi and Perlman, 1992).

The exact cellular origin of the OPs is still debated. However, intraretinal recording studies have identified the IPL as the most possible region involved in the OPs formation (Ogden, 1973; Wachtmeister and Dowling, 1978). Moreover, pharmacological experiments and depth recording techniques have suggested that the OPs may reflect extracellular electrical currents generated by negative feedback pathways between amacrine cells, ganglion cells and bipolar cells (Wachtmeister and Dowling, 1978; Yonemura and Kawasaki, 1979; Heynen et al., 1985). Interestingly, administration of pharmacological agents such as L-glutamatic acid diethyl ester and alpha-aminoadipate which antagonize the excitatory neurotransmitters glutamate and aspartate, respectively, and consequently blocks the synaptic transmission from the photoreceptors, attenuated the OPs while the a-wave was unchanged (Wachtmeister, 1981). This finding confirms the postreceptoral origin of the OPs.

From the clinical point of view, the OPs are considered as a valuable indicator of mild retinal ischemia in the inner retina (Speros and Price, 1981) that happens, for example, in diabetic retinopathy. With respect to the higher sensitivity of OPs to ischemia in localized retinal regions (Perlman, 2011), in pathological conditions where the ERG a- and b-wave are normal, OP recording can indicate the background retinal ischemia.

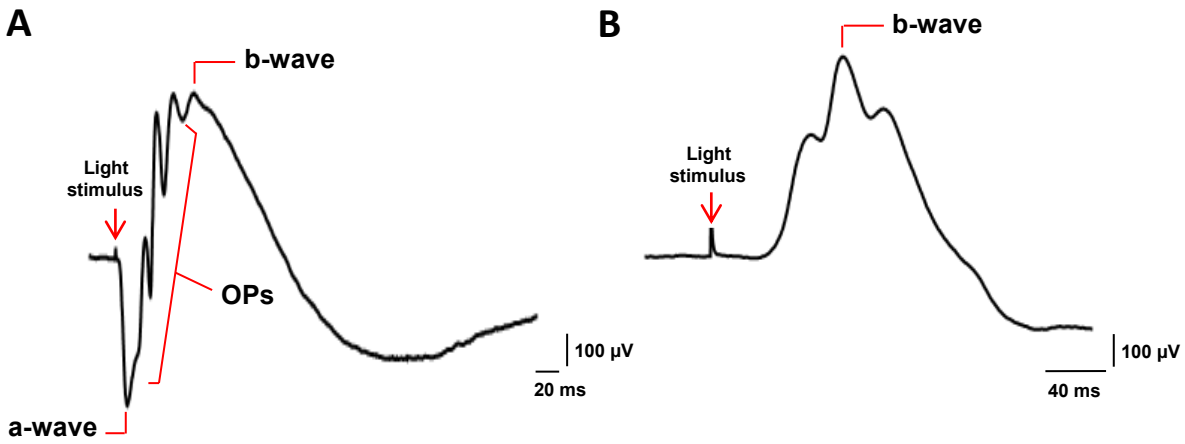

Figure 3: Representative ERG waveforms obtained from a 30 days-old albino Sprague-Dawley male rat in scotopic (**A**) and photopic (**B**) conditions.

It is of interest to note that in human subjects both scotopic and photopic ERGs contain a- and b-waves. In contrast, the light adapted (photopic) response recorded from rats (as shown in Fig.3 B) is only made of a b-wave and the a-wave is absent (Rosolen et al., 2004).

SECTION II

2. Sex-related differences in retinal function and structure and the role of estrogen

2.1 The role of biological sex on the retina

Biological sex is considered as a unique characteristic for living creatures. Undoubtedly, the sexual hormones play an important role in developing different biological functions in both sexes that may possibly affect the different tissues in the body. Similar to the other tissues of the human body, biological sex can also influence the function and structure of the retina. For example, the effect of biological sex on human retinal function, as determined with the ERG, has

been known for more than 50 years (Karpe et al., 1949). It has been claimed that the ERG amplitudes are larger in women compared to men (Birch and Anderson, 1992; Hébert et al., 1999). In a recent re-investigation of this claim, the amplitudes of scotopic and photopic ERGs of female subjects were reported to be on average 29% larger than their male counterparts (Brûlé et al., 2007). Interestingly, it seems that the sex-related differences is not only limited to the retinal function, but it would also impact on the retinal structure and thus explain the sex-related differences observed in the electrical activity of the retina. Several studies have shown significant differences in retinal thicknesses between male and female (Massin et al., 2002; Asefzadeh et al., 2007). One study, using spectral domain optical coherence tomography (SD-OCT) in 256 healthy eyes of men and women, showed that the ONL-OPL and the INL measured at the macular level were thicker in men, while the NFL was thicker in women (Ooto et al., 2011). Furthermore, Kashani et al. (Kashani et al., 2010), using the Stratus OCT, reported that the mean foveal thickness and center point foveal thickness were significantly thicker in male subjects compared to females. However, Asrani et al. (Asrani et al., 1999), through measurement retinal thickness in a small group of normal subject (n = 29), reported a small effect of biological sex on the retinal thickness. The sex-related functional and structural differences in body tissues, including the retina, might be attributed to male-female differences in sexual hormone profiles. It is of interest to note that some studies have also reported that a possible modulatory effect of the menstrual cycle and the accompanying hormonal fluctuations, especially estrogen, was observed on several ocular structures, including the retina (Barris et al., 1980; Bassi and Powers, 1986; Hébert et al., 1999).

The gonadal steroid 17β-estradiol (βE_2), also known as estrogen, is a fat-soluble hormone which enters the cells by direct interactions with phospholipids (Persky et al., 2000), and induces

its physiological effects through two different isoforms of estrogen receptors (ERs): ERα and ERβ (Giddabasappa et al., 2010). The expression of ERs has been reported in many systems including: primary and secondary reproductive, cardiovascular, immune, bone, gastrointestinal, respiratory, and central nervous systems (Deroo and Korach, 2006). It is of interest to note that the expression of ERs has been found in most retinal layers including the ganglion cell layer, the inner nuclear layer, inner and outer plexiform layers and photoreceptor layer (Kobayashi et al., 1998; Kumar et al., 2005). The distribution of ER subtypes (α and β) in different tissues is influenced by two biological factors: age and biological sex. In human, Ogueta et al. (Ogueta et al., 1999), using a mouse monoclonal antibody against the human ERα, detected ERα proteins in the neurosensory retina and retinal pigment epithelium of donor eyes obtained from premenopausal women, while no ERα protein was detected in tissues dissected from postmenopausal women and from men. In rodents, no sex-related differences were found in the localization of the ERα and ERβ throughout the retina (Kobayashi et al., 1998).

2.2 Estrogen as a multifunctional hormone

Estrogen, a major female sex steroid, is principally engaged in physiological functions such as sex differentiation and maturation. Interestingly, estrogen even plays a critical role in early sexual development of male offsprings (Wibowo and Wassersug, 2014). During fetal life, testicular-derived testosterone of male fetus can freely enter the brain. Within the brain, testosterone is converted into estradiol through the action of the P-450 enzyme aromatase (Lephart, 1996). Locally generated estradiol, paradoxically initiates the masculinization process, thus transforming embryonic brain regions to the male morphology (McCarthy, 2008). It is believed that because of the sex differences in gene expression, the male and female brain react differently to the same hormone (McEwen et al., 1984). Interestingly, the actions of estrogen are

not limited to the tissues which are involved in reproductive function. It also plays an important role in the development, growth, differentiation, maturation and function of different systems including cardiovascular, gastrointestinal, and respiratory systems (Chen et al., 2009; Bendale et al., 2013) as well as central and peripheral nervous system (Behl, 2002). Estrogen can also act as a neuroprotective factor that stabilizes neuronal function and, under certain conditions, can prevent neuronal death (Behl, 2002).

The neuroprotective effects of estrogen have been documented in various human and animal models of brain injuries (Wise et al., 2000). One animal study showed a reduction of ischemia-induced damages in the cerebral cortices of ovariectomized female C57Bl/6J mice that had been treated with 17β-estradiol prior to being exposed to hypoxia (Dietrich et al,, 2013). Interestingly, biological sex is considered as a crucial factor in determining the susceptibility of the brain to an ischemic event. It has been claimed that men are at higher risks for cardio-cerebrovascular events than women of the same age; however, the relative incidence of stroke between both sexes appears to normalize with advancing age (McCullough and Hurn, 2003). Higher vulnerability of female to ischemic events after menopause was related to a sudden decline in circulating estrogen which may lead to vascular dementia and Alzheimer's disease (Paganini-Hill and Henderson, 1994). Of note, the protective role of estrogen in ischemic events is related to the vaso-active properties of estrogen. It has been reported that estrogen increases the expression of endothelial nitric oxide synthase (eNOS) by binding to the estrogen receptors of the vascular endothelium (Deschênes et al., 2009; Haynes et al., 2000), thus exerting vasodilatory effect on the vascular tissues. The estrogen-dependent vasodilation that follows, increases the blood flow in different tissues, thus providing protection against ischemic events,

including ischemic cerebrovascular injuries (Finucane et al., 1993), cardiovascular diseases (Barrett-Connor and Bush, 1991) and central retinal vein occlusion (Group, 1996).

The discovery of estrogen receptors in the different retinal layers suggests that estrogen may play an important role in modulating biological functions of the retina and also protecting this neural tissue against oxidative-induced pathological conditions. From a clinical point of view, the role of estrogen on the occurrence of some ocular pathologies has been well documented. For instance, it has been reported that estrogen may have a protective effect against cataract (Bigsby et al., 1999). A study of 544 female subjects showed that an early onset of menopause was associated with a 2.9-fold increase in the risk of developing cataracts (Shibata et al., 1993). Of interest, the beneficial effect of (postmenopausal) estrogen replacement therapy on the incidence of cataracts supports the effectiveness of estrogen in preventing cataractogenesis (Klein et al., 1994; Benitez et al., 1997). Moreover, the incidence of primary open-angle glaucoma (POAG) is reported to be greater in men under 50 years old compared to age-matched women, while after the age of 50 the POAG incidence is nearly the same in both sexes (Armaly, 1965). Furthermore, one study showed that intraocular pressure (IOP) of pregnant women during the second and third trimester of pregnancy when the estrogen levels are highest, was significantly lower compared to non-pregnant control groups (Qureshi, 1994), confirming the regulatory effect of estrogen on IOP. The incidence of acquired macular degenerations is also reported to increase after menopause, presumably as a result of the abrupt decline in the circulating estrogen levels, and can be prevented with estrogen replacement therapy (Group, 1992).

2.3 The cellular mechanisms of estrogen action in neural tissues

2.3.1 Genomic mechanism

Through the genomic (classical) pathway, estrogen acts on gene transcription. In order to initiate gene transcription, the intracellular protein synthesis machineries have to be activated, thus resulting in a slow reaction (in the time-scale of hours) of prolonged duration (McEwen and Alves, 1999). With this mechanism, estrogen enters the cell either passively by diffusion across the cell membrane or by binding to transporter proteins (Beato et al., 1986), and binds to the nuclear or cytoplasmic ERs to form an intracellular E-ER complex. The E-ER complex binds to the specific DNA sequence in the estrogen-responsive promoter genes, which activates the expression of E-regulated genes and ultimately leads to the modulation of gene transcription, increased or decreased mRNA levels and protein synthesis (Gupta et al., 2005) (Figure 4).

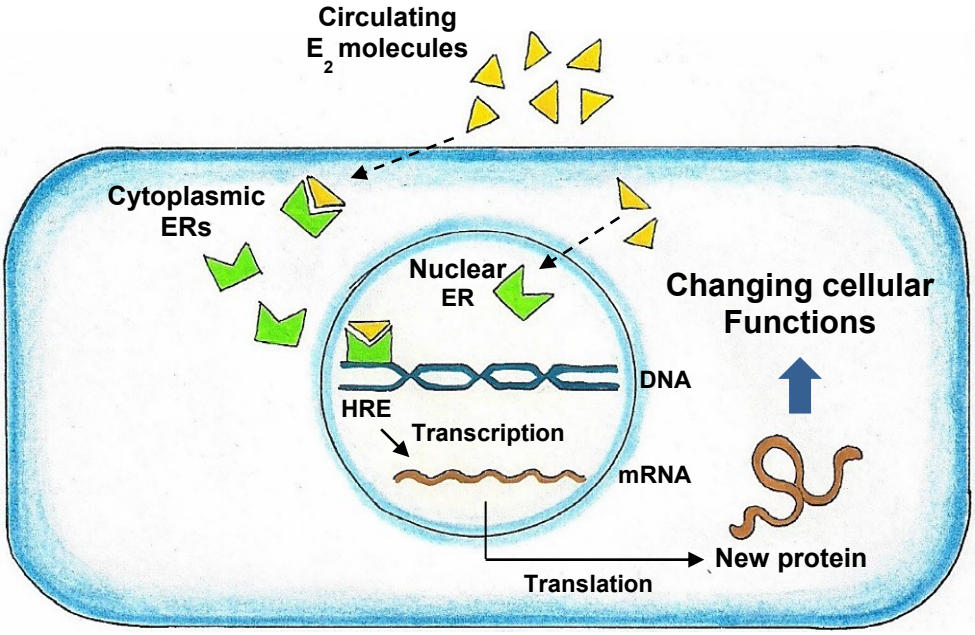

Figure 4: Schematic diagram of genomic pathway in a cell. ER: estrogen receptor; HRE: hormone response elements [Modified from (Gupta et al., 2005)].

2.3.2 Non-genomic mechanism

The non-genomic pathway is initiated as a rapid response (in the time-scale of minutes) of short duration (Lösel and Wehling, 2003). With this mechanism, estrogen activates the intracellular signaling pathways within the target cell through binding to the G protein coupled membrane receptors (Gupta et al., 2005). The non-genomic action of estrogen is mostly involved in generating intracellular second messengers such as cAMP, altering the ion fluxes particularly Ca^{2+}, opening or blocking the ion channels, or changing the activation of intracellular enzymes (Gupta et al., 2005) (Figure 5).

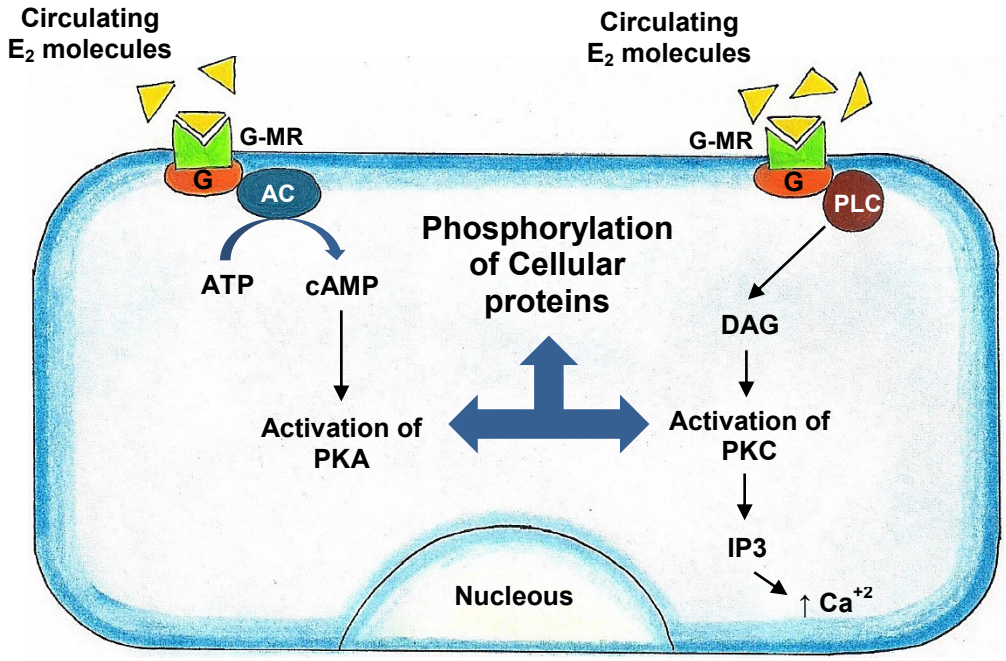

Figure 5: Schematic diagram of non-genomic pathway in a cell. G-MR: G protein coupled membrane receptor; AC: adenylyl cyclase; PLC: phospholipase C; cAMP: adenosine 3', 5'-cyclic monophosphate; DAG: diacyl glycerol; PKA: phosphokinase A; PKC: phosphokinase C; IP3: inositol trisphosphate [Modified from (Gupta et al., 2005)]

2.4 Estrogen as a potent antioxidant molecule

Estrogen can interact with intracellular signaling pathways and exert some antioxidant effects through suppressing peroxidation chain reactions (Bittner, 2001). It can also act as a free radical scavenger, thus preventing free radical-induced destructive effects (Behl, 2002). Direct free radical scavenging properties of estrogen is believed to be entirely independent of the activation of ERs and is related to the phenolic structure of its molecule (Behl et al., 1997; Moosmann and Behl, 1999). From a structural point of view, estrogen is a monophenolic compound that is analogous to the lipophilic free-radical scavenger α-tocopherol (Vitamin E) (Behl, 2002). The antioxidant properties of estrogen is attributed to the hydroxyl group on its phenolic A ring (Green et al., 1997), that can donate a hydrogen molecule in order to detoxify accumulating free oxygen radicals [such as the hydroxyl radical ($HO^{\cdot}$)] (Behl, 2002). Production and accumulation of free radicals, including reactive oxygen and nitrogen species (ROS and RNS), cause an oxidative stress to the cells leading to the extreme oxidation of cellular DNA, lipids and proteins (Behl, 2002). Accordingly, counteracting the effect of free radicals with estrogen plays a critical role in cellular protection against oxidative damage which is considered as the main cause of neurodegenerative disorders. In addition, it has been shown that estrogen increases the gene expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (Gpx) (Strehlow et al., 2003; Wang et al., 2014), thereby augmenting the cellular antioxidant defense and thus, protecting cells against oxidative damage.

From a physiological point of view, the retina has several properties which makes it an ideal target for the generation of reactive oxygen species (ROS), thereby enhancing its susceptibility to oxidative damage. Firstly, the outer segment membranes of photoreceptors contain a high concentration of polyunsaturated fatty acids (PUFA): docosahexaenoic acid (DHA; 22:6ω-3), arachidonic acid (AA; 20:4ω-6), and choline phosphoglyceride which are the

main targets of peroxidation and can be easily oxidized, thus triggering intracellular cytotoxic reactions (Bazan, 1988). Secondly, given that the retinal cells have a high metabolism rate, the consumption rate of oxygen is therefore extremely high in this tissue, and consequently ROS are abundantly produced as a by-product of oxygen metabolism during mitochondrial electron transport (Cringle et al., 2006). Thirdly, the RPE and the neurosensory retina contain plenty of photosensitizers including rhodopsin, melanin, lipofuscin, and cytochrome-c oxidase (a mitochondrial respiratory enzyme) all of which can initiate peroxidation following light absorption (Beatty et al., 2000). Fourthly, the outer retina is the site of phototransduction and photic reactions are believed to be a major source of oxygen radical formation (Wu et al., 2006). Finally, the phagocytosis of the outer segment of the photoreceptor by RPE is considered as an oxidative stress that can increase extracellular concentration of ROS such as H_2O_2 (Tate et al., 1995). Given the antioxidant properties of estrogen, its presence could have a beneficial effect on the retina and protect this vulnerable tissue from oxidative damage.

SECTION III

3. Animal models of oxidative retinopathies

In our laboratory, two animal models of postnatal oxidative stress-induced retinopathies, have been developed over the past decade. In first model, referred to as Oxygen-Induced Retinopathy (OIR), oxidative stress is induced by exposing the newborn Sprague-Dawley (SD) rats to the high level of oxygen (80 ± 5%). In second model, known as Light-Induced Retinopathy (LIR), the oxidative stress is developed by exposing the newborn SD rats to a bright luminous environment (l0,000 Lux). These two models are described in further details below.

3.1 Oxygen-Induced Retinopathy (OIR)

OIR is considered as a valuable animal model that mimics human proliferative ischemic retinopathies including Retinopathy of Prematurity (ROP) and diabetic retinopathy (Kermorvant-Duchemin et al., 2010). From the pathological point of view, OIR has two different phases: vaso-obliteration and vaso-proliferation (Sapieha et al., 2010). The vaso-obliterative phase is characterized by a suppression of retinal vascular development due to the high level of oxygen that inhibits the normal postnatal vascular growth towards the peripheral retina. One of the underlying pathological mechanisms during this phase is attributed to the suppression of the synthesis of the vascular endothelial growth factor (VEGF) by the retinal vasculature in response to the extra uterine hyperoxic environment (Connor et al., 2009; Kermorvant-Duchemin et al., 2010; Sapieha et al., 2010). Given that VEGF has a critical role in vascular development and retinal coverage (Stone et al., 1995), the absence of this factor causes an arrest in retinal vascular development, thus resulting in avascular regions particularly at the retinal periphery. The vaso-proliferative phase is manifested by a neo-vascularization due to the relatively hypoxic situation upon returning to a normoxic condition (Budd et al., 2010; Kermorvant-Duchemin et al., 2010; Sapieha et al., 2010). In fact, formation of new vessels is triggered by an insufficient retinal blood supply induced by the higher than normal levels of oxygen during the vaso-obliteration phase. Excessive retinal VEGF-A expression which is responsible for a variety of functional, vascular, and most probably irreversible cytoarchitectural changes in the retina, has been proposed as an important pathological factor in the second phase of OIR (Shih et al., 2003).

Of the different animal models, the newborn rat is one of the most studied animal models of OIR (Dorfman et al., 2011). Given that a rat pup has an immature retina at birth, said to be equivalent to that of a 24- to 26-week-old human fetus (Weidman and Kuwabara, 1969), it

provides an appropriate model to investigate the effects of postnatal hyperoxia on the developing retinal vasculature. In the rat model of OIR, permanent abnormalities in the retinal structure and function have been well documented (Lachapelle et al., 1999; Dembinska et al., 2002; Dorfman et al., 2008; Dorfman et al., 2009). The most striking retinal cytoarchitectural anomaly following post-natal hyperoxia is that of a significant decrease in the OPL thickness and loss of HC, while the other retinal layers remain nearly intact (Lachapelle et al., 1999; Dembinska et al., 2001). Moreover, it has been suggested that hypersensitivity of the retinal cells (mostly the horizontal cells) to oxygen or the relative hypoxia that follows hyperoxia may destroy these cells and trigger some degenerative processes leading to the interruption of synaptic transmissions between the retinal neurons (Connor et al., 2009). Functional retinal impairment detected with the ERG is manifested as a considerable decline of the b-wave amplitudes and also those of the OPs and a relative sparing of the a-wave (Lachapelle et al., 1999; Dorfman et al., 2006; Dorfman et al., 2008; Dorfman et al., 2009). The above-mentioned electroretinographic anomalies support the idea that the functional deficit of the retina in OIR is localized at the postreceptoral region, which is well correlated with the histological findings (Dembinska et al., 2001; Dorfman et al., 2006).

3.2 Light-Induced Retinopathy (LIR)

LIR is considered as a valid model for human retinal degenerations such as age-related macular degeneration (AMD) and some forms of Retinitis Pigmentosa (Organisciak and Vaughan, 2010). This type of retinopathy is described as a severe, rhodopsin-mediated, degeneration of the retina in which the rhodopsin acts as the main target for photoreceptor cell damage resulting from exposure to an intense light (Grimm et al., 2001; Noell et al., 1966). From a pathophysiological point of view, three possible hypotheses about light-induced retinal

damage have been discussed by Noell and his coworkers. They believed that the destructive effects of light upon the retina might be due to a metabolic disorder resulting from light exposure, a toxic photoproduct derived from vitamin D during contact to bright light and also light-induced oxidative chain reactions leading to ROS formation (Noell et al., 1966). Of interest, several factors including the duration of light exposure, the intensity of the environmental light, the wavelength of the light and the method of exposure (cyclic or constant) can influence the severity of retinal damage (De Vera Mudry et al., 2013). Moreover, it has been shown that in animal models of LIR, the age of animal at the time of light exposure could also affect the extent of retinal photodamage (De Vera Mudry et al., 2013). The latter was confirmed with a study where juvenile rats showed higher resistant to light damage compared to adult rats (Joly et al., 2006). Additionally, the body temperature during the light exposure has been reported as an intervening factor that can affect the severity of light damage as induced hyperthermia during light exposure increases the retinal damage (Organisciak et al., 1995).

Most studies of light damage have been carried out in albino rodents because of their extreme vulnerability to light damage (O'Steen et al., 1972). Although it has been shown that the sensitivity of photoreceptors to light damage is nearly similar in both albino and pigmented animals (Rapp and Williams, 1980a), pigmentation appears to play a protective role against light damage (Rapp and Williams, 1980b). The main pathological feature of LIR, leading to visual dysfunction and electroretinographic abnormalities, is the initial damage to the outer segments (OS) of the photoreceptors where the light photons are captured (Noell et al., 1966; Joly et al., 2006a; Joly et al., 2006b; Organisciak and Vaughan, 2010). Consequently, the OS damage triggered by an intense light and manifested with a shedding of the photopigment-coated disks acts as a pathological signal for apoptosis of the RPE cells (Hafezi et al., 1997). Then, it is

concluded that the most affected retinal cells in LIR are the photoreceptors and the pigmented epithelial cells. Given that the structural changes in LIR appear to be limited to the outer retina (i.e. photoreceptor layer), the loss of retinal function in LIR as evaluated with the ERG is characterized with a remarkable attenuation in amplitude of both a- and b-wave components of ERG (Joly et al., 2006a; Joly et al., 2006b).

