

**OXYGEN INDUCED RETINOPATHY IN THE
NEONATAL RAT: THE EFFECTS OF AGE,
STRAIN AND THERAPEUTIC INTERVENTION
ON RETINAL STRUCTURE AND FUNCTION**

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5. Dorfman AL, Chemtob S, Lachapelle P. Postnatal hyperoxia and the developing rat retina: Beyond the obvious vasculopathy. Doc Ophthalmol. 2009 (Epub ahead of print).

PREFACE: PART I

1 Contributions of authors on co-authored papers

1.1 Manuscript 1 (Chapter 2)

Dorfman A, Dembinska O, Chemtob S, Lachapelle P. Early manifestations of postnatal hyperoxia on the retinal structure and function of the neonatal rat. Invest Ophthalmol Vis Sci. 2008 Jan; 49:458-66.

For this study, Olga Dembinska carried out the exposure to hyperoxia, ERG recordings and histology preparation. I carried out the data analysis, literature review and wrote the manuscript. Dr. Pierre Lachapelle and Dr. Sylvain Chemtob helped with the revision of the manuscript.

1.2 Manuscript 2 (Chapter 3)

Dorfman AL, Dembinska O, Chemtob S, Lachapelle P. Structural and functional consequences of trolox C treatment in the rat model of postnatal hyperoxia. Invest Ophthalmol Vis Sci. 2006 Mar; 47:1101-8.

For this study, I exposed several cohorts to postnatal hyperoxia and injected them with 300µg/kg/50µl and 900µg/kg/50µl trolox C. I carried out all of the ERG recordings in these groups, analyzed the data and wrote the manuscript. Olga Dembinska performed ERGs and histology pertaining to the oxygen-exposed cohort administered with 600µg/kg/50µl trolox C. Dr. Pierre Lachapelle and Dr. Sylvain Chemtob helped with the revision of the manuscript.

1.3 Manuscript 3 (Chapter 4)

Dorfman AL, Polosa A, Joly S, Chemtob S, Lachapelle P. Functional and structural changes resulting from strain differences in the rat model of oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci*. 2009 May; 50:2436-50.

For this study, I exposed the albino Sprague Dawley and pigmented Long Evans rats to postnatal hyperoxia. I prepared all of the retinal flatmounts, recorded all of the ERGs and carried out all of the histology in both strains. Anna Polosa assisted me with the Western Blots and immunohistochemistry, and Dr. Sandrine Joly was instrumental in teaching us these two techniques. I also performed all of the data analysis and wrote the manuscript. Dr. Pierre Lachapelle and Dr. Sylvain Chemtob helped with the revision of the manuscript.

1.4 Manuscript 4 (Chapter 5)

Dorfman AL, Cuenca N, Pinilla I, Chemtob S, Lachapelle P. Cytoarchitectural Manifestations of OIR in the Neonatal Rat. *In Preparation*.

For this manuscript, I carried out the exposure of all animals to the various regimens of hyperoxia. I collected all of the retinal tissue and performed all of the cryosectioning and labeling with the TUNEL kit and anti-synaptophysin antibodies. I also performed all of the data analysis and wrote the manuscript. Dr. Nicolás Cuenca and Dr. Isabel Pinilla carried out the remainder of the immunohistochemistry studies and helped with revision of the manuscript. Dr. Pierre Lachapelle and Dr. Sylvain Chemtob helped with the revision of the manuscript.

1.5 Manuscript 5 (Chapter 6)

Dorfman AL, Joly S, Hardy P, Chemtob S, Lachapelle P. The effect of oxygen and light on the structure and function of the neonatal rat retina. *Doc Ophthalmol*. 2009 Feb; 118:37-54.

For this manuscript, I carried out the literature review, reported findings obtained in our laboratory and wrote the section of the manuscript pertaining to ROP and oxygen induced retinopathy. Dr. Sandrine Joly carried out the literature review and wrote the section of the manuscript pertaining to light induced retinopathy. Dr. Pierre Hardy and Dr. Sylvain Chemtob wrote the section of the manuscript that covered free radical mediators and Dr. Pierre Lachapelle helped with the revision of the manuscript.

1.6 Manuscript 6 (Chapter 7)

Dorfman A.L., Chemtob S., Lachapelle P. Postnatal hyperoxia and the developing rat retina: beyond the obvious vasculopathy. *Doc Ophthalmol*. December 2009 (Epub ahead of print).

For this manuscript, I carried out the literature review, reported findings obtained in our laboratory and wrote the manuscript. Dr. Pierre Lachapelle and Dr. Sylvain Chemtob helped with the revision of the manuscript.

PREFACE: PART II

1 Acknowledgements

This thesis would have never been possible without the patience, dedication and support of so many colleagues, friends and family members.

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2 List of abbreviations

4-HNE	4-hydroxynonenol
AA	Arachidonic acid
AC	Amacrine cell
AMD	Age-related macular degeneration
AMPA(S)	alpha-Amino-3-hydroxy-5-Methyl-4-isoxazolPropionic Acid
APB	2-amino-4-phosphonobutyric acid
ATP	Adenosine triphosphate
BB	Blue cone-specific bipolar
BC	Bipolar cell
BDNF	Brain-derived neurotrophic factor
BN	Brown Norway
cGMP	cyclic guanosine monophosphate
CNTF	Ciliary neurotrophic factor
CRALBP	Cellular retinoid binding protein
CSNB	Congenital stationary night blindness
DA	Dark agouti
DHA	Docosahexaenoic acid
DOPA	Dihydroxyphenylalanine
DR	Diabetic Retinopathy
E	Embryonic day
ERG	Electroretinogram
F344	Fischer 344
FeSO ₄	Iron Sulfate
FEVR	Familial exudative vitreoretinopathy
FGF-2	Fibroblast growth factor-2
FMB	Flat midget bipolar
GABA	γ -aminobutyric acid
GAP	GTPase activating protein
GC	Ganglion cells

GCL	Ganglion cell layer
GDNF	Glia-derived neurotrophic factor
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
Gt	Transducin G-protein
GTP	Guanosine triphosphate
H ₂ O ₂	Hydrogen peroxide
HC	Horizontal cell
IMB	Invaginating midget bipolar
INL	Inner nuclear layer
IPL	Inner plexiform layer
IRBP	Interstitial retinol binding protein
IS	Inner segments
L-cone	Long wavelength cone
LE	Long Evans
LGN	Lateral geniculate nucleus
LIR	Light Induced Retinopathy
M-cone	Medium wavelength cone
MC	Müller cell
MDA	Malondialdehyde
mfERG	Multifocal ERG
mGluR6	Metabotropic glutamate receptor-6
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Norrie disease
NDP	Norrie disease protein
NV	Neovascularization
O ₂ ⁻	Superoxide anion
OH [·]	Hydroxyl radical
OFF-GC	OFF-ganglion cell
OFF-HBC	OFF-hyperpolarizing bipolar cells
OIR	Oxygen induced retinopathy

ON-DBC	ON-depolarizing bipolar cells
ON-GC	ON-ganglion cell
ONL	Outer nuclear layer
OP	Oscillatory potential
OPL	Outer plexiform layer
OS	Outer segments
P	Postnatal day
PAF	Platelet activating factor
PaO ₂	Partial pressure of arterial oxygen
PDA	cis-2,3-piperidine-dicarboxylic acid
PDE	Phosphodiesterase
PEDF	Pigment epithelium derived factor
PKC- α	Protein kinase C- α
PL	Photoreceptor layer
PUFA	Polyunsaturated fatty acids
RLF	Retrolental fibroplasia
ROP	Retinopathy of Prematurity
ROS	Reactive oxygen species
RP	Retinitis Pigmentosa
RPE	Retinal pigment epithelium
S-cone	Short wavelength cone
SD	Sprague Dawley
SOD	Superoxide dismutase
STZ	Streptozotocin
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TxA ₂	Thromboxane A ₂
VEGF	Vascular endothelial growth factor
VEP	Visual evoked potential

PREFACE: PART III

1 Abstract

Retinopathy of prematurity (ROP) is a potentially blinding retinal disorder that affects small prematurely born infants which results from the combination of the immature retina and the high level of oxygen (hyperoxia) needed to keep these infants alive. A rat model of oxygen induced retinopathy (OIR) was developed to better understand what occurs in premature infants that are exposed to postnatal hyperoxia and previous studies have demonstrated that this model bears a striking resemblance to the human form of ROP, at least in its vascular component. The purpose of this study was to investigate beyond the retinal vasculature in order to better understand the functional [electroretinogram (ERG)] and cytoarchitectural (histology and immunohistochemistry) manifestations of this disease.

While structural and functional consequences of OIR in the albino rat had previously been evaluated in mature rats (at postnatal days 30 and 60, for example), the sequence of events leading up to these changes could only be inferred. Consequently, the purpose of our first study was to further characterize the early manifestations of this disease process, namely: 1- to evaluate changes in retinal vasculature throughout exposure to hyperoxia and, 2- to examine how early after the cessation of oxygen exposure we can demonstrate changes in retinal structure and function. Findings suggest that while the vascular growth process could repair itself while still subjected to a hyperoxic environment (from P0-12 and P0-14, for example), irreversible changes in retinal ultrastructure and function could still be evidenced, and are documented immediately following the cessation of the hyperoxic regimen (**Dorfman et al., 2008**).

It has been suggested that free radicals may be the source of the pathologic effects described above. Given that trolox C, a water-soluble analogue of vitamin E, was previously shown to limit the vasculopathy component of OIR, the aim of our next study was to determine whether it could prevent the functional and structural consequences of OIR. While trolox C could limit the damage

intrinsic to the rat model of OIR, it could not entirely prevent it from occurring. This suggests that either OIR is not solely caused by the action of free radicals or that trolox C is inadequate in treating all aspects of OIR (**Dorfman et al., 2006**).

We next sought to compare our findings using trolox C with those obtained using the pigmented Long Evans (LE) rat in which melanin is thought to play an antioxidant role. Our results show that melanin did not exert the predicted protective effect on retinal structure and function, where LE rats were affected earlier on and more drastically than albino Sprague Dawley rats following hyperoxic exposure (**Dorfman et al., 2009**). Specific markers for rod and cone bipolar cells (PKC- α and recoverin, respectively), amacrine cells (parvalbumin) and horizontal cells (calbindin) reveal that OIR is primarily an inner retinal disorder, the earliest consequences of which include cell death in the inner retina (TUNEL staining) and synaptic retraction in the OPL (synaptophysin) (**Dorfman et al., in preparation**).

Collectively, our results suggest that the rat model of OIR is not only useful for studying retinal vasculature, but can also be used to further elucidate the functional and structural sequelae that likely resemble the human form of ROP. Our ability to demonstrate persistent functional and cytoarchitectural damage despite vascular repair would suggest the importance of examining beyond the fundus in ROP patients, should these features also apply to humans. ROP is one of several types of proliferative retinopathies characterized by retinal ischemia followed by abnormal vessel growth. Studies such as these will undoubtedly be instrumental in a better understanding of these types of retinopathies and in the derivation of new therapeutic avenues aimed at controlling them.

2 Résumé

La rétinopathie du prématuré (RDP) est une maladie rétinienne qui potentiellement aboutie à la cécité des enfants de faible poids nés prématurément et qui est causée par la combinaison de l’immaturité de la rétine et du niveau élevé d’oxygène (hyperoxie) essentiel pour garder ces nouveau-nés en vie. Afin de mieux saisir l’effet de l’hyperoxie postnatale chez ces bébés prématurés, un modèle animal de rétinopathie induit par l’oxygène (RIO) a été développé chez le rat et a démontré une forte ressemblance à la forme humaine de la RDP, du moins dans sa composante vasculaire. Notre but était d’examiner au-delà de la vascularisation rétinienne afin de mieux comprendre les manifestations fonctionnelles [électrorétinogramme (ERG)] et cytoarchitecturelles (histologie et immunohistochimie) de cette maladie.

L’évaluation antérieure de la fonction et de la structure rétinienne chez les rats albinos matures (par exemple à 30 et 60 jours postnatals) de la RIO a uniquement permis de déduire la chaîne d’évènements menant à ces changements. Par conséquent, la première étude consistait à caractériser les signes hâtifs de la RIO, pour: 1 – évaluer la vascularisation rétinienne durant l’exposition et, 2 – savoir quand, au plus tôt, après l’exposition les changements de structure et de fonction peuvent être observés. Les résultats suggèrent que la croissance vasculaire pourrait se restaurer durant le stress hyperoxique (expositions de P0-12 et de P0-14, par exemple), malgré la présence des changements irréversibles de l’ultrastructure rétinienne et sa fonction survenant immédiatement après la fin du régime hyperoxique (**Dorfman et al., 2008**).

Les radicaux libres ont été suggérés d’être à l’origine des effets pathologiques décrits ci-dessus. Étant donné que trolox C, un analogue hydrosoluble de la vitamine E, a été démontré de limiter la composante vasculaire de la RIO, le but de l’étude suivante était de voir s’il pouvait aussi prévenir les séquelles fonctionnelles et structurelles. Nos résultats montrent que trolox C représente une alternative thérapeutique valide limitant les dommages, mais il

n'empêche pas la totalité des dégâts rétiens causés par l'oxygène. Ceci suggère que la RIO n'est pas juste provoquée par l'action des radicaux libres ou que le trolox C est inadéquat pour traiter tous les aspects de la RIO (**Dorfman et al., 2006**).

Nous avons ensuite comparé nos résultats de trolox C à ceux obtenus avec le rat pigmenté Long Evans (LE) dans lequel la mélanine est présumée de jouer un rôle antioxydant. Nos données montrent que la mélanine n'exerce pas l'effet protecteur prévu sur la structure et la fonction de la RIO, où les rats LE ont été affectés plus tôt et plus gravement que les rats albinos Sprague Dawley suivant l'exposition à l'oxygène (**Dorfman et al., 2009**). Le marquage spécifique pour les cellules bipolaires de bâtonnet et de cône (PKC- α et recoverin, respectivement), les cellules amacrine (parvalbumine) et les cellules horizontales (calbindine) indiquent que la RIO est une maladie de la rétine interne définie par une mort cellulaire à ce niveau (TUNEL) et par la rétraction synaptique dans la couche plexiforme externe (synaptophysine) (**Dorfman et al., manuscrit en préparation**).

En conclusion, nos résultats suggèrent que le modèle de rat de la RIO est non seulement utile pour étudier la vascularisation rétinienne, mais peut aussi être employé pour élucider les conséquences fonctionnelles et structurelles pouvant ressembler au modèle humain de la RDP. Notre capacité de démontrer les dégâts fonctionnels et cytoarchitecturels persistants en dépit de la réparation vasculaire suggérerait l'importance de l'examen approfondi chez les patients avec la RDP. La RDP est une forme des nombreuses rétinopathies prolifératives. Ces genres d'études seront certainement utiles pour une meilleure caractérisation de ces types de rétinopathies et dans la création de nouvelles méthodes thérapeutiques visant à mieux les contrôler.

CHAPTER 1: GENERAL INTRODUCTION

1 The retina

In the embryonic development of vertebrates, the retina and optic nerve extend as outgrowths of the forebrain and are therefore considered to be part of the central nervous system (Kolb 2003, Dowling et al. 1987). The retina is a complex, layered structure with several layers of neurons that are interconnected by two layers of synaptic connections (Kolb 1994). Not only does the retina include light sensitive neurons, but also the neural circuitry that is involved in the initial processing of images that are transmitted to the brain (Kolb 2003). Many of the specific retinal cell types are common among different mammalian species (Peichl et al. 1998, MacNeil and Masland 1998, Boycott and Wassle 1991, Wassle and Boycott 1991) with similar retinal organization into three layers of neurons and two areas of neuropil (Kolb 1994).

Histological techniques have been instrumental in better understanding the populations of different cell types. In fact, some of Ramón y Cajal's studies were focused on sorting the retinal neurons into functional types based on the Golgi staining technique. These early studies revealed that the shape of retinal neurons defined their connectivity and arborization within the retinal sublayers. More recent methods, including electron microscopy, microelectrode recording techniques, and pharmacology have allowed for greater insight into retinal circuitry (Kolb 1994).

1.1 Retinal cytoarchitecture

The retina is a thin tissue that lines the back of the eye between the choroid and vitreous, and spans a thickness of approximately half a millimeter (Kolb 2003). The vertebrate retina is made up of five neuronal cell types in addition to one glial cell type. The retinal cell bodies are found in three nuclear layers, while the synapses lie in the outer and inner plexiform layers (Dowling 1970). The

retinal pigment epithelium (RPE) is bordered by the choroid on one side and by the photoreceptor layer (PL) on the other. The photoreceptor layer includes both the outer segments (OS) and inner segments (IS) of the photoreceptors, while their nuclei lay in the outer nuclear layer (ONL). Following this is the first synaptic layer known as the outer plexiform layer (OPL), the inner nuclear layer (INL), the second synaptic layer known as the inner plexiform layer (IPL) and the ganglion cell layer (GCL). A schematic representation of the retina is shown in figure 1.

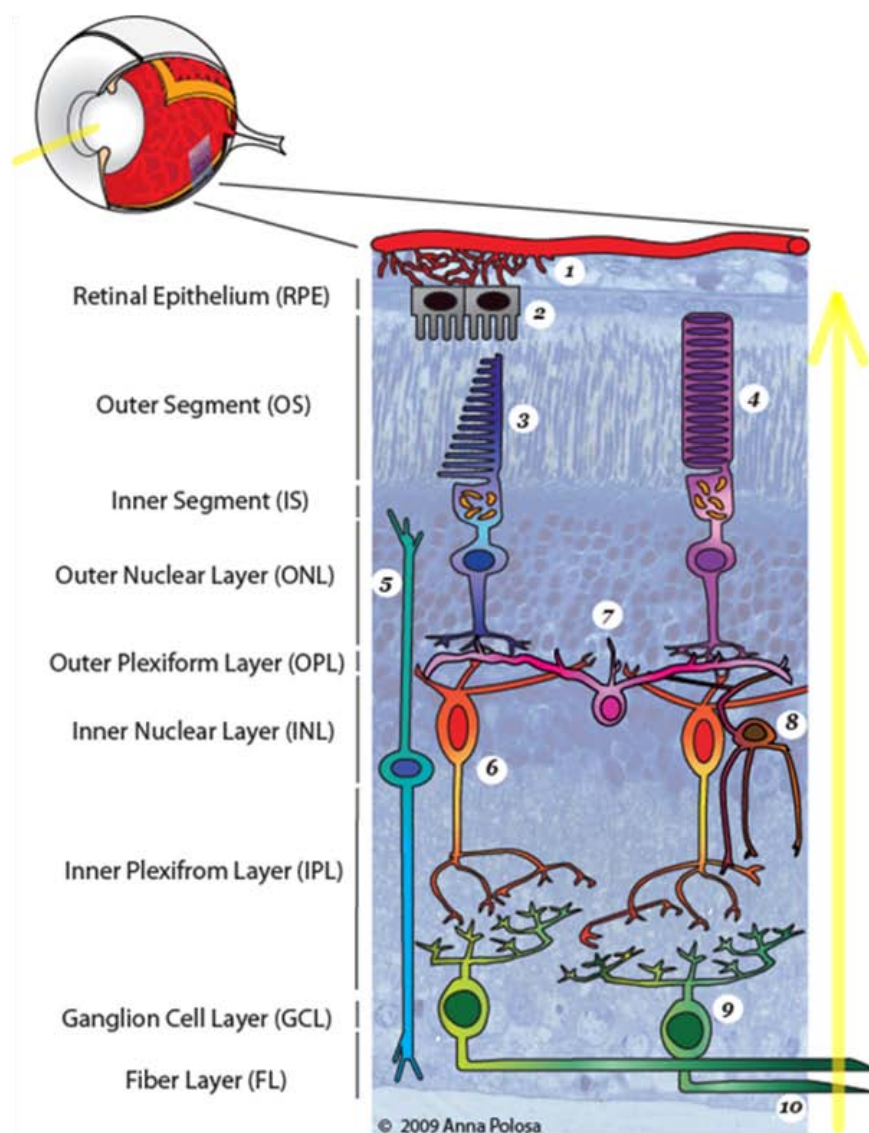


Figure 1: Schematic representation of the retinal structure. Retinal pigment epithelium (RPE), outer segments (OS) and inner segments (IS) of the photoreceptors, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), fiber layer (FL). Choroidal vessels (1), RPE cell (2), cone photoreceptor (3), rod photoreceptor (4), müller cell (5), bipolar cell (6), horizontal cell (7), amacrine cell (8), ganglion cell (9), optic nerve (10).

1.1.1 Retinal pigment epithelium (RPE)

The RPE lies adjacent to the neurosensory retina and is made up of a monolayer of cuboidal/columnar epithelial cells that extends from the optic nerve head to the ora serrata (Forrester et al. 2002). While the RPE is bordered on one side by the choroid, its long apical microvilli envelop the light sensitive outer segments of the photoreceptors on the other (Strauss 2005). The RPE plays an important role in nourishing the retinal cells and maintaining the normal visual process. For example, not only does the RPE secrete factors that benefit photoreceptor survival, but specific genes that are involved in melanogenesis in the RPE have also been shown to encourage retinal development (Jeffrey 1998).

Some of the specific functions of the RPE include 1- providing a selectively permeable barrier between the neural retina and choroid, 2- phagocytosis of shed outer segments of photoreceptors, 3- transport and storage of water, ions, metabolites and vitamins, 4- absorption of light and reduction of light scatter (Marmorstein 2001, Rizzolo 1997, Bok 1993, Steinberg 1985).

1.1.2 Photoreceptor layer (PL)

Situated in the outermost retina, the photoreceptors are the most abundant of the neuronal cells, with approximately 120 million rods and 5 million cones in the human retina. Vision in dim light is achieved through the rods which are more sensitive to light, while vision in a brighter environment, in addition to color vision, is achieved through the cones (Peichl 2005). Both rods and cones consist of an OS and inner segment IS, respectively, and their nuclei form the ONL. The OS is specialized for photon signal detection, while the IS contains the nucleus and other cellular organelles, including the mitochondria, golgi apparatus and

smooth endoplasmic reticulum, for example, that are required for cell metabolism (Kawamura et al. 2008).

Interestingly, the morphology of rod and cone outer segments is different. For instance, the visual pigment rhodopsin is embedded in stacks of disk membranes that are surrounded by a plasma membrane in rods, while the plasma membrane invaginates repeatedly to form a tightly stacked lamellar structure that is lined with visual pigment molecules, known as opsins, in cones (Kolb 2003, Baylor 1987, Cohen 1968). Since the photoreceptors lie adjacent to the RPE at the back of the eye, light must consequently travel through all of the retinal layers to reach their outer segments where photon absorption occurs.

Studies across all of the major vertebrate groups have established that there are spectrally distinct classes of cone pigments encoded by distinct opsin gene families in addition to the rod class of pigment (Bowmaker 2008), while there is only one type of rod visual pigment (Peichl 2005). Rods contain the rhodopsin visual pigment and are sensitive to blue-green wavelength of light with peak sensitivity at approximately 500 nm. The 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 wavelength sensitivity of L-cones, M-cones and S-cones peaks at 564nm, at 533nm and at 437nm, respectively (Kolb 2003, Dowling 1987).

The retinas of all mammals contain both rods and cones (Peichl 2005). Of interest, adaptations to a predominantly nocturnal or diurnal lifestyle are seen in different species. For example, nocturnal species have between 0.5% and 3% cones among their photoreceptors, crepuscular and arrhythmic species have between 2% and 10% cones, and diurnal mammals show a larger range that varies between 8% to 95% cones (Ahnelt and Kolb, 2000; Peichl et al. 2000).

1.1.3 Outer plexiform layer (OPL)

The OPL is the site of the first stage of information processing in the retina where the photoreceptors, bipolar cells (BC), and horizontal cells (HC) interact (Lukasiewicz 2005). This is the synaptic layer where connections between rod spherules and cone pedicles contact the processes of the vertically oriented BCs and horizontally oriented HCs which form a triad (composed of one BC and two HCs) (Kolb 1994). Rods and cones are distinguishable in the OPL based on their synaptic terminals. For example, rod spherules contain a single synaptic ribbon and a single complex of invaginated profiles where communication with BCs and HCs occurs (Kolb 1994), whereas cone pedicles have larger synaptic terminals that contain many synaptic ribbons and invaginated profiles for contact with second order neurons (Dowling and Boycott 1966). The wide spread of HC processes are thought to mediate lateral interaction in the OPL, while the bipolar cells are responsible for relaying visual information vertically from the OPL to the IPL (Dowling 1970).

Finally, a pathway that allows for crosstalk between cones and rods in the human retina is also known to exist through the telodendria on cone pedicles. These projections meet adjacent rod spherules and cone pedicles where they connect through gap junctions (Raviola and Gilula 1975).

1.1.4 Inner nuclear layer (INL)

The INL is made up of four distinct cell types that are closely packed together, but whose cell bodies lie in different stratifications. These cell types include HCs, BCs, amacrine cells (ACs) and müller cells (MCs). The HCs lie in the outermost margin of the INL and have cell bodies that take on a flattened appearance, while the ACs are found in the innermost part of this layer (Kolb

2003, Rodieck 1998, Dowling 1970). The nuclei of BCs and MCs are found in the distal half and the innermost area of the INL, respectively. Each of these cell types are described in further detail below.

1.1.4.1 Horizontal cells (HC)

HC processes extend outward to the OPL to synapse with photoreceptor terminals, while their cell bodies lay in the INL. HI, HII and HIII are three morphologically distinct HCs that have been described in the mammalian retina (Kolb 1994). While HI cells are known to synapse with both rods and cones, HII cells only synapse with cones. Rods synapse with at least two HCs, while cones synapse with three or four of each type of HC (Forrester et al. 2002). There is extensive overlap among HCs, which as a group play an important role in integrating and regulating input from photoreceptors. The release of γ -aminobutyric acid (GABA) from HCs plays an integral role in modulating the response by providing inhibitory feedback to the photoreceptors or inhibitory feed forward to BCs. The selectivity of HCs towards specific cone types is still debated. For example, it was suggested by Boycott and Wassle (1999) that HI and HII cells contacted S, M and L cones equally, while Anhalt and Kolb (2000) suggested that while HI cells were not selective towards any cone in particular, HII cells preferentially contacted S-cones. Their arrangement together with the ON-center and OFF-center BCs that receive input from the cones constitutes a form of lateral inhibition (Kolb 2003, Kolb 1994).

1.1.4.2 Bipolar cells (BC)

Different morphological subtypes of BCs exist in the retina, and various functions can be attributed to each of them (Forrester et al. 2002). While the cell

bodies of BCs lie in the INL, their single or multiple dendrites extend to synapse with photoreceptors while their single axon runs inward to synapse with ganglion cells (GCs), thus passing the information between these cells.

Studies using Golgi staining techniques have identified between nine and ten different morphological subtypes of bipolar cells in humans, where only one type is classified as a rod bipolar cell with the remaining classified as cone BCs (Kolb et al. 1992, Wassle and Boycott 1991). Cone BCs are further subdivided into diffuse cone BCs and midget BCs, depending on their shape, arborization and stratification in the IPL. Not only do the dendritic terminals of rod BCs synapse with a rod spherule as the central invaginating dendrite, but they also synapse with AII ACs (Kolb 1970). Diffuse cone BCs receive information from several different cones, as their dendrites extend into multiple cone pedicles. On the other hand, midget bipolar cells are a group of cells that can be further subdivided into invaginating midget bipolar (IMB) cells, flat midget bipolar (FMB) cells and blue cone-specific bipolar (BB) cells (Forrester et al. 2002, Kolb 1970). IMB cells synapse with the cone pedicle as central invaginating dendrites at ribbon synapses, while FMB cells synapse with the cone pedicle through semi-invaginating wide-cleft basal junctions (Forrester et al. 2002, Kolb 1970). Finally, BB cells have been identified in humans and primates (Kolb et al. 1992) and have been shown to make contact with an individual cone through several dendrites that converge on its pedicle.

Generally, rods synapse with metabotropic glutamate receptors, specifically metabotropic glutamate receptor-6 (mGluR6), found on ON-depolarizing bipolar cells (ON-DBC) only, while cones synapse with either mGluR6 receptors on ON-DBC, or with Amino-3-hydroxy-5-Methyl-4-isoxazolPropionic Acid (AMPA)-kainate receptors on OFF-hyperpolarizing bipolar cells (OFF-HBC) (Kolb 2003, Dhingra et al. 2000). This concept will be described in greater detail in the sections that follow on retinal function.

1.1.4.3 Amacrine cells (AC)

Approximately 40 different types of ACs have been identified through Golgi staining, and are very diverse with respect to morphology and function.

ACs are located in the inner portion of the inner nuclear layer and are easily distinguishable from the other cell types that reside there based on their large size and shape (Forrester et al. 2002). They are mostly divided through their different dendritic stratifications in the inner plexiform layer where they work laterally to affect the output of BCs. ACs can be classified as diffuse, starburst or stratified, for example (Kolb et al. 1992, Mariani 1990) and play an important role in modulating the signals that eventually reach the GCs. While not all ACs have been thoroughly described, a number of them have been characterized with respect to their neurotransmitters which include acetylcholine, dopamine, serotonin, GABA (A17, for example) and glycine (AII, for example) (Kolb 1994). The AII cell plays a particularly important role in transmitting information from rod ON-DBCs to cone bipolar circuitry from which it will eventually reach the GCs (Kolb 2003).