SECTION IV

4. General objectives

As described in the introduction above, it would appear that biological sex can influence the retinal function and structure of animals and human subjects as well, most probably due to the different sexual hormone profiles. The extent of this effect remains, however, to be determined. Also, it has been suggested that male and female react differently to oxidative stress, presumably due to the sex steroid hormones, particularly estrogen. As mentioned above, estrogen can act as a neuroprotective agent and due to its antioxidant properties it is able to protect the neural tissue, including the retina, against oxidative stress conditions. In a view of the above, the following two studies were conducted:

Study 1 (Chapter II):

- **Differences in retinal structure and function between aging male and female Sprague-Dawley rats are strongly influenced by the estrus cycle.**

This study was performed to investigate the sex- related differences on the retinal function and structure of albino SD rats. In addition, aging process is considered as a biological event leading to cumulative oxidative stress that can influence the function and structure of different tissues such as retina. Accordingly, in order to study the role of biological sex on the aging retina, retinal function and structure were investigated with the ERG and histology at five different ages. Also, ERGs recorded from premenopausal and menopausal female SD rats were compared in order to determine the effect of estrus cycle on the retinal function.

Study 2 (Chapter III):

- **Evaluating the neuroprotective effect of 17β-estradiol on the structure and function of the retina exposed to an intense oxidative stress.**

This study was conducted in order to investigate the neuroprotective role of estrogen on the retina of newborn male SD rats exposed to oxidative stress conditions induced either by hyperoxia (80 ± 5 % O_2) or bright light (10,000 Lux). Oxidative stress is considered as a major contributor to retinal degenerations such as AMD (Beatty et al., 2000) and ROP (Sapieha et al., 2010). In this study, OIR and LIR models were used as valuable animal models of the human oxidative retinopathies AMD and ROP, respectively.

CHAPTER II

MANUSCRIPT I

Preface to chapter II

In the first study, we obtained the ERG from male and female SD rats at different ages in order to investigate the effect of biological sex and age on the retinal function. The retinal histology was also performed at different ages to clarify the impact of these two biological factors on the retinal cytoarchitecture. In addition, in order to study the effect of estrus cycle on the retinal function, the ERGs recorded from premenopausal and menopausal female SD rats were compared.

Differences in retinal structure and function between aging male and female Sprague-Dawley rats are strongly influenced by the estrus cycle.

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1. ABSTRACT

Purpose: Biological sex and age are considered as two important factors that may influence the function and structure of the retina, an effect that might be governed by sexual hormones such as estrogen. The purpose of this study was to delineate the influence that biological sex and age exert on the retinal function and structure of rodents and also clarify the effect that the estrus cycle might exert on the retinal function of female rats.

Method: The retinal function of 50 normal male and female albino Sprague-Dawley (SD) rats was investigated with the electroretinogram (ERG) at postnatal day (P) 30, 60,100,200, and 300 (n=2-7 male and female rats/age). Following the ERG recording sessions, retinal histology was performed in both sexes. In parallel, the retinal function of premenopausal and menopausal female rats aged P540 were also compared.

Results: Sex and age-related changes in retinal structure and function were observed in our animal model. However, irrespective of age, no significant difference was observed in ERG and retinal histology obtained from male and female rats. Notwithstanding the above we did however noticed that between P60 and P200 there was a gradual increase in ERG amplitudes of female rats compared to males. Furthermore, the ERG of premenopausal female rats aged 18 months old (P540) was larger compared to age-matched menopausal female rats as well as that of male rats.

Conclusion: Our results showed that biological sex and age can influence the retinal function and structure of albino SD rats. Furthermore, we showed that cycled female rats have better retinal function compared to the menopausal female rats suggesting a beneficial effect of the estrus cycle on the retinal function**.**

2. INTRODUCTION

Age and biological sex are two of the most important regulators of our day-to-day body functions, including retinal function. The influence of biological sex on the human retinal function, as determined with the electroretinogram (ERG), has been known for more than 60 years (Karpe et al., 1950). Electroretinograms are usually reported to be of larger amplitudes in women compared to men (Birch and Anderson 1992; Hébert et al., 1999). In a recent study, the amplitudes of scotopic and photopic ERGs of female subjects were reported to be on average 29% larger than their male counterparts (Brûlé et al., 2007). From the structural point of view, spectral domain optical coherence tomography (SD-OCT) did reveal sex-related differences in the mean thickness of the retinal layers at the macula. It showed that the ONL-OPL as well as the INL were thicker in men, while the NFL was thicker in women (Ooto et al., 2011). It is thought that sex-related functional and structural differences in body tissues, including the retina, might be governed by the remarkable male-female differences in sex hormone profiles. Of interest, the presence of estrogen receptors in different retinal layers (Kobayashi et al., 1998; Kumar et al., 2008) suggests that this sexual hormone might play an important role in the normal functioning of this tissue (Yamashita et al., 2010; Yamashita et al., 2011). Furthermore, a possible modulatory effect of the menstrual cycle and the accompanying hormonal fluctuations, especially estrogen, was observed on several ocular structures, including the retina (Barris et al., 1980; Bassi and Powers, 1986; Hébert et al., 1999).

The effect of age on retinal function and structure has also been shown in human and animals (DiLoreto et al., 1995; Freund et al., 2011; El-Sayyad et al., 2014). Aging is one of the most important contributors to cumulative oxidative stress that could result in the gradual deterioration in function and structure of different tissues including the retina (Harman, 1981;

Harman, 1992; El-Sayyad et al., 2014). It has been reported that the antioxidant properties of some tissues such as the heart, kidneys, liver and brain was significantly higher in female rats explaining the longer lifespan in females (Katalinic et al., 2005). Given that estrogen has anti-oxidant effects on different tissues of the body and exerts neuroprotection without having to involve a receptor-mediated process (Weaver et al., 1997; Xia et al., 2002), it could be proposed that biological sex might also influence the normal aging process. Although the plasmatic levels of estrogen in men remain relatively constant throughout life, in women it fluctuates over a larger range (Seeman, 1997). At menopause, the ovaries stop producing sex hormones, while in men the testes continue to produce testosterone, which is partly converted into estradiol in the neural tissues (Seeman, 1997). It may explain the higher incidence of some age-related ocular pathologies such as cataract (Klein et al., 1992), glaucoma (Vajaranant et al., 2010) and acquired macular degenerations (Snow et al., 2002) in postmenopausal women that most probably result from the sudden decline in circulating estrogen. Supporting the latter claim, previous studies reported a reduced occurrence of cataract and age-related macular degeneration in women on estrogen replacement therapy compared to those not receiving hormonal supplementation (Group 1992; Klein et al., 1994; Cumming and Mitchell 1997; Snow et al., 2002).

In a view of the above, the purpose of the present study was to investigate the effect of biological sex and age on the retinal function and structure of aging male and female albino SD rats. Our results showed that in female rats there was an increase in ERG response amplitudes noticed between 2 and 6.5 months of age while in age-matched male rats the ERG amplitudes remained stable during the same period. The latter results would suggest a beneficial effect of the estrus cycle on the retinal function. Furthermore, our results also showed that premenopausal

female rats had larger ERG amplitudes compared to menopausal rats, further confirming the role of estrus cycle on retinal function.

3. METHODS

3.1 Animals

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and were approved by the McGill University-Montreal Children's Hospital animal care committee. Adult female SD rats (Charles River Laboratories, St-Constant, QC) were ordered at 15 days of gestation and kept in normal environment of animal facility where they were allowed food and water *ad libitum*. Rat pups remained with their mom until weaning at P21 when they were separated according to their sex.

3.2 Experimental groups

The retinal function of 50 normal male and female SD rats was investigated with the ERG at age P30, P60, P100, P200 and P300 [2-7 rats per sex and age groups] in order to evaluate the effect of sex and age on the retinal function. The retinal samples were collected right after each recording session at P30, P60, P100 and P300 [2-4 rats per each sex at each age] to assess the role of sex and age on the retinal structure.

In parallel, the ERGs of 6 normal female SD rats were obtained at 18 months (P540). Given that the menopausal age of laboratory rats is reported to be between 15 and 18 month (Sengupta, 2013), vaginal smears were taken from the female rats before the ERG recordings in order to study the effect of estrus cycle (equivalent to menstrual cycle in women) on the retinal function. Results were compared to those obtained from 5 male SD rats aged 18 months.

3.3 Vaginal smear sampling

Use of vaginal smear (VS) technique, allows one to identify the different estrus cycle phases (proestrus, estrus, metestrus and diestrus) based on the proportion among three types of cells: nucleated epithelial cells, cornified cells and leukocytes, the proportion of which varies according to the plasmatic level of estrogen (Marcondes et al., 2002).

In this study the VS technique was used in order to determine the menstrual status in female rats through observation of different cell types in the vaginal secretions. The six 18 month-old female rats were housed in standard cages, 2 per cage, in the animal care facility. They were kept in a controlled temperature room (22ºC), with 12hr light: 12hr dark cycle. Since the duration of the estrus cycle in rodents lasts 4-5 days, vaginal secretions were collected on five consecutive days between 10 and 11 AM with a plastic pipette filled with 10µL of normal saline (NaCl 0.9%) that was inserted into the vagina, but not deeply (Marcondes et al., 2002). The saline was then flushed into the vagina and sucked back into the pipette. The resulting fluid was placed on glass slides, stained with 0.1% toluidine blue, following which pictures were taken with a Zeiss microscope (Zeiss Axiophot, Zeiss microscope, Germany: 40X) equipped with a digital camera.

3.4 Electroretinography

ERGs were recorded with a data acquisition system (Acqknowledge®; Biopac MP100; Biopac System Inc., Goleta, CA, USA) as previously described (Dorfman et al., 2006; Joly et al., 2006; Dorfman et al., 2008). Briefly, the rats were kept in the dark for at least 12hr (i.e. overnight) before recording in order to enhance retinal sensitivity (Karpe and Tansley, 1948). The experimental procedure was done under a dim-red light. First, the animals were anaesthetized with an intramuscular injection of ketamine (85 mg/kg) and xylazine (5 mg/kg).

The pupils were dilated with one drop of 1% Mydriacyl, and the cornea was anesthetized with a drop of 0.5% Alcaine. The animals were laid on their right side in a recording box (Lachapelle and Blain, 1990) in which a rod desensitizing background light and a photo-stimulator had been installed (model PS 22, Grass Instrument, Quincy, MA). The retinal potential was captured at the cornea with a DTL fiber electrode (27/7 X-Static® silver coated conductive nylon yarn: Sauquoit Industries, Scranton, PA, USA) acting as the active electrode (Dawson et al., 1979). It was maintained on the cornea with an ophthalmic liquid gel (Tear-Gel®; Novartis Ophthalmic, Novartis Pharmaceuticals Inc., Canada), which was also used to prevent corneal dryness. Reference (E5 disc electrode; Grass Technologies, Quincy, MA, USA) and ground (E2 sub-dermal electrode; Grass Technologies, Quincy, MA, USA) electrodes were placed in the mouth and the tail, respectively. Twenty flashes of white light (flash duration: 20 μsec; inter-stimulus interval: 10 sec) at increasing intensities, starting from the lowest (- 6.3 $\log.cd.sec.m^{-2}$) to the brightest (0.9 $\log.cd.sec.m^{-2}$) were delivered to produce the scotopic ERGs luminance-response function. Following the scotopic ERG recordings, a background light of 30 $cd.m^{-2}$ was opened and, after 20 min of light adaptation, the photopic ERGs were recorded in response to a flash of 0.9 $\log.cd.sec.m^{-2}$ (average of 20 flashes with inter-stimulus interval 1 sec) in intensity.

3.5 Retinal histology

Following each recording session (P30, P60, P100 and P300) two to four male and female rats (per age groups) were euthanized with CO_2 asphyxiation and their retinas were collected for histology. Briefly, an orientation suture was placed on the nasal conjunctiva of both eyes then the eyes were enucleated and immersed for 3 hours in 3.5% glutaraldehyde for fixation. After the removal of the cornea and lens, the eyecups were placed in 3.5% glutaraldehyde overnight. On the following day, the eyes were immersed in a solution of 1%

osmium tetroxide (OsO_4) for 3 hours. Then, the eyes were sequentially dehydrated with 50, 85, 90, 95 and 100 % ethanol and propylene oxide, respectively. Finally, the samples were embedded in resin (Durcupan® ACM Fluka epoxy resin kit, Sigma-Aldrich, Canada) and kept in an oven at 55-60°C for 48 hours. The samples were cut along the vertical meridian of the eye passing through the optic nerve head (ONH) (Leica EM UC6 microtome, Leica microsystem, USA) in ultra-thin sections of 1.0 μm and stained with 0.1% toluidine blue. Retinal pictures were taken with a Zeiss microscope (Zeiss Axiophot, Zeiss microscope, Germany: 40X) equipped with a digital camera.

For each rat, the thickness of the entire retina as well as that of each retinal layer (i.e., RPE, OS/IS, ONL, OPL, INL, IPL, RGCL/FL) were measured on 6 different histological sections (taken at every 680 μm interval from the ONH) taken from the superior retina using the AxioVision® software (version 4.8.2.0; Carl Zeiss Microscopy GmbH, Jena, Germany). On each section thickness measurement was done at two to three randomly locations.

3.6 Data Analysis

ERG analysis was performed according to the standard practice (Marmor et al., 2009) as previously reported by us (Dembinska et al., 2002; Joly et al., 2006; Dorfman et al., 2008). The amplitude of the a-wave was measured from baseline to the most negative trough, while the amplitude of the b-wave was measured from the trough of the a-wave to the most positive peak of the retinal response. The maximal rod-mediated b-wave (rod V_{max}), representing the maximal function of the rods without the contribution of cones, was defined as the first ERG response (of the scotopic luminance response function) where a measurable a-wave was obtained. The mixed rod-cone a-wave and mixed rod-cone b-wave refer to scotopic ERGs evoked to the highest flash intensity available (0.9 log.cd.sec.m^{-2}). In order to investigate the possible age-related changes in

the retinal function over time, a maturation index of ERG parameters was calculated. This index represents the ratio between the amplitudes of the different ERG components measured at P60, P100, P200, P300 over those measured at P30 (i.e. P60/P30 x 100%). All values are represented as mean ± 1 standard deviation (SD). Two-way ANOVA followed by a post-hoc Tukey or Bonferroni test (for significant ANOVA results) was performed to evaluate the retinal function and structure of male and female rats at different ages. A $P < 0.05$ was considered as statistically significant. All statistical analyses were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA).

4. RESULTS

4.1 The effects of biological sex and age on retinal function

Figure 1 shows representative scotopic rod V_{max} responses (A), scotopic mixed rod-cone responses (B) and photopic cone responses (C) obtained from male and female rats at age P30, P60, P100, P200 and P300 (from left to right). Amplitude measurements of ERG parameters obtained from male and female rats at five different ages are reported in Table 1. Irrespective of age, no significant differences in amplitude and morphology were observed in ERG responses between males and females ($P > 0.05$). In both sexes, the scotopic and photopic ERGs were significantly attenuated at P60 compared to those at P30 ($P < 0.05$), whereas the amplitude drop measured for the rod V_{max} at P60 was not remarkable (Table 1).

Figure 2 shows the maturation index of the retinal function in male and female rats. Interestingly, the maturation indexes for ERG parameters in female rats gradually increased between P60 and P200, while those of males did not show this trend.

This is better exemplified at Figure 3 where the ERG responses of male and female rats measured at P200 were normalized to those obtained at P60. As shown at Figure 3, ERG responses in females increased noticeably between P60 and P200, while those of males decreased except for the cone-mediated ERG and rod V_{max} that showed small increases (Fig. 3C and 3D). At P200, the growth in amplitude of the rod-cone mediated ERG was significantly lower in male compared to female rats [scotopic a-wave: females, 116.41% vs. males, 85.55% ($P < 0.01$); scotopic b-wave: females, 125.58% vs. males, 91.58% ($P < 0.01$)] (Fig. 3A and 3B).

Afterwards, the retinal function in male and female rats decreased from P200 to P300. At P300, rod-cone mediated ERGs (scotopic ERGs) obtained from both sexes and the photopic ERGs of female rats were significantly attenuated compared to those obtained at P30 ($P < 0.05$) (Table 1).

4.2 The effect of estrus cycle on retinal function

Six 18 month-old female rats were categorized into premenopausal (N = 2) and menopausal (N = 4) groups according to their vaginal smears. Figure 4 shows representative vaginal smears of both groups. Estrus cycle phases were identified in premenopausal female rats, whereas no distinct phase could be observed in menopausal female rats. Representative scotopic and photopic ERG and rod V_{max} obtained from premenopausal and menopausal groups as well as from age-matched males are presented at Figure 5A. Group data are shown in Table 2.

Premenopausal females showed higher ERG responses compared to the menopausal females. The mixed rod-cone b-wave (scotopic b-wave) and rod V_{max} were significantly attenuated in the menopausal group compared to the premenopausal ($P = 0.0009$ and $P = 0.002$, respectively) (Fig. 5B). Interestingly, the retinal function of premenopausal females was better preserved compared to aged-matched males, while ERG responses in menopausal female rats

were of lower amplitude compared to those of age-matched males, suggesting an effect of estrus cycle on the retinal function of female rats.

4.3 The effects of biological sex and age on retinal structure

The maturation-induced changes in retinal function described earlier were also accompanied with changes in retinal structure. Representative retinal cross sections obtained at postnatal days 30, 60, 100, and 300 from male and female rats are shown in Figure 6. In both sexes the retinal thickness decreased with age. At P300, the retina of female rats was 20.14% thinner than P30 measures compared to 10.06% for males ($P > 0.05$).

As shown at Figure 7, the quantitative assessment of the different retinal layers did not evidence any significant difference in retinal layer thicknesses between male and female rats, irrespective of age. Interestingly, the ONL thickness in adult male and female rats aged P300 was significantly thinner than that measured at P30 [ONL thickness in male rats at P300 vs. P30: 29.003 ± 0.67 μm vs. 39.84 ± 3.07 μm ($P = 0.01$); ONL thickness in female rats at P300 vs. P30: 28.08 ± 1.98 μm vs. 43.48 ± 5.15 μm ($P = 0.001$)], suggesting that the photoreceptors were more vulnerable to the aging process. Moreover, of all the inner retinal layers, the RGCL/FL was that most affected by the aging process in females [RGCL/FL thickness in female rats at P300 vs. P30: 11.27 ± 3.9 vs. 20.02 ± 4.1 ($P = 0.02$)].

It is noteworthy that alterations in the thicknesses of the outer retinal layers (RPE, OS/IS, ONL and OPL) and INL showed nearly similar patterns in male and female rats over time. However, during the P100 to P300 period, the thicknesses of the IPL and RGCL/FL decreased more in female compared to male rats [IPL thickness at P300 vs. P100: male, 47.73 ± 9.4 μm vs. 52.21 ± 3.8 μm (8.59% decrease); female, 35.31 ± 7.4 μm vs. 48.92 ± 5.5 μm (27.83% decrease).

RGCL thickness at P300 vs. P100: male, 13.60 ± 3.2 μm vs. 14.42 ± 0.4 μm (5.69% decrease); female, 11.27 ± 3.9 μm vs. 16.69 ± 2.3 μm (32.49% decrease)]. (Fig. 7)

5. DISCUSSION

To our knowledge, this is the first study to explore sex-related differences in retinal function and structure during the normal aging process of albino SD rats. In this study, we showed that the retinal function of female rats was better preserved compared to age-matched male rats, particularly between ages P60 and P200. We believe that this difference might be explained by the positive effect of the estrus cycle on the retinal function, a claim also supported with our result showing ERG responses of larger amplitudes in premenopausal female rats compared to menopausal rats.

5.1 Age and sex-related changes in retinal function

In this study, we show that the normal aging process modulates the retinal function in both male and female SD rats, as shown with the attenuation of ERG amplitudes measured between P30 and P300 (Fig. 2). Age-dependent alterations in the ERG responses have been extensively investigated in both human and rat (Weleber, 1981; Birch and Anderson, 1992; DiLoreto et al., 1995). In human studies, it has been reported that the amplitudes of the ERG a- and b-waves decline with normal aging (Birch and Anderson, 1992), indicating age-dependent alterations in the outer and inner retinal function, respectively. However, the study of Freund et al. (Freund et al., 2011) on healthy subjects showed that despite a significant decrease in the amplitude of the dark adapted a-wave, the amplitude of the dark adapted b-wave did not decline with age resulting in an increased b/a wave ratio, indicating more vulnerability of the outer retinal function to the normal aging process. Several factors including subtle changes in ocular

media and/or reductions in photopigment contents (Birch and Anderson, 1992), or a gradual loss of retinal cells such as photoreceptor, bipolar or Müller cells in the aging retina (Dorey et al., 1989; Curcio et al., 1990) have been proposed as the main reasons for the age-dependent attenuation in ERG amplitudes.

Regardless of age, we did not observe any significant male-female differences in the electrical responses of the retina when female ERGs are compared with age-matched male controls. This finding is in agreement with the result of one animal study in which no significant differences were found in the photopic flash ERG obtained from male and female SD rats aged 9 to 12 weeks (Chen et al., 2010). However, while in male rats ERG amplitudes tended to remain stable between P60 and P200, in female rats there was an increase in all ERG parameters (Fig. 3). Of interest, this age episode (P60-P200) also corresponds to that where the frequency of estrus cycle starts to increase to reach maximum value at age P240 (Nelson et al., 1980), suggesting that the estrus cycle and accompanying circulating hormones, especially estrogen, might influence the retinal function in female rats. Given that the retina is a highly vascularized tissue, the discovery of estrogen receptors located in the different retinal layers (Kobayashi et al., 1998, Kumar et al., 2008) also supports the claim that the circulating estrogen may influence the retina. Moreover, it has been shown that the level of antioxidant enzymes such as catalase and superoxide dismutase (SOD) were remarkably increased in retinal tissues obtained from 6 months old albino Wistar rats (El-Sayyad et al., 2014) that is at an age where the estrus cycle frequency is nearly maximal (Nelson et al., 1980). Of interest, one study reported that estrogen enhanced the expression as well as the activity of extracellular SOD (ecSOD) and mitochondrial manganese SOD (mnSOD) by upregulating the transcriptional pathways (Strehlow et al., 2003). Regarding the modulatory effects of estrogen on the SOD level, our demonstration of a better

preservation of retinal function in female rats may have resulted from the gradual increase in the estrus cycle frequency and accompanying increase in the level of plasmatic estrogen, thus resulting in an increase in antioxidant activity within the retina, a tissue known to be highly susceptible to age-related oxidative damage (Rahman et al., 2012). Additionally, antioxidant properties of estrogen in neural tissue including retina (Prokai et al., 2003; Zhang et al., 2009; Giddabasappa et al., 2010) boost the antioxidative effects of SOD leading to better protection of retinal function against the gradual deterioration of functions due to age-associated oxidative insult.

The results obtained in the second part of our study confirm the impact of estrus cycle on the retinal function. It has been reported that the menopausal age of laboratory rats is between 15 and 18 month (Sengupta, 2013), accordingly, in the present study in order to determine the menstrual status of female rats at 18 months old, we studied the vaginal secretions of 18-month-old female rats collected on five consecutive days before ERG recording. As illustrated in this study (Fig. 5), the ERG amplitudes were larger in premenopausal compared to menopausal female SD rats and also larger than those obtained from age-matched male rats. In contrast, following menopause, the ERG responses in female rats declined noticeably compared to age-matched male rats. These findings suggest a beneficial effect of the estrus cycle and its related hormonal changes upon the retinal function that may explain the higher prevalence of age-related retinal degeneration in women after menopause (Snow et al., 2002). Of interest, previously reported preliminary data of ours showed that the ERG responses obtained from the female rats at proestrus phase [where the highest plasmatic levels of estrogen are measured (Marcondes et al., 2001)] and following estrus phase were larger compared to those recorded at metestrus and diestrus phases, suggesting that estrus cycle phases influenced the retinal function (Pollifrone et

al., 2004). Furthermore, the latter study also reported that β-estradiol injections to the ovariectomized SD rats increased ERG responses compared to the sham group, highlighting the neuromodulatory effect of estrogen on the retinal function.

Interestingly, it appears that the rod-mediated ERG is more influenced by the estrus cycle and accompanying hormonal fluctuations compared to the cone-mediated ERG. As shown in this study (Fig. 5B), in premenopausal rats the scotopic ERGs (rod V_{max} and mixed rod-cone responses) were significantly larger than those of menopausal rats, while the photopic ERG (representing cone function) did not show this remarkable discrepancy. This effect is also emphasized with the results at Figure 3 where we showed that in female rats the changes in rod V_{max} amplitudes measured between P60 and P200 were larger than those measured for photopic b-wave, suggesting a more important influence of the estrus cycle on rod function. Furthermore, at age P200, the difference between male and female responses was larger for scotopic rod V_{max} ERG compared to photopic ERG (Fig. 3), suggesting better preserved rod function in female rats.

5.2 Age and sex-related changes in retinal structure

Retinal histology obtained at P30, P60, P100 and P300 did not reveal significant differences in retinal layer thicknesses between male and female rats, irrespective of age. Interestingly, we found that the photoreceptor layer was the retinal structure most affected by the normal aging process of male and female rats. From a pathophysiological point of view, it is believed that age-associated photoreceptor loss might be attributable to age-dependent changes in the RPE (Katz and Robison, 1984; Katz and Robison, 1985) which has a crucial role in maintaining the cellular integrity of the photoreceptors (Zinn and Marmor, 1979). Any deterioration of the RPE function could affect the survival of retinal cells, including the

photoreceptors which are highly dependent upon the proper functioning of the RPE (Katz and Robison, 1986). Additionally, it has been hypothesized that the photoreceptor loss due to aging could be related to the damage induced by the excitatory amino acid, including glutamate (DiLoreto et al., 1995). In response to the light stimulus, the photoreceptors release glutamate (Kolb et al., 2009) which is then captured by the Müller cells. Aged Müller cells may not be as efficient to catch the released glutamate, therefore augmenting the extracellular levels of glutamate and, consequently, leading to a further increase in photoreceptor cell death at long term (DiLoreto et al., 1995). As illustrated in our study, except for the photoreceptor layer, the other layers of the outer retina (i.e.: RPE, outer and inner segments of the photoreceptors and OPL) of male and female rats did not change over time.