1.1.4.4 Müller cells (MC)

MCs are the main glial cells found in the retina, which stretch across the retina from the outer to the inner limiting membrane while their nuclei lie in the INL. The surface of the MC facing the pigment epithelium and subretinal space is expanded by many projections of the many apical villi projections which lie adjacent to the RPE, while their conical endfeet or “foot processes” are found adjacent to the inner limiting membrane. The numerous roles of the MC include protein synthesis, intracellular transport, management of neural waste, re-uptake of glutamate and structural support to the retina. MCs are also known to play a

role in regulating the ionic environment of neurons by taking up extracellular potassium and redistributing it within the retina (Newman and Reichenbach 1996).

Interestingly, the glycogen, mitochondria and intermediate filaments found in MCs are immunoreactive for glial fibrillary acidic protein (GFAP), which is known to be significantly upregulated throughout the cell following insult (Lewis et al. 1995, Guérin et al. 1990). Moreover, MCs have also been found to upregulate neurotrophic factors including fibroblast growth factor-2 (FGF-2) and ciliary neurotrophic factor (CNTF), for example, following exposure to environmental stresses such as bright light (Valter et al. 2005) and following mechanical stress (Cao et al. 2001, Wen et al. 1995).

1.1.5 Inner plexiform layer (IPL)

GCs receive synaptic inputs from presynaptic BCs and ACs in the IPL. The IPL is divided into two sublaminae, namely sublamina *a* (distal IPL) and sublamina *b* (proximal IPL), in order to separate information from the ON and OFF pathways from the GCs to the brain (Kolb et al. 2003, 2001, 1994). OFF-GC and ON-GC dendrites branch into sublamina *a* and *b* of the IPL, respectively. Certain ACs also communicate with BCs in the IPL by electrical synapses known as gap junctions (Kolb 1994).

1.1.6 Ganglion cells (GC)

The cell bodies of the GCs form the innermost nuclear layer of the retina. The GCs are the final link in the transmission and fine tuning of information from the retina to the brain (Rodieck 1998), and their axons which form the optic nerve

synapse with cells in the lateral geniculate nucleus (LGN) in the thalamus (Forrester et al. 2002). GCs can be differentiated both functionally and morphologically. For example, each type is particularly specialized for either the detection of contrast, color or spatial resolution, respectively. Their morphological diversity has resulted in classification of approximately 25 GC types in the mammalian retina (Forrester et al. 2002, Kolb 1992).

Some of the GC types include the midget and diffuse, respectively. Midget ganglion cells, which are also referred to as P-cells given that they project to the parvocellular layer of the LGN, are smaller in size and receive synapses from ACs and a midget BC. They are known to specifically relay information regarding color perception and shape (Kolb 1991a, 1991b). On the other hand, diffuse GCs, which are also referred to as M-cells given that they project to the magnocellular layer of the LGN, are larger in size and have a larger synaptic field with a variety of BC types. These cells are known to relay information about movement.

Interestingly, a rare subtype (approximately 1-3%) of GCs synthesizes the melanopsin photopigment which enables them to be directly activated by light. In addition to the rods and cones, these intrinsically photosensitive GCs make up a third class of photoreceptors in the mammalian retina. These cells have been shown to play a role in circadian rhythm and pupillary light reflex, for example (Fu et al. 2005, Berson et al. 2002).

2 Light induced electrical events and retinal physiology

2.1 Phototransduction

Phototransduction is the process by which a single photon is converted into an electrical signal, and while the cascade has been well described for rods, it is not as well understood in cones or photosensitive GCs of the retina.

Similar to other cell types, a potential difference exists along the surface of photoreceptors resulting in a more negative intracellular than extracellular environment due to the selective permeability of the membrane to ions. For example, a sodium-potassium ($\text{Na}^+\text{-K}^+$) exchange pump that is powered by adenosine triphosphate (ATP) is found in the IS, and it ensures that the intracellular concentration of K^+ ions is high (and low extracellularly), while the extracellular concentration of Na^+ is high (and low intracellularly). In dark conditions, a “dark current” exists, where channels in the outer segment of the photoreceptors allow for the inward flow of Na^+ ions, and where channels in the inner segment allow for the outward flow of K^+ ions. This circulating current results in a depolarized state for photoreceptors in the dark.

Upon capture of a photon, the channels that are located in the outer segment that normally allow for the inward flow of Na^+ ions (due to the dark current) will shut, and consequently result in the hyperpolarization of these cells as they become more negative intracellularly than extracellularly. More specifically, the rod outer segments contain many disk membranes that are surrounded by the plasma membrane and lined predominantly with the rhodopsin protein which is made up of an opsin and a chromophore (retinal). Once the visual pigment in the rods absorbs light, a conformational change in retinal from 11-*cis* retinal to the activated all-*trans* form is induced (Ridge and Palczewski

2007). This active form of rhodopsin, known as metarhodopsin II or R^* , signals changes to and activates the transducin G-protein (Gt). This causes transducin to dissociate from its normally bound guanosine diphosphate (GDP), and bind guanosine triphosphate (GTP). The α -subunit of transducin will then dissociate from the beta and gamma subunits, while GTP is still bound to the alpha subunit. The α -subunit GTP complex will subsequently activate the phosphodiesterase (PDE) enzyme which breaks down cyclic guanosine monophosphate (cGMP) to 5'-GMP. The reduced concentration of cGMP results in the closure of sodium channels while the ongoing potassium current continues. This ultimately results in the hyperpolarization of the photoreceptors and a decrease in the amount of glutamate that is released by the cell (Arshavsky et al. 2002).

The phototransduction cascade comes to a stop once R^* is phosphorylated by rhodopsin kinase, which enables it to bind to the arrestin protein where it had been bound to Gt, thus blocking any further interaction between R^* and Gt and also facilitating the breakdown to the opsin and all-trans retinal. Subsequently, R^* becomes inactive and GTPase Activating Protein (GAP) interacts with the α -subunit of Gt and hydrolyzes GTP to GDP, thereby making it inactive (Arshavsky et al. 2002, Baylor 1996).

The RPE plays an important role in regenerating the photopigment, as this is the site of conversion of all-trans retinal to 11-cis retinal prior to moving back into the photoreceptor OS where it again becomes conjugated to an opsin to once again form a functional visual pigment. Interstitial retinol binding protein (IRBP) is known to play a role in displacing the retinal between the RPE and photoreceptor outer segment, while cellular retinoid binding protein (CRALBP) is responsible for moving the retinal through the RPE.

2.2 Retinal circuitry and ON- and OFF-pathways

Photoreceptors maintain their depolarized state in the dark while they hyperpolarize in response to light. In the dark, glutamate is released from the synaptic terminals of the photoreceptors in their depolarized state, while a light stimulus will prevent this release once they are in their hyperpolarized state. The BCs are the first association neurons to which the electrical signal is relayed by the photoreceptors. BCs are known to either hyperpolarize (OFF-HBC), or depolarize (ON-DBC) in response to light. A dark environment results in the hyperpolarization of ON-DBCs as their cGMP-gated channels close, while the OFF-HBCs depolarize in the same environment as their channels open. OFF-HBCs express AMPA/kainate receptors (Devries 2000) and the hyperpolarization of the photoreceptors is conserved at this synapse. ON-DBCs, however, express a metabotropic receptor known as mGluR6 (Nomura et al. 1994), where glutamate tends to act as an inhibitory transmitter (Snellman et al. 2008) and the light-activated hyperpolarization of photoreceptors are inverted into a depolarization (Werblin and Dowling 1969).

The GCs also have ON-OFF properties and are the next of the neurons to receive the signal; ON-bipolar cells synapse with ON-centre, OFF-surround GCs, and OFF-bipolar cells synapse with OFF-centre, ON-surround GCs.

Two parallel streams of information run directly from the cones (the ON- and OFF-pathways), where information is passed to either ON-DBCs and then to ON-GCs or to OFF-HBCs, and then to OFF-GCs, respectively. It is thought that these different pathways might provide information to the GC with respect to contrast in visual perception (Kolb 1994, Schiller 1992). ON-DBCs along with ACs and ON-GCs synapse in the proximal part of the IPL, while cells that are involved in the OFF-pathway synapse in the distal part of the IPL.

In contrast to cones, the rods only transmit information through the ON-pathway. Rods synapse with ON-DBCs, which then synapse with AII ACs prior

to delivering the information to the GCs which generate action potentials in response to the graded changes in membrane voltage that occur in the retinal cells. The GC axons form the optic nerve which converges upon the chiasm, forms the optic tract, and terminates predominantly in the LGN and finally the visual cortex.

2.3 The electroretinogram (ERG)

The ERG is an evoked potential which measures the mass electrical response produced by the retina to a light stimulus. Electroretinography has proven to be a very accessible method to study retinal function, and recordings can be obtained in a very non-invasive manner. Briefly, the full-field ERG can be recorded with an active electrode on the corneal surface with a reference electrode placed elsewhere, such as the external canthus in humans, or the mouth in rodents. Following stimulation with a light source, a graphical representation of potential changes across the eye is obtained (Cameron 2008). The ERG has become a technique that is widely used both in the clinic as a diagnostic tool as well as for research purposes in humans and animals in order to better document the physiological integrity of the retina.

2.3.1 History of the ERG

In 1865, Holmgren showed that a light stimulus could provoke a change in the electrical potential of the amphibian eye (Holmgren 1865), a finding that was corroborated thereafter by Deward (1877) who revealed that a similar result could be obtained in the human eye when recorded from the cornea. The morphology of the ERG was further characterized by Gotch (1903), who reported that it consisted of an initial cornea-negative deflection that was followed by a larger cornea-positive waveform of greater amplitude, while Einthoven and Jolly (1908) had

also identified an additional slower positive wave. Einthoven and Jolly (1908) named these three waveforms the a, b and c waves, respectively, nomenclature which is still used to describe them today. Furthermore, fast oscillations of higher frequency on the ascending limb of the b-wave were first reported by Cobb and Morton (1954). These were later referred to as oscillatory potentials by Yonemura et al. (1962). Finally, in conditions where a longer flash duration is used, the d-wave is an additional corneal-positive wave that is recorded at the termination of the light flash. For example, the earlier waveforms are recorded at the onset of the light stimulus and therefore are considered components of the ON-potential, while the d-wave is recorded at the offset of the stimulus, thereby contributing to the OFF-potential. Flash intensity, rod-cone ratio or the methods used to obtain the recordings are all factors that can contribute to differences in ERGs among species.

The basis for much of our understanding of the ERG comes from studies in an anaesthetized cat, where Granit (1933) observed the gradual removal of the different ERG components as the level of anesthesia was deepened. Granit termed the different processes (P) in sequence of their disappearance: P-I, P-II and P-III which respectively describe 1) a slow cornea-positive wave (P-I), 2) a corneal-positive wave that rises quickly and then drops to an intermediate potential while the light stimulus is on (P-II) and 3) a cornea-negative wave that develops the fastest and remains that way while the light stimulus is on (P-III) (Granit 1933). These components account for the c-wave, b-wave and a-wave of the ERG, respectively.

2.3.2 Components of the ERG

While the ERG indeed represents the integrated activity of the retinal cells, several components that comprise it can be attributed to specific steps in the transmission of visual information.

2.3.2.1 a-wave

The a-wave is the early negative deflection found in the ERG and is thought to be generated by the hyperpolarization of the rod and cone photoreceptors following stimulation by light (Penn and Hagins 1969, Brown 1968). Interestingly, however, other studies have also confirmed that the a-wave might not solely be generated by photoreceptors alone. For example, Tomita (1950) reported that the a-wave is a composite of potentials from different retinal layers in the amphibian retina, findings which were corroborated by Murakami and Kaneko (1966) from in vitro studies conducted in the frog and turtle retinas which revealed that the a-wave originated postsynaptically to the photoreceptor layer. Furthermore, Bush and Sieving (1994) explored the contribution of OFF-HBCs and HCs to the ERG by using cis-2,3-piperidine-dicarboxylic acid (PDA) to block synaptic transmission between photoreceptors and second order neurons in the primate. Their finding of a significantly attenuated a-wave also supports that neuronal activity postsynaptic to photoreceptors contributes, at least in part, to the generation of the a-wave.

2.3.2.2 b-wave

The b-wave is a positive deflection that follows the a-wave and, though its origin is still debated, is thought to reflect BC and MC activities. Intra-retinal recordings carried out by Brown and Wiesel (1961) suggested that the maximal b-wave amplitude was reached at the level of the INL. Furthermore, the abolition of the b-wave that results following administration of 2-amino-4-phosphonobutyric acid (APB), a glutamate analogue which selectively blocks the ON-DBC response (Slaughter and Miller 1981), further strengthens the role of BCs in the generation of the b-wave (Stockton and Slaughter 1989).

As mentioned above, MCs have also been implicated in the generation of the b-wave. It was suggested, based on Current Source Density analysis, that the current sources and sinks related to the b-wave were likely due to ionic fluxes across the MC membrane (Faber 1969). Using intracellular recording techniques, Miller and Dowling (1970) reported that the latency and the time course of the MC light response are very similar to the b-wave. MCs are thought to depolarize when the extracellular K^+ concentration increases following the depolarization of retinal neurons, as their cell membrane is selectively and highly permeable to K^+ (Newman 1985, 1980).

Of interest, DL- α -amino adipate, a MC toxin, has also been shown to result in attenuation of the b-wave while other components such as the a-wave remain intact, thus further strengthening its role in the generation of the b-wave (Bonaventure 1981, Szamier 1981).

2.3.2.3 Oscillatory potentials (OPs)

The low-voltage, high frequency wavelets found on the ascending limb of the ERG b-wave are referred to as OPs. While the ERG is recorded between 1 Hz (low cutoff) and somewhere >300 Hz (high cutoff), the OPs can be extracted by raising the low cutoff to between 70 and 100 Hz (Marmor and Zrenner 1998, Wachtmeister 1998).

While the origin of the OPs is controversial, clinical and experimental evidence points to a post-receptoral location in the inner retina, such as the BCs, ACs and interplexform cells (Wachtmeister 1998). Wachtmeister and Dowling's studies in the mud puppy retina revealed that each OP was generated at a different retinal depth, where short-latency and long-latency OPs were generated in the proximal and the distal retina, respectively (Wachtmeister and Dowling 1978). Over time, it has become increasingly apparent that the GCs and MCs were not likely candidates as OP generators. For example, OPs remained unaffected

following the administration of tetrodotoxin which is known to block the GC action potentials, thus suggesting that they are unlikely to participate in OP generation (Ogden 1973). Furthermore, intra-retinal recordings carried out in the mudpuppy by Miller and Dowling (1970) revealed that Muller cells do not produce a response resembling that of OPs, thereby also making them an unlikely candidate.

While some have suggested that the formation of b-wave and OPs occurs independently from one another (Hamaski and Maguire 1985, Speros and Price 1981, Yonemura et al. 1962), the concept that the b-wave and OPs are more closely linked has also been put forth, as evidenced with clinical data from patients with Congenital Stationary Night Blindness (CSNB) and Cone Dystrophy. More specifically, the photopic ERG of CSNB patients is characterized by a square shaped a-wave and a truncated b-wave that results from the absence of the rising phase of the b-wave, along with an absence of OP₂ and OP₃, while OP₄ remains intact. In contrast, the Cone Dystrophy tracing, for example, is characterized by an attenuation in the latter part of the b-wave in addition to an attenuation and delay in OP₃ and OP₄, with a relatively intact OP₂ (Lachapelle et al. 1998). These findings would therefore suggest a close relationship between the genesis of the b-wave and that of the OPs, where each individual OP would actually contribute to a step in the development of the b-wave (Lachapelle et al. 1998). The results described above are corroborated by experimental evidence that revealed significant attenuation of both the b-wave and OPs following intravitreal injection of iodoacetic acid, APB and glycine (Lachapelle et al. 1990, Guité and Lachapelle 1990, Matthews et al. 1989).

2.3.2.4 Intensity-response function of the ERG

As the intensity of the flash stimulus is progressively increased from dim levels to brighter levels in dark-adapted (scotopic) conditions, the b-wave

amplitude will also progressively increase. The maximal rod response that is obtained to the exclusion of cone activity occurs at $2.4 \log \text{ cd} \cdot \text{sec} \cdot \text{m}^{-2}$ and is referred to as the rod Vmax (Naka and Rushton 1966). Any subsequent increase in flash intensity will generate further increases in the b-wave amplitude and the participation of both rod and cone pathways. Furthermore, the a-wave can only be obtained following stimulation with a flash intensity that is bright enough to elicit a mixed rod-cone response, usually $> 2.4 \log \text{ cd} \cdot \text{sec} \cdot \text{m}^{-2}$.

On the other hand, the light adapted (photopic) response is a pure cone response. In contrast to humans, mice and guinea pigs, for example, the light adapted (photopic) response obtained in rats is composed uniquely of a b-wave while the a-wave is absent (Racine et al. 2005, Rosolen et al. 2004).

3 Retinopathy of Prematurity (ROP)

3.1 History of ROP

ROP is a potentially blinding retinal disorder resulting from the exposure of premature infants to postnatal hyperoxia and has been characterized by the occurrence of two notable epidemics over the past 60 years. ROP, referred to earlier on as retrolental fibroplasia (RLF), was first observed clinically in 1942 by Terry in both eyes of a number of children born prematurely by approximately 8 weeks, and was characterized by a persistent fibroplastic sheath behind the crystalline lens (Terry 1942). Shortly thereafter, RLF was found to be associated with excessive oxygen use, and consequently, supplemental oxygen delivery to premature infants became very closely monitored (Patz et al. 1952, Crosse and Evans 1952, Campbell 1951). These clinical modifications resulted in a sharp decrease in the incidence of the disease, where the prevalence of blindness due to

RLF fell from 50% in 1950 to 4% in 1965 in the United States alone (Hatfield 1972). However, tight restrictions on oxygen use unfortunately led to an increase in morbidity and mortality due to respiratory distress and brain disease which were found to accompany the cessation of this first RLF epidemic (McDonald 2001, Avery and Oppenheimer 1960). Interestingly, despite increased control and monitoring of supplemental oxygen in preterm infants from the first wave of deaths resulting from RLF, a second epidemic nonetheless occurred in the late 1970s and 1980s. This time, however, the epidemic resulted from the increased survival of very low birth weight infants (750-999g), some of whom were born at even less than 27 weeks of gestation (Gibson et al. 1989, Phelps 1981). Despite the numerous attempts to regulate oxygen use, the number of infants with ROP has increased further, likely as a result of their increased ability to survive (Hussain et al. 1999, Phelps 1981), which therefore suggests a complex association of both oxygen-related and non-oxygen-related growth factors in generating the disease.

3.2 Vascular development of the human retina

In the human fetus, the development of the retinal vasculature normally begins within the fourth month of gestation (approximately 16 weeks) (Ashton 1970), starting at the optic disc in the posterior pole and gradually spreading peripherally as a wave of mesenchymal spindle cells towards the ora serrata. Endothelial cell proliferation and capillary formation follow the lead of these mesenchymal cells, and the new capillaries will subsequently form the mature retinal vessels. The nasal retina is completely vascularized by 36 weeks of gestation, while the temporal retina is vascularized by approximately 40 weeks of gestation (Michaelson 1948). The last 12 weeks in a normally occurring pregnancy are therefore crucial for the final stages of the developing retinal

vasculature and consequently the retina is almost completely vascularized in a baby that is born full term.

It has been suggested that retinal vascular growth proceeds from two distinct processes that occur at different times. First, an initial phase of vasculogenesis occurs that is responsible for the formation of new vessels by the transformation of vascular precursor cells from the optic nerve head, which ultimately results in a primitive vascular network. This is followed shortly after by a secondary phase of angiogenesis, where the superficial vascular plexus is expanded by the sprouting and branching of pre-existing vessels (Flynn and Chan-Ling 2006). Animal studies have suggested that the expression of vascular endothelial growth factor (VEGF) in macroglia such as astrocytes that are located at the inner surface precedes the formation of superficial retinal vessels, while that expressed by MCs located in the INL closely precedes the formation of the deep layer of retinal vessels (Stone et al. 1995). These macroglia come together to interact with various neuronal (Flynn and Chan-Ling 2006) as well as vasoformative elements. These include pericytes and smooth muscle cells, which have been implicated in vessel stabilization, limiting endothelial cell proliferation and preventing vessel regression (Hughes and Chan-Ling 2004) in order to form a retinal vasculature that meets the needs of the metabolically active tissue.

3.3 Pathogenesis of ROP

Two distinct phases of ROP have been described. Initially, the premature infant is born prior to the time at which retinal vascular development is complete, which normally occurs by the end of a full term pregnancy. This results in an incomplete retinal vasculature which would have normally occurred in utero, a loss of some of the vessels that had already fully developed and a peripheral avascular zone which largely depends on gestational age (Chen and Smith 2007, Smith 2004, 2003). Regression of existing retinal vessels, also referred to as vaso-

attenuation that is the hallmark of this first phase of ROP, is thought to result from the combined effects of a relatively hyperoxic extrauterine environment along with the supplemental oxygen that is administered to premature infants. The relatively hypoxic environment in utero, where blood is approximately 70% saturated and where the PaO_2 is 30mm Hg, helps to drive retinal vascular development, while the relatively hyperoxic extrauterine environment, where blood is 100% saturated and the PaO_2 is 60-100mmHg, makes the vascularization process rather difficult (Chen and Smith 2007, Saugstad 2006). Eventually as the infant continues to mature, the non-vascularized retina becomes increasingly metabolically active, and the tissue hypoxia that ensues resulting from the absence of sufficient vasculature marks the end of this initial phase of ROP (Chen and Smith 2007, Smith 2004, 2003).

The second phase of ROP is characterized by hyperoxia-induced retinal neovascularization (NV), where new vessels form at the interface between the vascularized retina and avascular zone. It was suggested early on by Michaelson (1948) that in the absence of sufficient oxygen levels, the increasingly metabolically active retina might release an angiogenic growth factor. Later on, this hypothesis was proven true with two studies which showed that hypoxia stimulated the production of VEGF which is currently believed to be responsible for inducing NV (Saugstad 2006, Shweiki et al. 1992, Ferrara et al. 1992). Eventually, the disorganized growth pattern which is characteristic of pathological NV, results in the development of a fibrous scar that extends from the retina throughout the vitreous and lens. It is the retraction of this scar that can lead to the separation of the retina from the RPE, ultimately resulting in blindness (Chen and Smith 2007, Saugstad 2006). In addition to the incomplete retinal vascularization and exposure of the immature retinal vessels to an abnormally high oxygen environment, other factors have also been thought to be related to the incidence of ROP including hypercapnia, acidosis, sepsis, blood transfusions, intraventricular haemorrhage, mechanical ventilation and respiratory distress syndrome (Dani et al. 2004, Hardy et al. 2000).

3.4 Predisposing factors

3.4.1 Birth weight

Although the pathogenesis of ROP is multifactorial, very low birth weight ($>1000\text{g}$ and $\leq 1500\text{g}$) and extremely low birth weight ($\leq 1000\text{g}$) of the baby as well as low gestational age have been consistently linked to the development of the disease. In fact, despite the careful monitoring of oxygen delivery since the link between its administration and ROP became apparent, as many as 30% of very low birth weight infants will progress to develop severe ROP and 8% will eventually become blind (Brown et al. 1998). Others have shown that as many as 52% of premature infants that weigh between 501-1250 grams at birth will develop the disease (Shohat et al. 1983).

3.4.2 Oxygen

The exact role that supplemental oxygen plays in the pathogenesis of ROP has been an important area of research ever since a link between the two was discovered (Campbell 1951). Whether it is the length of time over which oxygen therapy is administered or the wide fluctuations in oxygen saturation levels that affect the development and progression of ROP, is an area of great debate and controversy (Wheatley et al. 2002). The significance of excessive oxygen delivery stems from the inability of the choroidal vasculature in the newborn to autoregulate in response to increasing oxygen tensions (Hardy et al. 1996, Hardy et al. 1994, Chemtob et al. 1991) which is due to the combination of increased vasorelaxants and insufficient vasoconstrictors in neonates (Hardy et al. 1996). Subsequently, excess oxygen will move out of the choroid and into the retinal circulation, subjecting it to free radicals that it cannot easily dispose of due to the

decreased levels of free radical scavengers, including superoxide dismutase (SOD), α -tocopherol, catalase and glutathione peroxidase, for example, found in immature retinas (Huertas et al. 1998, Katz and Robinson 1988). The incompletely developed antioxidant system combined with the increased levels of polyunsaturated fatty acids (PUFAs) found in the highly metabolically active retina all render this tissue particularly vulnerable to free radical attack (Hardy et al. 2000). Furthermore, higher levels of free iron are found in newborn neural tissue in contrast to adults (Moncada et al. 1997, Stamler et al. 1992) and transition metals such as these can act as catalysts for reactions of the superoxide anion (O_2^-) with hydrogen peroxide (H_2O_2) to produce the very highly reactive hydroxyl radical ($OH\cdot$) (Hardy et al. 2000). DNA, protein and lipids are all susceptible to free radical attack, and new radicals are continuously generated. The propagation of peroxides that ensues (Hardy et al. 1996), combined with the effect of hyperoxia on the growth and maturation of spindle cells which results in gap junction formation and impaired vasculogenesis, eventually culminates in the initial vaso-obliterative phase that characterizes ROP (Hittner et al. 1984). Furthermore, the production of toxic metabolites such as malondialdehyde (MDA) and 4-hydroxynonenol (4-HNE), alkanes and alkoxyl radicals that result from metal-catalyzed Fenton reactions have also been shown to contribute to vascular permeability, edema, inflammation, cell death and ion channel dysfunction (Mylonas and Kouretas 1999, Keller et al. 1999, Comporti 1998, Esterbauer et al. 1991).

Given that free radicals appear to play a role, at least in part, in the pathogenesis of ROP, it is not surprising that clinicians and investigators have explored the possibility of antioxidant administration in preterm neonates in order to protect the retina from their deleterious effects. It was suggested that vitamin E supplementation might be beneficial to this end, especially when considering that plasma levels of this antioxidant are low in prematurely born infants (Nielson et al. 1988, Katz and Robison 1988, DeVito et al. 1986). Vitamin E levels are thought to increase with gestational age as photoreceptor outer segments lengthen

(Kretzer and Hittner 1985) and as IRBP levels increase in the subretinal space to facilitate its transport (Johnson and Kretzer 1985). It is thought that vitamin E naturally exists in the body to function as an antioxidant by quenching various free radicals as well as active oxygens such as singlet oxygen, for example.

Since retinal vascular endothelial cell membranes were thought to be affected by free radicals, it was thought that vitamin E could terminate these lipid peroxidation reactions given its ability to integrate into the lipid bilayer where they occur (Flower et al. 1984). However, the effectiveness of vitamin E supplementation in reducing the severity of ROP remains quite controversial (McColm and Fleck 2001). For example, intravenous treatment with vitamin E resulted in increased retinal and intraventricular haemorrhage without any beneficial effect on the development of ROP (Phelps et al. 1987). In addition, it has also been suggested that conflicting results may stem from the difficulty of delivering a lipid soluble molecule such as vitamin E to the immature retina (Nielsen et al. 1988). In other studies, however, a favourable effect of vitamin E on the incidence of ROP was reported (Liu et al. 2005, Johnson et al. 1995).

3.4.3 Genetic factors

While excessive use of oxygen has frequently been associated with the incidence of ROP resulting in curtailed use in the clinic, it is not the only factor involved. In fact, many premature infants that are treated with supplemental oxygen never develop ROP, while other children that are nearly full term who receive little or no treatment will eventually present with the disease (Brown et al. 1998). The factors that determine which infants will progress to retinal detachment remain unknown (Wheatley et al. 2002).

Studies have suggested that ethnicity is a significant factor which influences the epidemiology of ROP (Saunders et al. 1997, Schaffer et al. 1993) where, for example, a greater number of Caucasian infants have been shown reach

threshold ROP than African-American infants (Tadesse et al. 2002, Saunders et al. 1997). These studies have led to the conclusion that the African-Americans are protected against the development of severe ROP and that fundus pigmentation may play an instrumental role in this protection. Interestingly, however, other studies have suggested that an even greater incidence in the onset of threshold ROP exists in another darkly pigmented race, namely the Alaskan natives, when compared to that in other racial groups (Lang et al. 2005, Arnold et al. 1994). Evidently, the influence of race on the rate and severity of ROP is an area that requires further clarification, though it would seem that genetic, socioeconomic and possibly dietary factors might be involved (Saunders et al. 1997, Arnold et al. 1994).

Interestingly, the ocular characteristics that are observed in ROP patients, namely retinal folds, traction and detachment, are very similar clinical manifestations as those found in other diseases, including familial exudative vitreoretinopathy (FEVR) and Norrie Disease (ND) (Wheatley et al. 2002, McColm and Fleck 2001, Haider et al. 2001).

While the exact function of the Norrie Disease Protein (NDP), norrin, remains unclear, it is thought to be involved in the signalling cascade that is responsible for vascular development and maintenance of the inner ear and retina by providing the recognition signals for targeting neuronal and retinal connections and angiogenesis in these developing structures (Dickinson et al. 2006, Haider et al. 2001). While ND and FEVR are both X-linked disorders that occur in full term infants, the retinal changes that accompany them, which include abnormal vascularization, macular folds and retinal detachment, bear a striking resemblance to those observed in severe cases of ROP. Polymorphisms of the NDP gene have reportedly been found in patients with FEVR and a small population of ROP patients (Dickinson et al. 2006, Haider et al. 2001). The use of transgenic mice with specific defects in the Norrie gene will help to further elucidate the link between Norrie Disease and ROP.