Our results suggest that biological sex did not affect noticeably the age-related loss of photoreceptors since both sexes showed a nearly similar decrement in ONL thickness with age. This contrasts with DiLoreto et al.'s study (DiLoreto et al., 1994) which showed that compared to male, the retina of female Fischer 344 (F344) rats presented with a delayed and less severe photoreceptor degeneration with age. Interestingly, in their study, the higher rate of retinal degeneration in the male F344 rats was not observed in age-matched male SD rats. This finding suggests a strain difference in the severity of age-dependent retinal degeneration and may explain why in our study, we found an almost equal rate of degeneration in male and female SD rats.

It has been shown that the aging phenomenon is associated with the loss of neurons in the inner retina, particularly at the level of the ganglion cell and nerve fiber layers (Parikh et al., 2007, Harwerth et al., 2008). In one study conducted by Sandalon et al. (Sandalon and Ofri, 2012), it was shown that the significant RGC loss was initiated between the age 4 and 12 months

in rats, a finding in accord with the results presented herein. We found that the thinning of the inner retinal layer (INL, IPL, and RGC/FL) over time was more pronounced in female rats particularly after 3 months of age (P100). Moreover, the sex-related differences in IPL and RGCL/FL thicknesses between P100 and P300 appeared to be mostly responsible for the sex-related difference measured for the entire retina. As shown in this study (Fig. 7), the age-related deterioration of RGCL/FL was significantly noticed in female rats at age 10 months compared to 1-month-old female rats, a finding that might be related to the gradual decrease in estrus cycle frequency following its peak at 8 months of age (Nelson et al., 1980). In a recent study it was shown that the ganglion cells and nerve fiber layers suffered more from the normal aging process in female albino Wistar rats compared to their male counterparts (El-Sayyad et al., 2014). Of interest, these results could be explained with the findings of Kobayashi et al. (Kobayashi et al., 1998) who showed an enhanced expression of estrogen receptors in the inner retinal layers including the nerve fiber layer, the ganglion cell layer, and the inner nuclear layer of 8-week-old Lewis rats. Although, the latter study did not report significant sexual differences in the distribution of estrogen receptor protein in the rat retina, it could be that the responsiveness of estrogen receptor differs between male and female.

6. CONCLUSION

In summary, our results demonstrate that biological sex and age can influence the retinal structure and function in albino SD rats. Of note, it seems that estrogen, the most important fluctuating hormone of the estrus cycle, modulates the retinal function of female rats. This claim is supported with the larger ERG amplitudes that we obtained from female rats, particularly between 2 and 6 month of age. In addition, this claim is further confirmed with our result

showing ERG responses of larger amplitudes in premenopausal female rats compared to menopausal rats.

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8. CAPTIONS TO FIGURES AND TABLES

Figure 1. Representative scotopic rod V_{max} **(A)** and mixed rod-cone **(B)** ERGs and photopic ERG **(C)** recorded from a male and a female rat aged 30, 60, 100, 200 and 300 days old (age identified as P at the top up each column). Vertical arrows correspond to the stimulus onset. Horizontal calibration: 40 msec. Vertical calibration: 200 μV. Abbreviations: a: a-wave; b: b-wave. F: female rat. M: male rat.

Figure 2. Maturation of retinal function in male and female rats (expressed of P30 value in %) at 30, 60, 100, 200 and 300 days of age. Data was normalized to P30 values. Values are given as mean ± SD.

Figure 3. Percent changes in retinal function of male and female rats between P60 and P200 as determined with the retinal maturation index. The retinal maturation index represents the average (percentage) of the ratios (P200/P60 x 100) for ERG parameters (scotopic a-wave, scotopic b-wave, photopic b-wave, rod V_{max}). ** $P < 0.01$ indicates significant difference between male and female rats at P200. Values are given as mean ± SD.

Figure 4. Representative examples of vaginal smears obtained from premenopausal (the upper images) and menopausal (the bottom images) female rats aged 18 months collected over five consecutive days. A typical estrus cycle (with the four consecutive phases: proestrus, estrus, metestrus, and diestrus) could be identified in premenopausal rats. Proestrus phase was identified with the presence of nucleated epithelial cells (solid line in image taken at day 4); Estrous phase was recognized with the presence of cornified epithelial cells (dashed line in image taken at day

1); the other two phases (Metestrus and Diestrus) were mainly distinguished with the leukocyte infiltration (images taken at day 2 and 3). No equivalent distinct estrus phase could be observed in menopausal group. The images were taken with a 40x objective.

Figure 5. (A) Representative scotopic (mixed rod-cone and rod V_{max}) and photopic ERGs recorded from a male rat (first column), premenopausal and menopausal female rats (second and third column, respectively) at age 18 months. **(B)** Comparison of ERG amplitudes (scotopic a-wave, scotopic b-wave, photopic b-wave, and rod V_{max}) measured from male rats, premenopausal and menopausal female rats. ** $P < 0.01$; *** $P < 0.001$ indicate significant differences between premenopausal and menopausal female rats. Values are given as mean ± SD. Vertical arrows correspond to the stimulus onset. Horizontal calibration: 40 msec. Vertical calibration: 200 μV. Abbreviations: a: a-wave; b: b-wave.

Figure 6. Representative retinal sections obtained at 30, 60, 100, and 300 days of age from female **(A)** and male adult SD rats **(B)**. Images were taken between 1020 μm and 1700 μm from the optic nerve head in the superior retina. Abbreviations: RPE: retinal pigmented epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGCL/FL: retinal ganglion cell layer/fiber layer. Calibration bar: 75μm.

Figure 7. Quantitative assessment of retinal structure in male and female rats. Retinal layer thicknesses in female and male rats (N = 2-4/age) were measured at 30, 60, 100, and 300 days of

age. * $P < 0.05$; ** $P < 0.01$ indicate significant differences between the thicknesses measured at P300 and those at P30. Values are given as mean ± SD.

Table 1. Group data reporting the average amplitudes (in μV) of the scotopic ERGs, the photopic b-wave and rod V_{max} in male and female rats obtained at 30, 60, 100, 200 and 300 days of age. The number of animals per each age is shown in each column. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate statistical difference between the amplitudes recorded at the specified age and those at P30. Values are given as mean ± SD.

Table 2. Group data reporting the average amplitudes (in μV) of the mixed rod-cone a-wave (scotopic a-wave), mixed rod-cone b-wave (scotopic b-wave), photopic cone b-wave, and rod V_{max} measured for male rats, premenopausal and menopausal female rats at age of 18 months. ** $P < 0.01$ and *** $P < 0.001$ indicate statistically difference between premenopausal and menopausal female rats. Values are given as mean ± SD.

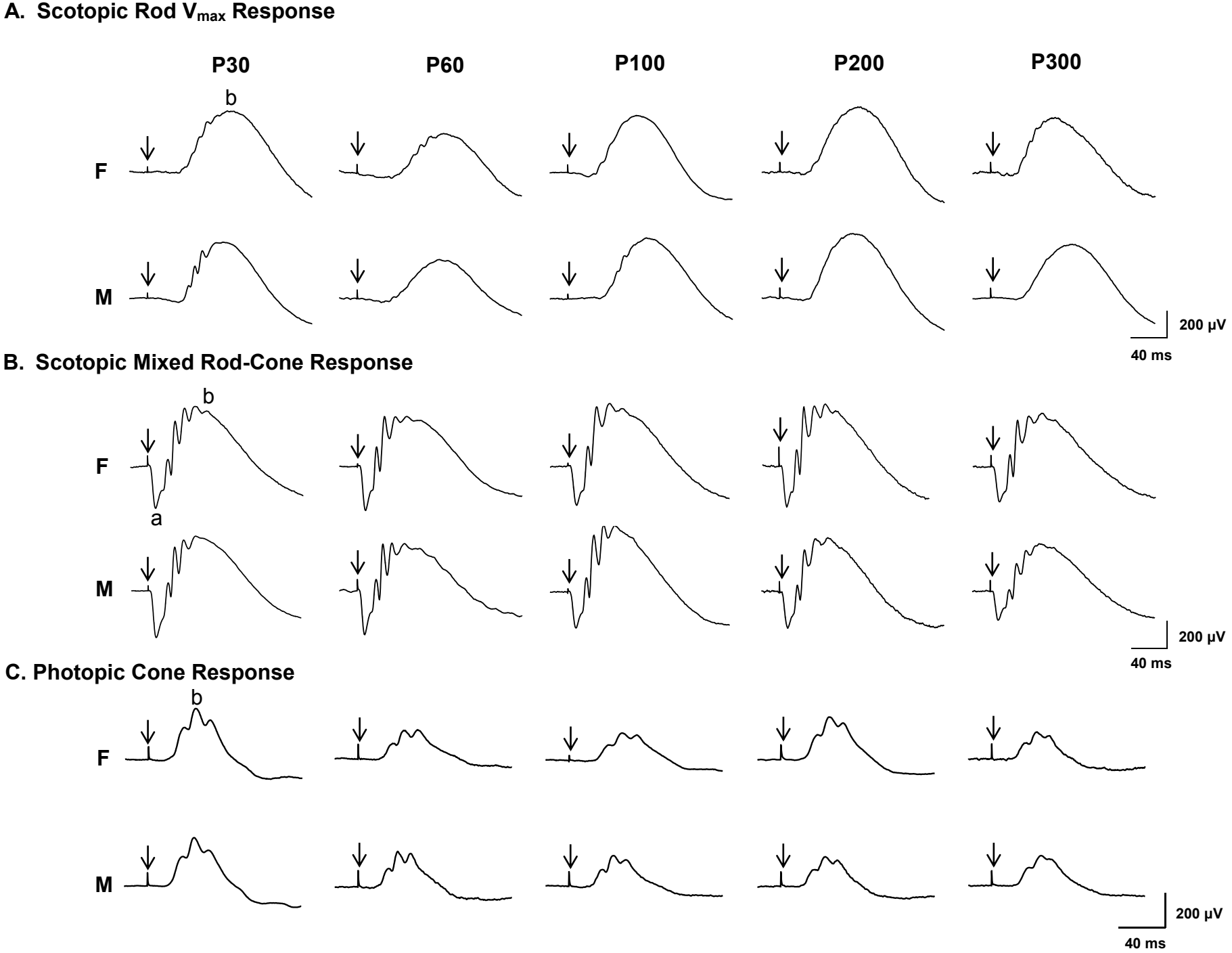
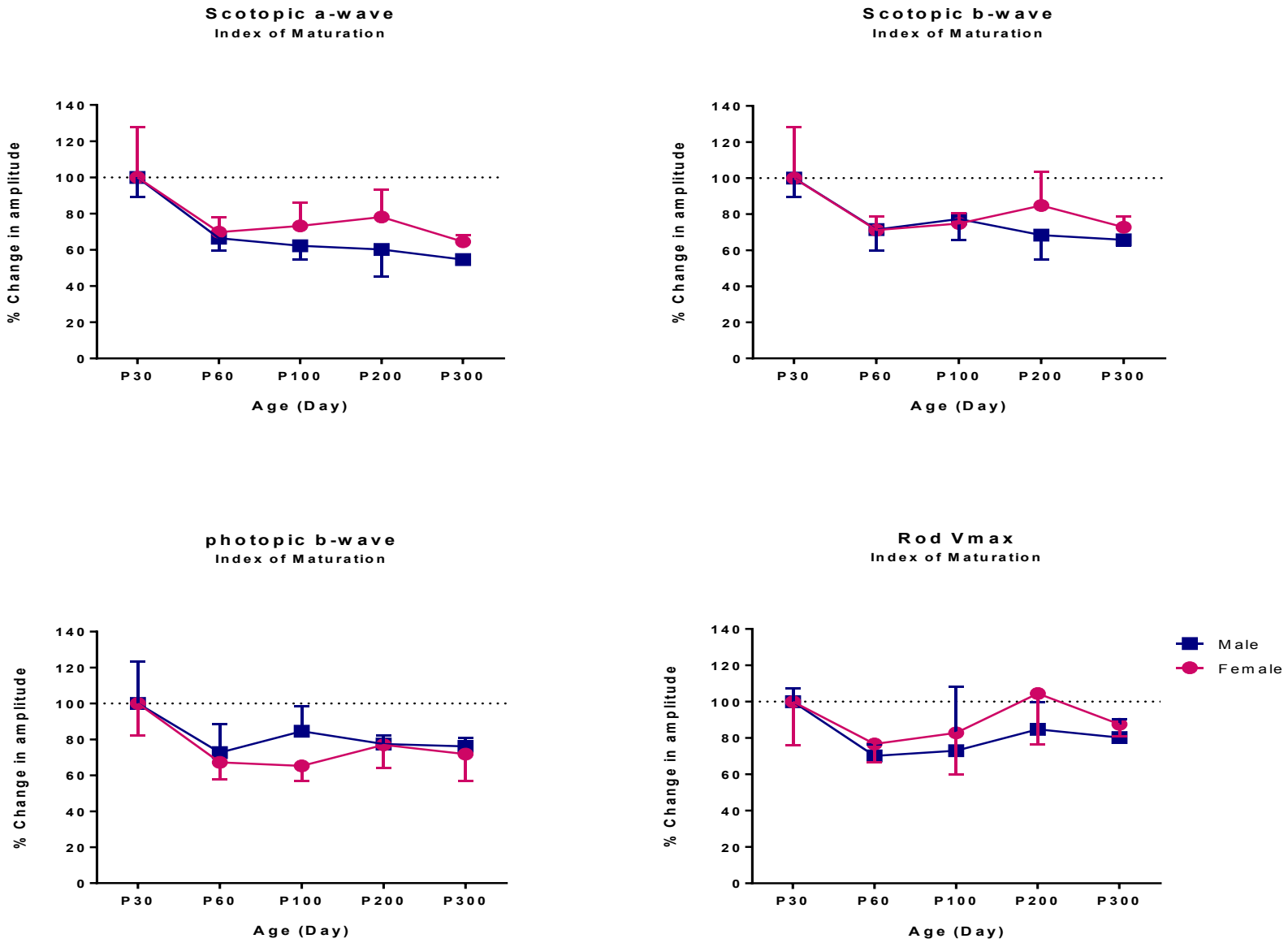

A. Scotopic Rod V_{max} Response
P30
P60
P100
P200
P300
b
F
M
200 µV
40 ms
B. Scotopic Mixed Rod-Cone Response
b
a
F
M
200 µV
40 ms
C. Photopic Cone Response
b
F
M
200 µV
40 ms

Figure 1



63

Figure 2

The number of animals: 2-7 rats per each sex at each age.
(The exact number of animals at each age is shown in Table 1.)

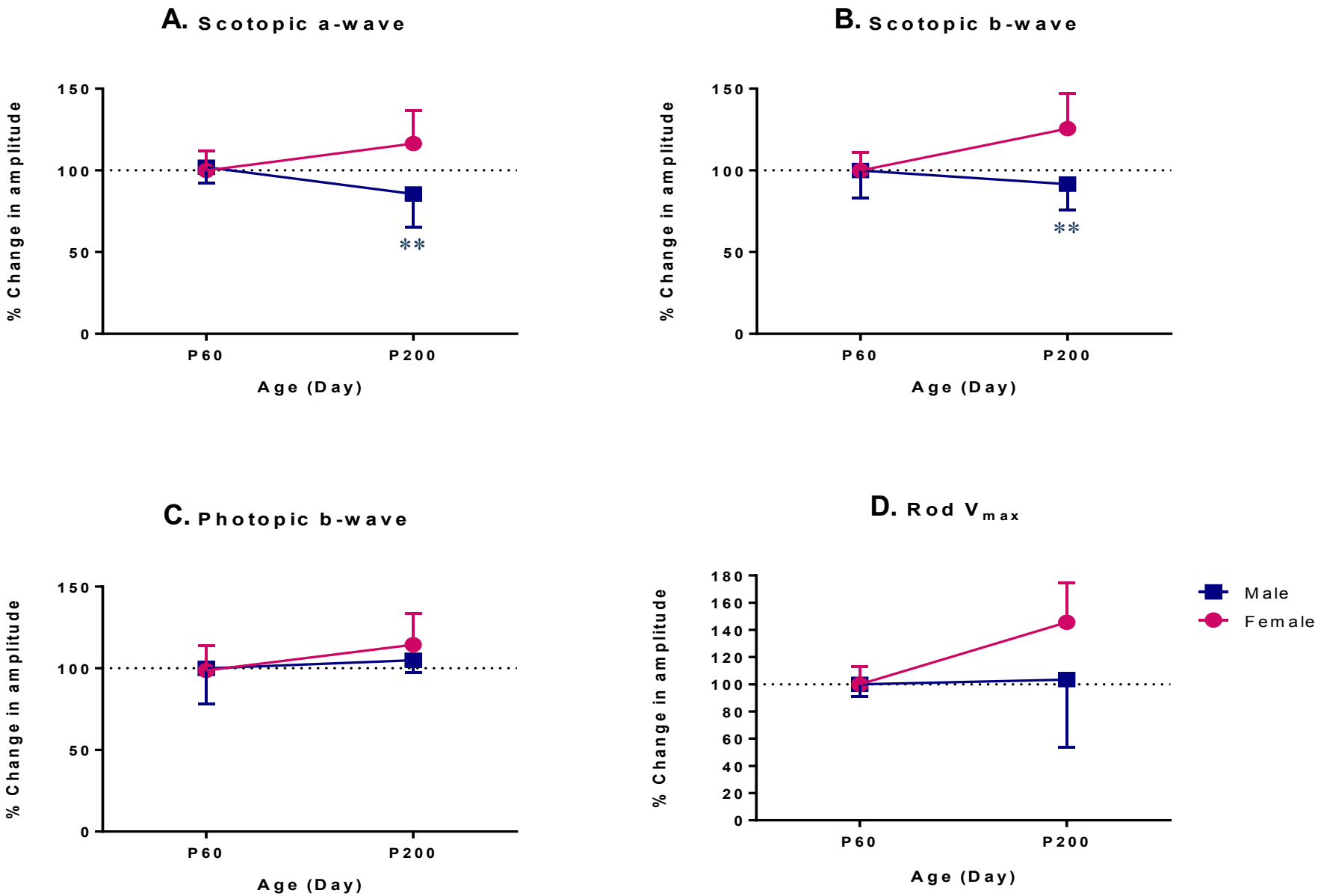

A. Scotopic a-wave
% Change in amplitude
150
100
50
0
**
P60
P200
Age (Day)
B. Scotopic b-wave
% Change in amplitude
150
100
50
0
**
P60
P200
Age (Day)
C. Photopic b-wave
% Change in amplitude
150
100
50
0
P60
P200
Age (Day)
D. Rod Vmax
% Change in amplitude
180
160
140
120
100
80
60
40
20
0
P60
P200
Age (Day)
Male
Female

Figure 3

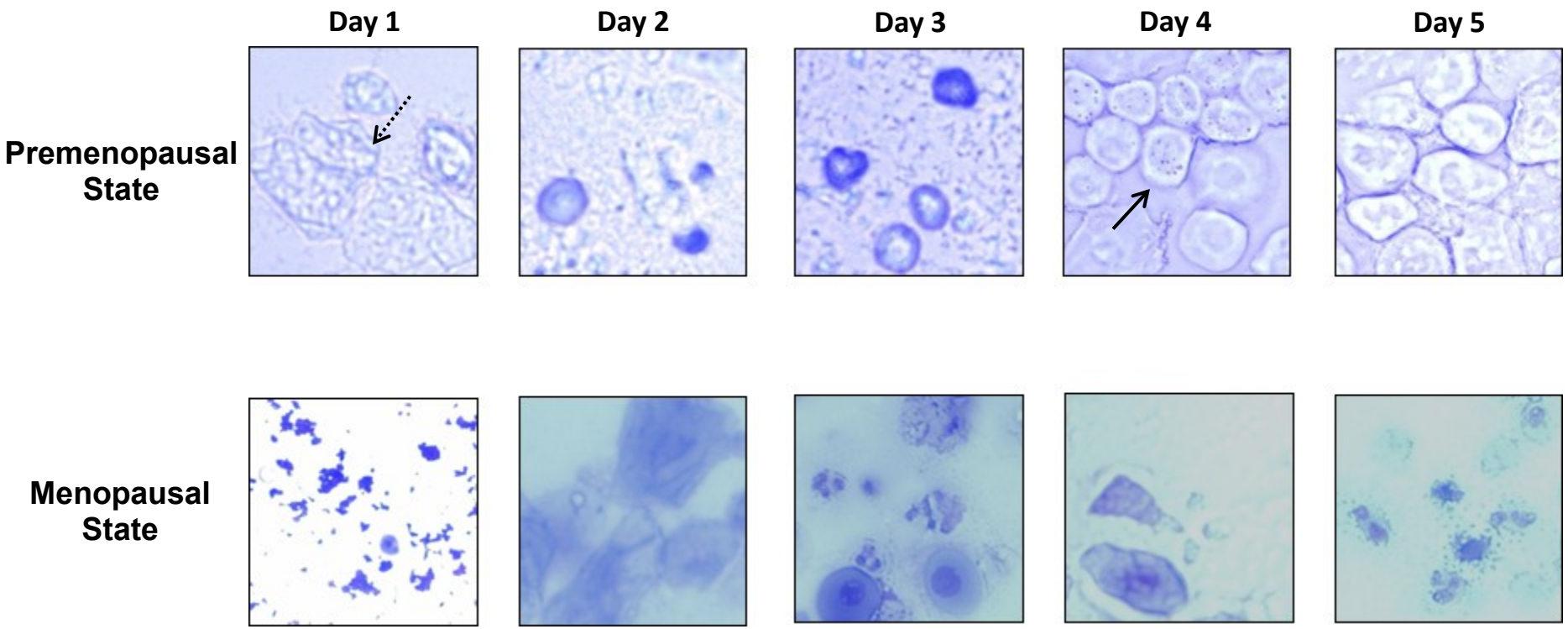

Day 1
Day 2
Day 3
Day 4
Day 5
Premenopausal State
Menopausal State

Figure 4

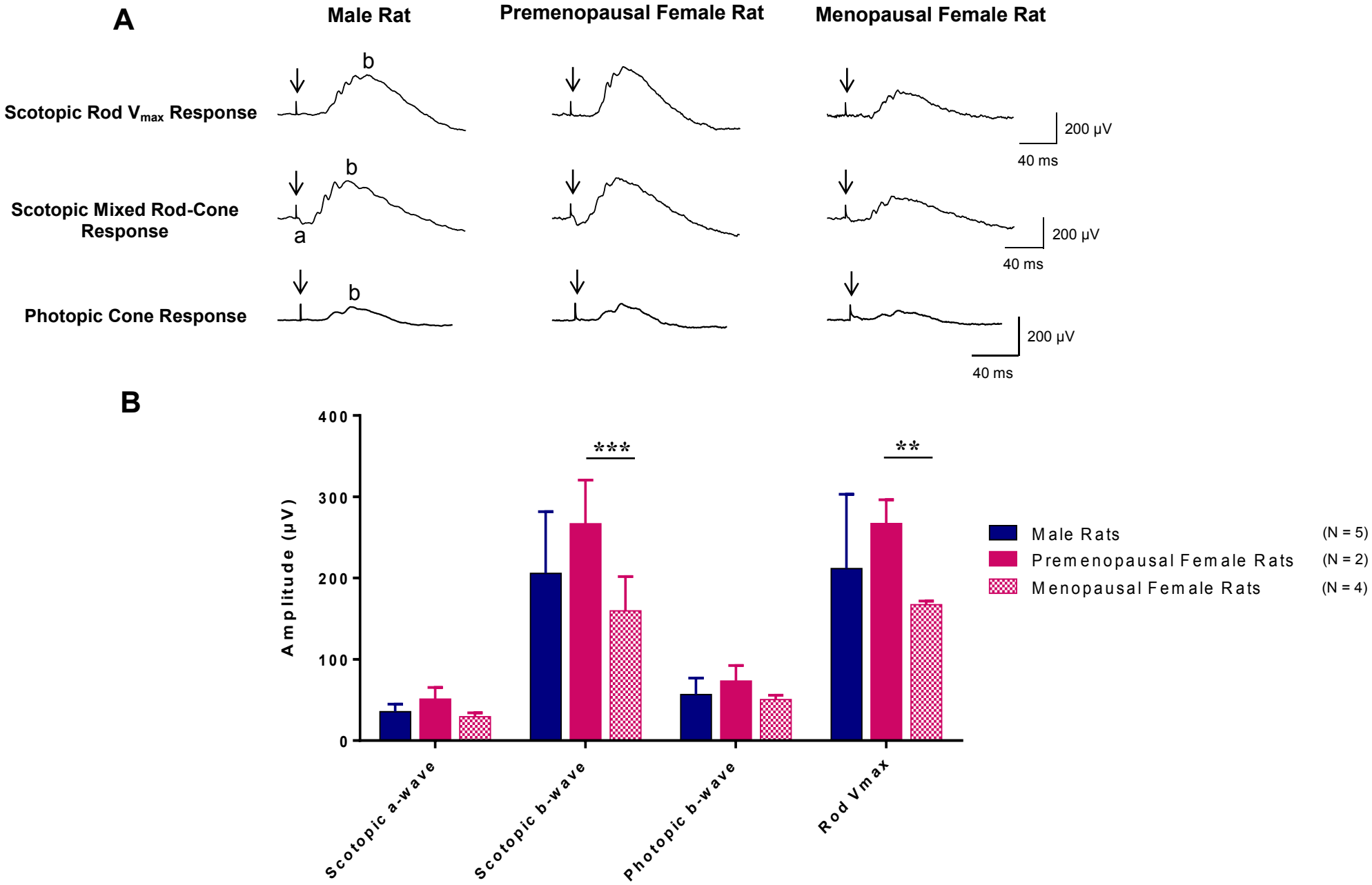

A
Male Rat
Premenopausal Female Rat
Menopausal Female Rat
Scotopic Rod V_{max} Response
Scotopic Mixed Rod-Cone Response
Photopic Cone Response
a
b
200 µV
40 ms
B
Amplitude (µV)
400
300
200
100
0
***
**
Scotopic a-wave
Scotopic b-wave
Photopic b-wave
Rod Vmax
Male Rats (N = 5)
Premenopausal Female Rats (N = 2)
Menopausal Female Rats (N = 4)

Figure 5

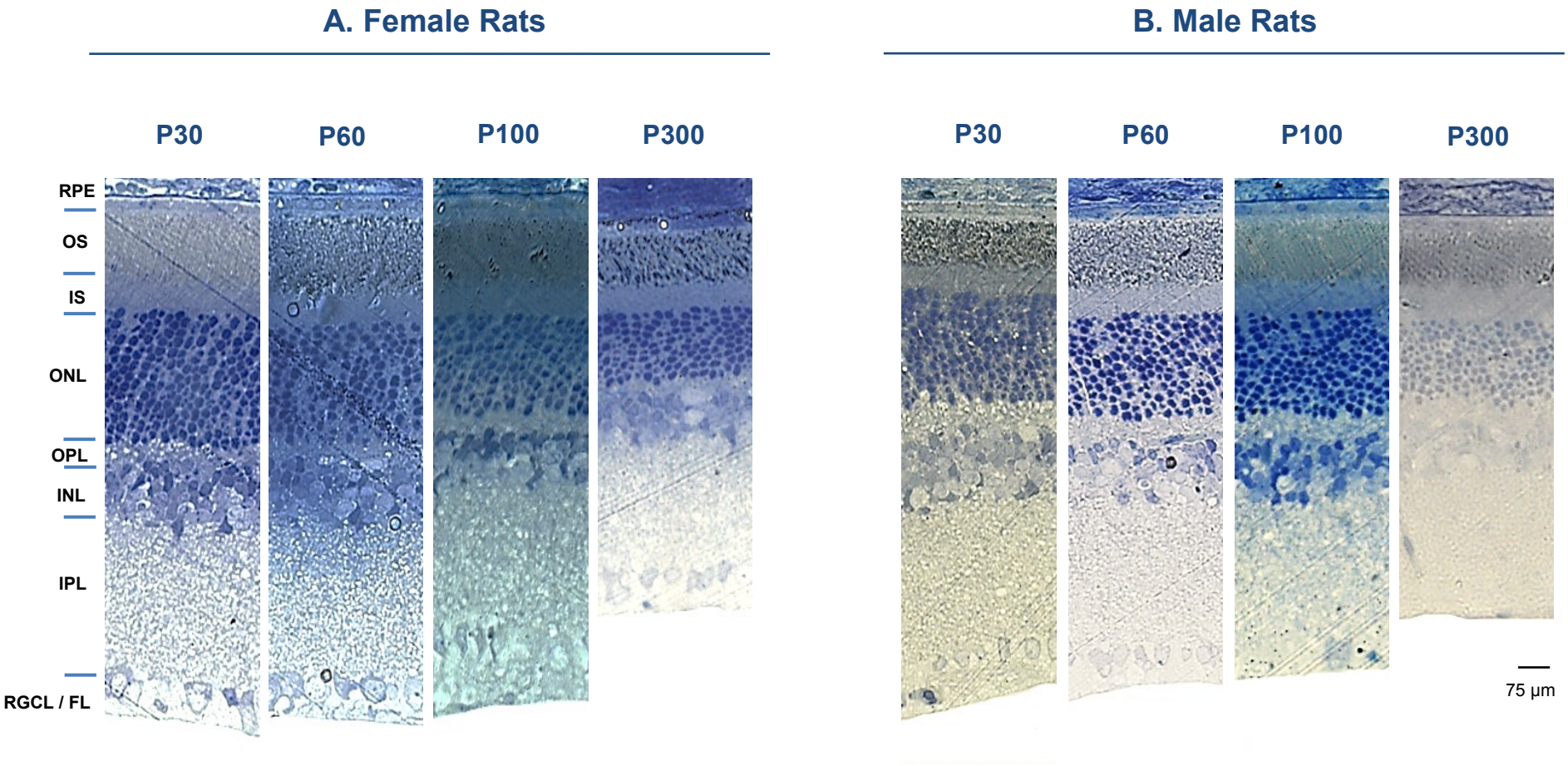

A. Female Rats
B. Male Rats
P30
P60
P100
P300
P30
P60
P100
P300
RPE
OS
IS
ONL
OPL
INL
IPL
RGCL / FL
75 µm

Figure 6

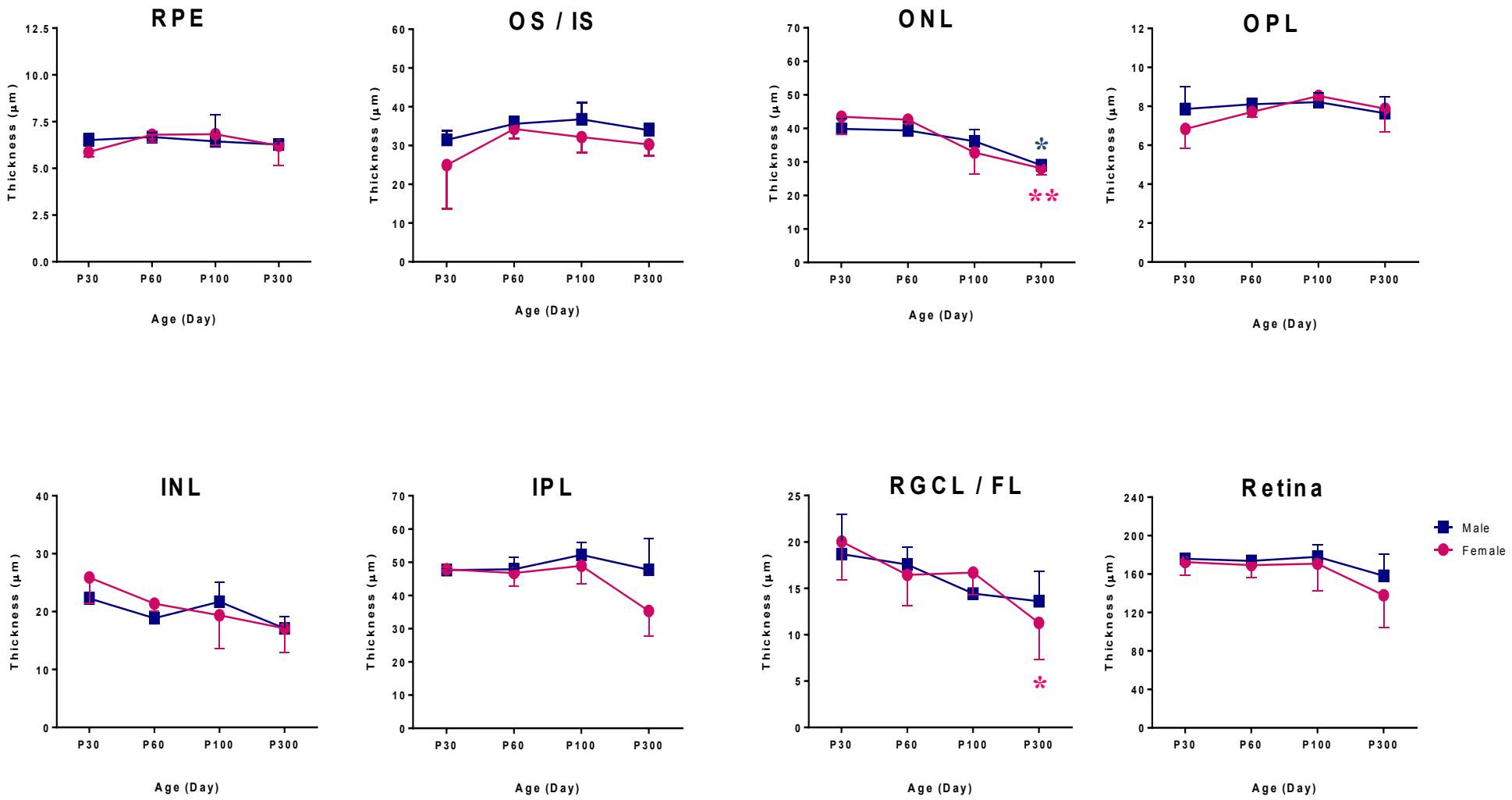


Figure 7

The number of animals: 2-4 rats per each sex at each age.

Table 1

Parameter	Gender	Age (Days)				
		30	60	100	200	300
Scotopic a-wave ± SD (µV)	F	440.176 ± 122.27 (N = 5)	307.026 ± 36.20 ** (N = 7)	322.326 ± 55.82 * (N = 5)	343.759 ± 67.40 (N = 7)	283.59 ± 16.61 ** (N = 5)
	M	488.977 ± 52.13 (N = 5)	324.89 ± 33.41 *** (N = 6)	304.300 ± 37.39 *** (N = 5)	294.463 ± 73.99 *** (N = 7)	266.941 ± 11.64 *** (N = 3)
Scotopic b-wave ± SD (µV)	F	971.910 ± 274.74 (N = 5)	691.912 ± 74.5 * (N = 7)	744.00 ± 61.47 (N = 5)	827.33 ± 177.84 (N = 7)	706.887 ± 57.89 * (N = 5)
	M	1075.498±111.75 (N = 5)	764.927 ± 128.17 ** (N = 6)	831.168 ± 126.55 (N = 5)	735.263 ± 144.24 ** (N = 7)	707.171 ± 32.71 ** (N = 3)
Photopic b-wave ± SD (µV)	F	254.042 ± 45.6 (N = 5)	170.904 ± 23.94 *** (N = 7)	165.924 ± 21.00 *** (N = 6)	195.660 ± 32.37 * (N = 7)	182.392 ± 38.14 ** (N = 5)
	M	225.158 ± 52.28 (N = 5)	163.722 ± 35.91 * (N = 6)	190.354 ± 31.67 (N = 5)	174.460 ± 10.81 (N = 7)	171.391 ± 10.35 (N = 3)
Rod V_{max} ± SD (µV)	F	550.58 ± 131.07 (N = 5)	422.267 ± 55.72 (N = 3)	456.100 ± 126.31 (N = 5)	575.343 ± 153.56 (N = 7)	481.840 ± 36.37 (N = 5)
	M	574.480 ± 41.15 (N = 5)	402.850 ± 36.92 (N = 3)	419.620 ± 202.26 (N = 5)	486.917 ± 85.43 (N = 6)	460.900 ± 59.11 (N = 2)

Table 2

	No. of Animals	Mixed Rod-Cone a-wave	Mixed Rod-Cone b-wave	Photopic Cone b-wave	Rod V_{max}
Male	N = 5	35.46 ± 9.4	205.75 ± 76.04	56.59 ± 20.2	211.54 ± 91.5
Premenopausal female	N = 2	51.14 ± 14.2	266.72 ± 53.85	73.3 ± 18.9	267.10 ± 29.1
Menopausal female	N = 4	29.36 ± 4.7	159.76 ± 41.9 ***	50.55 ± 5.2	167.3 ± 4.4 **

CHAPTER III

MANUSCRIPT II

Preface to chapter III

In the first study we noticed that biological sex could influence the retinal function and structure, possibly due to the modulatory effect of sexual hormones, including estrogen. Given the neuroprotective and antioxidant properties of estrogen on the retina, we conducted the second study to evaluate the protective effect of estrogen on the retinal structure and function in male newborn SD rats exposed to two oxidative conditions induced by hyperoxia and intense light.

Evaluating the neuroprotective effect of 17β-estradiol on the structure and function of the retina exposed to an intense oxidative stress

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1. ABSTRACT

Purpose: Apart from its major role in sexual differentiation, estrogen is also known to be a potent antioxidant agent that can protect the neural tissue against oxidative conditions. The discovery of estrogen receptors within the retina could suggest a possible protective effect of estrogen against oxidative retinopathies. The purpose of this study was to determine the effect of estrogen on the retinal function and structure in response to oxidative stresses resulting from hyperoxia or bright light exposure.

Methods: 32 male SD rat pups were exposed to hyperoxia (80 ± 5% O_2) from P8 to P14 (O_2E). In parallel, 32 male SD rat pups were exposed to an intense light (10,000 Lux) from P14 to P28 (LE). Control male pups were kept in normal air (21% O_2) and light (80 Lux). Either 17 β-Estradiol (βE_2) (4 and 12µg, IP, daily) in vehicle or vehicle alone were injected to exposed and control groups from P6-14 (O_2E) and P12-28 (LE). Retinal function was investigated with the ERG at P30 and P60, and retinal histology was performed at P60.

Results: In O_2E condition, at age P30, we found that the amplitude of ERG b-waves were significantly attenuated in hyperoxic rats treated with 12µg of βE_2 injection. At age P60, the ERG b-wave was also lower in O_2-exposed group treated with 12µg of βE_2 compared to control exposed animals. Retinal histology performed at age P60 showed a significantly thinner OPL (the most affected layer in oxygen-induced damage), in particular at the central region of the superior retina, in O_2-exposed rats treated with 12µg of βE_2 than control O_2-exposed group, indicating a detrimental effect of βE_2 on the retina exposed to postnatal hyperoxia. In LE condition there was no difference in ERG responses obtained from L-exposed rats (treated and untreated) at P30. However, we observed a protective effect of βE_2 injections (12µg) on the retinal function of L-exposed rats at P60. Retinal histology performed at P60 showed a

significantly thicker ONL (the most affected layer in light-induced damage), particularly at the central region of the superior retina, in L-exposed animals treated with 12µg of βE_2 compared to control exposed animals, suggesting a protective effect of βE_2 on the retina exposed to bright light.

Conclusion: Our results show that in condition where the primary target of the oxidative stress is the outer retina (i.e. the photoreceptors) estrogen can protect retina, while in situations where the inner retina (or retinal vasculature) is the main site of oxidative damage, estrogen may have a detrimental effect on the retina.

2. INTRODUCTION

Estrogen, a female sex steroid, plays an important role in physiological functions such as sex differentiation and maturation (Moosmann and Behl, 2002). Interestingly, the action of estrogen is not solely limited to the tissues which are involved in reproductive function. It also impacts on the development, growth, differentiation, maturation and function of different organs including the cardiovascular, gastrointestinal, and respiratory systems (Chen et al., 2009; Bendale et al., 2013), as well as the central and peripheral nervous system (Behl, 2002). Moreover, estrogen has been suggested to act as a neuroprotective factor that stabilizes neuronal function and, under certain conditions, can prevent neuronal death (Behl, 2002; Moosmann and Behl, 2002). It is suggested that its antioxidant activity would be exerted through suppressing free radical–induced peroxidation chain reactions and/or altering the activity of antioxidant enzymes (Persky et al., 2000; Wang et al., 2014). A neuroprotective effect of estrogen has been identified in neurodegenerative diseases such as Alzheimer's disease (Kimura, 1995) and Parkinson's disease (Sarkaki et al., 2008) as well as in various human and animal models of brain injury (Asl et al., 2013). One animal study showed reduced ischemia-induced damages in the cerebral cortices of ovariectomized female C57Bl/6J mice that had been treated with 17β-estradiol prior to being exposed to hypoxia (Dietrich et al., 2013). Interestingly, the discovery of estrogen receptors in different layers of the retina (Kobayashi et al., 1998) suggests a biological function for estrogen in this tissue.

Oxidative stress associated with high levels of free radical production has been proposed as an important factor in developing retinal injuries such as ROP and AMD (Beatty et al., 2000; Zhang et al., 2012). Supplemental oxygen therapy and the relative hypoxia that occurs upon return to normoxia are believed to be important risk factors involved in the severity and

progression of ROP (Sapieha et al., 2010). Similarly, it is suggested that excessive exposure to a bright luminous environment could be the major contributor to the development of AMD (Taylor et al., 1990). In both retinopathies, oxidative stress is considered as an important factor involved in creating retinal damage (Sapieha et al., 2010; Wang et al., 2014). Interestingly, it has been shown that estrogen, due to its antioxidant properties, could protect the retina against neurological damages induced by oxidative stress (Mo et al., 2013; Wang et al., 2014). The latter could explain sex-related differences in the incidence of retinal degenerations such as AMD (Smith et al., 1997; Friedman et al., 2004) and also highlight the role of hormone replacement therapy in decreasing the occurrence of AMD in menopausal women (Group 1992).

Given the antioxidant and free radical scavenging properties of estrogen, the purpose of this study was to elucidate the protective effects of 17β-estradiol (βE_2) in two animal models of oxidative stress-induced retinopathies namely oxygen-induced retinopathy (OIR) and light-induced retinopathy (LIR) that were generated by exposing male SD rat pups to 80 ± 5% oxygen or 10,000 lux light, respectively. Our results reveal that while βE_2 appears to protect the retina in the LIR model, its effect on the retinal structure and function would be detrimental in the OIR model.

3. METHODS

3.1 Animals

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and were approved by the McGill University-Montreal Children's Hospital animal care

committee. All experiments were conducted using only male rats as experimental subjects in order to avoid the confounding contribution of circulating estrogen in female rats as they age.

3.2 Oxygen-Induced Retinopathy (OIR) model

The OIR model was induced as previously described (Dembinska et al., 2002; Dorfman et al., 2008). Briefly, from P8 to P14 newborn male SD rats (Charles River Laboratories, St-Constant, St-Constant, Québec, Canada) along with their mothers were exposed daily to 80 ± 5% oxygen [measured with an oxygen meter ($MaxO_2$ Ceramatec, model OM25-ME; Medicana Inc., Montréal, Québec, Canada)] for a total of 22.5 hours that were interrupted by three 0.5-hour periods of normoxia (room air: 21% oxygen), following which they were returned to normoxia (normal animal care facility condition). In order to protect the mothers from pulmonary complications known to occur in adult rats raised in a hyperoxic environment, every 24 hours they were replaced with surrogate mothers belonging to the normoxia groups. The normoxia groups were kept in normal animal care facility conditions.

From P6 to P14, the pups received daily intraperitoneal injections of either 4µg or 12µg βE_2 (Sigma-Aldrich, MO, USA) dissolved in a vehicle [a solution of ethanol and phosphate-buffered saline (PBS)] in a volume of 0.05cc and 0.15cc, respectively. Maximal βE_2 dose selection was based on Barone et al. study (Barone et al., 2009). A total of 64 newborn male SD rats were randomly assigned to one of the following experimental groups: room air control (N = 8), room air treated with vehicle injections (N = 8), room air treated with 4µg βE_2 injections (N = 8), room air treated with 12µg βE_2 injections (N = 8), hyperoxia control (N = 8), hyperoxia treated with vehicle injections (N = 8), hyperoxia treated with 4µg βE_2 injections (N = 8), and hyperoxia treated with 12µg βE_2 injections (N = 8). The retinal function was investigated with the ERG at age P30 and P60, and retinal structure was assessed with histology at age P60.

3.3 Light-Induced Retinopathy (LIR) model

The LIR model was induced by exposing the male SD rat pups along with their mothers to a bright luminous environment of 10,000 Lux (12hr bright light/12hr dark) from P14 (the time of eyelid opening) to P28, following which they were returned to the normal condition of the animal care facility (80 Lux; 12hr ON/12hr OFF). The light intensity was adjusted with an IL 1700 Research light meter, the probe of which was placed at the bottom of the cage. The light exposure set-up consisted of 12 neon lights (Sylvania, 34W, cool-white neon light, Mississauga, ON, Canada) overlaying two lateral side and top of each cage (4 per each side). Control animals were kept in the normal lighting condition of the animal care facility as described above.

Daily intraperitoneal injections of either βE_2 in vehicle or vehicle alone were administered from P12 to P28. A total of 56 male SD rat pups were randomly assigned to one of the following experimental groups: normal lighting condition treated with vehicle injections (N = 8), normal lighting condition treated with 4μg βE_2 injections (N = 8), normal lighting condition treated with 12μg βE_2 injections (N = 8), control light-exposed rats (N = 8), light-exposed treated with vehicle injections (N = 8), light-exposed treated with 4μg βE_2 injections (N = 8), and light-exposed treated with 12μg βE_2 injections (N = 8). Of note, the same control group [i.e. exposed to normal room air (21% oxygen) and room light (80 Lux)] was used for both oxidative models (OIR and LIR). The retinal function was investigated with the ERG at age P30 and P60, while histological analysis of the retinal structure was conducted at age P60.

3.4 Electroretinography

ERGs were recorded with the Acqknowledge® data acquisition system (Biopac MP100; Biopac System Inc., Goleta, CA, USA). Following a 12 hour period of dark adaptation, the animals were anaesthetized, under a dim red light illumination, with an intramuscular injection

of ketamine (85 mg/kg) and xylazine (5 mg/kg). The pupils were maximally dilated with one drop of Mydriacyl 1%, and the cornea anesthetized with a drop of Alcaine 0.5%. The animals were then laid on their right side in a recording box of our design (Lachapelle and Blain, 1990) which included the rod desensitizing background light and the photo-stimulator (model PS 22, Grass Instrument, Quincy, MA). The retinal potentials were captured at the cornea with a DTL fiber electrode (27/7 X-Static® silver coated conductive nylon yarn: Sauquoit Industries, Scranton, PA, USA) acting as the active electrode (Dawson et al., 1979). The latter was maintained in place on the cornea with an ophthalmic liquid gel (Tear-Gel®; Novartis Ophthalmic, Novartis Pharmaceuticals Inc., Canada), that was also used to prevent corneal desiccation. Reference (E5 disc electrode; Grass Technologies, Quincy, MA, USA) and ground (E2 sub-dermal electrode; Grass Technologies, Quincy, MA, USA) electrodes were placed in the mouth and the tail, respectively. A scotopic luminance-response function was then generated using twenty different flash intensities (flash duration: 20 μsec; inter-stimulus interval: 10 sec; average of 5 flashes of white light per intensities) starting from the dimmest (-6.3 log.cd.sec.m^{-2}) to the brightest (0.9 log.cd.sec.m^{-2}) stimuli, the latter response will be referred to as the scotopic mixed rod-cone ERG. Following the recording of the scotopic ERGs, a background light of 30 cd.m^{-2} was opened and after 20 minutes of light adaptation, the photopic ERGs were recorded in response to a flash intensity of 0.9 log.cd.sec.m^{-2} (average of 20 flashes of white light with inter-stimulus interval of 1sec).

3.5 Retinal histology

Following the P60 ERG recording session, two to five animals from each of the experimental groups were euthanized with CO_2 asphyxiation and their retinas were collected for histology. Prior to enucleating, an orientation suture was placed on the nasal conjunctiva of both

eyes. The eyes were then enucleated and immersed for 3 hours in 3.5% glutaraldehyde for fixation. After the removal of the cornea and lens, the eyecups were returned in 3.5% glutaraldehyde overnight. On the following day, the eyes were immersed in a solution of 1% osmium tetroxide (OsO_4) for 3 hours, following which they were sequentially dehydrated in 50, 85, 90, 95 and 100 % ethanol and propylene oxide bathes, respectively. Finally, the samples were embedded in resin (Durcupan® ACM Fluka epoxy resin kit, Sigma-Aldrich, Canada) and kept in an oven at 55-60°C for 48 hours. The samples were cut along the vertical meridian of the eye passing through the ONH (Leica EM UC6 microtome, Leica microsystem, USA) in ultra-thin sections of 1.0 μm and then stained with 0.1% toluidine blue. Retinal pictures were taken with a Zeiss microscope (Zeiss Axiophot, Zeiss microscope, Germany: 40X) equipped with a digital camera. The thickness of the entire retina as well as that of each retinal layers (i.e., ONL, OPL, INL, IPL, RGC/FL) were measured using the AxioVison® software (Version 4.8.2.0; Carl Zeiss Microscopy GmbH, Jena, Germany).

The superior retina was reconstructed by cropping retinal sections of 75 μm in width taken at every 340 μm segment along the entire length of the superior retina (i.e. from the ONH towards the ora serrata) using Adobe Photoshop® (Adobe System Inc.). These sections were aligned, side by side, to represent the entire superior retina. The retinal reconstructions shown in this study were limited to the superior retina only, as this is the part of the retina that was previously shown to be the most susceptible to light damage in albino rats (Rapp et al., 1985; Organisciak and Vaughan, 2010). The same region of the retina was also evaluated in the OIR model.

The average thicknesses of the OPL (the most affected layer in OIR) and the ONL (the most affected layer in LIR) were measured in central and peripheral regions of the superior retina

in order to study the distribution of damage within these layers throughout the superior retina. Retinal sections comprised within a radius of 2040 μm from the ONH were considered as the central retina, while those outside of this radius to the ora serrata represented the peripheral retina.