3.4.4 Pigmentation

Studies have suggested that melanin pigmentation offers protection against the development of severe ROP, where African-American premature infants were less likely to develop the disease compared to other racial subgroups, thus suggesting that fundus pigmentation may play an instrumental role in this protection (Saunders et al. 1997, Monos et al. 1996). On the other hand, other studies have revealed that an even greater incidence in the onset of threshold ROP exists in another darkly pigmented race, namely native Alaskans, when compared with that in other racial groups (Lang et al. 2005, Arnold et al. 1994). Previous studies that compared pigmented and albino rats have shown that the former are less susceptible to ischemic retinal injury than are the latter (Safa and Osborne 2000), whereas others have demonstrated that certain pigmented strains develop increased vascular permeability and more severe retinal NV than albino rats (Gao et al. 2002, Zhang et al. 2001). Of interest, melanin is produced and stored in the retinal pigment epithelium (RPE) cytoplasm and melanocytes, and continuous exposure of the RPE to environmental stressors such as light and oxygen have been thought to dampen its antioxidant capacity (Hu et al. 2002). Furthermore, melanin has been known to take on the role of a pro-oxidant under these conditions, leading to cytotoxic activity associated with photoreceptor damage as well as age-related macular degeneration (Sarna et al. 2003, Sarna 1992). Further elucidation of the role of pigmentation and/or genetics is warranted in order to better understand these discrepancies which pertain to both humans and animal models.

3.5 Regression of ROP

ROP cases that are classified as stages 1 and 2 will most often undergo full regression. The rate of regression normally occurs as a function of the severity of

the lesion. Once the disease fails to progress, the ridge will begin to dissolve and may change from a pink to white colour, and the peripheral retina will become vascularized as the vessels begin to grow through the thinning ridge (Cryotherapy for Retinopathy of Prematurity Group 1991). However, cicatricial changes can occur in cases where the peripheral retina fails to fully revascularize. Areas of retinal avascularities, abnormal branching of vessels as well as increased or decreased pigmentation may be observed and retinal folds and detachment may also develop as complications of regressed ROP (An International Committee for the Classification of Retinopathy of Prematurity 2005).

Interestingly, however, despite the fact that ROP is considered a disease of the developing retinal vasculature and that retinal vascular repair indeed occurs in some cases, functional sequelae have been shown to persist nonetheless. For example, in patients with a history of ROP, significant abnormalities in the a- and b-waves of the ERG have been shown to persist into adulthood (Fulton et al. 2001, 1996, 1995a). Furthermore, elevations of the scotopic visual thresholds in children with a history of resolved, mild ROP are also suggestive of photoreceptor involvement (Hansen and Fulton 2000 and Reisner et al. 1997). Finally, significant deficits in amplitude and implicit time of the multifocal ERG (mfERG) response, which is a central retinal response, have also been shown to occur in subjects whose clinical ROP had completely resolved more than 10 to 20 years earlier (Fulton et al. 2005). These results would suggest that a history of ROP can alter central retinal development and therefore the most central responses of the mfERG. Given that ON- and OFF-BCs contribute to this central response, the attenuation in amplitude might therefore occur should ROP specifically target these cells and thereby cause for an altered summation of depolarization and hyperpolarization in these subjects (Fulton et al. 2005). Therefore, despite the resolution of vascular abnormalities which is a common occurrence in ROP patients, it is necessary to consider that neural dysfunction of the retina may still carry on throughout adulthood.

It is important to note that while ROP is the ophthalmic condition most often associated with preterm birth, a number of other visual manifestations can result from the challenges that prematurity imposes on the developing ocular system (O'Connor et al. 2007). These can include an increased incidence in refractive errors including myopia, astigmatism and anisometropia, as well as an increased incidence in strabismus. Aside from the effects of preterm birth on eye growth and movement, visual functions including visual acuity, contrast sensitivity, colour vision and visual fields may also be compromised, thereby significantly impacting psychological and educational development (O'Connor et al. 2007).

The advancement of neonatal technologies has resulted in the increased survival of extremely premature infants and consequently the incidence of severe ROP has begun to emerge as a major cause of childhood blindness. While we now have a greater understanding of the postnatal risk factors for the development of ROP, studies aimed at further elucidating the molecular pathogenesis of ROP are essential in order to identify new therapeutic modalities for neonatal care (Gilbert 2008). It is for this reason that researchers are continually investigating animal models that will most closely resemble the human form of ROP.

3.6 Current treatment for ROP

An appropriate course of treatment is often chosen based on the location and stage of the disease. As mentioned above, stage 1 and 2 ROP often only require observation of the infant, whereas more advanced cases of ROP, namely stage 3 through 5, require invasive surgeries on the eye such as laser treatment or cryotherapy, for example (O'Keefe and Kirwan 2006, Fielder and Reynolds 2001). The goal of the treatment is to destroy the areas of the retina that are devoid of vessels. This helps to shrink the newly forming abnormal vessels in the eye and prevents the formation of scarring that usually follows which leads to

increased traction on the retina, ultimately resulting in visual impairment, retinal distortion or retinal detachment. Laser treatment involves the use of a diode or argon laser that is delivered by transpupillary or transcleral routes, with a laser beam directed by a target light on specific retinal areas or with a probe on the external scleral surface that is viewed as an indentation of the retina with an indirect ophthalmoscope, respectively (Fielder and Reynolds 2001). This type of therapy will "burn away" the periphery of the retina. On the other hand, cryotherapy involves the use of instruments that generate freezing temperatures in order to touch spots on the surface of the retina that are affected. Both laser treatment and cryotherapy destroy the peripheral areas of the retina, thereby slowing or reversing the abnormal growth of retinal blood vessels. It has been suggested by some that laser therapy and cryotherapy are equally effective, while others have reported a better outcome of laser therapy with respect to visual acuity and refractive status (Connolly et al. 1999, 1998). While long term effects are often debated and still remain unclear, these are still considered to be the most effective treatments for ROP.

Other options that are available for the treatment of very advanced stages of ROP (4 or 5) include scleral buckling and vitrectomy. Scleral buckling involves tightening a silicone band around the eye which reduces the ability of the vitreous to pull the scar tissue, consequently allowing the retina to better settle against the wall of the eye. Vitrectomy, on the other hand, involves the removal of the vitreous gel and its replacement with a saline solution. Upon removal of the vitreous, retinal scar tissue is removed so that the retina can once again settle against the wall of the eye.

4 Oxygen Induced Retinopathy (OIR)

4.1 Animal models

Approximately ten years after discovering the link between oxygen and human RLF, researchers began to focus on developing an experimental animal model that could be used to better understand the pathophysiology of the disease.

Altricial animals, including rats, mice, dogs, cats and rabbits, are born with their eyes closed and a very immature visual system (Gresh et al. 2003, Gorfinkel and Lachapelle 1990, Hamasaki and Maguire 1985, Kurihara 1977, Gum et al. 1973, Braekvelt and Hollenberg 1970), while precocial animals, including guinea pigs, macaques and humans (Spira 1975, Smelser 1974 et al., Hollenberg and Spira 1972) are born with their eyes open and a more mature visual system. Consequently, altricial animals have been commonly used to study the pathogenesis of OIR.

The birth of retinal cells in altricial animals such as rats and mice, for example, has been shown to begin at approximately embryonic day (E) 10, beginning with GCs, cones, HCs and ACs while rods, BCs and MCs appear shortly after (Cepko et al. 1996). The rat is born at 21 days of gestation with the nerve fiber layer, the GCL, the IPL and an undifferentiated neuroblastic layer (Braekvelt and Hollenberg 1970). Retinal development and fine tuning in the rat continues ex-utero, and includes cellular migration and differentiation first in the inner retina and then in the outer retina, and the development of the OPL which appears at postnatal day (P) 5. While the retinal stratification is more or less complete at P9, eye opening typically occurs at P14, following which normal developmental cell loss in the ONL (Maslim et al. 1995) and re-organization of the IPL occur. These events usually take place up until P25-P30. Consequently, it is not surprising that an ERG cannot be recorded from rats at birth, and while

the various elements of the ERG are known to develop throughout the first month of life, only by the end of the first month is their retina considered to be fully mature with respect to function (Jacobson et al. 1987, Braekvelt and Hollenberg 1970). In contrast, retinal development in precocial animals largely occurs *in utero*, resulting in a retina that is mature at birth, both structurally and functionally.

One of the earliest studies on the consequences of high oxygen administration (60% O₂) in animals was carried out on the baby opossum. The immaturity of their eyes along with the difficulty of manipulating the overly protective mothers and their young made it difficult to carry out these experiments (Tasman et al. 2006).

The mouse was studied next and interestingly, intraocular haemorrhages, hyperplasia of the tunica vasculosa lentis along with occasional retinal detachment were observed following exposure to hyperoxia (Gyllenstein and Hellstrom 1952). In addition, proliferation of endothelial cells in the nerve fibre layer of the retina as well as that of the capillaries that penetrated the internal limiting membrane could be seen (Patz et al. 1953), similar to that observed in the human form of RLF. Interestingly, at approximately the same time that work was done, preliminary studies were also being carried out in the kitten. The kitten was chosen due to the fact that while vascular budding in this animal begins between the 35th and 45th day of gestation, it remains incomplete until approximately 3 weeks after birth. Therefore, a full-term kitten at birth could be considered comparable to a preterm infant (Ashton et al. 1953). The results of these experiments ultimately revealed for the very first time that exposure to high concentrations of oxygen resulted in an interruption of the normal process of vascularization of the kitten retina. This occurred initially through the irreversible obliteration of the immature retinal vessels and subsequently through the abnormal proliferation (including haemorrhages and growth of vessels into the vitreous) upon the return to room air (Ashton et al. 1953), which was later found to occur following the upregulation of angiogenic factors such as VEGF in the

relatively hypoxic environment (Stone et al. 1995). Eventually, however, the regression of NV over time, in addition to the lack of retinal detachment, have both been considered drawbacks to the kitten model of OIR.

The canine model of OIR has several characteristics that are similar to what is observed in the kitten model of OIR as well as human ROP. For example, retinal tractional folds, extensive vaso-obliteration and NV have been documented among all of them (McLeod et al. 1998). The canine model has also successfully shown a number of additional features observed in the human form of ROP, namely retinal scarring and detachment (McLeod et al. 1998, Flower et al. 1985), though these are features observed in only a small percentage of infants with ROP (Madan and Penn 2003). Furthermore, these results should be interpreted with caution given the fact that retinal detachments as well as retinal folds have been shown to occur spontaneously in canines (Flower and Blake 1981, Roberts 1959).

The rat is one of the best characterized of all animal models of OIR. Contrasting with other animal models that have been used to study the pathophysiology of this disease, the neonatal rat offers the advantage of an immature retina at birth that is comparable to that of a 4-5 month old human fetus (Weidman and Kuwabara 1969). As in humans, exposure of the rat pup to postnatal hyperoxia will cause severe vasoconstriction and vaso-obliteration that are followed by an abnormal proliferation of retinal vessels upon the return to room air (Reynaud and Dorey 1994, Penn et al. 1994). Other features that are reminiscent of the human form of ROP have also been observed in that rat model of OIR, including retinal haemorrhages, retinal folds and even retinal detachment in a small number of cases (Penn et al. 1992). These similarities in addition to other advantages of the rat, including the large litter sizes (approximately 15 pups per dam dependant upon the strain), low costs associated with developing the model and knowledge that has already been acquired from the rat with respect to various antioxidant, anti-angiogenic and anti-inflammatory therapeutics (Madan and Penn 2003) make it a very attractive model to study the pathogenesis of OIR.

and consequently better understand the sequelae of ROP. This was our model of choice for carrying out the experiments that will be described in this thesis.

4.2 Oxygen Exposure

4.2.1 Experimental protocols of oxygen exposure

A number of different oxygen exposure regimens have been used based on variations of oxygen concentration and duration of exposure in order to more closely represent what is observed in human neonates, though this is only one aspect of what these premature infants experience. Some of these regimens included uninterrupted exposure to 60% O₂ from P0-14 by Penn et al. (1994, 1988) and to 80% O₂ from periods varying from 5 to 10 days after birth by Ricci et al. (1988a, 1988b, 1987) which resulted in both significant reductions in vascular area and density. Smith et al. (1994) carried out another protocol whereby mice were exposed to 75% O₂ from P7-12, which successfully generated significant levels of NV. While models such as those described above resulted in a retinopathy that was followed by NV, they have not necessarily been able to very closely resemble the arterial oxygen fluctuations of the preterm infant (Cunningham et al. 2000). Multiple attempts have been made by investigators to establish an animal model that closely mimics human like vascular fluctuations given that the arterial oxygen level of preterm infants has been shown to fluctuate significantly. Studies have shown that severe ROP is associated with variability in transcutaneous oxygen within the first 2 weeks of life, suggesting that these variations in oxygen might interrupt the normal vascularization process which responds to hypoxic and hyperoxic triggers (Cunningham et al. 1995). Penn et al. (1993) attempted to use a cyclic oxygen regimen which fluctuated between 80% and 40% O₂ in order to more closely mimic human arterial oxygen fluctuations.

While this generated findings that were comparable with the continuous 80% O₂ exposure with respect to a decrease in vascular area, the cyclic exposure also generated a significantly greater degree of NV. Similar findings were reported following cyclic exposure fluctuating between 80% and 21% O₂, thus further strengthening the suggested correlation between the duration of hypoxic episodes and NV severity. Finally, the development of an oxygen delivery system in rats by Cunningham et al. (2000) produced changes in oxygen each minute, thereby very closely mimicking arterial oxygen changes of preterm infants. Fluctuations such as these have also resulted in reductions in peripheral retinal vascularity and concentration of capillaries in hyperoxic cohorts compared to those raised in room air.

4.2.2 Mechanism of action of oxygen

The retina is extremely vulnerable to oxygen mediated damage for a number of reasons. Retinal cells have a very high oxygen consumption rate, and oxygen radicals are normally formed in the mitochondria as oxygen is reduced along the mitochondrial electron transport chain. Under hyperoxic conditions, the number of oxygen-derived radicals is amplified and can disturb various cellular components such as lipids, nucleic acids and proteins, causing cytotoxicity (Hardy et al. 2005a). The retina is also composed of lipids that contain high levels of PUFAs such as docosahexaenoic acid (DHA; 22:6 ω -3), arachidonic acid (AA; 20:4 ω -6) and choline phosphoglyceride which are all targets for peroxidation. For reasons that remain unclear, retinal endothelial cells seem particularly susceptible to peroxidation-induced injury, whereas pericytes, smooth muscle cells, and perivascular astrocytes are relatively resistant (Kondo et al. 1996, Auge et al. 1995, D'Amore and Sweet 1987). Finally, the retina is also the site of phototransduction and photic excitation is known to initiate oxygen radical formation (Penn 1990). Another important aspect to consider in the development

of OIR is the status of the retina at birth. The retina of a full term rat is highly immature and it is thought to undergo an increase in metabolic rate in the second to third week of life which is accompanied by a significant maturation phase (Graymore 1960, 1959). Enhanced metabolism has been associated with increased electron leaks in the mitochondria and consequently with the facilitation of free radical generation.

4.2.3 Effect of oxygen on retinal vasculature

While human retinal vascular development largely occurs in utero resulting in an almost completely vascularized retina at birth, that in rats, as well as in other altricial species, develops to a greater extent postnatally (Fleck and McIntosh 2008). Consequently, exposure of the rat to hyperoxia from birth disrupts the normal retinal vascularization process. The first phase is characterized by the cessation of normal vascular growth and vaso-obliteration. Briefly, exposure of the retina to high concentrations of oxygen results in the cessation of normal vascular development and obliteration of large areas of the newly formed immature retinal vessels. These early stages result in decreased perfusion and ischemia. The second phase, where NV occurs, is largely dependent on the extent of the first phase of OIR, since hypoxia is thought to be the driving force of this process through modulating VEGF expression (Stone et al. 1996). Interestingly, higher VEGF levels have been found in vitreous samples from patients with NV (Mantagos et al. 2009), and consequently anti-VEGF therapies have been used to treat other ischemic retinopathies as well (Andreoli and Miller 2007).

4.3 Therapeutic avenues

Given that free radicals do, at least in part, appear to play a role in ROP, it is not surprising that efforts have been made by both clinicians and investigators to explore the use of antioxidant administration in preterm infants as a means of protecting the retina from their deleterious effects. Vitamin E supplementation has been thought to be beneficial to this end, especially when considering that plasma levels of this antioxidant are low in prematurely born infants (Katz and Robison 1988, Nielson et al. 1988, DeVito et al. 1986). Furthermore, since retinal vascular endothelial cell membranes are thought to be susceptible to free radical attack, it was thought that vitamin E could terminate these lipid peroxidation reactions given its ability to integrate into the lipid bilayer where they occur (Flower et al. 1984). However, the effectiveness of vitamin E supplementation in reducing the severity of ROP remains quite controversial (McColm and Fleck 2001). For example, in the vitamin E deficient rat, an increased lipid peroxidation was observed and was followed by increased cell membrane permeability and decreased photoreceptor function (Goss-Sampson et al. 1998). A favorable effect of vitamin E on the incidence of ROP (Liu et al. 2005, Johnson et al. 1995) and in iron or nitric oxide induced models of peroxidation (Siu et al. 1998) has been documented. In contrast, intravenous treatment with vitamin E resulted in increased retinal and intraventricular hemorrhage without any beneficial effect on the development of ROP (Phelps et al. 1987). It has also been suggested that conflicting results may stem from the difficulty of delivering a lipid soluble molecule such as vitamin E to the immature retina (Nielson et al. 1988).

Trolox C, a water soluble analogue of vitamin E with potent antioxidant activity, was used by Penn et al. (1997) in the rat model of OIR in order to determine its effect on the retinal vasculopathy that results from the exposure of neonatal rats to high oxygen concentrations. The retinas of rats treated with trolox C while exposed to oxygen had greater vascular coverage and higher capillary

density than those that were untreated, suggesting that trolox C could help prevent the vascular consequences of OIR. As described above, previous studies showed a lack of correlation between the vascular and the retinal structural and functional consequences of OIR, in which the retinal vasculature often resumed normalcy shortly after the return to normoxia, whereas the electroretinographic and cytoarchitectural anomalies became permanent features of the hyperoxic rats (Dembinska et al. 2001, Lachapelle et al. 1999, Fulton et al. 1999, Reynaud et al. 1995). Therefore, while retinal vascular anomalies of OIR rats were shown to be partly prevented by free radical scavengers (Penn et al. 1997), whether they could exert a similar protective effect on both structural and functional levels was unknown.

More recently, the role of omega-3 and -6 PUFAs in angiogenesis associated with OIR has also been studied, where increased retinal omega-3-PUFA levels inhibited NV. It should be noted that this protective effect was secondary to increased regrowth of vessels and not a result of a reduced oxygen-induced vessel loss during hyperoxia (Connor et al. 2007).

5 General Objectives

As described in the introduction above, ROP is one of a group of potentially blinding ischemic retinal disorders that affects small prematurely born infants. The rat model of OIR was developed to better understand what occurs in premature infants that are exposed to postnatal hyperoxia and previous studies have demonstrated that this model bears a striking resemblance to the human form of ROP, at least in its vascular component. However, despite the resolution of vascular abnormalities which is a common occurrence in ROP patients, neural dysfunction of the retina may still carry on throughout adulthood. Previous findings from our laboratory have shown that in addition to the vasculopathy that develops in the rat model of OIR, permanent retinal cytoarchitectural and

functional anomalies can also be documented (Dorfman et al. 2008, 2006, Dembinska et al. 2002, 2001, Lachapelle et al. 1999). The most notable changes in retinal ultrastructure using the albino Sprague Dawley (SD) rat model of OIR include OPL thinning and a decrease in HC count. Furthermore, attenuation in the amplitude of the ERG b-wave with relative sparing of the a-wave is also characteristic of this model (Dorfman et al. 2008, 2006, Dembinska et al. 2002, 2001, Lachapelle et al. 1999).

Finally, it is of interest to note that periods of increased oxygen susceptibility have been documented in the first week of life and in the third week of life for retinal vasculature in the rat (Benjamin et al. 1998) and rat photoreceptors (Maslim et al. 1995), respectively. A window of oxygen hypersensitivity has also previously been documented in our laboratory for the retinal structure and function of the albino SD rat, where early exposure to hyperoxia that included the second week of life or was initiated within the second week of life resulted in a more severe retinopathy than when limited solely to the first week of life (Dembinska et al. 2002, 2001).

While studies that have been aimed at better understanding the pathogenesis of ROP have been, and continue to be, instrumental in the improvement of neonatal technologies and survival of premature infants, a number of questions still remain unanswered. For example, only few studies set out to identify the earliest structural and functional manifestations of the disease process triggered by the postnatal hyperoxic shock in the albino rat (Liu et al. 2006a, 2006b, Fulton et al. 1999, Reynaud et al. 1995, Penn et al. 1988), where retinal function and structure were evaluated either at predetermined time points, such as at postnatal days [P]13 and P18 or P31, for example (Liu et al. 2006a, 2006b, Fulton et al. 1999), or on a weekly basis, beginning immediately after the exposure regimen (Penn et al. 1988). For the most part, studies have been performed at intervals that were further removed from the oxidative insult and at a time when the retina was considered to be mature (e.g., at postnatal days 30 and/or later) (Dorfman et al. 2006, Dembinska et al. 2002, 2001, Lachapelle et al. 1999)

and have therefore made it difficult to understand the pathophysiological progression of OIR. Consequently, our first study was aimed at examining the effects of postnatal hyperoxia on vascular development throughout hyperoxic exposure and we also attempted to determine how early after the cessation of hyperoxia we could observe changes in retinal structure and function that are known to characterize adult rats that had been exposed to the same conditions during the first 2 weeks of life.

Given that free radicals have been thought to play a role in the pathogenesis of ROP (Beauchamp et al. 2004, 2002, 2001, Hardy et al. 2000, 1996) and that the retinal vascular anomalies found in OIR rats were shown to be partly prevented by free radical scavengers, we next examined whether they could also exert a protective effect on a structural and functional level. Finally, although the albino rat model is traditionally used to better understand the pathogenesis of OIR, studies comparing the racial incidence of ROP have suggested that ocular pigmentation might offer protection against the development of severe ROP. We therefore decided to verify the structural and functional consequences of postnatal hyperoxia by comparing albino SD and pigmented Long Evans (LE) rats. This was complemented by a study that compared the cytoarchitectural manifestations of OIR in the SD and LE rat models using immunoblotting techniques and confocal microscopy in order to better understand the pathophysiological processes that characterize this retinopathy.

The general purpose of this work was to investigate beyond the retinal vasculature in order to further characterize the OIR model and therefore better understand the functional (ERG) and cytoarchitectural (histology and immunohistochemistry) manifestations of this disease. We believe that results from our studies will lead to a better and more complete understanding of the pathophysiology of ROP and thus suggest new therapeutic avenues to protect the developing visual system during this critical period. A total of six manuscripts have been incorporated into this thesis and are briefly described below:

Study #1: Previous studies carried out in our laboratory using the albino rat model of OIR have suggested that exposure to hyperoxia for the first 2 weeks of life caused widespread loss of the outer plexiform layer and horizontal cells of the retina and resulted in functional deficits (attenuated scotopic and photopic b-waves of the ERG). These studies were conducted in relatively mature rats at time points that were substantially far removed from the hyperoxic insult (at postnatal days 30 and 60, for example) and therefore the sequence of events leading up to these changes could only be inferred. The purpose of our first study was: 1- to evaluate changes taking place at the level of the retinal vasculature throughout exposure to hyperoxia, and 2- to examine how early after the cessation of oxygen exposure can we demonstrate changes in retinal structure and function known to characterize OIR. Our findings revealed reduced retinal vascular coverage that accompanied the hyperoxic exposure in neonatal rats and suggested an increased susceptibility of the very immature retina to hyperoxic episodes that occurred within the first week of life (P0-6 and P0-9). Furthermore, despite the fact that vasculature appeared to repair itself while still subjected to a hyperoxic environment (throughout exposures from P0-12 and P0-14, for example), irreversible cytoarchitectural and functional changes occurred, the consequences of which were documented immediately following the cessation of the hyperoxic regimen (Dorfman et al. 2007 *Invest. Ophthalmol. Vis. Sci.* 2008; 49: 458-466).

Study #2: It has been suggested that free radicals may be the source of the pathologic effects described above, namely on retinal vasculature, cytoarchitecture and function. Trolox C, a water-soluble analogue of vitamin E, was previously shown to limit the vasculopathy resulting from exposure to postnatal hyperoxia and the aim of our next study was to investigate whether it could also help prevent the functional (ERG) and structural (retinal histology) consequences associated with OIR. Our results showed that while trolox C limited the retinal functional and structural damages, there were still signs (albeit less severe) indicative of OIR. This suggested that the pathophysiology of OIR was not solely caused by

the action of free radicals or that trolox C was inadequate in treating all aspects of OIR (**Dorfman et al., *Invest. Ophthalmol. Vis. Sci.* 2006; 47: 1101-1108**).

Study #3: It is well known that melanin can act as a free radical scavenger. The purpose of this study was to compare the effect of OIR in albino and pigmented rats. Our results show that melanin did not prove to exert the predicted protective effect on retinal structure and function in OIR. Following postnatal oxygen exposure, LE pigmented rats were affected earlier on and more drastically than SD albino rats. (**Dorfman et al., *Invest. Ophthalmol. Vis. Sci.* 2009; 50: 2436-50**). These results support our previous demonstration that concurrent administration of trolox C, a synthetic free radical scavenger, showed only a minimal protective effect. It therefore still remains important to determine the role played by free radicals in causing ROP. One wonders if the more severe OIR observed in LE rats might result from melanin exerting its' pro-oxidant effect upon exposure of these rats to hyperoxic conditions, thus exacerbating the effect of O₂ radicals in the retina and lead to a degenerative path.

Study #4: Our previous findings have revealed that while structural and functional impairments are evident in the albino SD rat, including OPL thinning, reduced HC count along with attenuation in the b-wave amplitude of the ERG with relative sparing of the a-wave (Dorfman et al. 2006, Dembinska et al. 2002, 2001, Lachapelle et al. 1999), an even greater susceptibility of the pigmented LE strain to hyperoxia is noted. This extreme vulnerability is characterized by a higher vascular dropout, permanently altered retinal function that occurs following all exposure regimens and permanently altered retinal structure which not only involves the loss of the OPL and HCs as in SD rats, but also involves INL, IPL and GCL thinning (Dorfman et al. 2009a). Little is known, however, about the specific cytoarchitectural mechanisms that contribute to these findings. The purpose of this study was therefore to further investigate the impact of hyperoxia on retinal circuitry, using immunohistochemical techniques to study synaptic contact, cell morphology and cell death, which might be at the origin of strain specific differences. Various immunohistochemical markers, including

synaptophysin staining (presynaptic marker) as well as specific markers for rod and cone bipolar cells [Protein kinase C- α (PKC- α) and recoverin, respectively], for example, indicate that cell death in the inner retina and synaptic retraction in the OPL (the primary retinal synapse between the photoreceptors and bipolar cells) are the earliest consequences of hyperoxic exposure (**Dorfman et al., manuscript in preparation to be submitted to *Invest. Ophthalmol. Vis. Sci.* Febraury 2010**).

Finally, as a result of our expertise, the following two review articles were requested from us:

- 1) The effect of oxygen and light on the structure and function of the neonatal rat retina (Dorfman et al., *Doc Ophthalmol.* 2009; 118: 37-54)
- 2) Postnatal hyperoxia and the developing rat retina: Beyond the obvious vasculopathy (Dorfman et al., *Doc Ophthalmol.* 2009 [Epub ahead of print]).

Collectively, our results suggest that the rat model of OIR is not only useful for studying retinal vasculature, but can also be used to better understand the functional and structural sequelae that are associated with ROP. While the albino rat has been widely used as a model for studying ROP, our findings would suggest that the pigmented rat also represents a valid working model of OIR that demonstrates a slightly different and more exaggerated disorder. ROP is only one of several types of proliferative retinopathies [including diabetic retinopathy (DR) and age related macular degeneration (AMD), for example] whose pathologic course involves retinal ischemia followed by abnormal vessel growth. Studies such as these will undoubtedly be instrumental in a better characterization of these types of retinopathies and in the derivation of new therapeutic avenues aimed at controlling them.

CHAPTER II: MANUSCRIPT I

Dorfman A, Dembinska O, Chemtob S, Lachapelle P. Early manifestations of postnatal hyperoxia on the retinal structure and function of the neonatal rat. Invest Ophthalmol Vis Sci. 2008 Jan; 49:458-6.

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1 Preface to chapter II

Previous studies using the albino Sprague Dawley rat model of OIR largely focused on the consequences of postnatal hyperoxic exposure at time points that were substantially removed from the insult. Consequently, this has made it difficult to understand the pathophysiological progression of the disease. In order to address the above, we investigated the effects of postnatal hyperoxia on vascular development throughout hyperoxic exposure (at P6, P9, P12 and P14) and we examined retinal structure and function as early as one day after exposure to hyperoxia (at P15, P16, P17, P19, P24, P30 and P60) to determine how early after the cessation of hyperoxia we observe changes in retinal structure and function known to characterize adult rats that had been exposed to the same conditions during the first two weeks of life.