3.6 Data Analysis

ERG analysis was performed according to the standard practice (Marmor et al., 2009) as previously reported by us (Dembinska et al., 2002; Dorfman et al., 2008). The amplitude of the a-wave was measured from baseline to the most negative trough, while the amplitude of the b-wave was measured from the trough of the a-wave to the most positive peak of the retinal response. In the absence of an a-wave, the amplitude of the b-wave was measured from the pre-stimulus baseline to the most positive peak of the retinal response. In both models the maturation indexes of the different ERG parameters were determined for control and exposed groups. This index represents the ratio between the amplitudes of the different ERG components measured at P60 over those measured at P30 (i.e. P60/P30 x 100%). All values are represented as mean ± 1 standard deviation (SD). Two-way ANOVA followed by a post-hoc Tukey or Bonferroni test (for significant ANOVA results) was performed to evaluate the efficacy of βE_2 treatments. A $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA).

4. RESULTS

4.1 The effect of postnatal injection of βE_2 on the normal retina

As exemplified from the results shown at Figures 2 and 8, βE_2 (or vehicle) treatment had no significant effect on the amplitude of the ERG response recorded from control (unexposed)

rats at P30 or at P60, Thus irrespective of age, or treatment groups, we could not find any significant effect of βE_2 injection on the retinal function of normal rats.

4.2 The effect of postnatal injection of βE_2 on the retinal function in OIR model

Figure 1 shows representative scotopic (top 2 rows) and photopic (bottom 2 rows) ERGs obtained from rats exposed to room air (rows 1 and 3) or hyperoxia (rows 2 and 4). Representative ERGs were obtained from untreated control rat (first column) as well as from rats treated with either the vehicle (second column) or with 4μg (third column) or 12μg (fourth column) of βE_2 at 30 days (Fig. 1A) or 60 days of age (Fig. 1B). As illustrated, irrespective of age, the amplitude of the ERGs obtained from hyperoxic rats was remarkably reduced compared to age-matched controls. This reduction in ERG amplitude was further enhanced in hyperoxic animals treated with 12μg of βE_2, an effect most prominent in ERGs obtained at P60 (fourth column of Fig. 1B). Of interest, at P30, ERGs of hyperoxic animals that were treated with the vehicle (second column of Fig. 1A) had smaller b-waves compared to the untreated hyperoxic rats. The above findings are best exemplified with the group data reported in Figure 2. The amplitude of the scotopic and photopic b-waves were significantly attenuated in hyperoxic rats (treated or not) compared to their age-matched counterparts ($P < 0.001$).

At P30 (Fig. 2A), scotopic b-waves were significantly attenuated in exposed animals treated with 12μg of βE_2 compared to the untreated exposed rats ($P < 0.05$), while photopic b-waves were remarkably attenuated in exposed groups treated with either 4μg or 12μg of βE_2 ($P < 0.05$), suggesting a detrimental effect of βE_2 on inner retinal function as assessed with the ERG b-wave. Surprisingly, we also observed that the function of the inner retina of hyperoxic animals (i.e. scotopic and photopic b-waves) was markedly reduced by the vehicle compared with the

untreated hyperoxic control [scotopic b-wave: 510.63 ± 81.1 vs. 684.34 ± 83.1 ($P > 0.05$); photopic b-wave: 85.014 ± 27 vs. 172.45 ± 28.2 ($P < 0.01$)].

At P60 (Fig. 2B), the scotopic and photopic b-waves recorded from hyperoxic rats treated with 12μg βE_2 were slightly (but not significantly) smaller than those obtained from hyperoxic control rats. The outer retinal function, represented with scotopic a-wave, was significantly attenuated in hyperoxic rats treated with 4μg βE_2 compared to those treated with 12μg βE_2 ($P < 0.05$).

Figure 3 shows maturation indexes of ERG parameters measured from control and hyperoxic animals. As illustrated, the ERG responses obtained from control and exposed animals at P60 were of smaller amplitudes compared to those recorded at P30. The attenuation of the scotopic ERG of control animals that received treatment was significantly less compared to that of untreated control animals ($P < 0.05$) (Fig. 3A), while the photopic ERG did not show this difference (Fig. 3C). It appears that the outer retinal function in hyperoxic condition was significantly preserved with the highest dose of βE_2 (Fig. 3A), while the inner retinal function in photopic condition was significantly attenuated in hyperoxic animals treated with 12μg of βE_2 (Fig. 3C), suggesting opposing effects of βE_2 on the outer and inner retinal functions and also on the rod and cone functions.

4.3 The effect of postnatal injection of βE_2 on the retinal structure in OIR model

Hyperoxia-induced alterations in retinal function were also accompanied with changes in the retinal cytoarchitecture. Figure 4 shows representative histological sections of the superior retina obtained from control and oxygen-exposed (treated or not with 12μg of βE_2) rats at age P60. We have previously demonstrated that the OPL is the retinal layer that is most damaged following postnatal hyperoxia (Lachapelle et al., 1999; Dembinska et al., 2001; Dembinska et al.,

2002; Dorfman et al., 2008; Dorfman et al., 2009). As shown at Figure 5, the average thicknesses of the retinal layers confirm this finding. The OPL thickness was significantly reduced in the hyperoxic groups compared to the normoxic control group ($P < 0.05$) and even more in hyperoxic rats that had been treated with 12μg of βE_2. As further exemplified at Figure 6, the thinning of the OPL mostly affected the central retina, while the OPL of the peripheral retina was of control thickness. Interestingly, in the treated hyperoxic rats, the OPL was thinnest in the central retina compared to the peripheral retina ($P < 0.001$), suggesting that the effect of βE_2 injection was most detrimental to the OPL found in the central part of the superior retina.

4.4 The effect of postnatal injection of βE_2 on the retinal function in LIR model

Figure 7 shows representative scotopic (top 2 rows) and photopic (bottom 2 rows) ERGs obtained from rats exposed to room light (rows 1 and 3) or bright light (rows 2 and 4). Representative ERGs were obtained from untreated control rat (first column) as well as from rats treated with either the vehicle (second column) or with 4μg (third column) or 12μg (fourth column) of βE_2 at 30 days (Fig. 7A) or 60 days of age (Fig. 7B). As exemplified at Figure 7A, ERG responses obtained from the light exposed rats were severely attenuated thus making it difficult to identify an effect of treatment. However, results obtained at age P60 (Fig. 7B) suggests a protective effect of βE_2 treatment (12μg dose) compared to the untreated light exposed rats. At both ages, the a-wave measured in light exposed rats was remarkably reduced compared to that of controls, exemplifying the destructive effect that bright light exposure exerted on the photoreceptors. At P30, the b-wave measured from light exposed rats was also markedly smaller than that of controls. Of interest, at P60, the b-wave obtained from the light exposed rats treated with 12μg of βE_2 (fourth column of Fig. 7B) was nearly similar to that of controls, whereas the other light exposed rats disclosed remarkably smaller b-waves compared to controls.

Group data obtained at P30 and P60 are graphically reported in Figure 8. At P30, the amplitude of the mixed rod-cone ERGs and cone b-waves were, as expected, significantly attenuated in light exposed rats compared to control rats ($P < 0.001$) (Fig. 8A). At this age, we could not demonstrate any significant protective effect of βE_2 treatment on the retinal function of light exposed animals compared to the control.

At P60, the amplitude of the scotopic a-wave measured from light exposed rats was significantly reduced compared to control rats ($P < 0.001$). The ERG responses obtained from the light exposed rats treated with 12μg of βE_2 were of larger amplitude than those recorded from the untreated exposed rats. Of note, light exposed rats treated with the highest dose of βE_2 did not show the expected amplitude reduction of the ERG b-waves compared to control groups, while the other light exposed groups disclosed significant differences ($P < 0.05$) (Fig. 8B), suggesting a protective effect of βE_2 in LIR model.

Figure 9 represents maturation indexes of ERG parameters measured for control and light exposed animals. As shown, the maturation indexes obtained from control rats were significantly smaller compared to the light exposed rats, indicating improvement of retinal function in exposed rats over time. Of interest, the retinal function of exposed rats treated with 12μg of βE_2 was significantly preserved compared to that of exposed rats treated with 4μg of βE_2, suggesting a dose dependent effect of βE_2 in protecting the retina against light damage.

4.5 The effect of postnatal injection of βE_2 on the retinal structure in LIR model

Figure 10 shows representative retinal sections taken from the superior retina of control and light exposed rats (with and without 12μg of βE_2) at age P60. As shown in Figure 10A, the ONL of a normal two-month-old rat is made of densely packed photoreceptor nuclei. In comparison, retinas taken from light exposed rats revealed a less densely packed ONL (Fig. 10B

and 10C), as previously demonstrated by us (Joly et al., 2006a; Joly et al., 2006b). This is best evidenced with the data shown at Figure 11 where the average thicknesses of the retinal layers measured in control, treated and untreated light exposed rats are compared. As expected the ONL was significantly thinner in light exposed animals compared to controls ($P < 0.001$). The treated group did however yield a slightly thicker ONL. The ONL of untreated exposed rats, which was thinner in the central region of the superior retina (Fig. 10B), was significantly preserved in the βE_2-treated group (Fig. 10C). The latter is further confirmed with the group data analysis shown in Figure 12. As shown, the ONL was significantly thinner at the center and periphery of the superior retina in light exposed groups compared to the control. Although in light exposed rats, the light-induced photoreceptor degeneration was most prominent in the central retina, it was significantly ($P < 0.05$) less pronounced in rats treated with βE_2, suggesting a protective effect of estrogen against light damage (Fig. 11).

5. DISCUSSION

Exposure of neonatal rats to hyperoxia or bright light causes an oxidative stress to the retina which leads to either oxygen-induced retinopathy (OIR) or light-induced retinopathy (LIR), respectively. These oxidative retinopathies are claimed to be valuable animal models of human retinal diseases known as ROP and AMD, respectively. Given the antioxidant and free radical scavenging properties of estrogen, the present study aimed at evaluating the protective effect of 17β-estradiol (βE_2) on the retinal function and structure of male SD rat pups exposed to hyperoxia or bright light within the first month of life. Our results show that, although βE_2 injection could protect the retinal structure and function against the light-induced oxidative stress, it had a detrimental effect on the hyperoxic retina.

5.1 The effect of postnatal injection of βE_2 on the normal retina

In the present study, irrespective of the duration/dose of βE_2 treatment or age, we did not observe any significant effect of estrogen on the retinal normal function, suggesting that βE_2 does not in normal condition effect the normal functioning of the rat retina. In our study, βE_2 treatment was administered to male pups. Although no significant difference was reported in the distribution of estrogen receptors in the retina of male and female rats (Kobayashi et al., 1998), responsiveness of estrogen receptors might be different in male rats compared to female rats. Of interest, in our OIR model, the dark-adapted ERGs of control rats treated with βE_2, was remarkably preserved over time (Fig. 3A and 3B), a finding that could not be evidenced in the control rats of the LIR group. This could be explained by the different treatment duration in the two models where the longer duration of injection in the LIR model may have affected the retinal function at long term.

5.2 The effects of postnatal injection of βE_2 on the retinal function and structure in OIR model

Although several studies (in vivo and in vitro) reported a protective effect of estrogen on the retinal vasculature subjected to a hyperoxic stress (Miyamoto et al., 2002; Zhang et al., 2010; Zhang et al., 2012), none reported its effect on the retinal function and structure subjected to a similar oxidative stress. Results presented in this study indicate that intraperitoneal injections of βE_2 to rat pups did not protect the retinal function and structure against the oxidative stress generated with our OIR model. In fact βE_2 was found to be detrimental to the retina of animals exposed to hyperoxia. The deleterious effect of βE_2 on the retina of rats raised in a hyperoxic milieu might be explained by its vaso-active properties. It is well documented that estrogen is a potent vasodilator, thus causing an increase in blood flow within various organs and providing

protection against ischemic events such as ischemic cerebrovascular injuries (Finucane et al., 1993), cardiovascular diseases (Barrett-Connor and Bush 1991; Thompson et al., 2000) as well as central retinal vein occlusion (Group, 1996). From a physiological point of view, it was previously shown that estrogen augments the expression of endothelial nitric oxide synthase (eNOS) by binding to the estrogen receptors of the vascular endothelium (Caulin-Glaser et al., 1997; Rubanyi et al., 1997) and also induces phosphorylation and activation of eNOS through the intracellular phosphatidylinositol-3 kinase/ Akt (PI3 K/Akt) signaling pathway (Haynes et al., 2000), thus increasing the release of endothelium-derived nitric oxide that ultimately causes vascular dilation. Deschênes et al. (Deschênes et al., 2009), using a quantitative autoradiography approach, assessed the retinal blood perfusion in ovariectomized (OVZ) rats treated with βE_2 following an injection of [14C]-IMP (a diffusible blood flow tracer). In the latter study, they found that the tissue concentration of [14C]-IMP was higher in the OVZ-βE_2 rats compared to the OVZ rats treated with the vehicle, indicating a higher retinal blood perfusion in OVZ-βE_2 rats. In addition to its vasodilatory effect, estrogen can induce angiogenesis through promoting the expression of vascular endothelial growth factor (VEGF) (Ruohola et al., 1999; Suzuma et al., 1999). Estrogen can increase the plasmatic level of VEGF (Agrawal et al., 2000; Khazaei and Nematbakhsh, 2006) and up-regulate the expression of VEGF in target tissues including the breast (Garvin et al., 2006) and the endometrium (Fujimoto et al., 1999). Suzuma et al. (Suzuma et al., 1999) incubated cultures of bovine retinal endothelial cells (BREC) with βE_2 and found that estrogen augmented VEGF-induced DNA synthesis and also increased the expression of VEGF receptor 2 in BREC. Interestingly, VEGF plays an important role in the vascular development and retinal coverage (Stone et al., 1995) and its expression is suppressed by the high level of oxygen during the vaso-obliterative phase of OIR (i.e. the initial stage of the OIR

pathological process), thus resulting in reduced retinal vascularization (Alon et al., 1995). In order to investigate the effect of βE_2 on the retinal vascular pattern in a murine model of OIR, Zhang et al. (Zhang et al., 2010) obtained retinal flat mounts from the C57BL/6J mice treated with βE_2 while exposed to a hyperoxic condition. They showed a significant reduction in the percentage of avascular/total retinal area, an effect that was further confirmed in a recent study from the same group (Zhang et al., 2012).

Given that estrogen can act as a vasodilator and also enhance the expression of VEGF, thus influencing the retinal vasculature, we believe that these effects could possibly explain our results showing a deleterious effect of βE_2 in our model of OIR. βE_2 injection to hyperoxic rats may have caused a significant vasodilation of the developing retinal vessels, thus increasing the retinal blood flow and consequently cause an enhancement of oxygen delivery to the maturing retinal tissue. It is well known that the retina is extremely vulnerable to oxygen-mediated damage. Firstly, the consumption rate of oxygen is very high in retinal cells, and ROS are normally generated as by-products of oxygen metabolism during mitochondrial electron transport. Exposing the retina to hyperoxia would thus increase the production of oxygen radicals that could damage and even destroy retinal cellular components including lipids, nucleic acids and proteins, thus initiating an intracellular cytotoxic process (Hardy et al., 2005). Secondly, the retina has a high concentration of polyunsaturated fatty acids (PUFA): docosahexaenoic acid (DHA; 22:6ω-3), arachidonic acid (AA; 20:4ω-6), and choline phosphoglyceride which can be easily oxidized and initiate intracellular cytotoxic reactions (Bazan, 1988). Finally, the outer retina (photoreceptor layer) is the site of phototransduction and photic reactions are known to be a major source of oxygen radical formation (Wu et al., 2006). In addition, the retina of a full term rat pup is extremely immature at birth and continues to mature as the rat ages, resulting in an

increase in its metabolic rate during the second week (i.e. when the pups were subjected to the hyperoxic regimen in this study) to third week of life (Graymore, 1959). A greater metabolism is associated with an increase in electron leaks in the mitochondria, thus resulting in the acceleration of free radical formation. It seems that βE_2 administration to rat pups exposed to hyperoxia leads to higher oxygen delivery to the retinal tissue, thus augmenting the ROS formation in the retina and, as a result, causing severe damage as evidenced with the reduced ERG amplitudes (Fig. 2). Furthermore, as previously mentioned, estrogen causes vasodilation through increasing the endothelial-derived nitric oxide (NO). Interestingly, ROS can react with NO and produce highly reactive nitrogen species (RNS) including peroxynitrite, nitrogen dioxide and dinitrogen trioxide (Squadrito and Pryor, 1998) that can destroy the retinal microvasculature and damage cellular function (Gu et al., 2003; Beauchamp et al., 2004), thus providing further explanation for the destructive effect of βE_2 on the retina of hyperoxic rats in our study. Some studies have shown that high doses of βE_2 injected to mice while exposed to hyperoxia decreased the concentration of malondialdehyde (MDA), a biomarker of oxidative stress, within the retinal tissue (Zhang et al., 2010; Zhang et al., 2012), suggesting an antioxidant effect of βE_2 under such oxidative stress conditions. In the present study, we did not measure the blood concentration of estrogen in rat pups after daily intraperitoneal βE_2 injection. To our knowledge, other studies also failed to report it. However, it appears that the dose of βE_2 injected to hyperoxic rat pups in our study was not sufficient to counteract such a high level of free radicals resulting from oxygen-induced oxidative condition.

Surprisingly, at P30 we observed that injection of the vehicle alone significantly affected the inner retinal function (scotopic b-wave and photopic b-wave) of exposed animals (Fig. 2A). This unexpected finding might be explained by the extra stress due to the manipulation of

animals (i.e. injection) that are already subjected to a highly stressful condition. The possible effect of environmental stress in increasing the level of oxidative stress in the retina has been previously reported (Beatty et al., 2000). It has been shown that both acute and chronic stresses increase the release of hormones such as ACTH and prolactin (Neill, 1970; O'Steen and Brodish, 1985) that could influence the function and structure of neurons and neuroglial cells as well as cause neuronal cell death in the central nervous system (Landfield et al., 1978; Landfield et al., 1981). The studies of O'steen and coworkers (O'Steen and Brodish, 1985; O'steen et al., 1987) illustrated the detrimental effect of an environmental stress, induced by a signaled electric foot-shock, on the neural retina of Fischer-344 rats. However, we could not find any effect of vehicle injection in LIR model. The latter might be explained by the age difference of the pups at the time of exposure to the oxidative stress condition. In OIR model, the male pups were exposed to hyperoxia between ages P8 and P14 compared to ages P14-P28 for rat pups exposed to the bright light. Moreover, although there is no study to compare these two oxidative conditions (OIR and LIR) in terms of production level of MDA, for some reasons it would appear that the hyperoxic condition is more stressful to the nursing mothers and, consequently, could lead to a drastic increase in the plasmatic levels of stress hormones that could be easily passed to the pups through breastfeeding and, potentially, influence the retinal function. Firstly, the hyperoxic condition is extremely harmful to the pulmonary function of adult rats and to avoid fatality, the nursing mothers have to be replaced by the surrogate mothers every 24 hours. In contrast, the mothers exposed to bright light do not need to be replaced, thus removing the stress of changing mothers to the pups. Secondly, while animals were subjected to the bright luminous environment for only 12 hours per day, exposure to hyperoxia lasted 22.5 hours per day, thus resulting in a significantly greater stress for both, the nursing mothers and the pups. In addition to the above-

mentioned reasons, in LIR model the 14- to 28-day-old pups are gradually less dependent upon their nursing mothers (in fact weaning is usually done at P21) compared to the 8- to 14-day-old pups in OIR model. Taken all together, the relatively younger hyperoxic pups may have been more stressed by the exposure condition compared to the relatively older light-exposed pups and as a result, the impact of animal manipulation during injection might have enhanced that stress.

In this study the destructive effects of βE_2 on the hyperoxic retina were well documented with the retinal histology obtained at P60 (Fig. 3 and Fig. 4). We have previously reported that in OIR, the OPL is the most affected layer of the retina (Lachapelle et al., 1999; Dembinska et al., 2002; Dorfman et al., 2008; Dorfman et al., 2009) a finding in accord with the results presented herein (Fig. 4). Given that the cytoarchitectural anomalies in OIR model seem to be limited to OPL thinning while the photoreceptor layer remains intact, it is not surprising to obtain a nearly normal electrical function for this layer as determined with the ERG a-wave, thus suggesting that the OIR impairments occur at the postreceptoral level. The destroyed OPL prevents signal transmission between outer and inner retina leading to inner retinal dysfunction as evidenced with the reduced ERG b-wave (Fig. 2). Interestingly, it seems that the extent of structural damage following hyperoxia was not uniformly distributed throughout the retina. We noticed that the central region of the retina was more susceptible to hyperoxia as demonstrated with the significantly thinner central OPL of exposed albino SD rats compared to control (Fig. 6). Of interest, it was shown that in pigmented Long-Evans rats the central retina was that most damaged following postnatal hyperoxia while localized regions of normal synaptic contact and cell morphology could still be found in the peripheral retina (Dorfman et al., 2011). These discrepancies between the central and peripheral retinas might be attributable to differences in the rate of maturation of the blood supply to these regions. At birth, the retinal vasculature of a

newborn rat is extremely immature (Barnett et al., 2010). Soon after birth, the primary vascular plexus develops thoroughly from the optic disk towards the ora serrata (Connolly et al., 1988). Given that during hyperoxia the central retina is more vascularized compared to the peripheral retina, it is more prone to ROS-induced oxidative damage as demonstrated with its thinner OPL compared to that of the peripheral retina. It is of interest to note that the retina of hyperoxic rats treated with βE_2 was greatly vulnerable to the deleterious effects of hyperoxia as shown with the significantly thinner OPL of these rats compared to the untreated hyperoxic rats (Fig. 6). We also noticed that βE_2 treatment caused more damage to the OPL in the central retina compared to the peripheral region, indicating a prominent vaso-active effect of βE_2 in the central region that increased oxygen delivery and consequently augmented ROS formation in this region.

5.3 The Effects of postnatal injection of βE_2 on the retinal function and structure in LIR model

In this study, while 12μg of βE_2 preserved the retinal function at P60, we could not find any effect of βE_2 administration on the ERGs obtained from light exposed rats at P30 (Fig. 7A). Previous studies of ours (Joly et al., 2006a, Joly et al., 2006b) have shown that in the early stage of LIR the functional effect of bright light exposure is contaminated with the photostasis effect, whereby the sensitivity of the retina changes through altering the length of photoreceptor outer segments and reducing the rhodopsin content, thus leading to less photon absorption and, consequently a reduced light damage (Boulos and Terman, 1998). It would appear that the ERGs of all exposed rats (treated or not) at P30 were equally suppressed as a result of this effect. This would explain why we were not able to demonstrate any effect of βE_2 treatment in exposed rats at that age. However, at P60 (Fig. 7B), we found that the ERG responses of light exposed rats treated with the highest dose of βE_2 (12μg) were of larger amplitude compared to those obtained

from untreated exposed rats, suggesting a protective effect of βE_2 on the retinal function of light exposed animals. Of note, light exposed rats treated with the highest dose of βE_2 did not show the expected amplitude reduction of inner retinal function witnessed in other light exposed groups, but did so for the outer retinal function (Fig. 7B). In contrast, injection of the lowest dose (4μg) of βE_2 did not prevent the expected amplitude reduction of the outer and inner retinal functions, indicating a dose-dependent effect of βE_2 in its neuroprotective role against retinal light damage. Some studies have also reported a protective effect of βE_2 against photostress-induced retinal degeneration (Mo et al., 2013; Wang et al., 2014). We have previously reported (Dembinska et al., 2001; Dembinska et al., 2002; Joly et al., 2006a; Joly et al., 2006b) that the normal maturation of the rat retina (P30-P60) is manifested by a reduction in ERG amplitudes. However, as illustrated in Figure 9, the ERG responses obtained from the light exposed animals increased over time, presumably due to the regeneration of the outer segments.

The retinal cytoarchitectural anomalies in our rat model of LIR included significantly thinner ONL in exposed rats compared to control (Fig. 10) a finding in accord with previous reports (Joly et al., 2006a; Joly et al., 2006b). This explains the functional deficits reported in our LIR model manifested as a significant reduction of both scotopic and photopic ERG responses (Fig. 8). Photoreceptor loss resulting from bright light exposure caused significant scotopic a-wave reduction and the remaining photoreceptors are not efficient enough to provide an adequate output to the inner retina, thus leading to a decline in scotopic and photopic b-wave as well.

In this study, we chose the superior retina to investigate the effect of βE_2 on the retinal structure in our LIR model. It is believed that the inferior retina is less vulnerable to light damage because it contains shorter rod outer segment and a lower rhodopsin level compared to those found in the superior retina (Rapp et al., 1985). We noticed that the central part of the superior

retina was that most susceptible to light damage. It was previously shown that the destructive effect of bright light is most pronounced in a region 1-2 mm superior to the optic nerve head (Rapp and Williams, 1980), a distance that is compatible with the most damaged region found in our exposed rats (Fig. 10). It is thought that the remarkable resistance of the peripheral retina to light damage is due to a decrease in retinal irradiance within this region (Organisciak and Vaughan, 2010). Furthermore, a high concentration of basic fibroblast growth factor (bFGF), a neuroprotective factor, has been found in the peripheral sector of the retina correlating with reduced light-induced photoreceptor degeneration in that area (Stone et al., 1999). Of interest, we found that daily βE_2 injections (12μg) significantly preserved the photoreceptors against light damage in exposed rats compared to the untreated exposed rats, as indicated with the significantly thicker ONL measured in the central retina (Fig. 12). This finding is in accord with previous studies which showed that βE_2 administration to adult SD rats exposed to bright light protected the photoreceptors against light-induced damage and maintained the thickness of ONL (Mo et al., 2013; Wang et al., 2014).

Different action mechanisms of βE_2 might be involved in protecting the function and structure of the retina exposed to an intense light environment. Estrogen could exert its neuroprotective properties by acting as an anti-apoptotic and/or an anti-oxidative molecule. Apoptosis is one of the main pathways of photoreceptor and RPE cell death in light-induced degeneration (Remé et al., 1998; Hao et al., 2002). An increase in intracellular caspase-mediated activity is considered as a precursor to DNA degradation which is demonstrable several hours following light exposure (Organisciak and Vaughan, 2010). A study by Mo et al. (Mo et al., 2013) showed that an intravitreal administration of βE_2 protected the retina of adult SD rats from the deleterious effect of an exposure to 8000 lux of white light by preventing the retinal neural

apoptosis as determined with DNA ladder assay. In the latter study, they also reported that the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway played an important role in βE_2-mediated retinal protection by augmenting the nuclear translocation of NF-κB that inhibited the caspase-3 cleavage. Activation of caspase-3 in SD rats has been reported to be involved in the apoptosis of photoreceptor cells induced following exposure to 400 to 480 nm light for 6 hours (Wu et al., 2002). The anti-oxidative effects of E_2 on the neural tissue have been well documented (Persky et al., 2000; Behl, 2002). Oxidative stress is known to be an early event in the retinal light damage leading to free radical formation (Organisciak and Vaughan, 2010). Accordingly, the activation of intracellular anti-oxidative enzymes seems to play an important role in protecting the retinal cells against phototoxic damage. Interestingly, a study by Wang et al. (Wang et al., 2014) showed that intravitreal injection of βE_2 to adult SD rats significantly reduced the light-induced production of MDA, a by-product of lipid peroxidation, which is considered as a biomarker of cellular oxidative stress. They demonstrated, in animals placed in a bright luminous environment, that the anti-oxidant effect of βE_2, as evidenced with a decreased MDA production, was mediated through the up-regulation of gene expression of anti-oxidant enzyme including superoxide dismutase (SOD) 1, SOD 2, catalase (CAT), and glutathione peroxidase (Gpx) 1, Gpx 2, and Gpx 4 detected by qRT-PCR.

6. CONCLUSION

In summary, our results reveal that estrogen can protect the retina in pathological conditions where the primary target of the oxidative stress is the outer retina (i.e. the photoreceptors) as in LIR, while it appears to have a detrimental effect in conditions where the inner retina is the main target, such as in OIR. Although it has been suggested that estrogen

could be used as a prophylactic agent in ischemic retinopathies such as retinopathy of prematurity (ROP) (Miyamoto et al., 2002), our results suggest that it could also deteriorate retinal function and structure in an animal model of ROP. Of interest, the protective effect of βE_2 in our animal model of LIR provides valuable evidence in support the role of estrogen in reducing the incidence of retinal degeneration such as AMD in women before menopause state.

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8. CAPTIONS TO FIGURES

Figure 1. Representative scotopic (top 2 rows) and photopic (bottom 2 rows) ERGs recorded from rat pups exposed to room air (rows 1 and 3) or hyperoxia (rows 2 and 4). Representative ERGs were obtained from untreated control rats (first column), rats treated with vehicle (second column) and rats treated with either 4μg of βE_2 (third column) or 12μg of βE_2 (fourth column) at age P30 **(A)** or P60 **(B).** Vertical arrows correspond to the stimulus onset. Horizontal calibration: 40 msec. Vertical calibration: 200 μV. a = a-wave; b = b-wave.

Figure 2. ERG amplitudes in μV (scotopic a-wave, scotopic b-wave, photopic b-wave) were measured at **(A)** 30 days of age (upper graphs identified P30) and **(B)** 60 days of age (lower graphs identified P60) in control and hyperoxic groups. Four different subgroups in control and exposed groups were identified with different colors. *** $P < 0.001$ indicates significant difference between hyperoxic groups and their equivalent control groups. *$* $P < 0.05$ shows significant difference between hyperoxic rats and hyperoxic rats treated with vehicle. Values are given as mean ± 1 SD.

Figure 3. Maturation index of ERG parameters measured for control (unexposed) and hyperoxic groups. This index represents the ratio between the amplitudes of the different ERG components measured at P60 for control and hyperoxic rats over those measured at P30 (i.e. P60/P30 x 100%). * $P < 0.05$ shows significant difference between control rats treated with βE_2 and untreated control rats. † $P < 0.05$ indicated significant difference between hyperoxic rats treated with 12μg of βE_2 and those treated with 4μg of βE_2. *$* $P < 0.05$ shows significant difference

between hyperoxic groups and controls. § $P < 0.05$ shows significant difference between hyperoxic treated rats with 4μg of βE_2 and untreated hyperoxic rats.

Figure 4. Representative sections taken from the superior retina of a control rat **(A),** hyperoxic rat **(B)** and hyperoxic rat treated with 12μg of βE_2 **(C).** The 12 retinal sections (75 μm in width) were taken at every 340 μm segment along the entire length of the superior retina from the optic nerve head (ONH) towards the ora serrata. These sections were aligned, side by side, to reconstruct the entire superior retina. Retinal layer abbreviations: RPE: retinal pigmented epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGCL/FL: retinal ganglion cell layer/fiber layer.

Figure 5. The average thicknesses (in μm) of the different retinal layers were measured in the superior retina for three different groups as indicated with the accompanying legend. Retinal layer abbreviations: Retina: thickness of the entire retina; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC/FL: retinal ganglion cell/fiber layer. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences between hyperoxic and control rats. Values are given as mean ± 1 SD.

Figure 6. The average thicknesses (in μm) of the OPL was measured within the central region **(**Up to 2040 μm away from the optic nerve head) and compared to those measured within the peripheral region (from 2040 μm to 4080 μm towards ora serrata) in the superior retina for control rats, untreated hyperoxic rats and hyperoxic rats treated with 12μg 17β-E_2. *** $P < 0.001$

indicates significant differences between hyperoxic and control rats. †† $P < 0.01$ shows a significant difference between the central region of untreated hyperoxic rats and that of treated hyperoxic rats. *$$$* $P < 0.001$ indicates significant difference between the thicknesses of OPL at the central and the peripheral regions of the superior retina in hyperoxic rats treated with 12μg of βE_2. Values are given as mean ± 1 SD.

Figure 7. Representative scotopic (top 2 rows) and photopic (bottom 2 rows) ERGs recorded from rat pups exposed to room light (rows 1 and 3) or bright light (rows 2 and 4). Representative ERGs were obtained from untreated control rats (first column), rats treated with vehicle (second column) and rats treated with either 4μg of βE_2 (third column) or 12μg of βE_2 (fourth column) at age P30 **(A)** or P60 **(B).** Vertical arrows correspond to the stimulus onset. Horizontal calibration: 40 msec. Vertical calibration: 200 μV. a = a-wave; b = b-wave.

Figure 8. ERG amplitudes in μV (scotopic a-wave, scotopic b-wave, photopic b-wave) were measured at **(A)** 30 days of age (upper graphs identified P30) and **(B)** 60 days of age (lower graphs identified P60) in control and light exposed groups. Four different subgroups in control and light exposed groups were identified with different colors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate significant differences between light exposed groups and their equivalent control groups. Values are given as mean ± 1 SD.

Figure 9. The maturation index of ERG parameters was measured for control (unexposed) and light exposed groups. This index represents the ratio between ERG amplitudes obtained at P60 for control and light exposed rats compared with the same ERG component measured at P30 (i.e.

P60/P30 x 100%). * $P < 0.05$ indicates significant difference between control and light exposed rats. $ $P < 0.05$ shows significant difference between light exposed rats treated with vehicle and 4μg of βE_2 and untreated exposed rats. † $P < 0.05$ and †† $P < 0.01$ indicate significant differences between light exposed rats treated with 12μg of βE_2 and those treated with 4μg of βE_2 and vehicle.

Figure 10. Representative sections taken from the superior retina of a control rat **(A),** light exposed rat **(B)** and light exposed rat treated with 12μg of βE_2 **(C).** The 12 retinal sections (75 μm in width) were taken at every 340 μm segment along the entire length of the superior retina from the optic nerve head (ONH) towards the ora serrata. These sections were aligned, side by side, to reconstruct the entire superior retina. Retinal layer abbreviations: RPE: retinal pigmented epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGCL/FL: retinal ganglion cell layer/fiber layer.

Figure 11. The average thicknesses (in μm) of the different retinal layers were measured in the superior retina for three different groups as indicated with the accompanying legend. Retinal layer abbreviations: Retina: thickness of the entire retina; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC/FL: retinal ganglion cell/fiber layer. *** $P < 0.001$ indicates significant difference between light exposed rats and control rats. Values are given as mean ± 1 SD.

Figure 12. The average thickness (in μm) of the ONL was measured within the central region **(**Up to 2040 μm away from the optic nerve head) and compared to that measured within the peripheral region (from 2040 μm to 4080 μm towards the ora serrata) in the superior retina for control rats, untreated light exposed rats and light exposed rats treated with 12μg of βE_2. *** $P < 0.001$ indicates a significant difference between light-exposed and control rats. † $P < 0.05$ shows a significant difference between the central region of untreated light exposed rats and that of treated light exposed rats. *$* $P < 0.05$ and *$$$* $P < 0.001$ display significant differences between the central and the peripheral regions of the superior retina in control rats and untreated light exposed rats, respectively. Values are given as mean ± 1 SD.

A. ERG responses obtained at P30 in OIR model

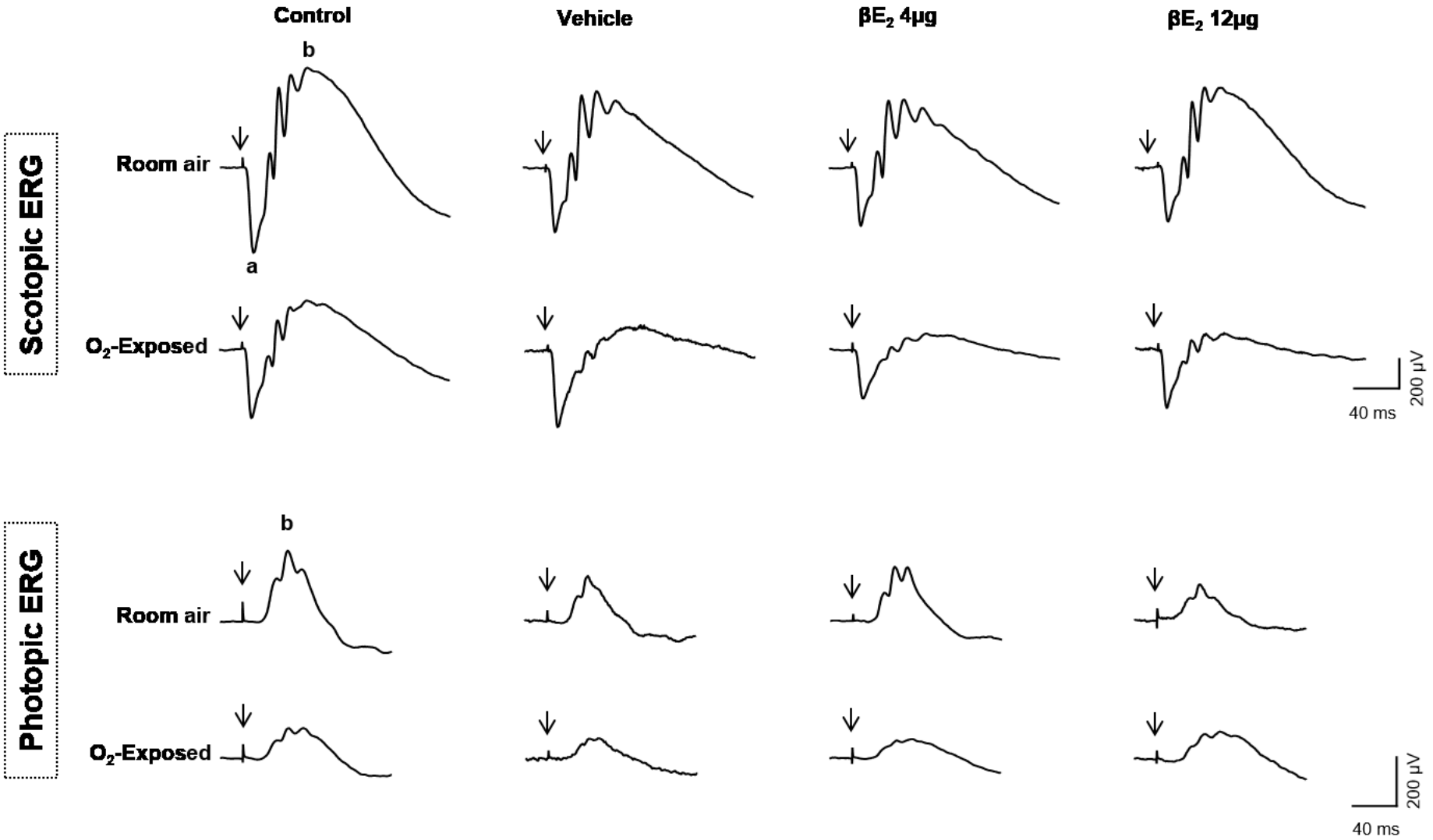


Figure 1-A

B. ERG responses obtained at P60 in OIR model

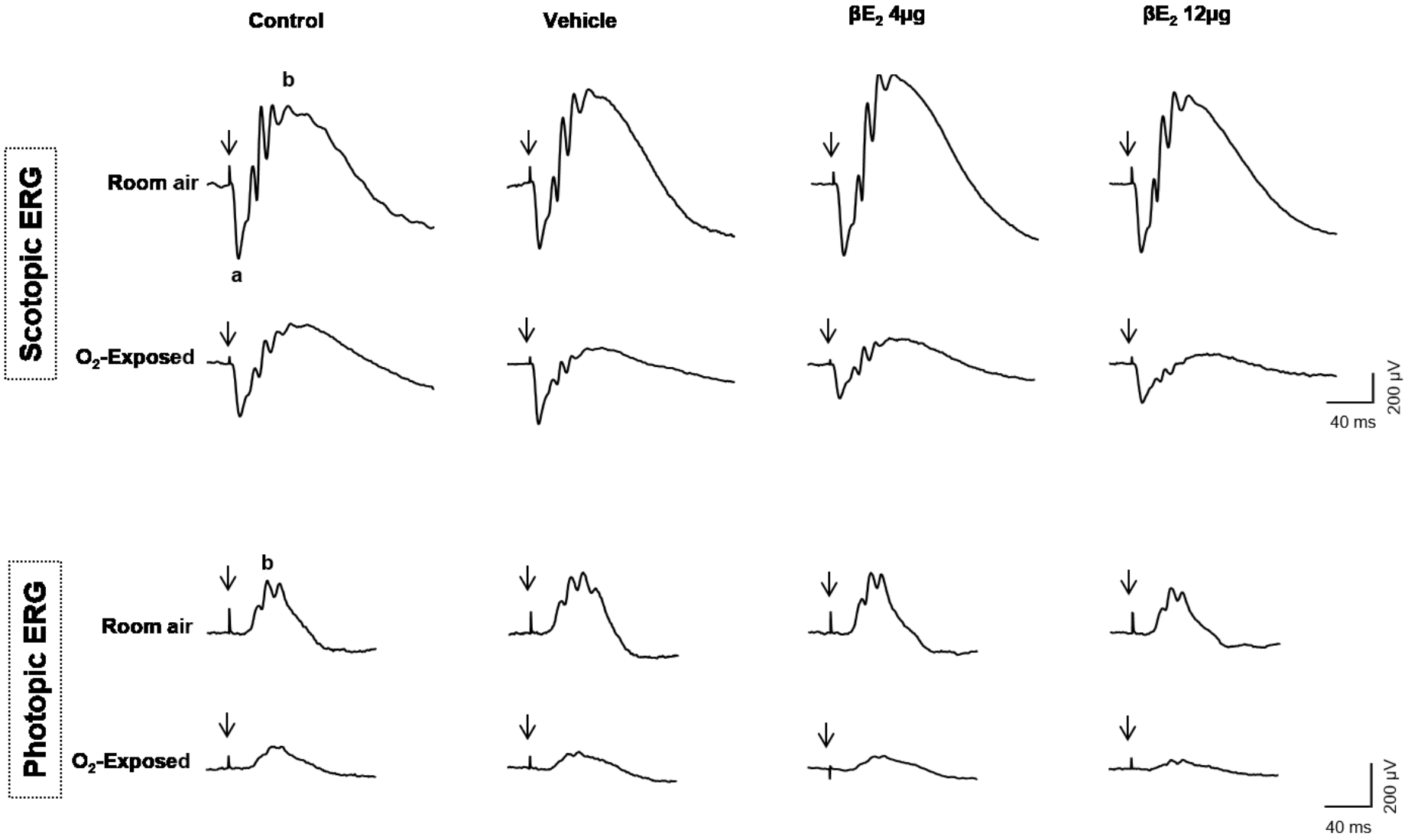


Figure 1-B

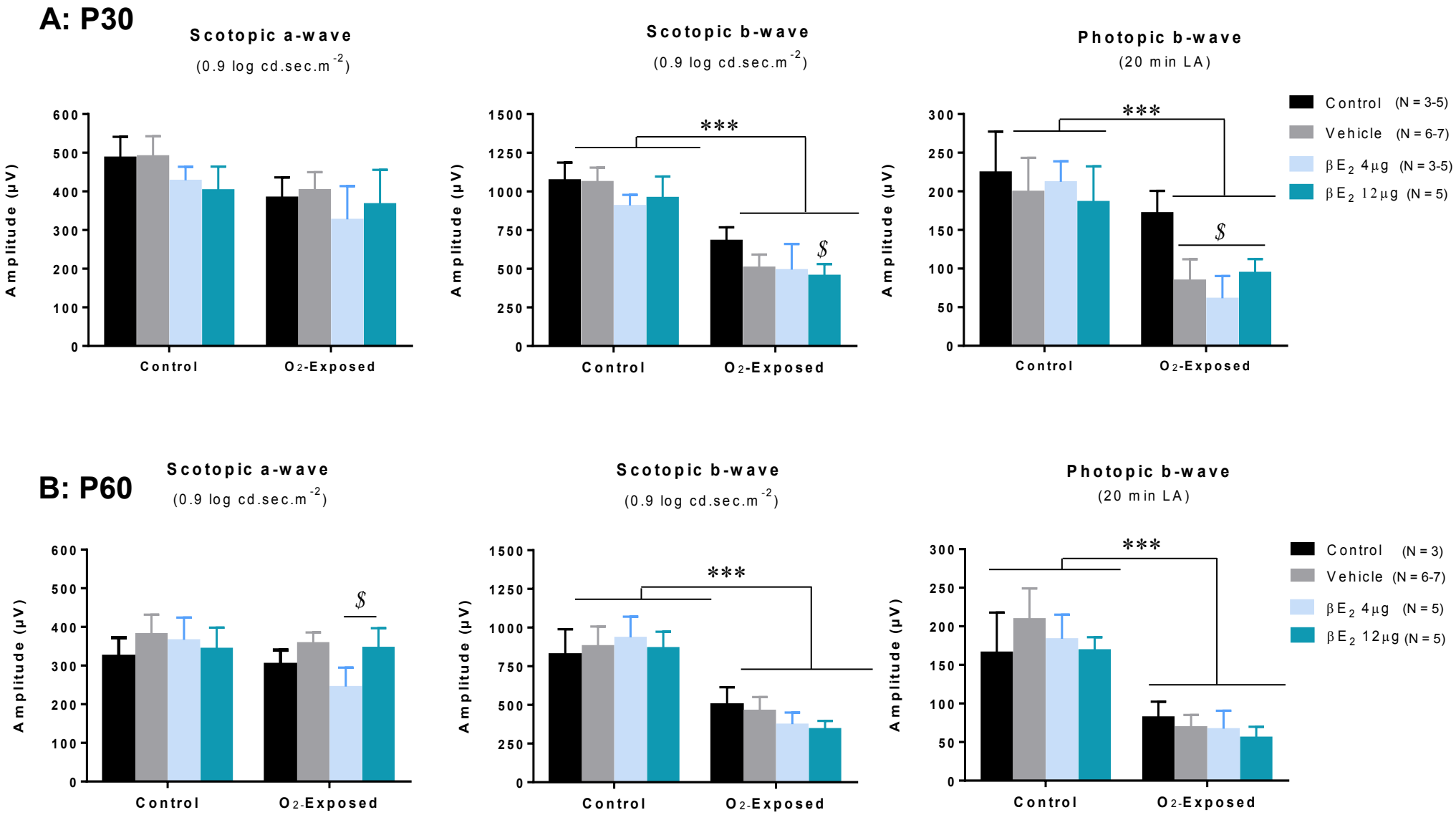

A: P30
Scotopic a-wave
(0.9 log cd.sec.m-2)
Scotopic b-wave
(0.9 log cd.sec.m-2)
Photopic b-wave
(20 min LA)
Amplitude (µV)
Control
O2-Exposed
***
$
Control (N = 3-5)
Vehicle (N = 6-7)
βE2 4µg (N = 3-5)
βE2 12µg (N = 5)
B: P60
Scotopic a-wave
(0.9 log cd.sec.m-2)
Scotopic b-wave
(0.9 log cd.sec.m-2)
Photopic b-wave
(20 min LA)
Amplitude (µV)
Control
O2-Exposed
***
$
Control (N = 3)
Vehicle (N = 6-7)
βE2 4µg (N = 5)
βE2 12µg (N = 5)

Figure 2

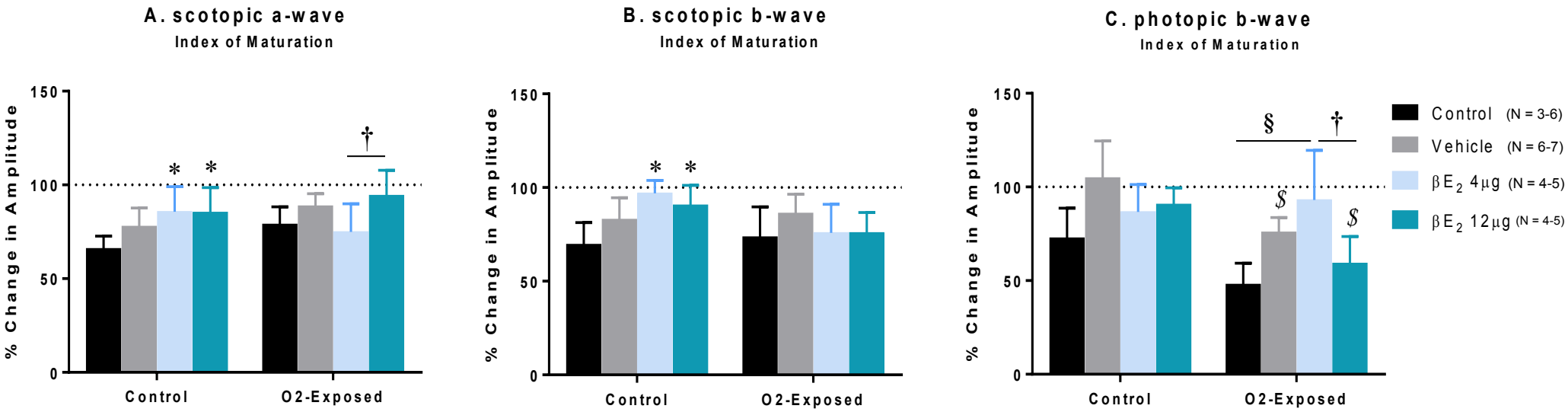

A. scotopic a-wave
Index of Maturation
% Change in Amplitude
150
100
50
0
Control
O2-Exposed
*
*
†
B. scotopic b-wave
Index of Maturation
% Change in Amplitude
150
100
50
0
Control
O2-Exposed
*
*
C. photopic b-wave
Index of Maturation
% Change in Amplitude
150
100
50
0
Control
O2-Exposed
§
†
$
$
Control (N = 3-6)
Vehicle (N = 6-7)
βE2 4µg (N = 4-5)
βE2 12µg (N = 4-5)