2 Abstract

Purpose: Postnatal hyperoxia in rats causes an arrest in growth of retinal blood vessels, along with severe changes in retinal ultrastructure and function. Previous studies focused on consequences of postnatal hyperoxia at time points substantially removed from the hyperoxic insult. In this study, the earliest manifestations of this retinopathy were examined. **Methods:** Newborn rats were exposed to 80% O₂ from birth to postnatal day 14. The retinas were collected for vascular assessment at postnatal days 6, 9, 12, and 14, and electroretinograms were recorded at postnatal days 15, 16, 17, 19, 24, 30, and 60, after which retinal histology was performed. **Results:** Hyperoxia significantly attenuated vascular development, especially after 6 and 9 days of exposure which resulted in 64% and 72% of normal coverage, respectively. Vascular growth continued despite hyperoxic exposure, reaching 87% of normal by postnatal day 14. Electroretinograms of hyperoxic rats retained very immature features throughout with nearly abolished b-waves and relatively preserved a-waves. Finally, while retinal structure was virtually complete in the control animals by postnatal day 15, hyperoxic rats always showed a significantly thinner outer plexiform layer (OPL) and lower horizontal cell count ($P < 0.05$), irrespective of the duration of exposure. **Conclusion:** The findings confirm previous reports of reduced retinal vascular coverage that accompanies the earliest manifestation of postnatal hyperoxia in rats and suggest increased retinal susceptibility to hyperoxia within the first week of life. However, despite the fact that vasculature appears to repair itself, irreversible cytoarchitectural and functional changes occur, the consequences of which are documented immediately after the cessation of hyperoxia.

3 Introduction

The rat model of oxygen-induced retinopathy (OIR) has been widely used as an animal model of the human form of this disease, retinopathy of prematurity (ROP). ROP is a vasoproliferative disease that, in its most severe form, can result in childhood blindness.^{1 2 3} In contrast with other animal models that have been used to study the pathophysiology of this disease, the neonatal rat offers the advantage of a highly immature retina at birth that is comparable to that of a 24- to 26-week-old human embryo.⁴ As in humans, exposure of the rat pup to postnatal hyperoxia causes severe vasoconstriction and vaso-obliteration, followed by an abnormal proliferation of retinal vessels on the return to room air.^{5 6 7 8 9} Accompanying this vasculopathy are permanent structural and functional abnormalities, as documented with retinal histology and electroretinography, respectively.^{10 11 12 13 14 15 16 17 18} Of interest, however, in only a few of these studies did the researchers try to identify the earliest structural and functional manifestations of the disease process triggered by the postnatal hyperoxic shock,^{10 11 16 17 18} where retinal function was evaluated either at predetermined time points (at postnatal days [P]16 and P29,¹¹ P31,¹⁰ or P13 and P18,^{11 16} for example) or on a weekly basis, beginning immediately after the exposure regimen.¹⁸ These and other studies that were performed at intervals that were further removed from the oxidative insult and at a time when the retina was considered to be mature (e.g., at postnatal days 30 and/or later)^{12 13 14 15} have made it difficult to understand the pathophysiological progression of OIR. Therefore, the purpose of the present study was to examine first the effects of postnatal hyperoxia on vascular development throughout hyperoxic exposure and, second, to determine how early after the cessation of hyperoxia we observe changes in retinal structure and function that are known to characterize adult rats that have been exposed to the same conditions during the first 2 weeks of life.

4 Methods

All experimental procedures were approved by the McGill University/Montreal Children's Hospital Research Institute Animal Care committee according to the guidelines of the Canadian Council on Animal Care and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Newborn litters of Sprague-Dawley rats (Charles River Laboratories, St-Constant, Quebec, Canada) were exposed to 80% oxygen immediately after birth (mixture of medical grade 100% O₂ and room air measured with an oxygen meter; MaxO₂ Ceramatec, model OM25-ME; Medica Inc., Montreal, Quebec, Canada), as previously described.^{12 13 14 15} Briefly, exposure to hyperoxia (80% O₂) persisted from birth until postnatal day 14 for 22.5 hours daily, interrupted with three intervals of 30 minutes' duration under normoxic conditions (21% O₂). Animals were euthanatized for vascular assessment at P6 ($n = 3$), P9 ($n = 3$), P12 ($n = 3$), and P14 ($n = 3$) throughout exposure to 80% O₂ and compared with that in animals raised simultaneously in room air (21% O₂, $n = 3$). After hyperoxic exposure, animals were assigned to one of seven experimental groups to be studied at age P15 ($n = 6$), P16 ($n = 6$), P17 ($n = 6$), P19 ($n = 6$), P24 ($n = 6$), P30 ($n = 8$), and P60 ($n = 7$). Data were compared with that obtained from control age-matched animals raised simultaneously in normoxic conditions (21% O₂) on P15 ($n = 6$), P16 ($n = 6$), P17 ($n = 6$), P19 ($n = 6$), P24 ($n = 6$), P30 ($n = 14$), and P60 ($n = 14$). The rats were maintained in a cyclic lighting environment (80 lux; 12 hours dark/12 hours light). Finally, mothers of the litters were alternated between normoxic and hyperoxic conditions every 24 hours so that pulmonary complications known to arise in adult rats raised in a hyperoxic environment could be avoided.

4.1 Retinal flatmounts

After the rats were euthanized, the eyes were enucleated, the anterior segments dissected, and the eyecups fixed overnight in 4% formalin. The retinas were subsequently isolated, and flatmounts were prepared for staining with adenosine diphosphatase (ADPase), as previously described.^{15 19 20} Specimens were mounted and photographed (40x, Axiophot microscope; Carl Zeiss Meditec, GmbH, Oberkochen, Germany). To quantify the extent of vascular coverage (in normoxia- and hyperoxia-raised animals), the ratio of total area of vascularized retina over the total retinal area was calculated (Image-Pro Plus 4.1 software; Media Cybernetics, Silver Spring, MD).

4.2 Electrophysiology

Electroretinograms (ERGs) and oscillatory potentials (OPs) were recorded with a data acquisition system (Acknowledge; Biopac MP 100WS; BioPac Systems, Inc. , Goleta, CA), according to a technique previously reported by us^{12 13 14 15} on rats aged 15, 16, 17, 19, 24, 30, and 60 days. After a period of 12 hours of dark-adaptation, the rats were anesthetized, under dim red light, with an intramuscular injection of ketamine hydrochloride (85 mg/kg) and xylazine (6 mg/kg). The pupils were dilated with a 1% cyclopentolate hydrochloride solution (Mydracil; Alcon Laboratories, Fort Worth, TX) and a DTL silver-coated conductive nylon yarn (27/7 X-Static; Sauquoit Industries, Scranton, PA) electrode was placed on the cornea to serve as the active electrode. The latter was maintained in place on the cornea with 2% hydroxymethylcellulose (Gonioscopic solution; Alcon Laboratories) which also prevented corneal desiccation. Reference and ground electrodes were placed in the mouth (model E5 disc electrode; Grass Technologies, Quincy, MA) and tail (model E2 subdermal needle

electrode, Grass Technologies), respectively. The rats were then placed in a light-proof recording chamber of our design that included the light stimulus and background light.^{12 13 14 15} Full-field ERGs and OPs were recorded simultaneously (ERG: 10,000x, 1–1,000-Hz bandwidth, 6 dB of attenuation and OPs: 50,000x, 100–1,000 Hz, 6 dB of attenuation) using analog preamplifiers (model P511; Grass Technologies). Scotopic responses were evoked by flashes of white light spanning a 6.0-log-unit range in 0.3-log increments (maximum intensity: $0.6 \log \cdot \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$; average, two to five flashes; interstimulus interval, 10.240 seconds). Photopic ERGs (average, 20 flashes, interstimulus interval: 1 second) were evoked by flashes of white light of $0.9 \log \cdot \text{cd} \cdot \text{sec} \cdot \text{m}^{-2}$ delivered after more than 15 minutes of light adaptation (after the dark-adaptation period) to a background of $30 \text{ cd} \cdot \text{m}^{-2}$ to avoid the light adaptation effect.^{21 22}

4.3 Retinal histology

After the P15, P17, P19, and P24 ERG recording sessions, three to four animals per group were killed, and their retinas were collected for histology. Sections of the central retina were embedded in epoxy resin (Epon 812; Mecalab, Montreal, Quebec, Canada) and ultrathin sections ($0.7 \mu\text{m}$) were stained with toluidine blue, according to a method previously described.^{12 15} Measurements of retinal thickness were obtained on 250- μm wide sections with the use of an ocular with a calibrated grating. Three retinas per group were analyzed and an average of 10 different retinal measurements was obtained from each. Pictures were taken with a microscope (40x, Axiophot; Carl Zeiss Meditec, GmbH).

4.4 Data analysis

ERG components were measured, as previously described by us.^{12 13 14} Briefly, the amplitude of the a-wave was measured from the baseline to the most negative trough and the amplitude of the b-wave was measured from the trough of the a-wave to the most positive peak of the response. The amplitude of the b-wave was plotted against the corresponding flash intensity to generate the scotopic luminance–response function curve that was fitted to a sigmoidal regression curve (Prism, ver. 3.00 for Windows; GraphPad Software, San Diego, CA) from which the rod $V_{\max}$ (maximum pure rod response) was derived. Also, the ERG response evoked by the brightest flash delivered in scotopic conditions (the so-called mixed rod–cone response) was also included in the analysis. Finally, since no a-wave can be identified in the photopic responses of rats (Fonteille VL, et al. *IOVS* 2005;46:ARVO E-Abstract 2246),^{12 13 14 23 24} the photopic b-wave was measured from baseline to the peak of the wave. A one-way ANOVA ($P < 0.05$) followed by the Tukey honestly significant difference (HSD) or a Student's *t*-test was used to compare the effect of postnatal hyperoxia on retinal structure (vasculature and histology) and function. The data are presented as the mean $\pm$ 1 SD.

5 Results

5.1 Retinal vasculature

Figure 1 shows representative examples of retinal flatmounts obtained at P6 (Fig.1A), P9 (Fig.1B), P12 (Fig.1C), and P14 (Fig.1D) from animals raised in normoxia (top) and hyperoxia (bottom) from P0 to P6 (Fig.1E), P0 to P9 (Fig.1F), P0 to P12 (Fig.1G), and P0 to P14 (Fig.1H). The extent of retinal vasculature

(percent of total retinal surface) is represented graphically in Figure 2. In normal rats, vascular growth gradually progressed from the center to the periphery as the rat pup aged from P6 ($71.56\% \pm 3.91\%$ of coverage as per Fig.2) to P12 ($98.93\% \pm 1.05\%$). By P12, vascular coverage was complete, and no further changes were observed at P14 ($99.07\% \pm 0.95\%$). In comparison, hyperoxia significantly hampered the growth of the retinal vasculature. Hyperoxic exposure between P0 and P6 (Fig.1E) significantly limited the vascular coverage to $46.50\% \pm 2.95\%$ (Fig. 2), representing 64.9% of age-matched normal values ($P < 0.05$), which were not yet completely vascularized, as just mentioned. However, despite the hyperoxia regimen, the retinal blood vessels continued to grow and reached $87.90\% \pm 2.07\%$ of coverage at P14, a value that was still significantly less than in the control rats ($P < 0.05$).

5.2 Maturation of the scotopic ERG

Figure 3 shows representative scotopic and photopic ERG waveforms obtained at P15, P16, P17, P19, P24, P30, and P60 from control (Fig.3A) and hyperoxic (Fig.3B) rats, respectively. Group data are graphically reported in Figure 4. In normal rats, the scotopic ERG obtained on eye opening (P15) was of low voltage with a negative morphology, due to a prominent a-wave and underdeveloped b-wave. The ascending limb of the b-wave was also devoid of well-demarcated OPs. As the rat aged (P16–P17), the a-wave continued to dominate the response, despite the growth in amplitude of the b-wave and the appearance of easily identifiable OPs. Maximum a-wave amplitude was reached at P19, whereas the b-wave and OPs continued to grow until P30. Responses obtained at P60 suggest that a decline in ERG amplitude takes place during the second month of life. The latter findings are further supported by the group data illustrated in Figures 4A, 4B, 4C, which show a rapid and significant growth in a-wave amplitude to a maximum reached at P19 (P15: $175.45 \pm 52.20 \mu\text{V}$; P19:

485.88 $\pm$ 44.45 μ V; $P < 0.05$), representing a 176.9% increase. At P60, the amplitude of the a-wave (366.16 $\pm$ 55.18 μ V) was 75.4% of that measured at P19. Similarly, the amplitude of the rod $V_{\max}$ increased from 160.81 $\pm$ 58.86 μ V measured at P15 to a maximum of 684.12 $\pm$ 136.64 μ V reached at P24, representing a significant 325.4% increase ($P < 0.05$), whereas the rod–cone b-wave was augmented by 428.3% ($P < 0.05$) from 212.42 $\pm$ 97.52 μ V at P15 to a maximum of 1122.11 $\pm$ 174.52 μ V at P30, suggesting a delayed maturation compared with the rod $V_{\max}$. At P60, the amplitudes of the rod $V_{\max}$ (609.74 $\pm$ 73.64 μ V) and of the rod–cone b-wave (919.77 $\pm$ 103.65 μ V) were 89.1% and 82.0% of maximum, respectively, most probably as a result of the normal aging process, as is documented elsewhere.^{10 12 13 14 25}

Postnatal hyperoxia markedly hampered the normal maturation process of the scotopic ERG signal, as exemplified in Figure 3B. As in normal rats, responses obtained from hyperoxic rats on eye opening (P15) were of negative morphology due to a prominent a-wave and a nearly absent b-wave. Again, the rising phase of the remnant b-wave was devoid of OPs. With time, there was a gradual growth in amplitude of the ERG components to reach maximum amplitude at P24, where the morphology of the resulting waveform was reminiscent of the ERG recorded at P17 in control rats. The increase was then followed by a gradual decline in ERG amplitude, which was most pronounced for the b-wave and OPs. Group data analysis (Figs.4A, 4B, 4C) confirmed the relative resistance of the a-wave maturation process to postnatal hyperoxia. Results indicate a rapid and significant growth in a-wave amplitude to a maximum reached at P24 (P15: 166.42 $\pm$ 49.81 μ V; P24: 445.23 $\pm$ 57.40 μ V, $P < 0.05$) representing a 167.5% increase ($P < 0.05$). It is worth mentioning that, although delayed by 5 days, the maximum a-wave amplitude reached in hyperoxic rats is not significantly different from that reached in normal rats (445.23 $\pm$ 57.40 μ V and 485.88 $\pm$ 44.45 μ V, respectively; $P > 0.05$). At P60, the amplitude of the a-wave (314.21 $\pm$ 47.29 μ V) was 70.6% of that measured at P24 and 85.8% of that measured in age-matched control animals. Similarly, the amplitude of the rod

$V_{\max}$ increased from nonrecordable at P15 to a maximum of $290.70 \pm 99.52 \mu\text{V}$ at P24 (42.5% of age-matched control amplitude; $P < 0.05$), whereas the rod–cone b-wave was augmented by 295.2% ($P < 0.05$) from $121.35 \pm 52.41 \mu\text{V}$ at P15 (57.1% of control amplitude) to a maximum of $479.55 \pm 101.32 \mu\text{V}$ at P24 (47.2% of age-matched control amplitude; $P < 0.05$).

5.3 Maturation of the photopic ERG

Similar to the results reported for the scotopic ERG parameters, the course of maturation of the photopic b-wave did not occur at the same rate among animals raised in the different oxygen environments (Fig.3) .

In normal rats, the photopic ERG obtained on eye opening (P15) was identifiable, though it was of low voltage and lacking well-demarcated OPs. As the rat aged, the amplitude of the photopic b-wave and OPs grew gradually to reach a peak at P30, after which it declined. As shown in Figure 4D , there was a significant (529.7%) increase in amplitude of the photopic b-wave from eye opening to age P30 (P15: $41.50 \pm 21.18 \mu\text{V}$; P30: $261.34 \pm 31.77 \mu\text{V}$, $P < 0.05$). At P60, the amplitude of the photopic b-wave ($230.82 \pm 19.50 \mu\text{V}$) declined to 88.3% of the maximum.

Again, postnatal hyperoxia markedly halted the normal maturation process of the photopic ERG signal as shown in Figure 3B. From nearly undetectable at eye opening (P15), the photopic b-wave grew gradually to reach maximum amplitude at P24, at which point the morphology of the resulting waveform was reminiscent of that recorded at P17 in control rats (Fig.3A). As with the scotopic responses, the photopic ERG recorded at the end of the second month of life (P60) was smaller than that obtained 1 month earlier (P30). Group data analysis (Fig.4D) confirms the high susceptibility of the photopic b-wave maturation process to postnatal hyperoxia. Results indicate a gradual growth in photopic b-wave amplitude to a maximum reached at P24 ($78.82 \pm 31.36 \mu\text{V}$), a value that is

35.4% of age-matched control subjects ($P < 0.05$). Of interest, this peak in amplitude occurred 6 days earlier than that observed in the normoxic cohort (P30). Finally, at P60, the amplitude of the photopic b-wave ($77.44 \pm 35.95 \mu\text{V}$) is 98.2% of that measured at P24 ($P > 0.05$) and 33.5% of that measured in age-matched control rats ($P < 0.05$).

It is noteworthy that in control rats the reduction in amplitude noted between P30 and P60 is significant for the a-wave, the rod-cone b-wave, and the cone b-wave, whereas it was not for the rod V_{max} . In contrast, in exposed rats, only the a-wave's amplitude was reduced significantly between P30 and P60, whereas the other ERG parameters remained basically the same.

Finally, to identify which of the ERG components was the most affected by the postnatal hyperoxic regimen, a ratio comparing the maximum amplitude in exposed rats with the maximum amplitude in normal rats was used. Results obtained are graphically represented in Figure 5. Calculations were performed on a-wave and rod V_{max} data generated at P19 and P24, respectively, and on rod-cone b-wave and cone b-wave data at P30, since these time points correspond to each respective peak in the normal maturation process, as described earlier. The resultant a-wave ratio of $75.06\% \pm 7.06\%$ at P19 confirms our initial impression that the a-wave is only minimally susceptible to postnatal hyperoxia. This ratio further increased to $91.50\% \pm 11.85\%$ when re-evaluated at P24, suggesting that although the peak in a-wave maturation after hyperoxia was delayed by 5 days (occurring at P24 instead of P19, as in normal eyes), it nonetheless almost completely developed, as observed in Figure 1. In comparison, the development of the scotopic b-wave, including the rod V_{max} and mixed rod-cone response, was compromised to a greater extent, as evidenced with ratios of $42.50\% \pm 14.52\%$ and $39.69\% \pm 13.85\%$, respectively. Of interest, however, despite its attenuated amplitude, the rod V_{max} ratio is significantly greater ($P < 0.05$) than that of the cone b-wave ($25.40\% \pm 9.70\%$), suggesting an overall greater susceptibility of cone function to postnatal hyperoxia compared with pure rod function, a finding that supports previous claims of ours.^{12 13}

5.4 Effect of hyperoxia on retinal cytoarchitecture

The maturation-induced changes in retinal function described earlier were also accompanied by changes in retinal structure. Representative retinal cross sections obtained at postnatal days 15, 17, 19, and 24 from control and oxygen-exposed animals are shown in Figure 6. Given that our previous studies^{12 14 15} showed that postnatal hyperoxia affects only the thickness of the outer plexiform layer (OPL) and the horizontal cell (HC) count, data analysis was limited to these two cytoarchitectural features. Group data are graphically reported in Figure 7. In control rats, the thickness of the OPL grew slightly, but not significantly, from eye-opening to P24 (P15: $6.82 \pm 2.09 \mu\text{m}$, P24: $9.62 \pm 1.06 \mu\text{m}$; $P > 0.05$). In contrast, the OPL of the hyperoxic-cohort reached maximum thickness at P16, after which a gradual and significant thinning occurred (P16: $5.42 \pm 0.84 \mu\text{m}$, P24: $2.39 \pm 1.44 \mu\text{m}$, $P < 0.05$). Furthermore, animals that were raised in 80% O₂ always showed a thinner OPL than that in age-matched control rats, which reached significance at P16 (68.1% of control at P15 to 24.8% of control at P24; $P < 0.05$), confirming our previous demonstration of OPL susceptibility to postnatal hyperoxia.^{12 14 15} This hyperoxia-induced OPL thinning was also accompanied by a significant reduction in the total number of HCs, as reported in Figure 6. In control rats, we noticed a slight but significant attrition of the HC count with age (P15: 3.53 ± 0.12 cells, P24: 2.40 ± 0.35 cells; 68.0% of P15, $P < 0.05$). The above contrasts with the severe reduction in HC count noted in the hyperoxic cohort from P15: 0.93 ± 0.23 cells (26.3% of age-matched control HC count, $P < 0.05$) to P24: 0.28 ± 0.25 cells (11.7% of age-matched control HC count, $P < 0.05$).

6 Discussion

Previous studies have shown that postnatal exposure to hyperoxia severely damages retinal structure (e.g., significant thinning of the OPL and attrition of the HC count) and function (significant reduction in amplitude of photopic and scotopic ERGs).^{10 11 12 13 14 15 16 17 18} These irreversible sequels are preceded by a typical and reversible vasculopathy reminiscent of that known to characterize the human form of this retinal disorder, retinopathy of prematurity (ROP). Although previous studies have also evidenced significant reductions in vascular coverage after postnatal hyperoxia in rats,^{5 8 9 19 26 27 28} few studies have examined the differential susceptibility of retinal vasculature to postnatal hyperoxia as a function of the degree of vascular maturity reached at the time of hyperoxic exposure. To our knowledge, however, our study is the first to show that despite the damage that is observed within the first week or so of exposure (e.g., P0–P6 and P0–P9 in Figs. 1 and 2), the retinal vascular growth process appeared to fight this oxidative stress, to achieve nearly full coverage while remaining under hyperoxic exposure throughout the first 2 weeks of life. The window of blood vessel plasticity described by Benjamin et al.²⁹ would further support our findings, in which early postnatal exposure to hyperoxia (P4–P6) similarly resulted in vaso-obliteration. This phenomenon is explained by the increased susceptibility of immature vessels that have not yet acquired pericytes at such an early age, whereas vessels that are more mature appear to better withstand an equivalent stress that occurs later on.²⁹ Furthermore, VEGF has been shown to play a pivotal role in vascular development and retinal coverage, and hyperoxia counteracts its expression, which inevitably results in underdeveloped retinal vasculature.³⁰ In fact, exogenous VEGF administration has been shown to act as a vascular survival factor, rescuing vasculature from hyperoxia-induced destruction.³¹ Of interest, however, other studies have also shown that prolonged hyperoxia (during which VEGF levels are suppressed) paradoxically results in an enhanced revascularization process,^{19 32} thus placing less of an emphasis on a role for

VEGF. For example, vaso-obliteration occurred to a greater extent in rats exposed to hyperoxia from P7 to P9 than in those exposed from P7 to P12.¹⁹ Similarly, mice that are maintained in a continuous hyperoxic environment undergo more rapid revascularization than those that are returned to room air after exposure, thought perhaps to result from the preservation of astrocytes and Müller glia in these conditions.³² Concomitant increases in NO levels via increased eNOS expression with early hyperoxia-induced vaso-obliteration and with subsequent revascularization after prolonged hyperoxia have also suggested a cytotoxic and cytoprotective role for NO, respectively, in each of these conditions.^{19 33} Whether similar mechanisms are responsible for enabling the retina to overcome vascular deficits that are initiated early on in our model of continuous exposure, however, requires further elucidation. Nevertheless, previous studies, in addition to our findings reported herein, have shown that despite full vascular coverage, permanent structural and functional damage inevitably occurs.^{10 11 12 13 14 15} It is important to keep in mind, however, that many of the results referred to from previous studies were obtained from mature or nearly mature animals (ranging from P18 to P60) and at a time interval remote from the cessation of the oxidative insult. With this study, we confirm that the pathophysiological sequence of events that ultimately yields the clinical picture described earlier was initiated while the rats were still in the hyperoxic regimen, at least when the retinal cytoarchitecture is considered. Given that these cytoarchitectural anomalies appear to be limited to OPL thinning and reduced HC counts, it is not surprising that there is no evidence of significant functional deficits, as determined with the ERG a-wave in the first 2 days or so after the opening of the eyes (and consequently after the cessation of the hyperoxic regimen, as per our protocol), while scotopic and photopic b-waves already tend to be attenuated in amplitude. These findings would therefore suggest that OIR is initiated at a postreceptoral level, where both outer retinal structure and function remain intact, whereas synaptic impairment at the level of the OPL prevents signal transmission to the inner retina. Our results are in accord with previous studies that documented early differences between control and hyperoxia-exposed animals on eye opening at P13 and P14, 2 days after and

immediately after the cessation of hyperoxic exposure, respectively.^{17 18} While hyperoxia resulted in attenuated b-wave amplitudes compared with those in control rats raised in room air, the a-wave appeared to be either similarly¹⁷ or less¹⁸ affected. Evaluation at further time points revealed even greater scotopic b-wave attenuations in hyperoxic rats compared with control as evidenced at P17,¹¹ P18,¹⁷ and at P21,¹⁸ whereas the a-wave amplitude improved with age, reaching values virtually identical with the control at P18,¹⁷ at P21, and beyond.¹⁸ These findings of impaired postreceptoral function that occur shortly after the hyperoxic episode may therefore be explained by the thinning of the OPL, which we have identified as early as eye opening (P15).

Previous studies of ours also suggested a normal age-related decline in amplitude of scotopic and photopic ERG parameters between P30 and P60,^{12 13 14 15 25} findings that are corroborated with results reported herein (Fig.4). Of interest, only the a-wave showed a similar age-dependent amplitude attenuation after exposure to hyperoxia, whereas the rod V_{max} , rod-cone b-wave, and photopic b-wave did not. A study by Penn et al.¹⁸ revealed a similar decline in the scotopic b-wave amplitude (by ~40%) between control animals aged 5 and 9 weeks (from P35 to P63, time points that resemble those used in the present study), whereas no such findings were evident in the hyperoxic cohort. No other significant gain or decline in amplitude was observed in recordings obtained throughout 16 weeks of age (P112), thereby excluding the likelihood of any delay in this age-related decline. On the other hand, the a-waves obtained from animals raised in the different oxygen environments within that age-range were both similarly attenuated, and when taken together with the results just reported, further support our current findings. These results collectively suggest that whereas normal developing animals are subjected to a decline in retinal function [which is also accompanied by a normal thinning of the retinal layers between the time of eye opening (approximately P15) through adulthood],³⁴ a different picture emerges after exposure to hyperoxia. The thinning of the retina, which is likely to be at the root of the functional attenuation that takes place with age, most probably occurs

through apoptotic mechanisms or otherwise, as a part of the normal aging process. As described earlier, hyperoxia that occurs from birth throughout P14 is of only little consequence on the a-wave, suggesting that the photoreceptor layer is resistant to this stress. Consequently, the normal cellular refinement of the outer retina that occurs with time (between P30 and P60) can still progress under conditions of excess oxygen. In contrast, hyperoxic exposure culminated in a compromised b-wave along with considerable damage to the generating cells, and of note, the irreversible reorganization of retinal structure and/or cell death that occurred in the postreceptoral retinal layers of exposed animals appeared to be stationary once maximum amplitude was attained. This finding suggests that the damage triggered by hyperoxia precludes further deterioration of the retina, such as that which occurs in the normal aging process. The steps involved in this "protective" mechanism remain to be fully understood.

Unlike precocial species (such as humans, for example) that are born with eyes opened and relatively mature retinal structure and function,^{35 36 37 38} altricial animals (such as the rat and mouse) are born with their eyes closed and a relatively immature retina,^{39 40} comparable to a prematurely born human infant at 24 to 26 weeks of gestation.⁴ Consequently, the susceptibility of the different retinal elements to the oxidative insult could depend on the level of maturity that they reached at the time of the hyperoxic exposure and/or the duration of the hyperoxic exposure that they suffered (also dependent on their birth date). For example, it has been shown in mice that cones, ganglion cells, amacrine cells, and HCs are the first identifiable retinal cells at E14, whereas rods and Müller cells appear at birth.⁴¹ In a normal environment, this results in a well-organized maturation of retinal function, where development of saturated rod a- and b-wave amplitudes has been shown to occur between P12 and P30, which is related to rod outer segment (ROS) length and rhodopsin content levels.⁴² Similarly, photopic OPs have also been shown to reach maturity much later (P30) compared with the earlier maturation of scotopic OPs at P17.⁴³ Results obtained in our present study are in accordance with these findings, given that we observed a peak in the normal

rod $V_{\max}$ amplitude at P24, whereas that of the photopic b-wave occurs at P30. Surprisingly, however, the peak for both parameters after hyperoxic exposure is found at P24, suggesting that despite the attenuation in both rod and cone responses, the rod $V_{\max}$ still follows a normal developmental time course after hyperoxia, in contrast to the cone and rod–cone b-waves whose maturation peaks tend to fall short of normal. Furthermore, the attenuation in the cone b-wave amplitude at the height of maturation (as a function of normal, as per Fig. 5) is significantly more pronounced than that of the rod $V_{\max}$. When taken together with the normal process of retinal cellular development, these findings suggest that the earliest existing structures (cones and HCs, for example) are those that are most susceptible to maturation-induced changes that follow postnatal hyperoxia. This concept could also explain the more severe impact of oxidative stress on HCs and the OPL (the layer of synaptic contact between photoreceptors and HCs and bipolar cells), as the former are more developed at birth, and would consequently be exposed to the hyperoxic regimen for a longer duration. Of interest, previous studies have described a role for free radicals in generating the pathogenesis of OIR.^{5 6 12 27 28 44 45} It has also been suggested that endogenous antioxidant defense systems may differ between rods and cones, rendering the latter system more susceptible in the face of reactive oxygen species induced by hyperoxia,¹³ or FeSO_4 ,⁴⁶ for example. Furthermore, our finding of a more potent effect of Trolox C, a vitamin E analogue with antioxidant capacity, on cone-mediated function in the hyperoxic retina, suggests to us that treatment is targeted to the areas that need it most.¹² Collectively, these results support the hypothesis whereby devastation of the cone pathway (which begins its development in utero) occurs to a greater extent than that of the rod pathway (which begins its development postnatally).

Our ability to demonstrate altered retinal function (with the exception of the a-wave) immediately after cessation of hyperoxic exposure would put less emphasis on the contribution of neovascularization to these manifestations, since this aggravated angiogenic process is known to be triggered after recovery in room air.^{8 9 47 48 49 50} Although in the present study we have not tried to quantify the

level of neovascularization, our findings suggest that it may play a negligible role in the initial impairment of retinal function (at birth) compared with the devastating structural consequences (e.g. retinal detachment) that it could cause, should it fully manifest itself. If the above scenario also applies to the human counterpart, care will have to be taken when evaluating the functional consequences of hyperoxic exposure in human premature infants, despite the lack of significant evidence of underlying neovascularization.

In conclusion, posthyperoxia events, including the return to normoxia (e.g., relative hypoxia), and the resulting neovascularization have been deemed largely responsible for the pathogenesis of OIR and its human form, ROP. The results obtained with the present study suggest that alterations in retinal cytoarchitecture concurrent with hyperoxic exposure in addition to the arrest in functional maturation that directly follows the exposure regimen in neonatal rats may play an equally important role. Whether the above are induced as a result of the vasculopathy or occur independently remains to be further elucidated.

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9 Captions to figures

Figure 1: Representative retinal vascular flatmounts stained with ADPase from control animals (*top*) euthanatized at postnatal day 6 (**A**), 9 (**B**), 12 (**C**) and 14 (**D**), respectively, and from animals (*bottom*) that were euthanatized immediately after 6 (P0–6, **E**), 9 (P0–9, **F**), 12 (P0–12, **G**), and 14 (P0–14, **H**) days of oxygen exposure. Magnification, x40.

Figure 2: Graphic representation of retinal vasculature coverage (ordinate) as a function of age (days, abscissa) in the normoxic-cohort (*open symbols, dashed line*) compared with that observed in the hyperoxic-cohort (*shaded symbols, solid line*). Results are expressed as a percentage of completely vascularized retinas at P14 (control) $\pm$ 1 SD. *Significant differences from control at a given age; the Student's *t*-test ($P < 0.05$).

Figure 3: Representative scotopic (mixed rod–cone, flash intensity: $0.60 \log \cdot \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$) and photopic (flash intensity: $0.9 \log \cdot \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$, background: $30 \text{ cd} \cdot \text{m}^{-2}$) ERGs obtained from animals raised in normoxia (21% O_2 , *top tracings, A*) and hyperoxia (80% O_2 , *bottom tracings, B*) from postnatal day 0 through 14.

Recordings were obtained at postnatal days 15, 16, 17, 19, 24, 30, and 60, respectively. Calibrations: horizontal, 20 ms; vertical, 200 μ V for scotopic mixed rod-cone responses and 50 μ V for photopic responses. All tracings include a 20-ms prestimulus baseline; *vertical arrows*: flash onset.

Figure 4: Graphic representation of a-wave (**A**), rod $V_{\max}$ (**B**), rod-cone b-wave (**C**), and photopic b-wave (**D**) amplitudes (ordinate in microvolts) obtained at postnatal days 15, 16, 17, 19, 24, 30, and 60, respectively (abscissa in days). Amplitudes from control animals raised in normoxia (*open symbols, dashed line*) are compared with those obtained from animals raised in hyperoxia (*shaded symbols, solid line*). *Significant differences between normoxia- and hyperoxia-reared groups for each given age; Student's *t*-test ($P < 0.05$). Significant differences within normoxic or hyperoxic cohorts were identified with a one-way ANOVA followed by a post hoc Tukey test. (•) Values found to be significantly different from those obtained at P15, or the time at which the earliest recordable response was obtained ($P < 0.05$). Results are given as the mean amplitude ± 1 SD.

Figure 5: The mean ratios of individual ERG measures from exposed groups/control mean (at maximum value) were calculated for each parameter and are represented graphically. Results were obtained based on a-wave and rod $V_{\max}$ data generated at P19 and P24, respectively, and on rod-cone b-wave and cone b-wave data at P30, which are the times corresponding to each of their respective peaks in maturation. A one-way ANOVA followed by a Tukey post hoc test comparing data obtained from each group was used to detect statistically significant differences between ratios. *Significantly different from the a-wave ratio ($P < 0.05$); (•) significantly different from the rod $V_{\max}$ ratio ($P < 0.05$). Results are given as the mean ratio ± 1 SD.

Figure 6: Representative retinal histologic sections (central retina) obtained from animals at P15 (control, **A**; oxygen-exposed, **F**), P16 (control, **B**; oxygen-exposed, **G**), P17 (control, **C**; oxygen-exposed, **H**), P19 (control, **D**; oxygen-exposed, **I**), and P24 (control, **E**; oxygen-exposed, **J**). *Oblique arrows*: HCs located within the INL. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 50 μ m.

Figure 7: The mean thickness (in micrometers) of the OPL (**A**) and the mean HC count (**B**) in animals raised in normoxic and hyperoxic environments. Measurements were taken at P15, P16, P17, P19, and P24, respectively. *Significant differences between normoxia- and hyperoxia-reared groups at each given age found using the Student's *t*-test ($P < 0.05$). Significant differences within normoxic or hyperoxic cohorts were identified with a one-way ANOVA followed by Tukey post hoc test comparing data obtained from all age groups. (•) Significantly different values from those obtained at P15 ($P < 0.05$). Results are given as the mean thickness or cell count $\pm$ 1 SD.

10 Figures

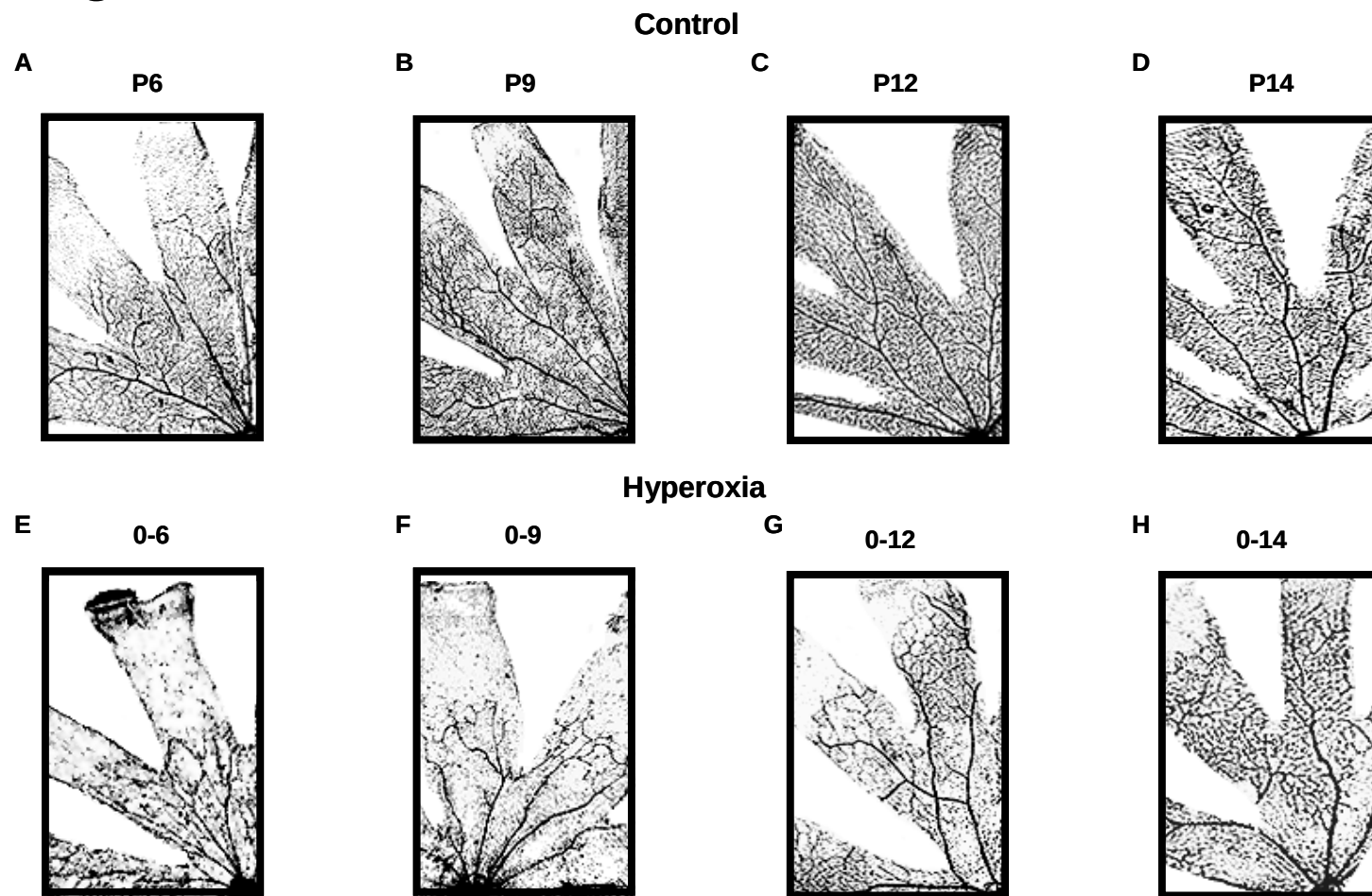


Figure 1

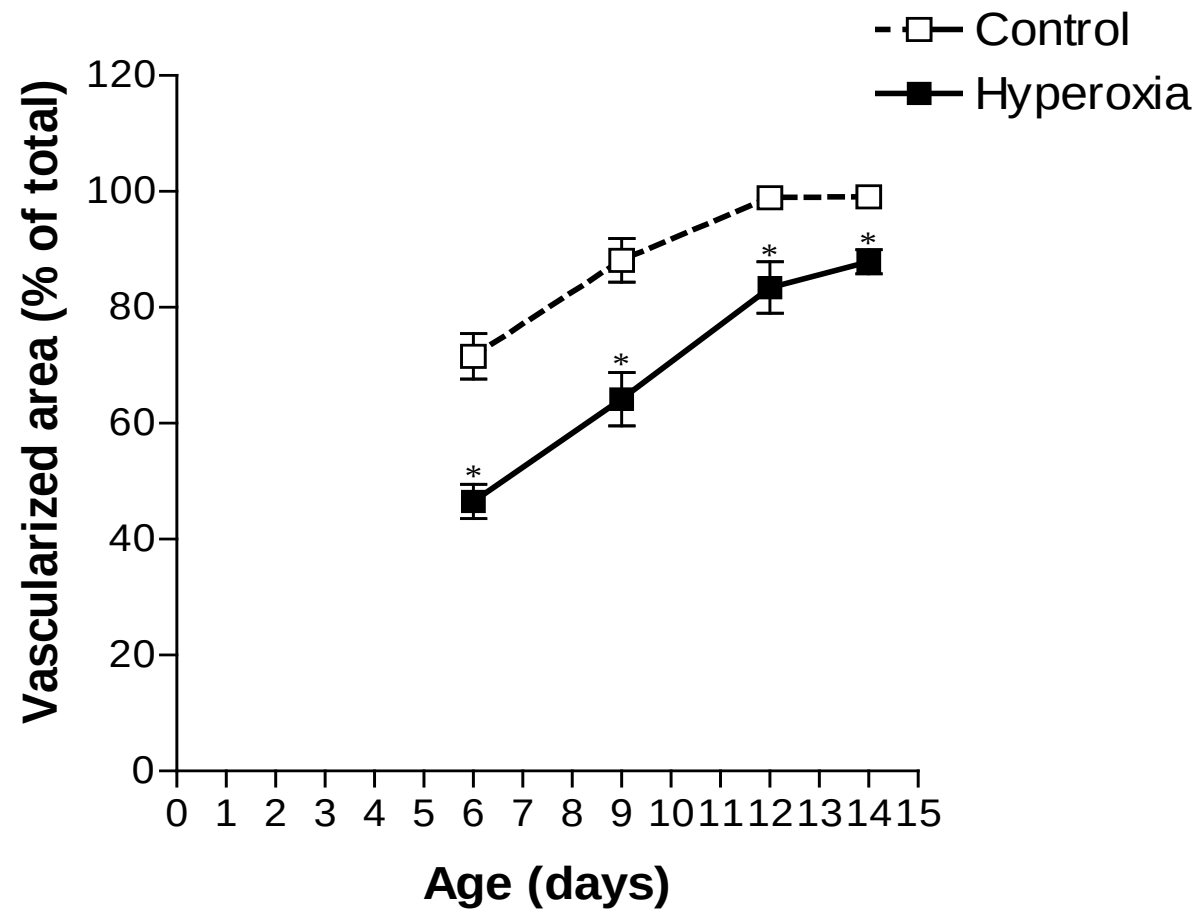
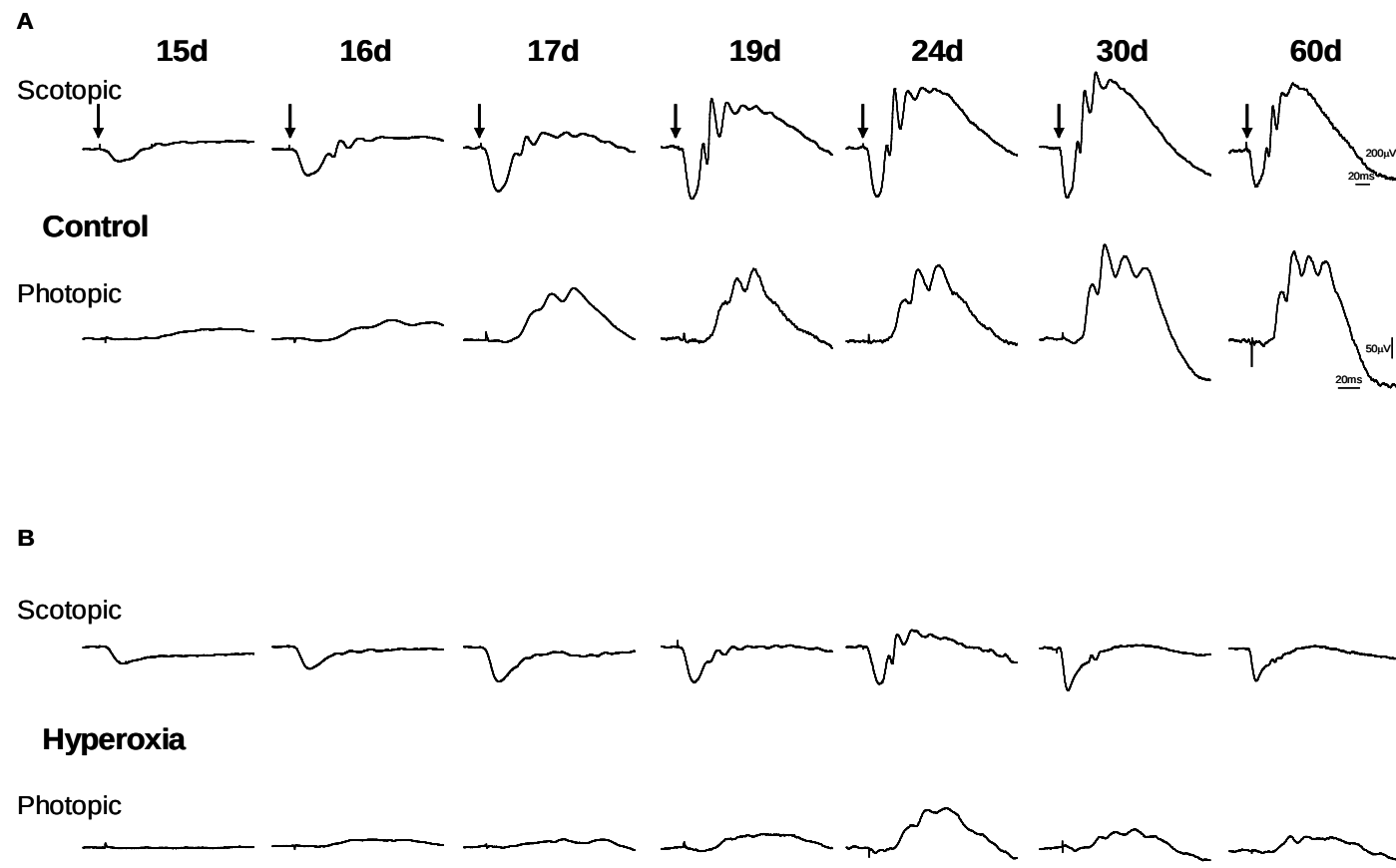


Figure 2

**Figure 3**

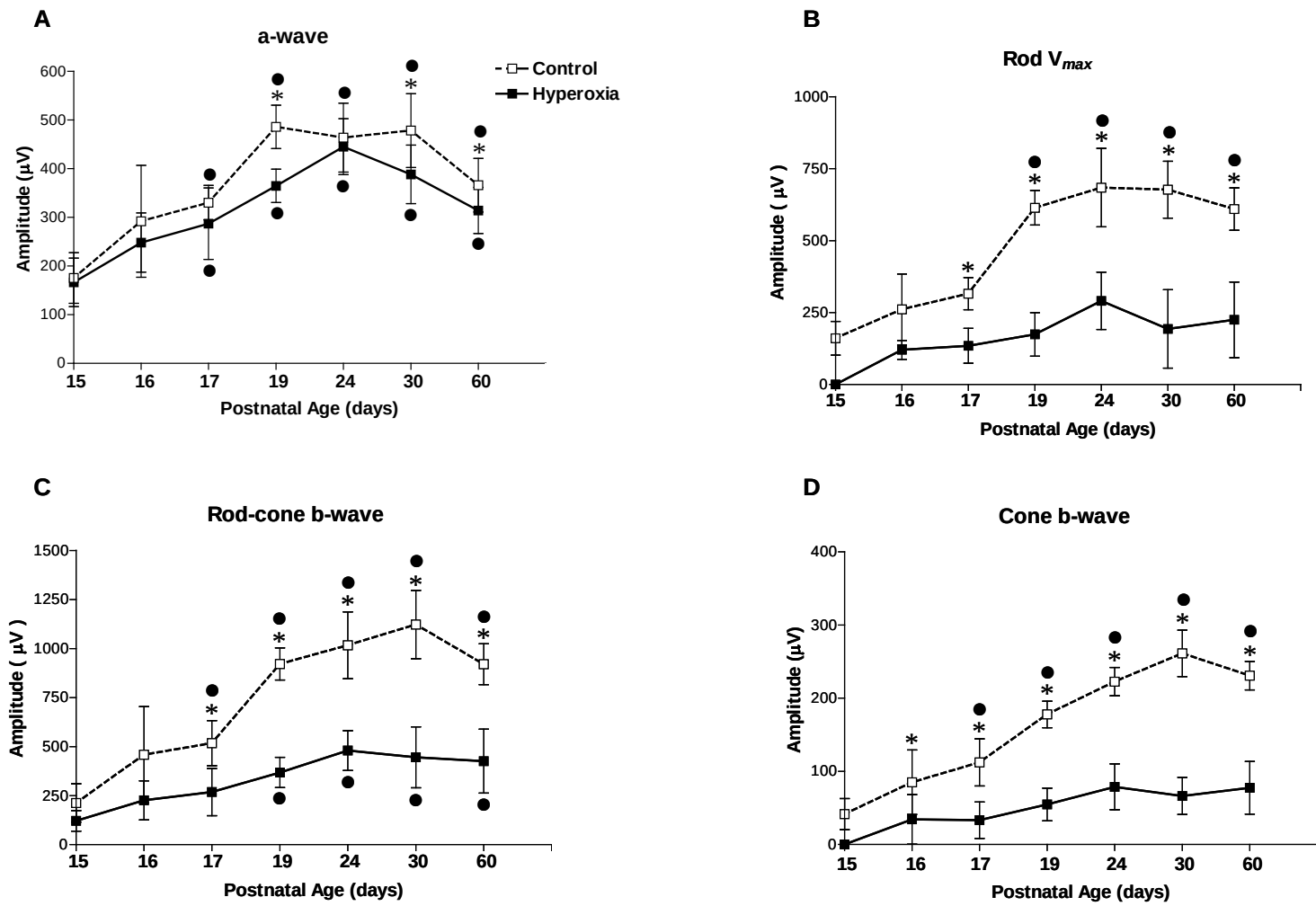


Figure 4

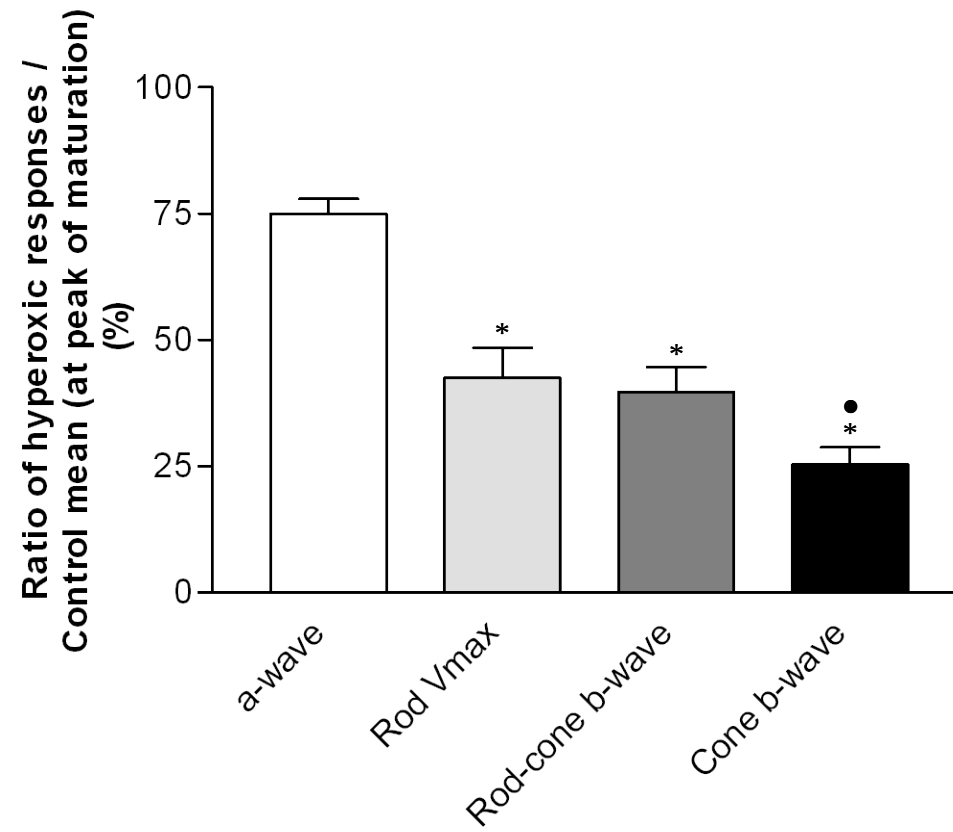
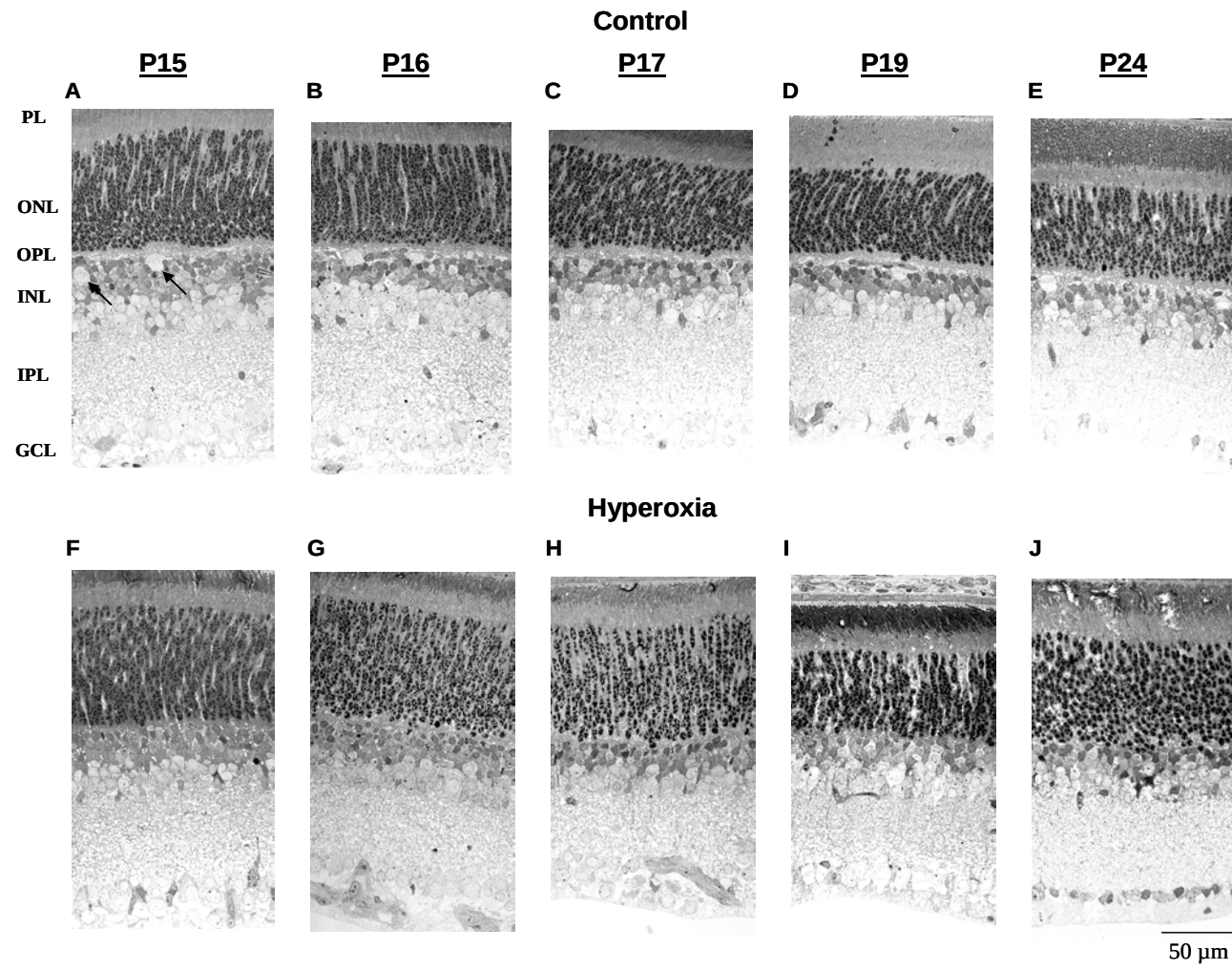


Figure 5

**Figure 6**

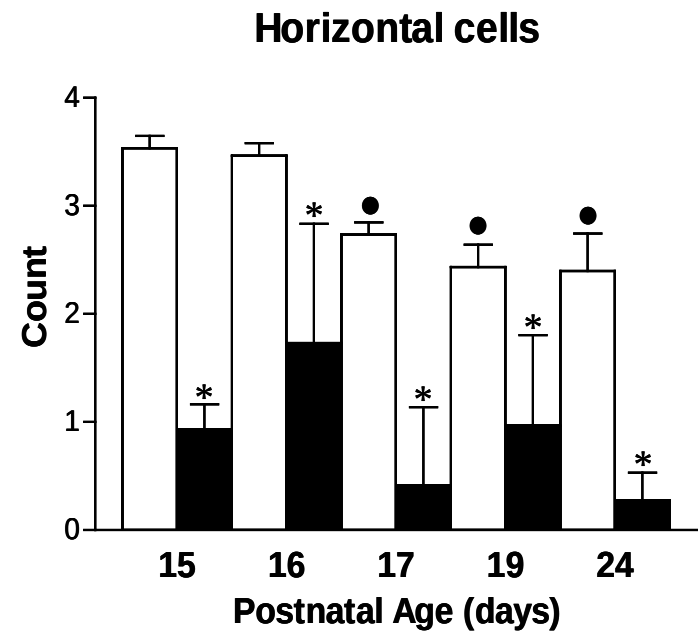
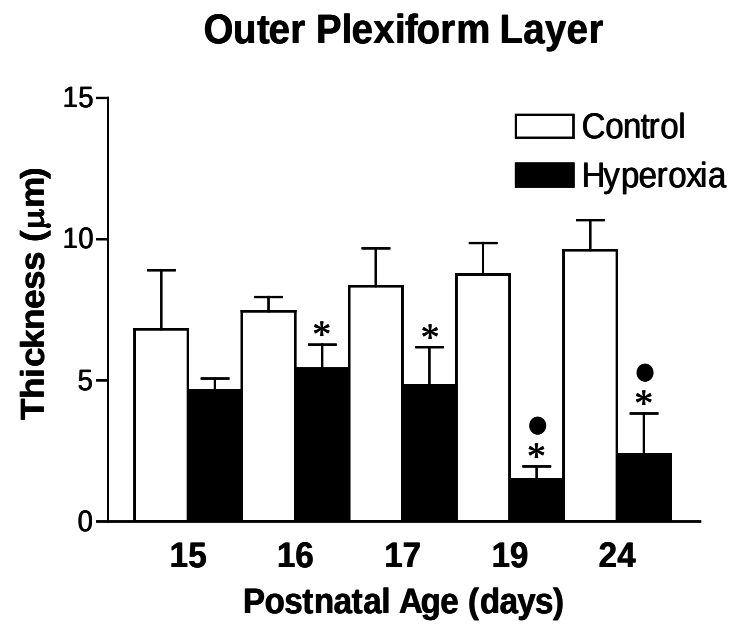


Figure 7

CHAPTER III: MANUSCRIPT II

Dorfman AL, Dembinska O, Chemtob S, Lachapelle P. Structural and Functional Consequences of Trolox C Treatment in the Rat Model of Postnatal Hyperoxia. Invest Ophthalmol Vis Sci. 2006 Mar; 47:1101-8.