Figure 3

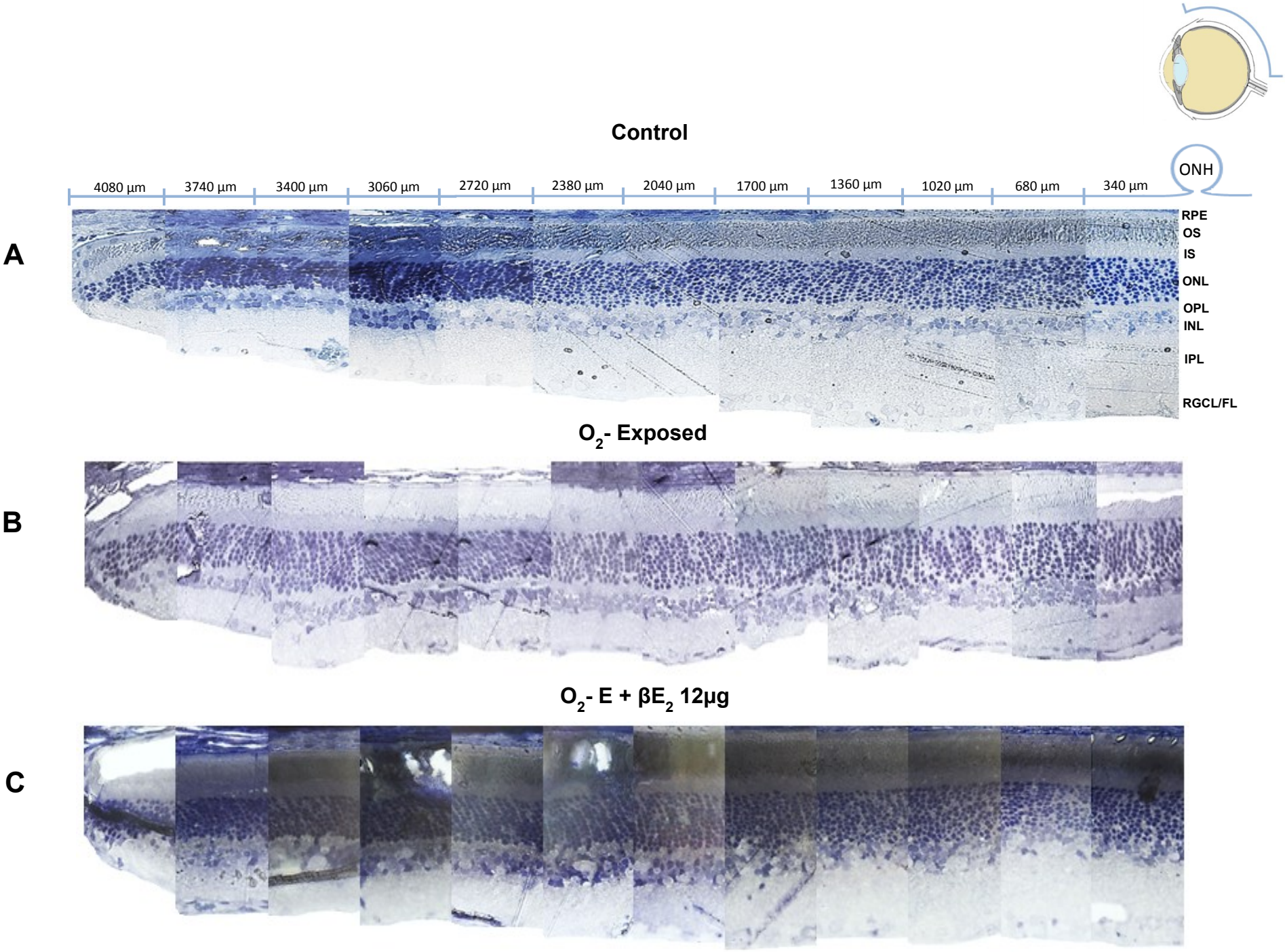

Control
ONH
4080 µm
3740 µm
3400 µm
3060 µm
2720 µm
2380 µm
2040 µm
1700 µm
1360 µm
1020 µm
680 µm
340 µm
A
RPE
OS
IS
ONL
OPL
INL
IPL
RGCL/FL
O2- Exposed
B
O2- E + βE2 12µg
C

Figure 4

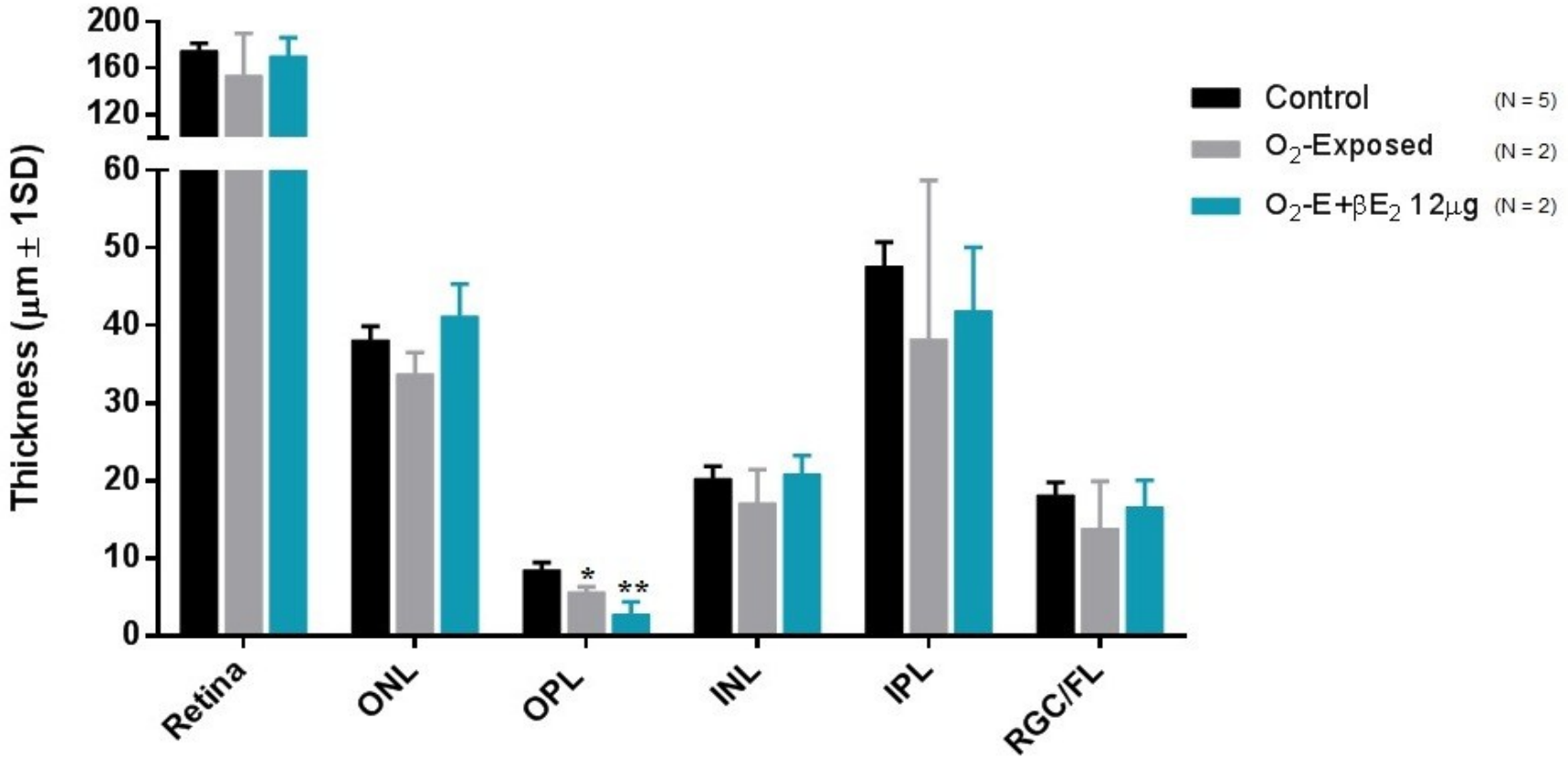

Thickness (µm ± 1SD)
200
160
120
60
50
40
30
20
10
0
*
**
Retina
ONL
OPL
INL
IPL
RGC/FL
Control (N = 5)
O2-Exposed (N = 2)
O2-E+βE2 12µg (N = 2)

Figure 5

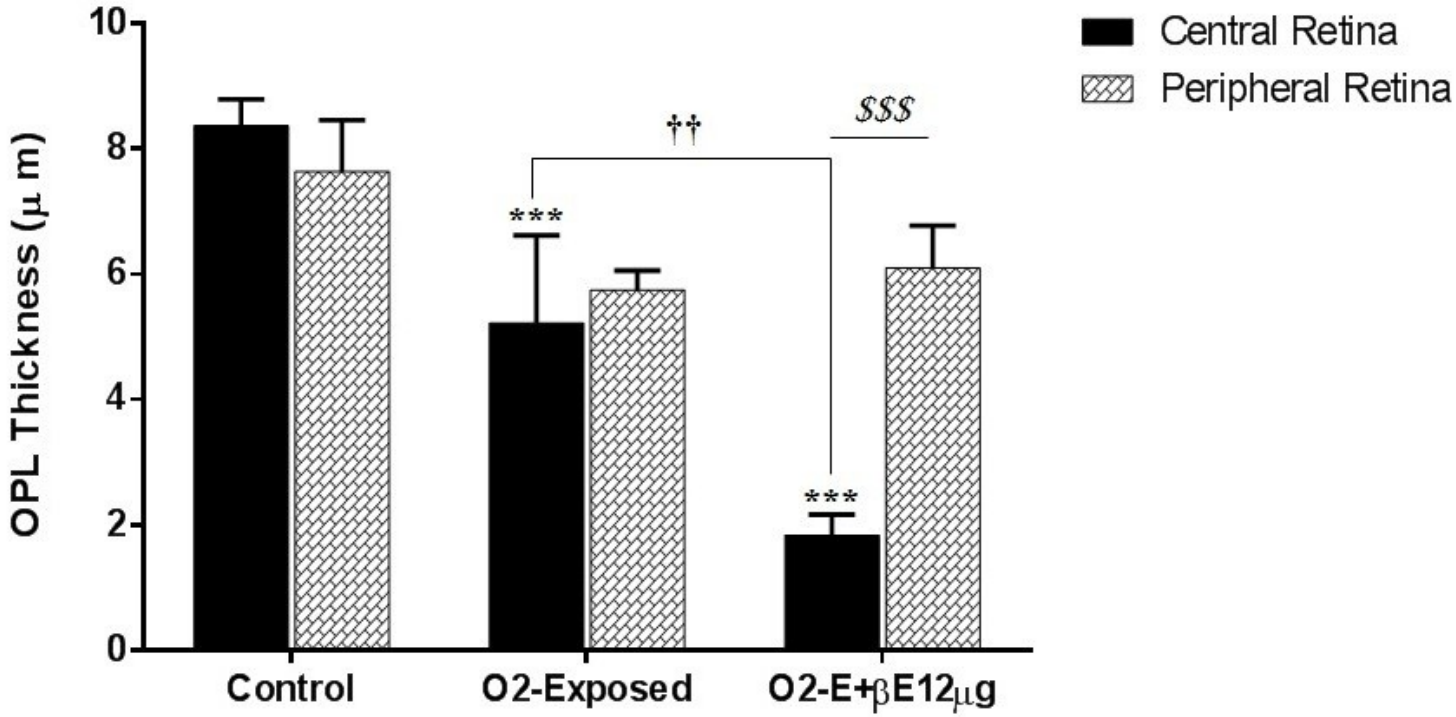

Central Retina
Peripheral Retina
OPL Thickness (μ m)
10
8
6
4
2
0
***
***
††
$$$
Control
O2-Exposed
O2-E+βE12μg

Figure 6

A. ERG responses obtained at P30 in LIR model

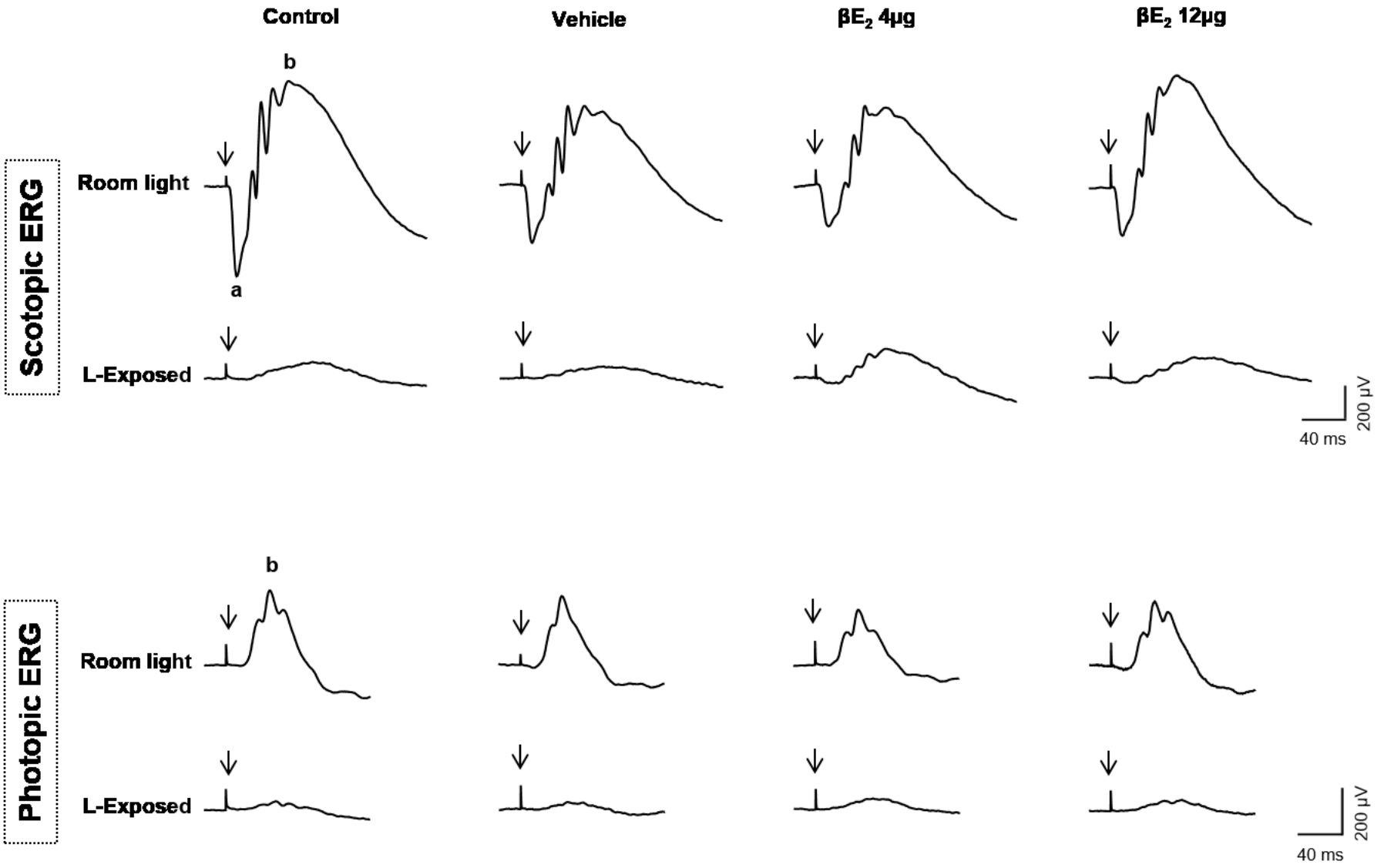


Figure 7-A

B. ERG responses obtained at P60 in LIR model

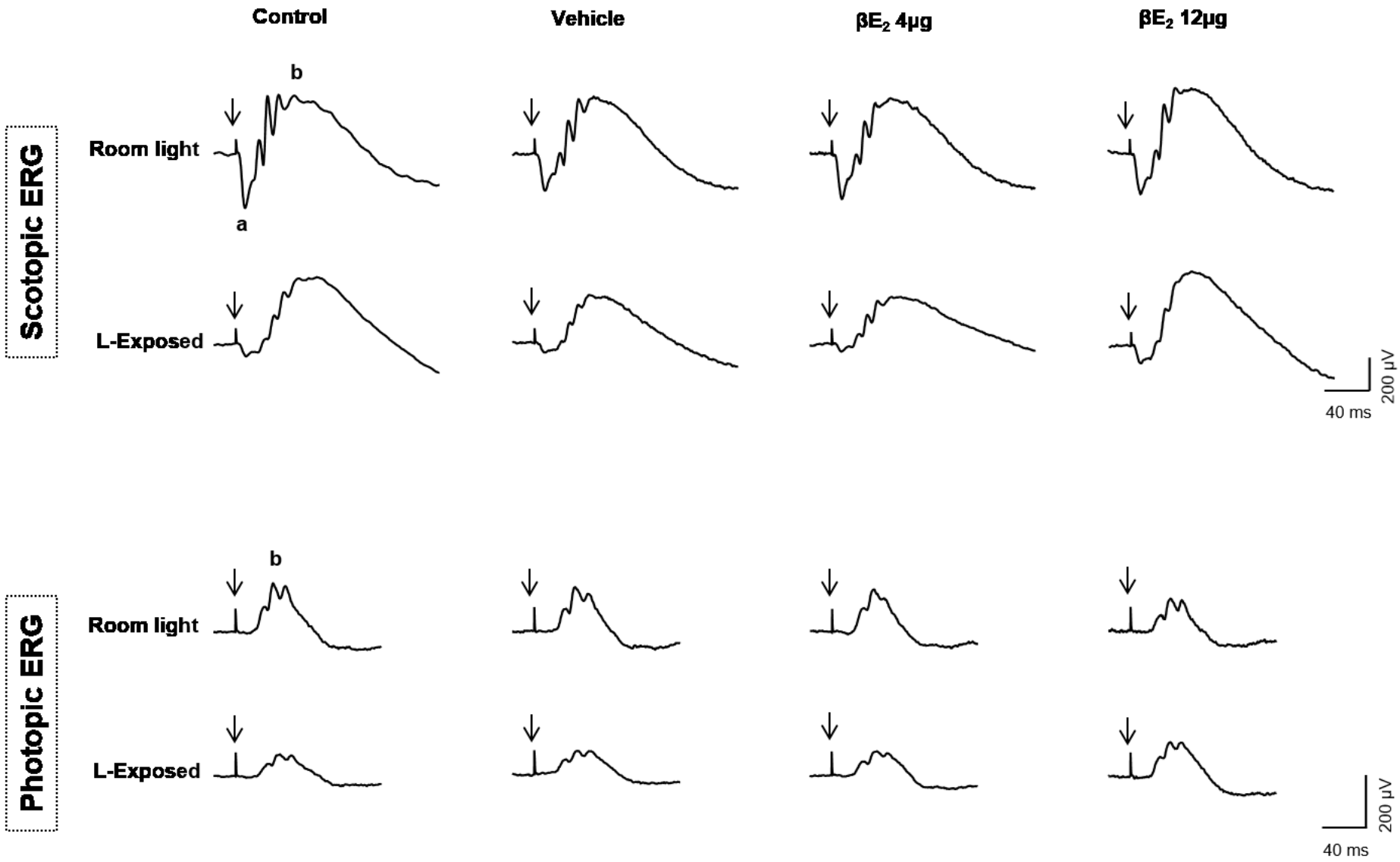
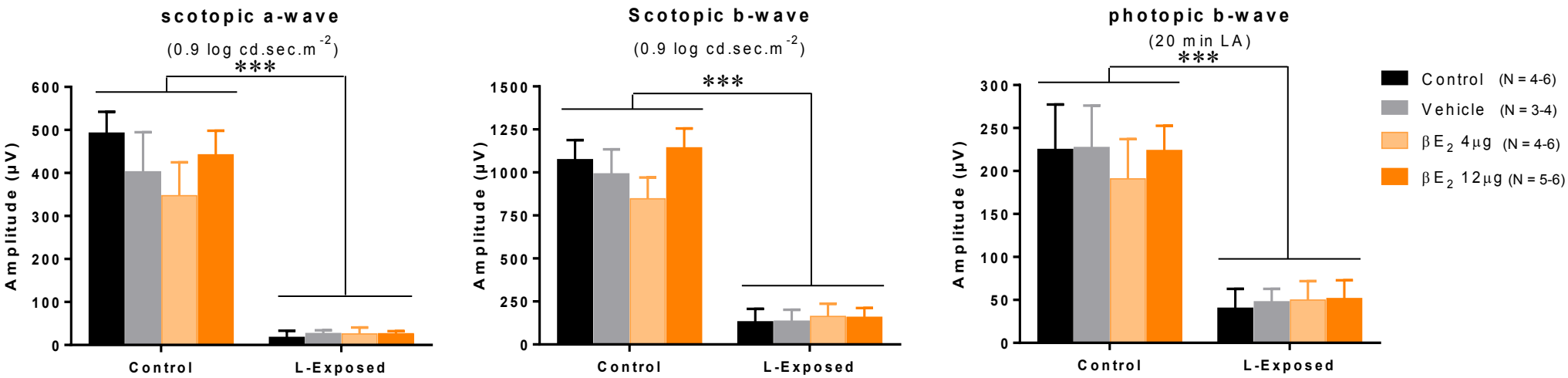


Figure 7-B

A: P30



B: P60

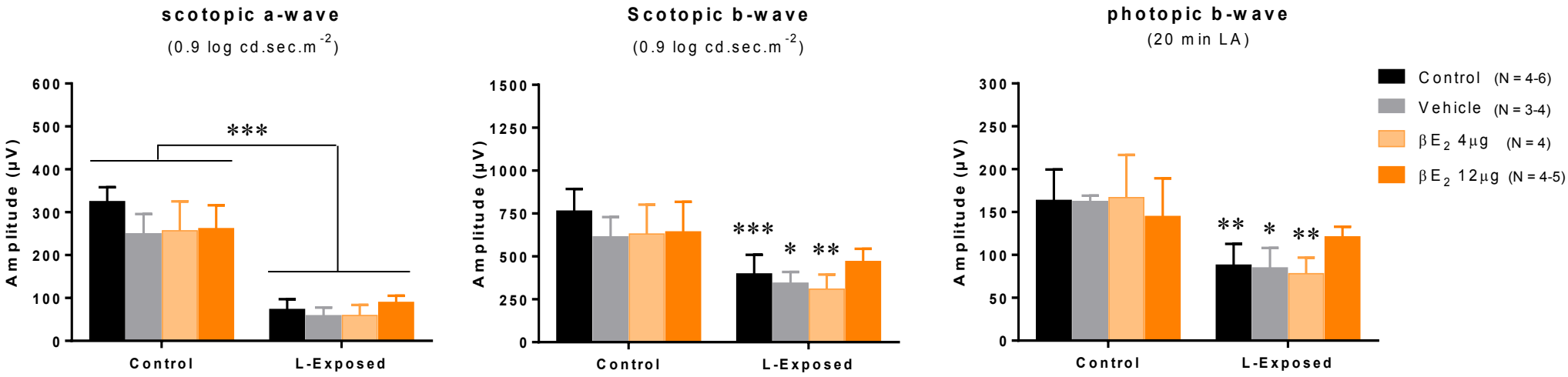


Figure 8

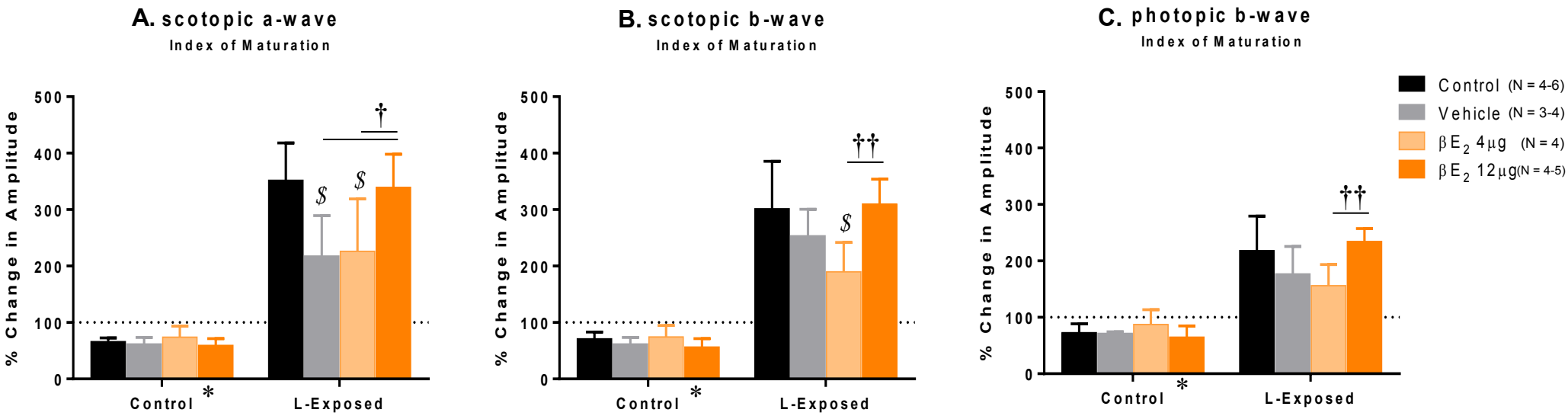
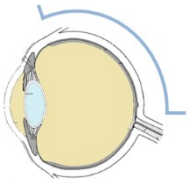

A. scotopic a-wave
Index of Maturation
% Change in Amplitude
500
400
300
200
100
0
Control
L-Exposed
*
$
$
†
B. scotopic b-wave
Index of Maturation
% Change in Amplitude
Control
L-Exposed
*
$
††
C. photopic b-wave
Index of Maturation
% Change in Amplitude
Control
L-Exposed
*
††
Control (N = 4-6)
Vehicle (N = 3-4)
βE2 4μg (N = 4)
βE2 12μg (N = 4-5)