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1 Preface to chapter III

Previous studies have suggested that while retinal vasculature abnormalities are the hallmark of OIR, permanent changes in retinal structure and function can also be evidenced. While the use of vitamin E therapy in ROP is controversial, trolox C is a water soluble analogue of vitamin E that has been used as an antioxidant in diseases where reactive oxygen species play a role, such as myocardial injury and diabetic retinopathy, for example. It has been suggested that free radicals may be the source of the pathologic effects described above, namely on retinal vasculature, cytoarchitecture and function. Trolox C was also previously shown to limit the vasculopathy resulting from exposure to postnatal hyperoxia and consequently the aim of this study was to investigate whether it could also help prevent the functional (ERG) and structural (retinal histology) consequences associated with OIR.

2 Abstract

Purpose: Previous studies have shown that newborn rats exposed to hyperoxia within the first 2 weeks of life develop vasculopathy in addition to permanent changes in retinal structure and function. It has also been suggested that free radicals may be the source of these pathologic effects. Trolox C, a water-soluble analogue of vitamin E, was previously shown to limit the vascular consequences of exposure to postnatal hyperoxia. The aim of this study was to investigate whether trolox C could also help prevent the functional (electroretinography) and structural (retinal histology) consequences associated with oxygen-induced retinopathy (OIR). **Methods:** Newborn albino Sprague-Dawley rats exposed or not exposed to hyperoxia received daily injections of trolox C in doses of 300, 600, and 900 $\mu\text{g/kg}$ (total volume, 50 μL). The effect of treatment was evaluated through electroretinography and retinal histology. **Results:** Although trolox C tended to have a retinoactive effect on the normal retina, normalization of the hyperoxia-treated group to hyperoxic control and of the normoxia-treated group to normoxic control revealed that the a-wave remained relatively unaffected by hyperoxia exposure and by treatment with trolox C, the efficacy of trolox C at doses of 600 and 900 $\mu\text{g/kg}$ largely outweighed the retinoactive effect, and the oscillatory potentials (OPs) benefited to the greatest extent from trolox C treatment. Furthermore, trolox C was able to limit the reduction in outer plexiform layer thickness but not the concomitant reduction of the horizontal cell count, each of which is associated with OIR. **Conclusion:** These results show that, as had been previously demonstrated with retinal vasculature, trolox C limited the retinal functional and structural damages inherent in the rat model of OIR. However, despite treatment, there were still signs (albeit less severe) indicative of OIR. This suggests, as previously advanced, that the

pathophysiology of OIR is not solely caused by the action of free radicals or that trolox C is inadequate in treating all aspects of OIR.

3 Introduction

Retinopathy of prematurity (ROP) is a potentially blinding retinal disorder that affects small prematurely born infants.¹ Supplemental oxygen is often delivered to these infants to maintain adequate blood levels and stable pulmonary status.² It has been suggested that the formation of free radicals in the retina may result from the exposure of these premature infants to excessive oxygen, given that they are deficient in antioxidant defenses.³ Some consequences associated with the formation of free radicals include vasoconstriction and vaso-obliteration of the developing retinal vessels, which are often followed by neovascularization on the return to normoxia.^{4 5} These clinical signs are used to identify and grade the severity of the human and animal forms of this retinopathy.^{5 6 7 8 9 10 11 12 13 14 15} It is the later stages that cause the most severe consequences of postnatal hyperoxia.

Previous studies have shown that vitamin E therapy results in decreased vaso-obliteration of retinal blood vessels in newborn rats exposed to hyperoxic conditions.^{15 16} However, high doses of vitamin E treatment were necessary to produce a measurable protective effect against free radicals, a factor potentially leading to toxicity. It thus appears that to be effective, the antioxidant of choice must not only be able to enter the cells and subcellular compartments in which reactive oxygen species (ROS) exist¹⁷ but, more important, must be safe to use.

Trolox C is a water-soluble vitamin E analogue previously used for antioxidant therapy in various models in which ROS are formed, including myocardial injury¹⁸ and diabetic retinopathy.¹⁹ This was also the antioxidant used by Penn et al.²⁰ in the rat model of oxygen-induced retinopathy (OIR), by

which they showed that the retinas of newborn rats exposed to oxygen while receiving trolox C had greater vascular coverage and higher capillary density, suggesting that trolox C could help prevent the vascular consequences of OIR. However, apart from vasculopathy, OIR was also shown to include cytoarchitectural and functional anomalies of the retina. Newborn Sprague–Dawley rats exposed postnatally to hyperoxia were shown to develop severe electroretinographic anomalies characterized by marked attenuation of the b-wave and oscillatory potentials of the electroretinogram (ERG) and relative sparing of the a-wave, suggesting that the functional deficit in OIR was located at a postreceptoral site.^{21 22 23 24 25 26} This finding correlated well with the reported decrease in the number of horizontal cells and the reduction in thickness of the outer plexiform layer (OPL) along with the sparing of the photoreceptor layer, which was also shown to characterize this type of retinopathy.^{21 22} Of interest, previous studies also showed a lack of correlation between the vascular and the retinal structural–functional consequences of OIR, in which the retinal vasculature often resumed normalcy shortly after the return to normoxia, whereas the electroretinographic and cytoarchitectural anomalies became permanent features of the hyperoxic rats.^{21 22 23 24} Therefore, although the retinal vascular anomalies of OIR rats were shown to be partly prevented by free radical scavengers,²⁰ it is not yet established whether they can also exert a protective effect on both structural and functional levels. Because of these discrepancies, this study was conducted to determine whether trolox C could also protect the structure and function of the retina after postnatal hyperoxia.

4 Methods

The experimental protocol was approved by the McGill University/Montreal Children’s Hospital Research Institute Animal Care committee according to the guidelines of the Canadian Council on Animal Care.

Experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Newborn litters of Sprague-Dawley (SD) rats (Charles River Laboratories, St. Constant, Quebec, Canada) were exposed to 80% oxygen (mixture of medical grade 100% O₂ and room air measured with a MaxO₂ ceramtec oxygen meter, model OM25-ME; Medicana Inc, Montreal, Canada) from postnatal day 0 through postnatal day 14 for 22.5 hours daily, interrupted three times for a 30-minute period of normoxia (21% oxygen).²¹⁻²⁶ There were eight experimental groups in total. The hyperoxic cohort (postnatal exposure to 80% O₂) was subdivided into four experimental groups: one that received oxygen only (i.e. control hyperoxic group, $n = 16$) and three that were exposed to oxygen (80%) while receiving trolox C (hyperoxia-treated group) at concentrations of 300 ($n = 8$), 600 ($n = 11$), and 900 ($n = 8$) µg/kg, respectively. The normoxic cohort (postnatal exposure to 21% O₂) consisted of one group that was raised under normoxic conditions without receiving the antioxidant treatment (i.e., control normoxic group, $n = 16$) and three groups also raised in normoxic conditions while receiving trolox C (normoxia-treated group) at concentrations of 300 ($n = 6$), 600 ($n = 5$), and 900 ($n = 5$) µg/kg, respectively. Because it was previously shown that postnatal hyperoxia occurring within the first week of life had a negligible effect on retinal structure and function²¹⁻²⁶ and to minimize trauma to the newborn pups, intraperitoneal injections of trolox C (300, 600, and 900 µg/kg, respectively), dissolved in 10% EtOH, were administered starting at postnatal day 5 and continuing through postnatal day 14. Finally, to avoid pulmonary complications that are known to develop in adult rats raised in a hyperoxic environment, mothers of the litters were alternated between normoxic and hyperoxic conditions every 24 hours.

4.1 Electrophysiology

ERGs and oscillatory potentials (OPs) were recorded with a data acquisition system (Biopac MP 100WS; Biopac Systems Inc. , Goleta, CA) using a method that was previously reported.^{21 22 26} Briefly, after a period of 12 hours of dark adaptation, rats were anesthetized (under dim red light illumination) with intramuscular injections of ketamine hydrochloride (85 mg/kg) and xylazine (6 mg/kg). Pupils were dilated with 1% cyclopentolate hydrochloride (Mydracyl solution; Alcon Laboratories, Fort Worth, TX), and full-field ERGs were recorded (10,000x, 1- to 1000-Hz bandwidth, 6-dB attenuation) along with OPs (50,000x, 100- to 1000-Hz bandwidth, 6-dB attenuation) using analogue preamplifiers (P511; Grass Instruments, Quincy, MA). A fiber electrode (DTL; 27/7 X-Static silver-coated conductive nylon yarn; Sauquoit Industries, Scranton, PA) was placed on the cornea and was used as the active electrode. Hydroxymethylcellulose (2%; Gonioscopic solution; Alcon Laboratories) was placed on the cornea thereafter to prevent corneal desiccation and to hold the DTL fiber in place. A disc electrode (model E6GH; Grass Instruments) was placed in the mouth, and a needle electrode (model E2; Grass Instruments) was inserted into the tail to serve as the reference and to ground electrodes, respectively. The rats were then placed in a light-proof recording chamber of our design that included the light stimulus and background light.^{21 26} Scotopic responses were evoked to flashes of white light spanning a 7.2-log unit range in 0.3-log increments (maximal intensity, 0.6 log candela·s/m² [log cd·s/m²]; average, 2–5 flashes; interstimulus interval, 10.24 seconds), whereas photopic responses were evoked to flashes of white light of 0.9 log cd·s/m² delivered after more than 15 minutes of light adaptation to a background of 30 cd/m² (average, 20 flashes; interstimulus interval, 1 second) to avoid a light adaptation effect.²⁷ A first set of recordings was obtained at 30 days of age, at which point rod and cone functions are known to be fully mature.^{28 29 30} Furthermore, to determine whether the functional

abnormality that was created was permanent, a second set of recordings was obtained at postnatal day 60.

4.2 Histology

Structural changes were assessed on specimens collected at 60 days of age. Histologic sections (2- μ m cross-sections) of whole-eye mounts were embedded in epoxy resin (Epon; Resolution Performance Products, Houston, TX) and stained with toluidine blue according to a method previously described.²² Measurements of OPL thickness and horizontal cell counts were performed on 250- μ m-wide fields of the central retina. Three retinas per group were analyzed, and an average of 10 measurements from each was obtained. Pictures were obtained using a photograph microscope (Acti; Zeiss, Oberkochen, Germany).

4.3 Data Analysis

Amplitudes of ERG components were measured according to a method previously described.^{21 26} Briefly, the amplitude of the a-wave was measured from baseline to trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Oscillatory potentials were measured in a similar fashion from the preceding trough to the peak of the OP evaluated and were reported individually or as the sum of OPs ($SOP = OP_2 + OP_3 + OP_4 + OP_5$).

For each animal, scotopic luminance-response function curves were derived by plotting b-wave amplitudes against flash intensities. A sigmoidal-intensity response regression curve was then used to fit the data (Prism 3.00 software; GraphPad, San Diego, CA), from which the rod V_{max} (maximal rod

response) was calculated. In addition, the ERG response evoked to the brightest flash delivered in scotopic conditions (the mixed rod-cone response) was also included in the analysis. Two-way repeated measures ANOVA ($P < 0.05$) with Bonferroni posttests were used to determine the effect of hyperoxia and trolox C treatment on the different parameters of the ERG and on retinal histologic structures, with maturation (30 and 60 days) as the repeated factor and treatment group as the independent factor. Data are presented as the mean $\pm$ 1 SD.

Finally, because ERG measurements were repeated at 30 and 60 days of age, we devised the retinal maturation index as a means to determine the change in retinal function resulting from the normal aging process. This index was calculated by taking the average of the percentage difference between ERGs obtained at 30 and 60 days for each individual rat. One-way ANOVA followed by Tukey's honest significance difference (HSD) test was used to detect significant differences. Data are presented as mean percentage change in amplitude $\pm$ 1 SD.

5 Results

Figure 1 shows representative scotopic (rod $V_{\max}$, mixed rod-cone ERGs, and mixed rod-cone OPs) and photopic (cone ERG and cone OPs) retinal responses obtained from four of the eight study groups. The first group illustrated in Figure 1 consists of rats raised in room air (normoxic control). They are compared with rats raised in room air while receiving 600 $\mu\text{g/kg}$ trolox C from postnatal day 5 through postnatal day 14 (normoxia-treated), with rats raised in a hyperoxic environment from birth through postnatal day 14 (hyperoxic control), and with rats exposed to postnatal hyperoxia for 14 days from birth while receiving 600 $\mu\text{g/kg}$ trolox C (hyperoxia-treated) from postnatal day 5 through postnatal day 14. ERG responses were obtained twice, at 30 days (upper tracings) and at 60 days (lower tracings) of age. Amplitude measurements are reported in Table 1 .

Amplitude attenuation of the scotopic and photopic b-waves, scotopic a-wave, and OPs can be observed in the hyperoxic control rat compared with the normoxic control rat. As shown in Table 1, amplitudes of the rod $V_{\max}$ and the scotopic and photopic SOPs measured from hyperoxic rats treated with 600 $\mu\text{g/kg}$ trolox C were nearly double the size of those measured from hyperoxic control rats ($P < 0.05$). There was also a slight, but not significant, enhancement ($P > 0.05$) of the rod-cone b-wave and photopic b-wave amplitudes, whereas the amplitude of the a-wave was unaltered by trolox C treatment (Table 1).

Results presented in Table 1 also suggest that trolox C might alter the functioning of the normal (unexposed) retina. The scotopic ERG responses (rod $V_{\max}$ and rod-cone b-wave) of control treated rats were larger (but not significantly; $P > 0.05$) than those of untreated controls, whereas the photopic SOPs were significantly smaller ($P < 0.05$) than untreated controls. Consequently, to dissociate the protective effect that trolox C exerted on the retinas of newborn rats exposed to hyperoxia from its retinoactive effect observed in the normal retina, we normalized ERG amplitude measurements obtained from each normoxia-treated rat (trolox C 300-, 600-, and 900- $\mu\text{g/kg}$ groups, respectively) to the mean amplitude of the normoxic control group (taken as 100%). Similarly, ERG amplitude measurements obtained from each hyperoxia-treated rat (trolox C 300-, 600-, and 900- $\mu\text{g/kg}$ groups, respectively) were normalized to the mean amplitude of the hyperoxia control group (also taken as 100%). Results obtained from this data manipulation are shown in Figure 2, in which data obtained at postnatal days 30 and 60 are also compared. Our results confirm our initial impression that, despite the lack of an effect on the a-wave (Figs.2A, 2B), treatment with trolox C does indeed have an impact on retinal function. Amplitude ratios generated from hyperoxia-treated rats always tended to be greater than those obtained from normoxia-treated rats, suggesting that the therapeutic effect of trolox C outweighed its (normal) retinoactive effect. In fact, when normoxia-treated and hyperoxia-treated rats are compared, significant differences favoring the therapeutic effect of trolox C are seen for the photopic b-

wave at postnatal days 30 and 60 and for scotopic and photopic SOPs at postnatal days 30 and 60 (Figs.2G, 2H, 2I, 2J, 2K, 2L). Similar tendencies can also be seen for the rod $V_{\max}$ and the rod-cone b-waves. As mentioned, OPs have been shown to be particularly susceptible to postnatal hyperoxia. The dose-dependent effect observed on the SOPs after the administration of trolox C is, therefore, not surprising. Once normalized to their respective controls, scotopic and, more important, photopic SOPs measured in the hyperoxia-treated group are significantly different from those obtained from the normoxia-treated rats, indicating that the OPs clearly benefited from the therapeutic effect of trolox C. This holds true for groups that received as little as 300 $\mu\text{g}/\text{kg}$ (Figs.2J, 2K, 2L).

In previous studies, we show that the amplitude of ERG responses decreases with age when data obtained from 30- and 60-day-old rats are compared.^{21 26} This was further exemplified in Figure 3, with its comparison of the maturation-induced ERG amplitude decrease in normoxic and hyperoxic rats regardless of whether they received trolox C treatment. To facilitate the comparison, we devised a maturation index that represented the average of the percentage difference between ERGs obtained at 30 days and those obtained at 60 days for each rat as measured with the ERG a-wave and scotopic (combined rod $V_{\max}$ and mixed rod-cone) and photopic b-waves. Figure 3A shows the index of maturation for the a-wave, which was similar for normoxic control and hyperoxic control rats ($23.19\% \pm 21.14\%$ vs. $24.18\% \pm 16.40\%$; $P > 0.05$). Furthermore, despite some variability, responses obtained from animals raised in either environment while receiving trolox C, irrespective of concentration, were not significantly different from those of respective controls, suggesting that oxygen exposure alone or with concomitant trolox C treatment did not result in maturation-induced attenuation of the a-wave that exceeded what was considered the expected normal range. This result further supported a previous claim of ours that, compared with the other ERG components, the a-wave is minimally affected after postnatal hyperoxia.^{21 26} On the other hand, the scotopic b-wave (including

the rod $V_{\max}$ and mixed rod-cone responses) appeared to have matured differently depending on whether the rats were exposed to the hyperoxic environment or not, as shown with the maturation indices in Figure 3B. The index of maturation obtained from the normoxic control rats ($13.49\% \pm 16.12\%$) was similar ($P > 0.05$) to that obtained from normoxia-treated rats, irrespective of concentration, indicating that trolox C did not impair the normal maturational process. In contrast, the negative ($-14.73\% \pm 88.95\%$) index of maturation found for the hyperoxic control rats suggested that the hyperoxic environment tended, albeit not significantly ($P > 0.05$), to alter the normal course of the maturation process. Interestingly, however, a positive (normoxic-like) index of maturation was reinstated if the hyperoxic rats were treated with 600 and 900 $\mu\text{g/kg}$ trolox C but not with the 300- $\mu\text{g/kg}$ dose. A similar trend was also observed with the photopic b-wave index of maturation (Fig.3C), though in these conditions, the lowest dose, 300 $\mu\text{g/kg}$, already tended to return the index of maturation closer to normal, generating results similar to what was obtained using 600 $\mu\text{g/kg}$, whereas the 900- $\mu\text{g/kg}$ dose yielded a maturation index identical with that measured in normoxic control rats.

5.1 Effect of trolox C on the retinal cytoarchitecture

Illustrated in Figure 4 are representative cross-sections of retinas obtained from control (with and without 600 $\mu\text{g/kg}$ trolox C supplementation) and oxygen-exposed (with and without 600 $\mu\text{g/kg}$ trolox C supplementation) rats at 60 days of age. We had previously demonstrated that rats exposed to hyperoxic conditions undergo gradual thinning of the OPL and reduction in the number of horizontal cells.²¹ Retinal sections shown in Figure 4 confirm these findings. OPL thickness is significantly reduced in the hyperoxic control group (Fig.4C) compared with that in the normoxic control rats (Fig.4A). Hyperoxia-treated rats (600 $\mu\text{g/kg}$ trolox C; Fig.4D) showed a better-preserved OPL than was observed in the

hyperoxic control rats (Fig.4C). In contrast, no changes in OPL thickness were observed in the normoxia-treated group (Fig.4B). Measurements of OPL thickness and horizontal cell counts are graphically presented in Figure 5. Hyperoxic control rats showed a significant ($P < 0.05$) 55% reduction in the thickness of the OPL that was accompanied by a significant ($P < 0.05$) 60% reduction in horizontal cell count. Concomitant treatment of oxygen-exposed rats with 600 µg/kg trolox C limited the reduction of the OPL thickness to 75% of control ($P < 0.05$) but had no impact on the horizontal cell count. It should be noted that trolox C alone (without hyperoxia), though not altering the thickness of the OPL layer, still significantly reduced the number of horizontal cells by 30%.

6 Discussion

Our findings suggest that trolox C represents a valid therapeutic alternative in limiting the structural and functional damages intrinsic to the rat model of OIR. When retinal function is considered, our results (Fig.2) show that the a-wave was not impaired by hyperoxic exposure or by trolox C treatment (irrespective of dosage), the therapeutic effect of trolox C (especially at higher doses) outweighed its retinoactive effect, and the OPs are the ERG components that benefit most from the therapeutic effects of trolox C. Furthermore, results presented in Figure 2 also suggest that the photopic (cone-mediated) function benefits significantly more than the scotopic (rod-mediated) function from the therapeutic effects of trolox C. Given that the cone-mediated function was previously shown to be relatively more impaired than the rod-mediated function after postnatal hyperoxia,²⁶ a feature also evidenced in the present study (Table 1), our finding of a more potent effect of trolox C therapy on cone-mediated signals indicates that the treatment is targeted to where it is needed. The therapeutic efficacy of trolox C is further evidenced by the fact that it never modified the ERG a-wave, a component that was also unaffected by postnatal hyperoxia. Trolox C minimized the cytoarchitectural damage inherent in postnatal hyperoxia in that it limited the

reduction in OPL thickness that is associated with OIR.²¹ In contrast, its use had no significant impact on the reduction in horizontal cell count that also is associated with this retinopathy, suggesting either that trolox C might preferentially target specific cells whose processes contribute to OPL structure rather than target the horizontal cells or that the only two cytoarchitectural manifestations of the disease process involved in OIR might proceed from a different pathophysiological path.

Interestingly, the effect that trolox C exerted on the ERG signal appeared to depend on whether ROS were present. In the hyperoxic group, trolox C treatment enhanced the cone- and rod-mediated signals (compared with the untreated hyperoxic group), whereas in the normoxia-treated group, its use enhanced the rod response but reduced the cone-mediated ERG (compared with the normoxia-untreated group). Trolox C has been reported to have a dual mode of action—antioxidant and prooxidant—depending on the method by which peroxidation is induced. For example, when the oxidative stress is induced by Cu^{2+} or Fe^{3+} , trolox C acts preferentially as a toxic prooxidant and is converted to an α -tocopheroxyl radical as cellular functions deteriorate.³¹ In contrast, trolox C exerts its antioxidant role after metal-independent, peroxy radical–induced oxidation.³² This dual mode of action could apply to our model of OIR by which, in the presence of free radicals and, after exposure to postnatal hyperoxia, trolox C would carry out its antioxidant role. In the normal unexposed retina, in the absence of free radicals, trolox C could take on its more toxic role as a prooxidant. As with the mechanism by which cone function is more susceptible than rod function to postnatal hyperoxia (in the absence of any antioxidant), as previously reported by us,²⁶ the toxic effect of trolox C, as a prooxidant in the normal retina, could also be more devastating to cone function.

In addition, it has been shown that different mechanisms, namely apoptosis and necrosis, could be implicated in the pathophysiology of OIR and its human counterpart ROP.³⁴ Recent in vitro studies carried out on cultured bursal cells and neurons in which cell death occurred resulting from oxidative stress suggest that

trolox C could have a protective effect against necrosis but none against apoptosis.^{35 36 37} Other studies, however, have concluded that trolox C was successful, for example, in reducing apoptosis in mouse thymocytes, rabbit myocytes, and anterior pituitary cells.^{38 39 40} It will be useful to investigate the presence of apoptotic and necrotic markers in OIR to better understand the predominant mode of neuronal cell death taking place in this model and, more specifically, the implications on rod and cone pathways. Future studies in this area will also help to further elucidate the role of trolox C on these mechanisms of cellular death. Finally, though the present study indicates a beneficial effect on retinal structure, retinal function was spared to a lesser degree in animals that receive trolox C, suggesting that changes in retinal vasculature, retinal structure, and retinal function after exposure to postnatal hyperoxia might have proceeded in a cascade of events. For example, as previously described,^{4 5} postnatal exposure to hyperoxia generates a reduction in vasculature because of vasoconstriction followed by vaso-obliteration. Consequently, this inadequate blood supply could have made it difficult for the inner retina to receive adequate nourishment to carry out its proper function. It is possible that we observed loss or retraction of synapses that would lead to a thinning of the OPL only after cell function was lost. This could suggest a lag time between functional and structural impairment and perhaps an important role for vasoconstriction and vaso-obliteration as the trigger of the pathogenesis of OIR because this appeared to be the stage at which the cascade began. Given that each phase appeared to be interdependent (beginning with early changes in retinal vasculature and ending with changes in retinal cytoarchitecture and function), it seems logical to target the first step of this pathologic cascade. This would further support the use of a free radical scavenger such as trolox C. Furthermore, it is possible that though trolox C aids in facilitating vasculogenesis under hyperoxic conditions (thereby improving retinal function), its ability to take on the role of an antiapoptotic mediator prevents synaptic death that leads to a thinning of the OPL.

In summary, though we observed a therapeutic effect of trolox C, its use could not fully prevent oxygen-induced retinal damage from occurring. This could suggest, as previously proposed,²¹ that free radical formation is likely not the sole cause of the damage in retinal structure and function observed in OIR or that a significant proportion of the retinal damage induced by ROS occurs in hydrophobic domains in which trolox C would lose its efficacy because of its water solubility. For example, trolox C could act in limiting the circulating (plasmatic) ROS before lipid peroxidation. Should that be the case, a combination of liposoluble and hydrosoluble free radical scavengers (such as vitamin E [at a nontoxic level] + trolox C) could represent an interesting therapeutic alternative for further investigation.

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9 Captions to table and figures

Table 1: Group Data

Figure 1: Representative ERGs obtained at 30 and 60 days of age from a control rat subjected to normoxic conditions (Control), a control rat treated with 600 µg/kg trolox C (Control + Trolox C), a rat subjected to hyperoxia from postnatal days 0 to 14 (Oxygen), and a rat subjected to hyperoxia and treated from postnatal days 5 to 14 with 600 µg/kg Trolox C (Oxygen + Trolox C). Both scotopic (rod $V_{\max}$ and mixed rod-cone responses) and photopic (cone) ERGs with corresponding OPs are shown. Calibrations: horizontal, 20 ms; vertical, 400 µV for rod $V_{\max}$ and mixed rod-cone ERGs, 75 µV for scotopic OPs, and 100 and 10 µV for photopic ERGs and OPs, respectively. All tracings include a 20-ms prestimulus baseline; *vertical arrows* indicate flash onset.

Figure 2: Graphic representation of the dissociation between the protective effect of Trolox C on animals reared in hyperoxia and the retinoactive effect on the

normal retina. Data manipulation involved normalizing individual ERG amplitude measurements from normoxia-treated rats to the normoxia control mean, which was taken as 100%. Hyperoxia-treated animals were similarly normalized to the hyperoxia control group that was taken as 100%. Each ERG parameter is compared at 30 and 60 days, and individual graphs (**A–L**) compare normoxia-treated (*shaded symbols, solid line*) and hyperoxia-treated (*open symbols, dashed line*) animals (a-wave, **A–B**; rod $V_{\max}$, **C–D**; rod-cone b-wave; **E–F**; photopic b-wave; **G–H**; scotopic SOPs; **I–J**; photopic SOPs, **K–L**). Student's *t* test was used to compare the effect of trolox C on animals raised in a normoxic with those raised in a hyperoxic environment. *Asterisks* indicate significant differences between normoxia- and hyperoxia-treated groups. Results are given as mean amplitude change $\pm$ 1 SD.

Figure 3: Retinal maturation indices for the a-wave (**A**), scotopic b-wave (including rod $V_{\max}$ and mixed rod-cone responses) (**B**), and photopic b-wave (**C**). This index represents the average of the percentage difference between ERGs obtained at 30 and 60 days [% Δ (30/60)] for all individual rats. All values are given as mean $\pm$ 1 SD. *Asterisks* indicate significant difference from normoxic control.

Figure 4: Photomicrograph of cross-sections of the central retinas from (**A**) 60-day-old control (C), (**B**) control-treated with 600 $\mu\text{g/kg}$ trolox C (C+600), (**C**) oxygen-exposed (O_2), and (**D**) oxygen-exposed treated with 600 $\mu\text{g/kg}$ trolox C (O_2 +600) rats. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 10 μm .

Figure 5: Graphic representation of the number of horizontal cells (**A**) and the mean thickness (in micrometers) of the OPL (**B**) for 60-day-old control (C),

control with trolox C (C+600), oxygen-exposed (O₂), and oxygen-exposed with trolox C (O₂+600) rats. Error bars represent ± 1 SD. *Asterisks* indicate significant difference from normoxic control, and *filled symbols* indicate significant difference from hyperoxic control.

10 Table

Parameters	Age	Control	Control+Trolox (600µg/kg)	Oxygen	Oxygen+Trolox (600µg/kg)
Scotopic a-wave	30d	485.2±78.3	468.5±78.5	363.1±74.4*	376.9±72.0*
	60d	358.4±61.5§	317.8±42.8§	294.1±50.7§	282.8±86.9*§
Rod Vmax	30d	688.0±96.7	859.3±80.3‡	229.2±125.9*	390.6±123.8*†
	60d	606.7±71.9	701.4±117.5‡	214.4±110.4*	347.7±117.2*
Rod-Cone b-wave	30d	1142.3±171.7	1147.5±169.3‡	435.0±134.7*	599.5±144.6*
	60d	913.9±102.4§	922.1±122.3‡	407.5±137.6*	530.1±162.3*
Scotopic SOP_s	30d	469.4±60.0	456.8±62.0‡	140.2±87.7*	271.6±92.2*†
	60d	354.9±66.6§	352.8±68.3‡	120.8±54.7*	199.3±82.6*
Photopic b- wave	30d	262.0±30.0	241.6±37.0‡	86.3±31.8*	111.2±26.5*
	60d	229.0±20.0	161.9±21.9*§	78.7±28.9*	110.2±38.1*
Photopic SOP_s	30d	112.7±26.0	81.0±18.8*‡	27.9±15.3*	43.7±13.2*†
	60d	100.3±15.7	34.9±8.1*§	32.0±10.0*	39.5±14.9*

Table 1

11 Figures

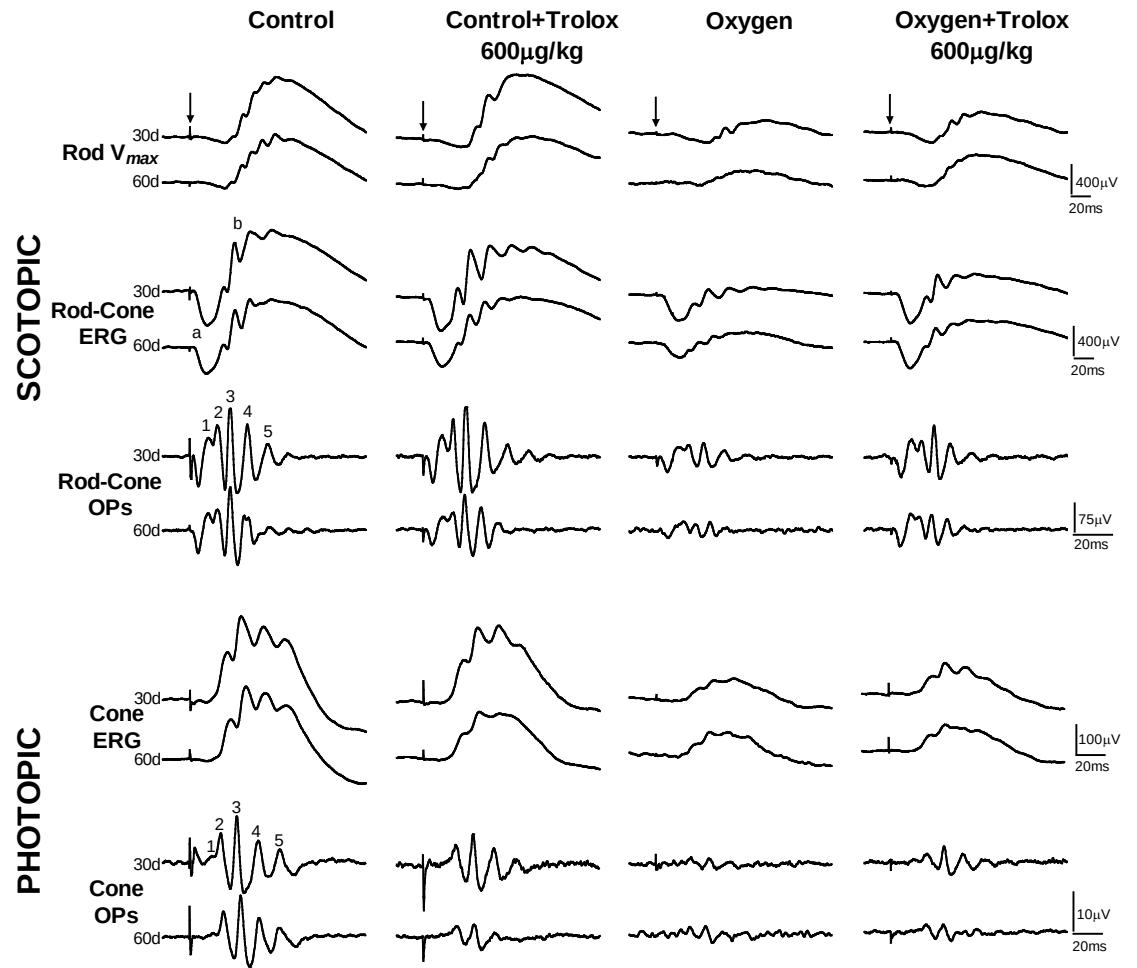


Figure 1

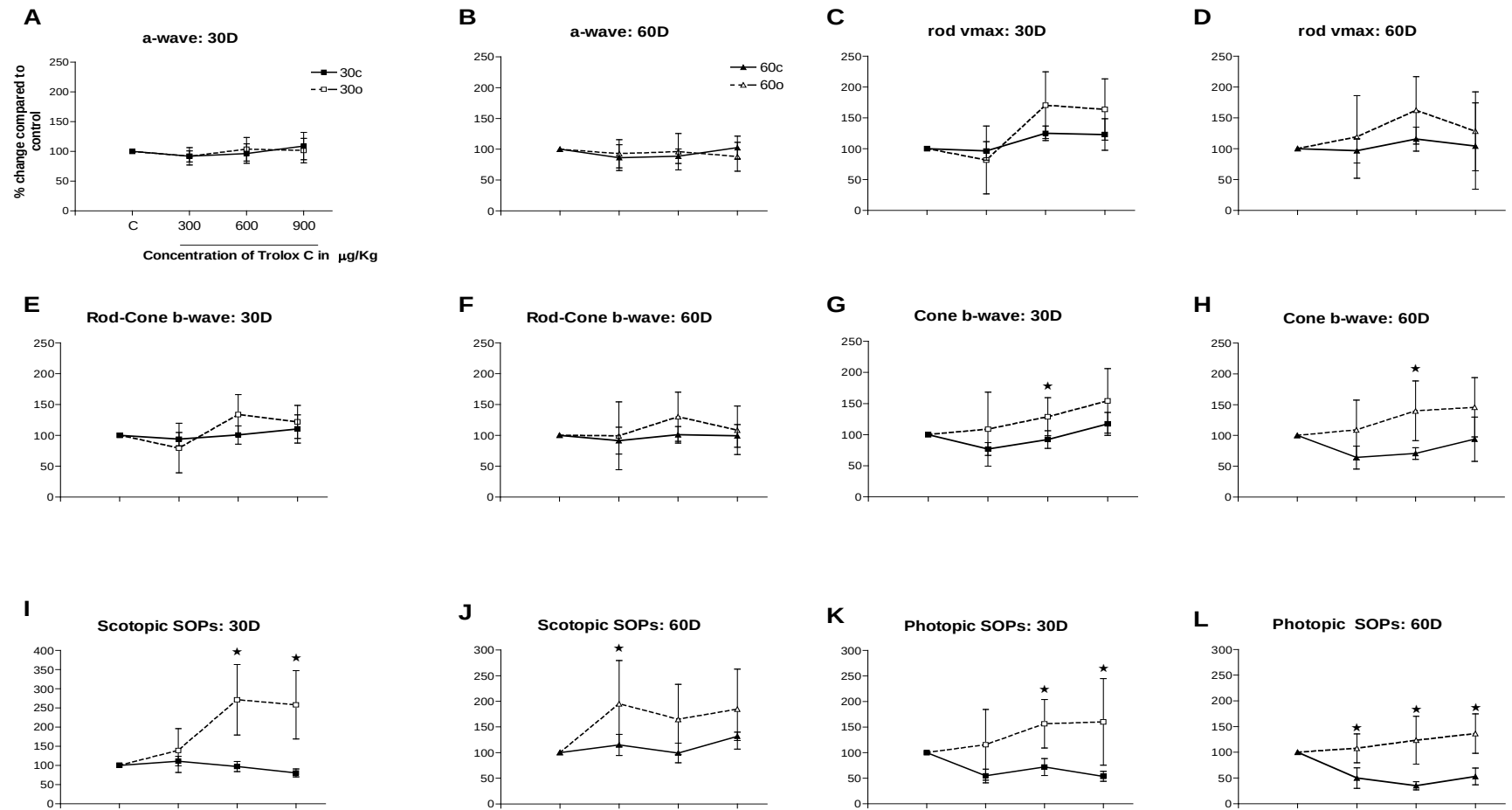


Figure 2

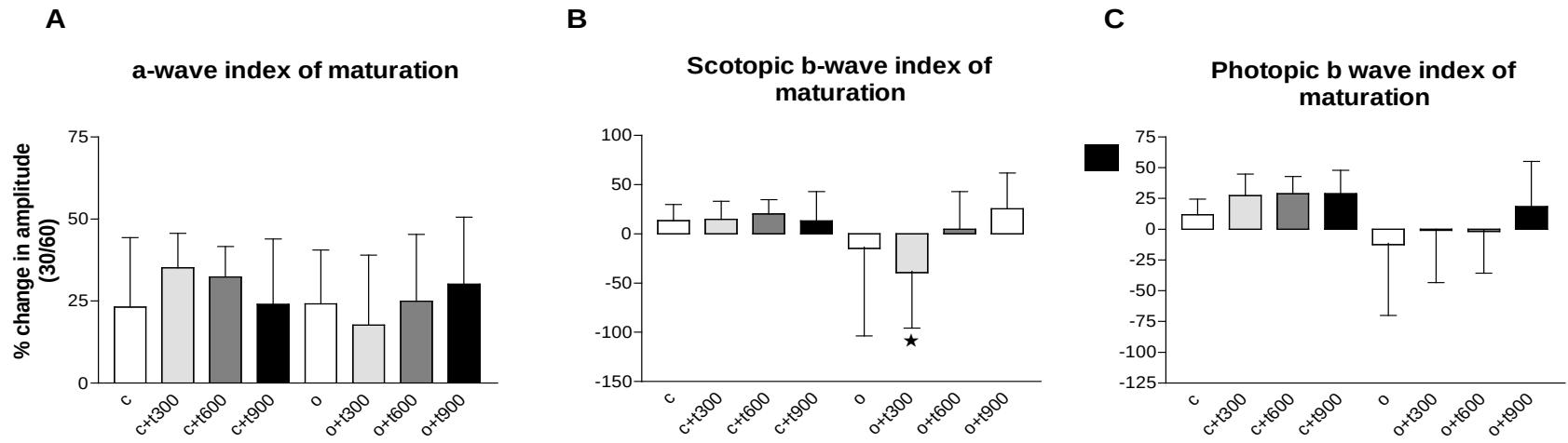


Figure 3

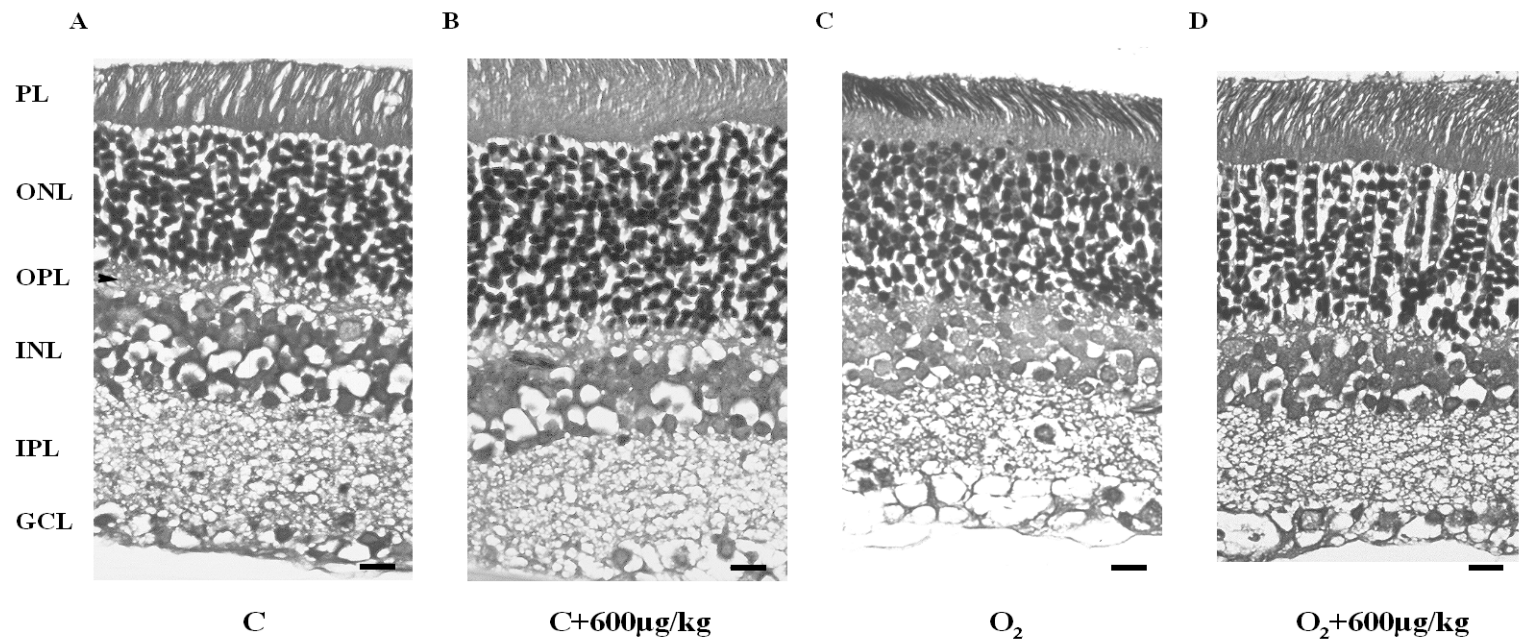
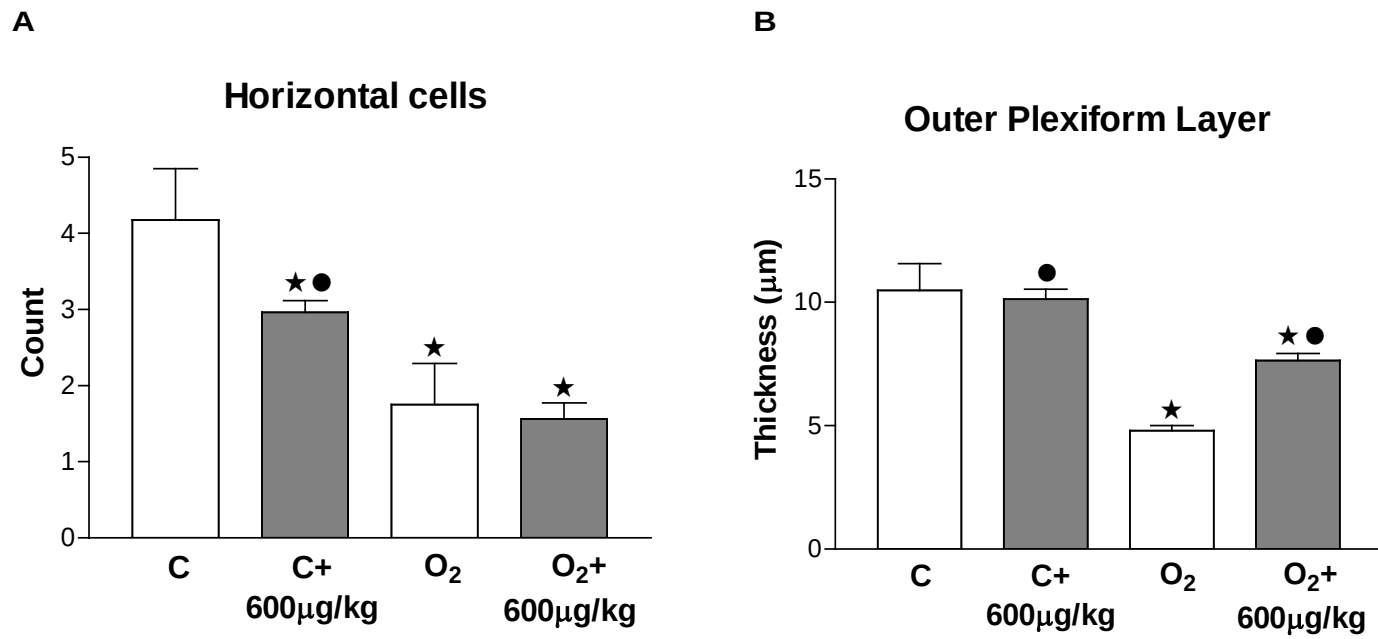


Figure 4

**Figure 5**

CHAPTER IV: MANUSCRIPT III

Dorfman AL, Polosa A, Joly S, Chemtob S, Lachapelle P. Functional and structural changes resulting from strain differences in the rat model of oxygen-induced retinopathy. Invest Ophthalmol Vis Sci. 2009 May; 50:2436-50.

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1 Preface to chapter IV

While the studies described in chapters II and III were carried out in the more conventionally used albino rat, we next sought to compare our findings using trolox C, an exogenous free radical scavenger, with those obtained using the pigmented rat in which melanin could play an antioxidant role, as an endogenous free radical scavenger. Of interest, previous studies comparing the racial incidence of ROP have also suggested that ocular pigmentation might play a protective role against the development of severe ROP. The structural (retinal vasculature and histology) and functional (ERG) consequences of postnatal hyperoxia were compared in albino Sprague-Dawley (SD) and pigmented Long-Evans (LE) rats at P30 and P60 in order to verify this finding. The role of neurotrophic factor expression (FGF-2 and CNTF) in OIR is also explored.

2 Abstract

Purpose: Results of studies that compared the racial incidence of retinopathy of prematurity (ROP) suggested that ocular pigmentation might offer protection against the development of severe ROP. The structural and functional consequences of postnatal hyperoxia (oxygen-induced retinopathy; OIR) were compared in albino Sprague-Dawley (SD) and pigmented Long-Evans (LE) rats to verify this finding. **Methods:** Newborn rats were exposed to 80% O₂ during selected postnatal day intervals. The severity of the OIR was determined by examining retinal flatmounts (retinal vasculature assessment), protein level quantification and cellular localization of fibroblast growth factor (FGF)-2 and ciliary neurotrophic factor (CNTF; Western blot analysis and immunohistochemistry, respectively), retinal histology, and photopic and scotopic electroretinograms (ERGs). **Results:** Irrespective of the parameter considered, structural and functional deficits resulting from postnatal hyperoxia were significantly more pronounced in LE rats. Although FGF-2 levels in LE rats had a tendency to increase after hyperoxia compared with normoxic littermates, it did not reach statistical significance. A similar finding was observed in SD rats. Of interest, however, baseline levels of FGF-2 were approximately four to five times higher in SD rats than in LE rats. There was a similar, hyperoxia-induced increase in CNTF levels between SD and LE rats. **Conclusion:** The findings suggest an increased susceptibility of newborn LE rats to postnatal hyperoxia in comparison with SD rats. Whether a pro-oxidant rather than antioxidant role of melanin or other genetic factors can explain these differences in oxygen susceptibility of the animal model of this retinopathy remains to be determined.

3 Introduction

Despite many advances in the clinical monitoring of oxygen administration in neonatal intensive care units, retinopathy of prematurity (ROP) continues to be a major cause of morbidity affecting prematurely born infants. Similar to other types of proliferative retinopathies, such as diabetic retinopathy and sickle cell retinopathy, ROP is characterized by retinal neovascularization.^{1 2 3}

⁴ It is this abnormal growth of new retinal vessels that can lead to the most devastating consequences of the disease, namely retinal detachment and eventually blindness. Through years of research, it has become increasingly evident that ROP is a very complex, multifactorial disease. Some of the risk factors known to be associated with the incidence of ROP include low birthweight and low gestational age^{5 6 7} and, notably, relative hyperoxia (compared with oxygen tension in utero).¹ Other studies have also shown that race is a significant factor that influences the epidemiology of ROP^{8 9}; for example, a greater number of Caucasian than African-American infants have been shown to reach threshold ROP.^{8 10} These studies suggest that melanin pigmentation offers protection against the development of severe ROP, suggesting that fundus pigmentation may play an instrumental role in this protection.^{6 8} On the other hand, other studies have suggested that an even greater incidence in the onset of threshold ROP exists in another darkly pigmented race, namely native Alaskans, when compared with that in other racial groups.^{11 12} Despite the fact that several of these studies have controlled for birth weight, gestational age, length of oxygen therapy, and ethnic group in multivariate analysis, the influence of race on the rate and severity of ROP is evidently an area that requires further clarification.

The rat model of oxygen-induced retinopathy (OIR) mimics what occurs in the human form of ROP and continues to provide insight into the pathogenesis of this disease. Findings of ours have shown that in addition to the vasculopathy that develops in the rat model of OIR, permanent retinal cytoarchitectural and

functional anomalies can also be documented.^{13 14 15 16 17} The most striking changes in retinal ultrastructure that we have evidenced in the albino SD rat model of OIR include outer plexiform layer (OPL) thinning and a decrease in horizontal cell count. Attenuation in the amplitude of the electroretinogram (ERG) b-wave with relative sparing of the a-wave are also features that are characteristic of this model.^{13 14 15 16 17} Previously published studies that compared pigmented and albino rats have shown that the former are less susceptible to ischemic retinal injury than are the latter,¹⁸ whereas others have demonstrated that certain pigmented strains develop more severe retinal neovascularization.^{19 20} Consequently, the purpose of this study was to determine whether similar strain differences might also extend to retinal vasculature (flatmounts), structure (histology), and function (ERG), in addition to neurotrophic factor expression. In the present study, we therefore sought to explore these factors in two rat models of OIR: the albino SD and the pigmented LE strains.

4 Methods

4.1 Animals

All experiments were approved by the McGill University-Montreal Children's Hospital Research Institute according to the guidelines of the Canadian Council on Animal Care and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Within a 24-hour period after birth, newborn litters of Sprague-Dawley (SD) and Long Evans (LE) rats (Charles River Laboratories, St. Constant, Québec, Canada) were placed, with their mothers, in room air (21% O₂) or in a hyperoxic (80% O₂) environment (mixture of medical grade 100% O₂ and room air measured with an oxygen meter [MaxO₂ Ceramtec, model OM25-ME; Medicana Inc., Montréal, Québec, Canada]). A controlled cyclic light environment was maintained

throughout the exposure (12 hours light–dark). Exposure to hyperoxia lasted for 22.5 hours daily and was interrupted for three 0.5-hour periods of normoxia (21% O₂), as per previously published protocols.^{13 14 15 16 17} Casualties were more frequently observed throughout hyperoxic exposure of the pigmented LE strain, a finding that might be explained by interstrain differences in the rat genome that contribute to variations in ventilation, metabolism, and ventilatory responses to inspired levels of O₂ and CO₂.^{21 22} Based on previous studies in SD rats that suggested unequal susceptibilities to hyperoxia throughout different developmental time points,^{15 16} the hyperoxic cohort of each strain was divided at birth into seven different exposure protocols: from birth through postnatal day 6 ([P0–P6]: LE, *n* = 24 [P30], *n* = 5 [P60]; SD, *n* = 6 [P30], *n* = 6 [P60]); birth through postnatal day 9 ([P0–P9]: LE, *n* = 11 [P30], *n* = 5 [P60]); birth through postnatal day 12 ([P0–P12]: LE, *n* = 7 [P30], *n* = 6 [P60]); and birth through postnatal day 14 ([P0–P14]: LE, *n* = 21 [P30], *n* = 6 [P60]; SD, *n* = 11 [P30], *n* = 7 [P60]); or from postnatal day 6, 9, or 12 through postnatal day 14 (P6–P14: LE, *n* = 22 [P30], *n* = 6 [P60]; SD, *n* = 8 [P30], *n* = 8 [P60]; P9–P14: LE, *n* = 17 [P30], *n* = 9 [P60]; and P12–P14: LE, *n* = 15 [P30], *n* = 7 [P60]). Select time points within the first and second weeks of life were taken for comparing SD rats to LE rats, as we had documented the effects of postnatal hyperoxia in the former strain elsewhere.^{15 16} After hyperoxic exposure, animals were returned to room air (21% O₂) until P30 when retinal function was first assessed. Data were compared with respective age-matched control litters raised in normoxic conditions (LE, *n* = 21; SD, *n* = 22).

4.2 Malondialdehyde assay

Malondialdehyde (MDA), a product of peroxidation, was determined as previously described.^{23 24} LE and SD rats were killed immediately after exposure to hyperoxia (P0–P6 and P6–P9, *n* = 3 to 4 per group), given that levels (relative

to normoxia) have been shown to subside as the hyperoxic period is extended for longer periods.²³ Each retina was harvested less than 1 minute later, ground twice for 30 seconds each with a homogenizer (Omni International, Marietta, GA) in a buffer containing butyl-hydroxytoluene (BHT; 5 mM Tris, 0.02% ASA, 0.5 mM EGTA), and centrifuged at 1000*g* for 10 minutes. To each sample was added 0.33% 2-thiobarbituric acid (TBA) in water mixed with glacial acetic acid and then the sample was heated at 95°C for 60 minutes. HPLC-grade *n*-butanol was added, and samples were vortexed and spun down at 1000*g* for 10 minutes. The upper phase was read at 532 nm with a spectrophotometer.

4.3 Retinal flatmounts

Groups of animals killed on P6 and P14 after exposure from P0 to P6 (SD, *n* = 3; LE, *n* = 3) and P6 to 14 (SD, *n* = 3; LE, *n* = 3), respectively. After enucleation and dissection of the cornea and lens, eye cups were collected and fixed overnight at 4°C in 4% vol/vol formalin. Retinas were isolated, and flatmounts were prepared for adenosine diphosphatase (ADPase) staining, according to a method that was previously described.^{17 25 26}

Sequential, overlapping images of the entire retina were subsequently collected and arranged to construct a montage of the complete retina (Photoshop, ver. 6.0; Adobe Systems Inc., San Jose, CA). Vascularized areas were manually outlined and measured as a percentage of the total retinal area to assess vascular extent. Density analysis obtained from oxygen-exposed groups was compared with that from animals raised in normoxia, for which values were set at 100% (ImagePro Plus, ver. 4.1; Media Cybernetics, Silver Spring, MD).

4.4 Retinal immunohistochemistry

Other groups of animals were assigned to be killed on postnatal day 6 after exposure from P0 to P6 (LE, $n = 3$; SD, $n = 3$) and on P14 and P60 after exposure from P6 to P14 (P14: LE, $n = 3$; SD, $n = 3$; P60: LE, $n = 3$), respectively, for detection of endogenous levels of FGF-2 and CNTF neurotrophic factors that were found to be upregulated in another model of oxidative stress—namely, light-induced retinopathy (LIR).²⁷ Animals were euthanatized immediately after removal from hyperoxia and were perfused intracardially with 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer (PB; pH 7.4). After removal of the cornea and lens as described, eye cups were immersed in 4% PFA in 0.2 M PB (pH 7.4; 2 hours) and were cryoprotected in sequential sucrose solution gradients (10%, 20%, and 30% in PB for 30 minutes, 1 hour, and overnight, respectively) at 4°C. Eye cups were subsequently embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and were frozen in a bath containing 2-methylbutane/liquid nitrogen. Fourteen-micrometer-thick cryosections were collected on polylysine-S-coated slides for immunohistochemical analysis. To block any nonspecific binding of the antibodies, we incubated retinal sections in 3% normal goat serum, 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in 0.1 M PBS (pH 7.4) for 30 minutes at room temperature. The sections were then incubated overnight at 4°C with the following primary antibodies: monoclonal anti-human FGF-2 (type II, clone bFM-2, 10 µg/mL; Upstate Biotechnology, Lake Placid, NY), monoclonal anti-mouse CNTF (5 µg/mL; Chemicon, Temecula, CA), and polyclonal anti-rabbit GFAP (1:100; Sigma-Aldrich). Normally, GFAP expression in Müller cells is very limited^{28 29} but increases after neuronal cell death. In fact, studies conducted in the rat model of OIR have suggested that increasingly intense GFAP reactivity is observed once animals are returned to room air after 14 days of hyperoxia, the most intense of which is seen at approximately 10 weeks of age.³⁰ Consequently, we chose to conduct our colocalization studies with GFAP in retinas from rats

exposed to hyperoxia from P0 to 14, collected at P60. The next day, sections were incubated with the appropriate secondary antibody for 1 hour at room temperature, washed in PBS, and mounted with an antifade reagent (SlowFade; Invitrogen-Molecular Probes, Eugene, OR). Photographs were taken with a microscope (40x objective, Axiophot; Carl Zeiss Meditec, Oberkochen, Germany).

4.5 Western blot

Retinas from right and left eyes of LE and SD control and oxygen-exposed rats (80% from P0 to P6, P6 to P14, and P0 to P14) were isolated ($n = 3$ to 4 per group), independently homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (0.1% sodium dodecyl sulfate, 20 mM Tris [pH 8.0], 135 mM sodium chloride, 1% NP-40, 10% glycerol supplemented with protease inhibitors), and then pooled. Retinal lysates were kept on ice for 30 minutes and then centrifuged at 14,000 rpm for 15 minutes, after which the supernatant was collected. Protein concentration was determined by the Lowry method (Bio-Rad Life Science, Mississauga, ON, Canada). For each sample, 100 μ g of protein was resolved by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad Life Science), as previously described.²⁷ After nonspecific signal blocking in 10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20 (TBST), and 5% lyophilized skim milk for 1 hour at room temperature, the membranes were incubated overnight at 4°C with the primary antibodies used according to the following conditions: monoclonal anti-human FGF-2 (type II, clone bFM-2, 2.5 μ g/mL; Upstate Biotechnology) and monoclonal anti-mouse CNTF (5 μ g/mL; Chemicon). The membranes were subsequently washed with TBST and incubated with anti-mouse peroxidase-linked secondary antibodies (Amersham Pharmacia, Baie d'Urfé, Québec, Canada) for 1 hour at room temperature. A chemiluminescent reagent (ECL; GE Healthcare, Piscataway, NJ) was used for detection of protein signals, followed by the exposure of the membranes to autoradiograph film (X-OMAT; Eastman Kodak, Rochester, NY).