Figure 9



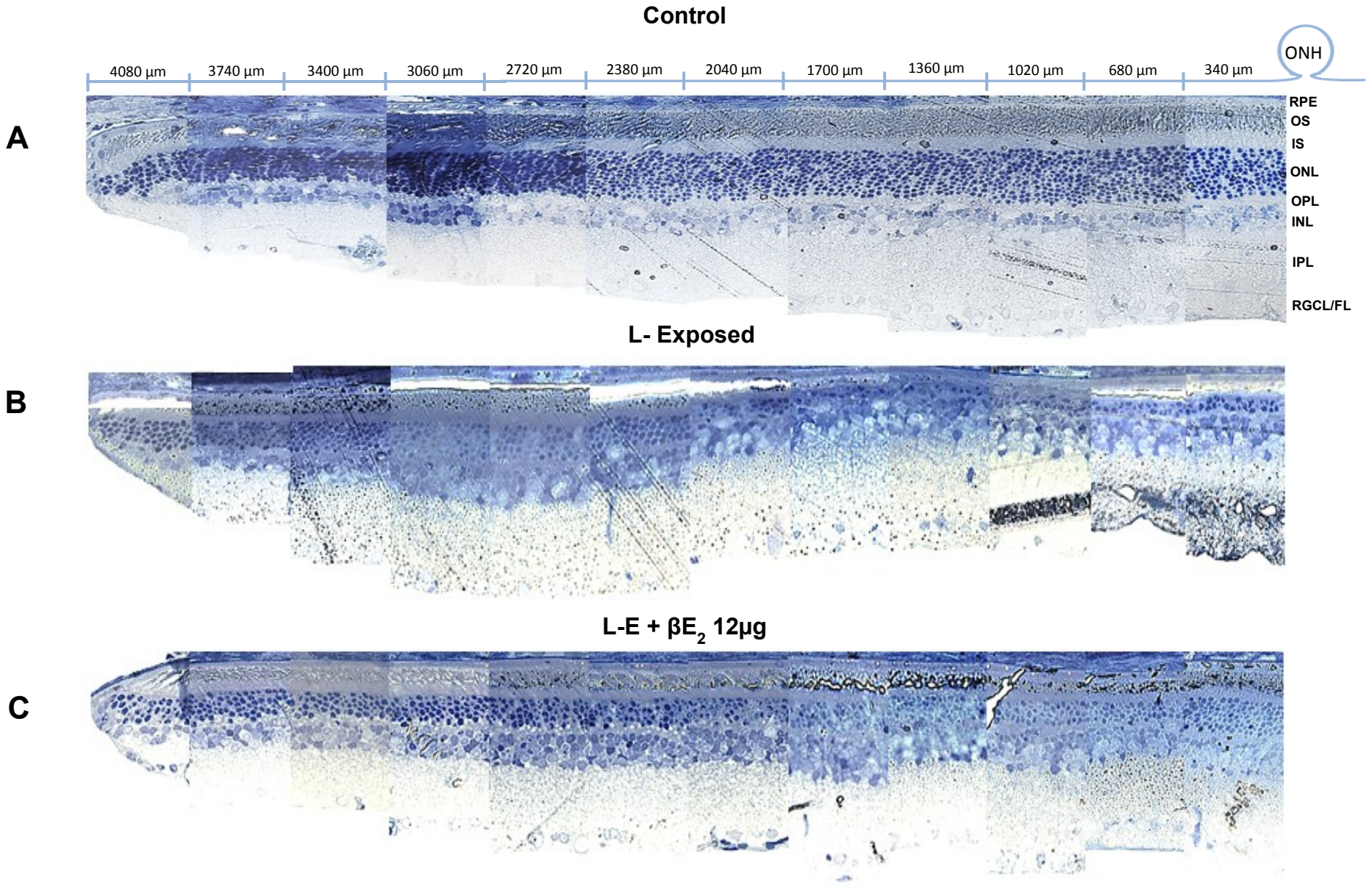

Control
4080 µm
3740 µm
3400 µm
3060 µm
2720 µm
2380 µm
2040 µm
1700 µm
1360 µm
1020 µm
680 µm
340 µm
ONH
A
RPE
OS
IS
ONL
OPL
INL
IPL
RGCL/FL
L- Exposed
B
L-E + βE2 12µg
C

Figure 10

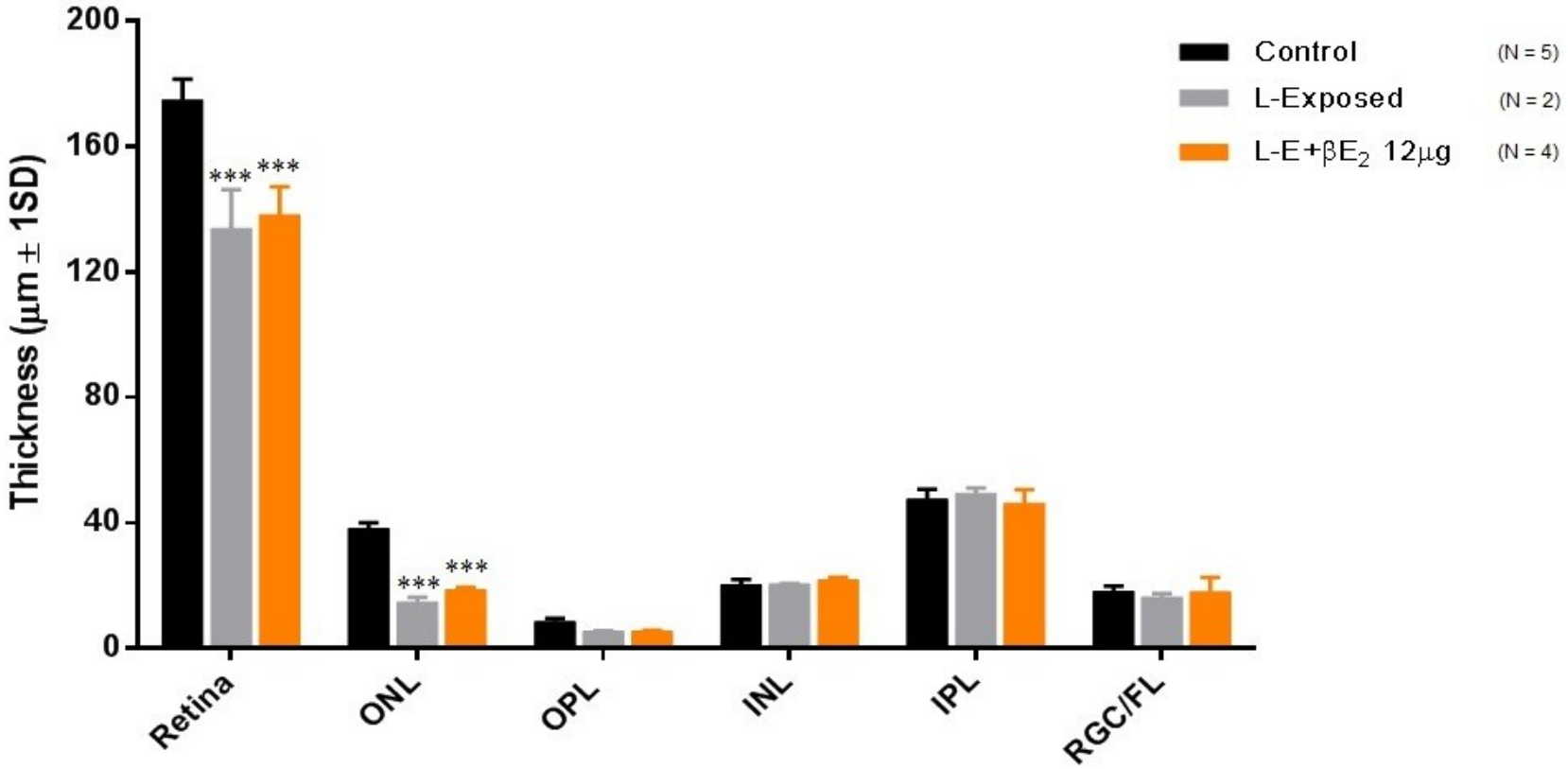

Thickness (µm ± 1SD)
200
160
120
80
40
0
***
***
***
***
Retina
ONL
OPL
INL
IPL
RGC/FL
Control (N = 5)
L-Exposed (N = 2)
L-E+βE2 12µg (N = 4)

Figure 11

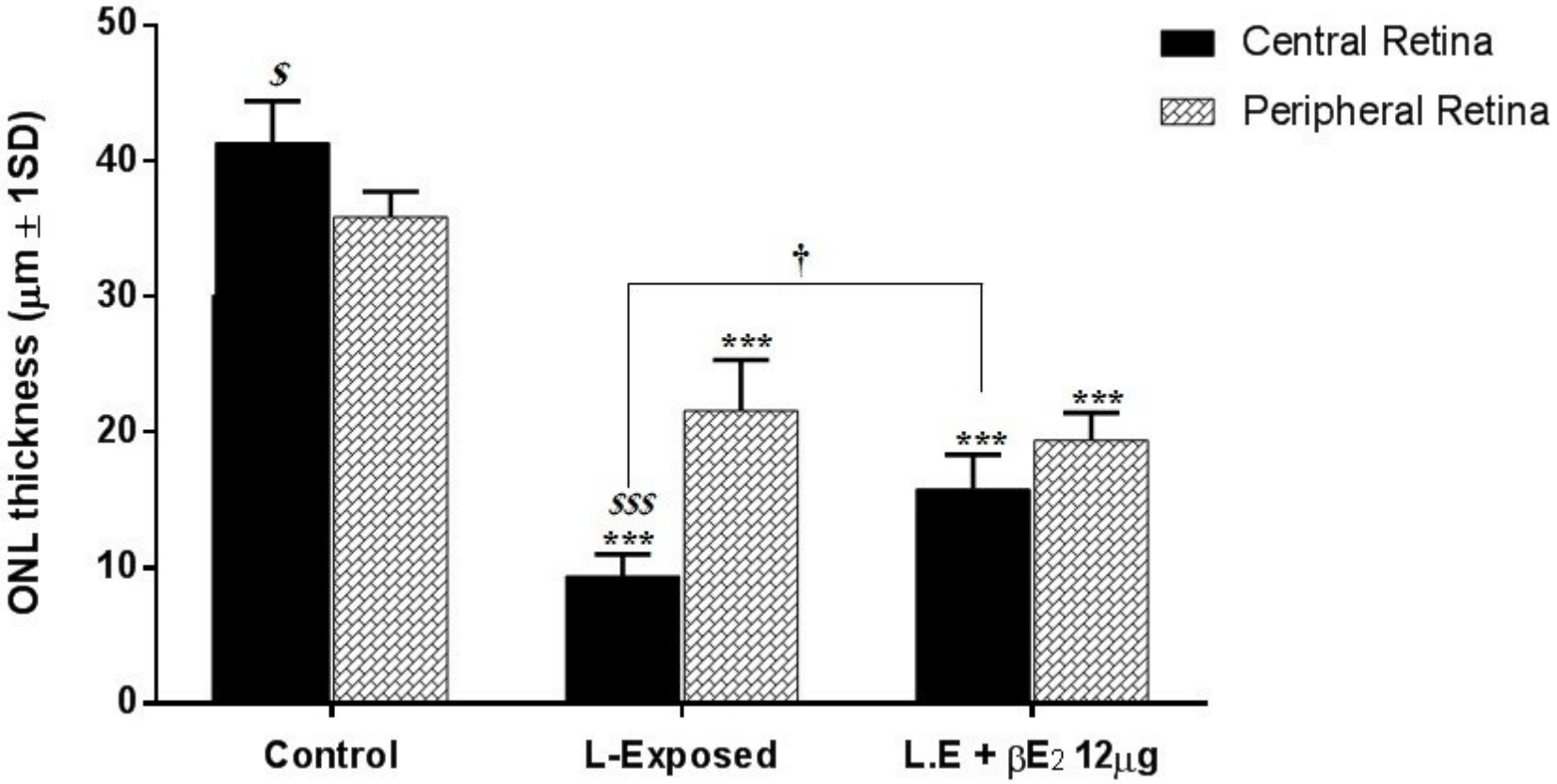

Central Retina
Peripheral Retina
ONL thickness (μm ± 1SD)
50
40
30
20
10
0
$
$$$
***
†
Control
L-Exposed
L.E + βE2 12μg

Figure 12

CHAPTER IV

GENERAL DISCUSSION

&

CONCLUSIONS

1. GENERAL DISCUSSION

The two manuscripts included in this thesis report the results of my MSc. research project which examined the impact of biological sex on the retinal structure and function of normal rats [Study # 1 (Chapter II)] and also the role of postnatal injection 17β-estradiol (βE_2) injections in protecting the retinal function and structure against two oxidative stress conditions induced by exposing neonatal rats to hyperoxia or a bright luminous environment [Study # 2 (Chapter III)].

1.1 The role of biological sex on the retinal function and structure

Our first study is, to our knowledge, the first one to investigate sex-related differences in retinal structure and function of aging rodents. In this study, the retinal function of adult male and female SD rats were investigated with the ERG at five different ages (P30, P60, P100, P200 and P300), while the retinal structure was examined at four different ages (P30, P60, P100 and P300) in order to evaluate the role of biological sex and age on the retinal function and structure. In this study, we did not find any significant age matched differences in the retinal function and structure between adult male and female SD rats. However, we did observe that between P60 and P200 the growth in ERG responses of female rats was significantly larger compared to that of male rats (Fig. 3 in study # 1). It was reported that in rodents the frequency of estrus cycles increases after the age of two months and peaks at around 8 months of age (Nelson et al., 1980). Of interest, the increase in ERG responses observed in our female rats is concomitant with the reported increase in estrus cycle frequency in rodents, suggesting a possible effect of the estrus cycle (and the accompanying hormonal fluctuation, including estrogen) on the retinal function of female rats. The assessment of the retinal function of 18-month-old female SD rats reported in the second part of this study, would strongly support our claim of an impact of the estrus cycle

on the retinal function. In this part of our study, we showed that the ERG responses obtained from premenopausal female rats were of larger amplitudes compared to those recorded from menopausal female rats as well as from age-matched male rats, while the ERG responses of menopausal rats were smaller than those obtained from the age-matched males, results that further confirmed the effect of estrus cycle on the retina (Fig. 5 and Table 2 in study # 1). It is of interest to note that the menstrual cycle and accompanying hormonal fluctuations were also shown to have some modulatory effects on the retina of women (Barris et al., 1980; Bassi and Powers, 1986; Hébert et al., 1999; Brûlé et al., 2007). The neuromodulatory effect of estrogen on the retinal function was also shown to yield in women larger ERG responses during the follicular phase of the menstrual cycle (with the high plasmatic level of estrogen) compared to the luteal phase (Brûlé et al., 2007)**.**

In our study, we found that age-related retinal changes were more pronounced in female rats, presumably due to fluctuations in the plasmatic level of estrogen. Aging is a biological event that is associated with cumulative oxidative stress due to a disturbance in antioxidant/pro-oxidant balance. The antioxidant properties of estrogen, the major female sex hormone, have been well documented (Persky et al., 2000; Wang et al., 2014). Accordingly, we believe that the better ERG responses of female rats between P60 and P200 and also those of premenopausal female rats is presumably due to the antioxidant effect of estrogen, an effect that could not be observed in menopausal female rats. Interestingly it has been reported that estrogen increases the expression of intracellular antioxidant enzymes (Strehlow et al., 2003; Wang et al., 2014), thereby enhancing the intracellular antioxidant defence. Better tolerance to age-related oxidative damage in female, presumably due to antioxidant properties of estrogen, might be a good explanation for the longer lifespan in females (Katalinic et al., 2005). Our results of this study

showing the possible effect of estrus cycle and accompanying sexual hormones, including estrogen, on the retina confirmed that the incidence of age-related retinal degeneration, such as AMD, increased after menopause in women (Snow et al., 2002)**,** a claim that was further supported with studies reporting a lower incidence of AMD in menopausal women who received estrogen replacement therapy (Haan et al., 2006).

In this study, we did not find any significant differences between retinal layer thicknesses in male and female rats, irrespective of age. However, as illustrated at Figure 7 of study # 1, we found that between P100 and P300 the progressive thinning of the inner retinal layers (including IPL and RGC/FL) was more pronounced in female rats, a finding that might be due to the gradual decrease in the estrus cycle frequency and subsequently reduction of plasmatic level of sexual hormones, in particular estrogen. Given that estrogen has a vasodilatory effect through enhancing the expression of endothelial nitric oxide synthase (Rubanyi et al., 1997) leading to increase the blood flow in different tissues, including retina (Deschênes et al., 2009), a reduction of circulating levels of this hormone may influence the blood supply of the inner retina. This may result in subtle ischemic events in this part of the retina, thus leading to tissue damage manifested as a reduction in thicknesses of inner retinal layers, including IPL and RGC/FL.

Results obtained from the first study suggest that the retina of female rats is influenced by the estrus cycle and its accompanying sexual hormones such as estrogen that might explain better response of female rats. Given the antioxidant properties of estrogen, this finding encouraged us to conduct our second study which aimed at investigating the neuroprotective effect of estrogen on the retinal function and structure in two well-documented retinal oxidative conditions. In this study, two doses of βE_2 (4µg and 12µg) were injected intraperitoneally to male SD rat pups

while exposed to an oxidative stress condition either induced by a high level of oxygen (80 ± 5 % O_2) or bright light (10,000 Lux).

1.2 The effect of estrogen on the retina in rodent models of oxidative retinopathies

1.2.1 Oxygen-Induced Retinopathy (OIR) Model

The rat model of OIR is a valid animal model of the human neonate ROP which helps us better understand the retinal consequences of exposing premature neonates to hyperoxia in the days following birth in order to compensate for their pulmonary immaturity. OIR is, in fact, a vasculopathy that can also influence the retinal function and structure. Free radical generation, one of the most important pathological consequences of postnatal hyperoxia (Sapieha et al., 2010), will cause an intracellular oxidative stress that will, if important enough, result in the deterioration of the function and structure of the retina. The main target of oxidative stress in OIR is the inner retina; this explains the characteristic functional and structural defects reported to occur following postnatal hyperoxia. We have previously shown that in OIR, functionally, the ERG b-wave (a measure of inner retinal function) is significantly depressed, and, structurally, the OPL is the most affected layer of the retina (Lachapelle et al., 1999; Dembinska et al., 2002; Dorfman et al., 2008; Dorfman et al., 2009), findings in accord with the results presented in study # 2. We found that the amplitude of the b-wave obtained from hyperoxic rats were significantly lower compared to those recorded from the controls at ages of P30 and P60 (Fig. 2 in study # 2). In addition, retinal histology obtained from hyperoxic rats at age P60 revealed a significantly thinner OPL than that measured in control rats (Fig. 4 and Fig. 5 in study # 2). Of note, in exposed rats the OPL was thinner in the central part of the retina (Fig. 6 in study # 2),

indicating a more detrimental effect of hyperoxia, possibly due to the fact that this region is initially (at the time of hyperoxia exposure) more vascularized than the peripheral retina.

Although previous studies have shown a protective effect of βE_2 on the retinal vasculature under hyperoxic condition (Miyamoto et al., 2002; Zhang et al., 2010; Zhang et al., 2012), to our knowledge, our study is the first one that investigated the effect of βE_2 treatment on the function and structure of the retina in rat pups subjected to a similar oxidative stress. We showed that not only postnatal βE_2 injections could not protect the retinal function and structure, but it could have a detrimental effect on the function and structure of the retina exposed to postnatal hyperoxia, possibly due to the vaso-active properties of estrogen. It has been reported that estrogen enhances endothelial nitric oxide synthase (Caulin-Glaser et al., 1997; Rubanyi et al., 1997), thus acting as a potent vasodilator that increases the blood flow in target tissues. Furthermore, estrogen can promote the expression of VEGF, thus augmenting angiogenesis (Ruohola et al., 1999; Suzuma et al., 1999). The expression of VEGF, which plays a critical role in the development of the retinal vasculature (Stone et al., 1995), is believed to be suppressed by the high level of oxygen during the initial stage of the OIR model (i.e. vaso-obliterative phase) (Alon et al., 1995). Accordingly, the administration of another angiogenic factor (such as estrogen) during the course of hyperoxic exposure would counteract the inhibitory effect of hyperoxia on the retinal vascularization and thus increase the vascularization of the retina subjected to hyperoxia.

The above-mentioned vaso-active properties of estrogen (vasodilatory and angiogenic effects) result in greater delivery of oxygen to the maturing retinal tissue which is extremely susceptible to oxygen-mediated oxidative damage. In fact, higher levels of oxygen at the retinal tissue will enhance the generation of ROS, which are normally generated as by-products of

oxygen metabolism during mitochondrial electron transport, thus increasing ROS-related retinal damages. From a pathological point of view, formation of free radicals during the hyperoxic condition plays an important role in the destructive effects in the OIR model (Sapieha et al., 2010). It seems that administration of βE_2 to rat pups subjected to a hyperoxic condition could result in enhancing the ROS formation due to an extra delivery of oxygen to the developing retinal tissue, and subsequently cause even more damage to the retina. In our study, the detrimental effect of βE_2 was manifested on the retinal function as reduced ERG b-waves in exposed rats treated with βE_2 compared to untreated rats (Fig. 2 in study # 2), and on the retinal structure as a significant thinner OPL, in particular at the central region of the retina (Fig. 6 in study # 2). Our finding of a detrimental effect of estrogen on the retina could explain the result of Seiberth et al. (Seiberth et al., 1990) who showed a significantly higher incidence of ROP in girls. Although they suggested some factors including different ventilation rates in premature girls and boys and also different mortality rates between preterm girls and boys that may influence the sex ratio of ROP, differential effects of maternal estrogen on the retinal tissues of premature male and female infants cannot be ignored.

1.2.2 Light-Induced Retinopathy (LIR) Model

The rat model of LIR shares some common pathological features with AMD and some forms of Retinitis Pigmentosa. LIR is a rhodopsin-mediated retinal degeneration in which the significant bleaching of rhodopsin caused by the intense light exposure will produce high levels of reactive oxygen species resulting in damage to intracellular components such as lipids, proteins and nucleic acid (Organisciak and Vaughan, 2010). Accordingly, the oxidative stress plays an important role in the pathological consequences of LIR which can be prevented with antioxidant agents such as vitamin E and melatonin (Siu et al., 1998). The main target of

oxidative stress in LIR is the outer retina (photoreceptors and RPE), thus explaining the structural and functional defects that we report following exposure to a bright luminous environment. As illustrated in Figures 10 and 11 of study # 2, the photoreceptor layer is the most affected layer following bright light exposure, confirming the destructive impact of intense light on the outer retina. We also found that all the ERG responses were significantly attenuated in light exposed rats compared to controls at age P30 and P60 (Fig. 8 in study # 2). However, the light exposed rats treated with a high dose of βE_2 (12µg) did not display the expected amplitude reduction of inner retinal function observed in other exposed groups. Of interest, we found that, between P30 and P60, the amplitude of all the ERG parameters significantly increased in light exposed rats compared to the decline measured in control rats (Fig. 9 in study # 2). This is most probably due to the photostasis phenomenon where the photoreceptors reduce the length of their outer segments in bright light condition, thus resulting in reduction of rhodopsin content and, consequently less photon will be absorbed and light damage will be reduced (Boulos and Terman, 1998). In fact, due to the photostasis effect, we could not find any effect of βE_2 injection on the retina subjected to intense light at age P30, while at P60, we observed that light exposed rats treated with 12µg of βE_2 had larger ERG amplitudes compared to untreated exposed rats (Fig. 8B in study # 2), suggesting that, in contrast to OIR model, βE_2 injection could protect the retinal function against light damage. From a structural point of view, we found that βE_2 injection could significantly preserve the photoreceptors in light exposed animals, in particular those located centrally (Fig. 12 in study # 2).

Some studies have shown a protective effect of βE_2 on the adult rat retina subjected to an intense light (Mo et al., 2013; Wang et al., 2014). Mo et al. (Mo et al., 2013) showed that the intravitreal injection of βE_2 protected the retina of adult SD rats from the destructive effect of an

exposure to 8000 lux of white light by suppressing the retinal neural apoptosis as determined with the DNA ladder assay. Furthermore, Wang et al, (Wang et al., 2014) showed that the βE_2 injection protected the SD rat retina against light damage by up-regulating the genes of antioxidant enzymes such as superoxide dismutase (SOD) 1, SOD 2, catalase, and glutathione peroxidase (GPX) 1, GPX 2, and GPX 4, thus augmenting the antioxidant defence of retinal cells against light-mediated damage. The above-mentioned studies suggest an anti-apoptotic and an anti-oxidative role for estrogen that could protect the function and structure of the retina when subjected to bright light condition.

Although, we were able to demonstrate the preservation of retinal function in light exposed rats treated with 12µg of βE_2, we believe that the long duration of light exposure (14 days) combined with the high intensity of light (10,000 Lux) used in our study could have masked the more subtle protective effect of βE_2 on the retinal function of rat pups subjected to a light stress.

2. GENERAL CONCLUSIONS

Based on the results we obtained from both studies, we conclude that biological sex can influence the retinal function and structure of rodents. We also demonstrate that the retinal function of female SD rats is strongly influenced by the estrus cycle and presumably its accompanying hormonal fluctuations. Among the female sexual hormones the neuroproective role of estrogen has been well documented and may explain the better retinal responses of female rats. The neuroprotective effect of estrogen on oxidative stress-related neurodegenerative disorders such as Alzheimer's disease (Kimura, 1995) and Parkinson's disease (Sarkaki et al., 2008) has been shown. However, it appears that the neuroprotective properties of estrogen on the retina depend on the main site of oxidative stress. As we showed with the LIR model, where the main target of oxidative damage is the outer retina, estrogen can protect the retinal function and structure, whereas with the OIR model, where the oxidative damage is most pronounced in the inner retina, estrogen will potentiate the detrimental effect of oxidative stress on the function and structure of the retina. Based on our results, estrogen is an effective antioxidant agent that can protect the retina against light-induced oxidative damage. In contrast, although it was claimed that vasoactive properties of estrogen could make it an appropriate candidate as a prophylactic agent in ischemic retinopathies such as ROP (Miyamoto et al., 2002), our results in OIR model suggest that, in fact, the vasoactive properties of estrogen could be detrimental to the function and structure of the hyperoxic retina.

CHAPTER V

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CHAPTER VI

APPENDIX

1. OTHER CONTRIBUTION FROM THE AUTHOR

1.1 Published Manuscript I

Systemic inflammation perturbs developmental retinal angiogenesis and neuroretinal function.

Tremblay S, Miloudi K, ***CHAYCHI S****, Favret S, Binet F, Polosa A, Lachapelle P, Chemtob S, Sapieha P.* Investigative Ophthalmology and Visual Science, 54: 8125-39, 2013.

In this study, I performed the ERG recordings for all the experimental animals. I also analyzed the ERG data and produced the figures related to the ERG.