The membranes were incubated in a stripping solution (200 mM glycine [pH 2.8], 500 mM NaCl, and 0.7% β -mercaptoethanol) at 55°C for 1 hour and then reprobed with an anti-actin polyclonal antibody (1:10,000; generous gift from

J. Chloe Bulinski) followed by incubation with an anti-rabbit peroxidase-linked secondary antibody (Amersham Pharmacia Biotech).

The γ -actin protein was chosen as a reference instead of β -actin, given that the latter isoform patterns vary with age.^{31 32 33} Ponceau-S (Sigma-Aldrich) was used to ensure equal protein loading. Densitometry analysis was performed on scanned autoradiographic films and quantified according to pixel intensity (Quantity One 4.1.0 software; Bio-Rad Laboratories). The densitometry values obtained for each neurotrophic factor were subsequently normalized to the γ -actin level in the same blot.

4.6 Electoretinography

ERGs and oscillatory potentials (OPs) were recorded (AcqKnowledge Data Acquisition System, Biopac MP 100WS; BIOPAC Systems Inc., Goleta, CA) as previously described^{13 14 15 16 17} from control and hyperoxic cohorts at postnatal days 30 and 60. Briefly, after 12 hours of dark adaptation, rats were anesthetized, under a dim red light illumination, with an intramuscular injection of ketamine (85 mg/kg) and xylazine (5 mg/kg). The pupils were subsequently dilated with 1 to 2 drops of 1% cyclopentolate hydrochloride, and the rat was placed on its side in a recording chamber that included both the flash stimulator and background light. A DTL fiber electrode (27/7 X-Static silver-coated conductive nylon yarn; Sauquoit Industries, Scranton, PA) that served as the active electrode was placed over the corneal surface and was kept in place with 2% methylcellulose (Gonioscopic solution; Alcon Laboratories, Fort Worth, TX), which also helped to prevent corneal desiccation. Grass disc (model E6GH; Grass Instruments, Quincy, MA) and subdermal needle electrodes (model E2; Grass Instruments) were placed in the mouth as the reference and inserted in the tail as the ground, respectively. Broadband ERGs (10,000x, 1–1000 zHz bandwidth, 6 dB of attenuation; P511 analog preamplifiers; Grass Instruments) and OPs

(50,000x, 100–1000 Hz, 6 dB of attenuation; P511 analog preamplifiers) were recorded simultaneously with the data acquisition system (AcqKnowledge; Biopac MP 100 WS; Biopac System Inc.). Scotopic luminance–response functions were evoked by flashes of white light spanning a 6-log-unit range in 0.3-log increments (maximal intensity, $0.6 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$, average, two to five flashes, interstimulus interval, 9.60 seconds). Photopic (cone-mediated) signals were evoked to flashes of white light of $0.9 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$ that were delivered after more than 15 minutes of light adaptation to a background of $30 \text{cd} \cdot \text{m}^{-2}$ (average, 20 flashes; interstimulus interval, 1 second) to avoid the previously described light adaptation effect.³⁴

4.7 Retinal histology

Retinal histology was performed at P60. After the completion of ERG recordings, the rats were euthanatized with an anesthetic overdose followed by intracardiac perfusion with 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The eyes were enucleated and postfixed in 3.5% glutaraldehyde for 3 hours. The corneas were then dissected, the lenses were removed as described earlier, and the eyes were subsequently placed in 3.5% glutaraldehyde overnight (12 hours). The eye cups were then immersed in a 4% osmium tetroxide solution with 0.1 M phosphate buffer. After sequential dehydrations in ethanol baths (50%, 70%, 90%, 95%, and 100% ethanol) and propylene oxide, the specimens were embedded in epoxy resin (Durcupan ACM Fluka epoxy resin kit; Sigma-Aldrich). Once polymerization was completed (48 hours at 58°C), the tissue was cut in 0.7- μm ultrathin sections that were mounted on glass slides and stained with 0.1% toluidine blue. Images were obtained with a digital camera (RR slider spot; Diagnostic Instruments Inc., Sterling Heights, MI) that was mounted on a microscope (Axiophot, 40x; Carl Zeiss Meditec). Three retinas per group were analyzed, and an average of 10 measurements was obtained from each.

4.8 Data analysis

Retinal histology and ERGs were obtained from the same group of animals, whereas retinal flatmounts, MDA assay, and neurotrophic factor analysis through Western blot analysis and immunofluorescence were all performed independently. Amplitudes of ERG components along with their peak times were measured as previously described.^{13 14 15 16 17} Briefly, the a-wave amplitude was measured from baseline to the most negative trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Similarly, oscillatory potentials were measured from the preceding trough to the peak of the OP under evaluation, with the exception of OP₂, which was measured from the baseline to its peak. OPs were reported as the sum of OPs (SOP = OP₂ + OP₃ + OP₄ + OP₅).

Scotopic luminance–response function curves were derived for each animal by plotting b-wave amplitudes against flash intensities. A sigmoidal intensity–response regression curve was then applied to fit the data (Prism 3.00 software; GraphPad, San Diego, CA), from which the rod $V_{\max}$ (maximal rod response) could be calculated. Furthermore, the ERG response evoked to the brightest flash delivered in scotopic conditions (the mixed rod-cone response) was also analyzed. To determine the effect of the different oxygen regimens on retinal structure and function, we used one-way analysis of variance (ANOVA; $P < 0.05$) and the Dunnett multiple comparison as a post hoc test. Functional data obtained from each exposure regimen at each recording session (P30 and P60) in addition to retinal vascular findings and protein analysis (immunoblotting) were compared by Student's *t*-test ($P < 0.05$). Finally, Student's *t*-test ($P < 0.05$) was also used to compare values obtained at P30 and P60 from LE and SD rats from preselected oxygen exposure regimens.

5 Results

5.1 Effect of postnatal hyperoxia on MDA concentration

Given the effects of the redox state on peroxidation^{35 36} we measured levels of malondialdehyde (MDA), a product of peroxidation. Although a 31.9% increase in MDA levels was observed in SD rats after early hyperoxia (P0–P6, 3.06 ± 0.34 [80%] compared with 2.32 ± 0.09 [21%]; $P < 0.05$), a strikingly greater difference in levels was found in LE rats after the same regimen, resulting in a 91.5% increase (P0–P6, 3.63 ± 0.13 [80%] compared with 1.90 ± 0.25 [21%]; $P < 0.05$). After hyperoxic exposures that extended into the second week of life (P6–P9), MDA levels were reduced to near normal levels (1.99 ± 0.35 [80%] compared with 2.50 ± 0.65 [21%] in SD rats and 2.47 ± 0.40 [80%] compared with 3.37 ± 0.69 [21%] in LE rats; $P > 0.05$).

5.2 Effect of postnatal hyperoxia on retinal vasculature

Retinal vasculature in the neonatal rat undergoes significant development from birth through P14, beginning in the central retina and moving outward to the periphery.¹³ Retinal flatmounts reveal that normoxia-raised LE and SD rats are similarly vascularized at P6 ($78.50\% \pm 8.11\%$ and $71.67\% \pm 2.89\%$, respectively; Figs.1A–1E), whereas vascular growth to the periphery is complete (100% vascularized) by P14 (Figs.1C, 1G). On the other hand, hyperoxia (P0–P6 exposure) significantly attenuated the postnatal vascular growth, reducing the vascularized area to 28.7% ($P < 0.05$) and 57.2% ($P < 0.05$) of control in LE and SD rats, respectively (Figs.1B, 1F, 2A). This attenuation in vascular growth was accompanied by a marked reduction in vascular density for the same exposure

regimen, resulting in $51.03\% \pm 4.25\%$ ($P < 0.05$) and $66.70\% \pm 3.96\%$ ($P > 0.05$) of normal density in LE and SD rats, respectively (Fig. 2C). Similarly, exposure from P6 to 14 resulted in a vascularized area that was also reduced in LE rats to $57.90\% \pm 28.15\%$ of normal coverage ($P < 0.05$), whereas SD rats were not significantly affected ($94.76\% \pm 5.61\%$ of normal, $P > 0.05$; Figs. 1D, 1H, 2B). As shown in Figure 2D, this trend was also reflected in vascular density measurements ($57.03\% \pm 18.72\%$ and $113.36\% \pm 16.46\%$ of normal in LE [$P < 0.05$] and SD [$P > 0.05$] rats, respectively).

5.3 Effect of postnatal hyperoxia on retinal function

Figure 3 shows representative scotopic (Figs.3A, 3B, 3C, 3D, 3E, 3F) and photopic (Figs.3G, 3H) ERGs (Figs.3A, 3B, 3C, 3D, 3E, 3G) and oscillatory potentials (Figs.3F, 3H) recorded from control (21% O₂) and hyperoxic rats (80% O₂) at P60. Exposure durations are indicated in postnatal day intervals. Group data are graphically reported in Figure 4. Although parallel experiments for LE and SD rats were performed, retinal function characteristics of SD rats have been reported by us^{13 14 15 16 17}; hence, a more elaborate description of retinal function parameters will be focused on LE rats, and comparisons concluded for strain differences. In normal LE rats, the use of a progressively brighter flash stimulus (from bottom to top) resulted in a gradual increase in the a- and b-wave parameters, a trend also observed in rats that were exposed postnatally to each of the hyperoxic regimens.

A comparison between the scotopic (a-wave, rod V_{max} , rod-cone b-wave, and SOPs) and photopic (b-wave and SOPs) ERG parameters of LE and SD rats obtained at P30 and P60 is graphically represented in Figure 4. Group data along with statistical analysis for LE and SD rats are shown in Tables 1 and 2, respectively.

5.4 Effect of postnatal hyperoxia on scotopic function

In all instances, there is a gradual decline in the amplitude of ERG parameters at P30 and P60, culminating in maximum attenuation after exposures to hyperoxia from P0 to P14 and P6 to P14. After hyperoxia initiated at birth, a dose–response effect was observed between the duration of hyperoxia and the amplitude of the resulting a-wave amplitude in LE rats (Fig.4A) for measurements obtained at P30 and P60. Although a tendency for a decline in a-wave amplitude was observed on exposure to hyperoxia in the first postnatal week (Fig.4A), a more pronounced attenuation was observed when hyperoxic exposure extended into the second week of life; this culminated in a decline to 51.9% and 45.6% of control values at P30 and P60, respectively ($P < 0.05$). Regimens that were limited to the second week of life also resulted in an attenuation of the a-wave amplitude to 63.4% and 42.9% of P30 and P60 control values, respectively ($P < 0.05$). Notably, the a-wave is considerably more affected in LE than SD rats exposed to hyperoxia, where a decline to 79.7% and 65.7% of control values, respectively, was observed at P30 and P60 in SD rats, compared with 51.9% and 45.6% of control values at P30 and P60, in LE rats (Fig.4G).

A similar dose–response correlation between the duration of hyperoxia and the amplitude of the rod $V_{\max}$ in LE rats was also observed in P30 and P60 recording sessions (Fig.4B). The amplitude of the rod $V_{\max}$ was already attenuated by hyperoxia from P0 to P6 to 84.9% (P30) and 74.4% (P60) of control; this parameter continued to decline when oxygen exposure was extended into the second week of life. Similar findings were also observed when hyperoxia was initiated in the second week resulting in attenuations to 18.2% (P30) and 13.0% (P60) of control ($P < 0.05$). As seen for the a-wave, the rod $V_{\max}$ attenuation after maximum hyperoxia was notably less severe in SD rats (Fig.4H) than in LE rats.

A comparable trend was also observed for the rod-cone b-wave of LE rats (Fig.4C), where significant attenuations in amplitude were observed after hyperoxic exposure from P0 to P6 to 68.7% of control at P30 ($P < 0.05$) and 59.9% of control at P60 ($P < 0.05$). Exposures from P0 to P14 further reduced the amplitude of the rod-cone b-wave. When initiated later in life, hyperoxia caused similar amplitude attenuations that were most pronounced after exposure from P6 to P14 (reductions to 21.7% of control at P30 and 17.6% of control at P60; $P < 0.05$).

Finally, given that there was no specific effect of hyperoxia on any particular oscillatory potential (OP), we are reporting them collectively as the sum of OPs (SOPs). Scotopic SOPs (Fig.4E) in LE rats were all significantly attenuated after hyperoxia, irrespective of the exposure regimen. Attenuations in SOP amplitude were observed in exposures from P0 to P6 (59% of control [P30], and 68.7% of control [P60]; $P < 0.05$). After maximum exposure from P0 to P14, SOP amplitudes were significantly ($P < 0.05$) reduced to 6.2% and 12.7% of control amplitude at P30 and P60, respectively. Hyperoxia resulted in an almost equivalent attenuation after P6 to P14 as it did after a 2-week oxygen exposure.

Again, the rod-cone b-wave (Fig.4I) along with the scotopic SOPs (Fig.4K) were less attenuated after postnatal hyperoxia in SD rats where maximum rod-cone b-wave attenuations were 36.9% (P30) and 40.3% (P60) of control compared with 18.3% (P30) and 20.5% (P60) of control for the scotopic SOPs ($P < 0.05$).

5.5 Effect of postnatal hyperoxia on photopic function

The photopic b-wave and SOPs of LE rats raised in a hyperoxic environment were also markedly affected. A striking decline in the photopic b-wave amplitude (Fig.4D) was observed after P0 to P6 exposure to 53.0% and 51.6% of control at P30 and P60, respectively ($P < 0.05$); this trend continued

during extended hyperoxia (P0–P14). Oxygen exposure regimens initiated in the second week of life also resulted in significant amplitude attenuations. Photopic SOPs were similarly affected after postnatal hyperoxia (Fig.4F). As described for the scotopic condition, the cone-mediated function of the SD rats also appeared to be less susceptible to the hyperoxic insult than was that of the LE rats. Along these lines, after hyperoxia from P0 to P14, the photopic b-wave of the SD rats (Fig.4J) was attenuated to 27.3% and 32.7% of control at P30 and P60, respectively, compared with 11.9% and 20.3% in the LE rats.

5.6 Effect of postnatal hyperoxia on retinal cytoarchitecture

The most striking ultrastructural consequences of postnatal hyperoxia, reported in the neonatal SD rat model included the near disappearance of the OPL after maximum exposure to approximately 20.0% of control along with a significant reduction in horizontal cell count to approximately 20.0% of the control, both of which were most obvious after regimens that extended hyperoxia into the second week of life^{13 14 16 17} (Figs. 5F, 5G). Added to this, postnatal hyperoxia in LE rats also damaged other retinal structures as exemplified with representative histologic sections shown in Figures 5A, 5B, 5C, 5D, 5E. These are characterized by a marked thinning of the inner retina as well as outer retinal damage limited to the outer nuclear layer (ONL). Group data are graphically presented in Figure 6. As shown in Figures 5B and 6, early exposure from P0 to P6 resulted not only in a reduction in OPL thickness to 65.6% of control ($P < 0.05$), but also in a significantly thinner ONL, inner nuclear layer (INL), and inner plexiform layer (IPL), reduced to 61.5%, 80.2%, and 70.9% of the respective controls. Progressive increases in duration of oxygen exposure, particularly when encompassing the second week of postnatal life, resulted in even greater structural anomalies. As shown in Figures 5C and 6, exposures within the first 2 weeks of life resulted in maximum thinning of the ONL, OPL, INL, and IPL to 67.6%,

0.7%, 26.0%, and 45.8% of control, respectively ($P < 0.05$). When hyperoxia was initiated in the second postnatal week (P6–P14 and P12–P14; Figs. 5D, 5E, 6), the thinning of the retinal layers was more important than that observed after exposures in the first postnatal week, but not as severe as that observed during 2 weeks of hyperoxia (P0–P14).

5.7 Neurotrophic factor expression after postnatal hyperoxia

Previous studies have suggested that mechanical injury to the retina,^{37 38 39} in addition to light-induced damage,^{40 41 42} can lead to the upregulation of neurotrophic factors. Furthermore, studies conducted in our laboratory have suggested that an increase in endogenous levels of some of these factors, such as FGF-2 and CNTF for example, plays a role in the neuroprotection of juvenile rat retinas against another environmental oxidative stress—namely, light-induced damage.²⁷ To determine whether a similar protective mechanism might explain the difference between SD and LE rats, we examined the levels of retinal FGF-2 and CNTF (Fig. 7) by Western blot analysis. LE and SD rat retinas were collected immediately after early oxygen exposure (P0–P6) at P6 and late oxygen exposure (P6–P14) at P14 and were compared with retinas collected from age-matched control rats at P6 and P14, respectively. Although FGF-2 levels in LE rats had a tendency to increase after exposures from P0 to P6, P6 to P14, and P0 to 14 compared with their respective controls (Fig. 7A), results did not reach statistical significance. Similarly, FGF-2 levels also tended to increase in SD rats after postnatal hyperoxia (Fig. 7B) after each exposure regimen compared with control. It is interesting to note, however, that control SD rats appeared to have approximately four to five times higher endogenous levels of FGF-2 than did the LE rats. This tendency extended during exposure to hyperoxia. In all hyperoxic cohorts of LE and SD rats studied, CNTF levels appeared higher compared with their corresponding controls (Figs. 7C, 7D).

Retinal immunohistochemistry was performed to determine the cellular localization of FGF-2 and CNTF proteins in LE and SD rats after exposure to hyperoxia. Figures 8A and 8B reveal that, as described above, FGF-2 immunoreactivity was more pronounced during exposure to hyperoxia compared with the control. Immunofluorescence in both strains was limited to the INL and ganglion cell layer (GCL). Basal levels of CNTF staining were observed in the INL and GCL of the control animals and were markedly increased after exposure to hyperoxia in both LE and SD rats (Figs.8C, 8D) . The localization of CNTF suggested that these were Müller cells (GFAP positive, Fig. 9).

6 Discussion

Previous studies showed that postnatal hyperoxia in the albino SD rat model of OIR resulted in severe structural and functional damage, including the permanent loss of the OPL, significant reduction in the horizontal cell count, and permanent attenuation in retinal function, as observed with the scotopic and photopic ERGs.^{13 14 15 16 17} Results from the present study suggest an even greater susceptibility of the pigmented LE rat to postnatal hyperoxia, as evidenced with higher vascular dropout throughout exposure, more severely depressed retinal function and permanently altered retinal structure that was not only limited to the loss of the OPL and horizontal cells as in SD rats, but also extended into the inner retina, resulting in significant loss of the INL and IPL.

These alterations in retinal cytoarchitecture are likely to be at the root of the functional anomalies, such as the severe attenuation of the ERG b-wave we are reporting. The most critical, and most permanent, sequelae of OIR in the SD rat model occurred in the more inner part of the retina. However, the tendency for the shortening of inner segments of the photoreceptors which is accompanied by a significantly attenuated a-wave in LE rats after hyperoxia is suggestive of a photoreceptor involvement as well. Therefore, the short-term and long-term

consequences of postnatal hyperoxia on the retinal structure and function were significantly more pronounced in LE rats than in SD rats.

Free radicals that are generated through lipid peroxidation have been thought to play an important role in the pathogenesis of ROP and its animal counterpart, OIR.⁴³ It has been suggested that the formation of free radicals in the retina may result from the exposure of premature infants to a hyperoxic environment, particularly due to their deficiency in antioxidant defenses, such as superoxide dismutase, α -tocopherol, catalase, and glutathione peroxidase, for example.^{44 45} Reactive oxygen species (ROS) have also been shown to contribute to the vaso-obliterative phase that is associated with OIR^{46 47 48 49} either through direct cytotoxic effects on endothelial cells or through an initial phase of vasoconstriction which ultimately results in reduced tissue perfusion. Of interest, treatment with vitamin E, a free-radical scavenger, has been shown to protect against the loss of superoxide dismutase in cats exposed to hyperoxia⁵⁰ and has successfully attenuated the severity of vaso-obliteration in the rat model of OIR.⁴⁹

⁵¹ A previous study of ours revealed that administration of the water-soluble antioxidant Trolox C to SD rats prevents only some of the structural and functional sequelae associated with OIR, suggesting that free radical formation may not be the sole cause of the damage.¹⁴ Similarly, our current results suggest that melanin would be insufficient in having any beneficial protective effect under similar circumstances; and moreover, it appeared as though it might even potentiate the effect of ROS. Melanin is produced and stored in the retinal pigment epithelium (RPE) cytoplasm and melanocytes, and continuous exposure of the RPE to environmental stressors such as light and oxygen have been thought to dampen its antioxidant capacity.⁵² Furthermore, melanin has been known to take on the role of a pro-oxidant under these conditions, leading to cytotoxic activity such as photoreceptor damage and even age-related macular degeneration.^{53 54} This would explain the severity of OIR in LE rats, including a significantly injured photoreceptor layer, given its proximity with the RPE. Our findings of increased MDA levels in LE compared with SD rats (after hyperoxic exposure

initiated at birth throughout the first week of life) support this inference. Recently, we showed that despite the damage to retinal vasculature that is observed within the first week or so of exposure of SD rats (e.g., P0–P6 and P0–P9), the retinal vascular growth process appears to be able to fight this oxidative stress to achieve nearly full coverage while remaining under hyperoxic exposure throughout the first 2 weeks of life,¹³ a finding that is further corroborated by the results in the present study. A different picture emerges in the pigmented rat, however, where vascular dropout and extent are so severely compromised that they never achieve a normal appearance (Fig 1). Taken together, these anatomic characteristics are inconsistent with a protective role of melanin in the retinal vasculature and inner structures. This result suggests that other mechanisms partake in the increased vulnerability of LE compared with SD rats, as observed in the present study. It is of interest to note that the complex series of events that characterize retinal development occur differently in albino and pigmented animals, where retinal maturation is delayed in the absence of melanin.⁵⁵ For example, studies have revealed a temporal delay in the central-to-peripheral pattern of cell production⁵⁶ along with a delay in cell death of the INL and GCL in the albino rat retina.⁵⁷ One wonders whether the more "mature" retina of the LE rat might result in a greater difficulty in overcoming hyperoxic stress, whereas the more plastic immature retina of the albino rat is better able to overcome a similar stress. Variations in the responses of different rat strains to hyperoxia, including the increased susceptibility of the Dark Agouti and Hooded Wistar rats compared with several albino strains,⁵⁸ as well as that of the pigmented offspring of Fischer 344 X Dark Agouti compared with the albino progeny,⁵⁹ further support the association between ocular pigmentation and vulnerability in OIR. Furthermore, although no difference in the time course of retinal vascular development was observed between age-matched control Brown Norway and SD rats in a study by Gao et al.,¹⁹ those raised in hyperoxia showed significant differences in vascular permeability and retinal neovascularization. For example, retinal artery constriction along with large avascular regions in the retina were observed in Brown Norway rats at P12—that is, 1 day after their removal from the hyperoxic

environment. In contrast, SD rats studied under the same conditions revealed a lower degree of retinal artery constriction and larger areas of vascular perfusion throughout the retina.¹⁹ Increased vascular permeability followed by a more severe neovascularization in the Brown Norway strain^{19 60 61} was also observed. This effect was characterized by tortuosity and dilatation of retinal vessels, neovascular tufts, and hemorrhage when compared with the SD rats, which were lacking neovascular tufts and evidence of microaneurysms.¹⁹ Moreover, the Brown Norway model of OIR revealed higher retinal VEGF levels and more severe retinal vascular leakage than did the SD strain.⁶² Taken together with our current results and considering that the secondary neovascular phase of OIR is thought to be a mirror image of the initial vasoconstriction and vaso-obliteration which takes place, these findings suggest that retinal vascular development along with the neovascularization phase that ensues is significantly more affected by hyperoxia in pigmented than albino SD rats. Variations in the regulation of the antiangiogenic pigment epithelium-derived factor (PEDF) and the angiogenic vascular endothelial growth factor (VEGF) among strains are thought to contribute to these discrepancies, which may be genetically regulated,¹⁹ not to mention other strain-dependant factors that may be involved in regulating the effects of oxygen tension on retinal angiogenesis, such as glial cell sensitivity to hyperoxia, for example.⁶³

Evidently, despite using identical oxygen exposure protocols, the pathophysiological consequences of OIR were much more severe in the pigmented LE model than in the albino SD rat model. Not only did the hypothesized free-radical-scavenging role of melanin fail to protect the LE cohorts that were exposed to hyperoxia postnatally, but significantly greater levels of the MDA product of peroxidation were also evidenced in the LE model. We therefore sought to further understand the mechanisms by which SD rats remained significantly more resistant to a hyperoxic stress by exploring the potential involvement of specific neurotrophic factors throughout the hyperoxia. In a previous study, we showed that the relative (compared with adult) resistance of

juvenile SD rats exposed after birth to a bright luminous environment (not combined with oxygen), another oxidative stress, might be explained by the upregulation of neurotrophic factors such as FGF-2 and CNTF. In adult retinas, FGF-2 and CNTF levels are also upregulated after mechanical injury. Furthermore, intravitreal injection of the above-mentioned factors in addition to brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor have all been shown to delay photoreceptor cell death in animal models of retinal degeneration.^{64 65 66 67 68 69} We therefore hypothesized that the expression of similar survival factors might explain the relative resistance of SD rats to (1) photoreceptor damage (as evidenced by the relatively intact a-wave) and to (2) more severe thinning of the inner retinal layers. Of note, our results suggest that although only limited variations in FGF-2 occur in both LE and SD models after hyperoxic exposure, higher levels (approximately twofold) are observed for each exposure regimen in SD rats. Furthermore, although CNTF levels are only significantly higher after exposure regimens that include the second week of life in LE rats, they are already significantly greater in SD rats after exposure within the first week (e.g., from P0–P6). These results would therefore suggest that SD rats are better equipped with survival factors in the face of a hyperoxic challenge. The involvement of CNTF and FGF-2 may explain the preservation of photoreceptor function in SD rats as well as the relative resistance to functional and structural damage that follows hyperoxia from P0 to P6. Moreover, previous studies have shown that, in the adult retina, light-induced damage provokes Müller cell gliosis,⁷⁰ which in turn releases several neurotrophic factors.^{38 39 40 71 72 73} Microglia have been thought to play an integral role in this process as they have been shown to secrete factors that diffuse to the outer retina.⁷⁴ Given the relatively intact inner retina that is observed in SD rats after hyperoxic exposure, the above may indeed be a plausible mechanism of photoreceptor survival in this model. The severely compromised inner retina of LE rats exposed to the same conditions, however, would make it quite difficult for a similar protective mechanism to take place and may consequently explain their increased susceptibility, particularly where photoreceptor function is concerned.

Conflicting results have been obtained using gene transfer strategy in Prph2Rd^{2/Rd2} mice receiving subretinal injections with adenoviral (Ad)-vectored CNTF that resulted in the preservation of ERG amplitudes^{75 76} and with adenoassociated virus (AAV)-vectored CNTF that resulted in accelerated deterioration of ERG amplitudes,⁷⁷ suggesting that therapeutics of this nature still require further study. More recently, however, Dong et al.⁷⁸ demonstrated that double transgenic mice with inducible glial-derived neurotrophic factor (GDNF) provides protection from loss of retinal function (a- and b-waves of the ERG) generated by three different sources of oxidative stress, including one where mice were exposed to 75% oxygen for 3 weeks. It may therefore also be worthwhile to explore the potential antioxidant activity (or other) that GDNF exerts on pigmented and albino models of OIR and in other models of disease in which oxidative damage plays a role.⁷⁸

As described earlier, there is an apparent discrepancy between some clinical studies of human ROP which have shown, for example, that a greater number of Caucasian infants reach threshold ROP compared with African-American infants^{8 10} and the animal model, which suggests a positive correlation between pigmentation and the severity of OIR.

Of interest, however, the greater susceptibility of the SD strain to vascular attenuation after hyperoxia compared with other albino strains such as the Fischer 344, Wistar-Furth, and Lewis strains would further support a role for factors other than ocular pigmentation.⁵⁵ Given that genetic factors appear to influence the angiogenic response of different rat strains, it would not be surprising that they also play a role in the increased incidence and severity of the retinopathy observed on a functional and structural level. Of interest, studies have shown that ventilatory responses are thought to be genetically as well as environmentally regulated^{21 22} resulting in varying responses to changes in O₂ and CO₂ levels, for example.²² In fact, our findings of an increased mortality rate of Brown Norway rats after exposure to the same hyperoxic protocol used to obtain our current results may be explained by these strain-related differences, which suggests an

unequal susceptibility of different rat strains. It is also of interest to mention that differences in the incidence and severity of OIR were observed among Sprague-Dawley rats obtained from independent commercial sources, suggesting an important role of genetics, even within a given strain (Kitzmann AS, et al. *IOVS* 2002;43:ARVO E-Abstract 1256). Of course, studies aimed at further elucidating the genetic and racial factors affecting the incidence of ROP in humans are even further complicated by several confounding variables including sex, socioeconomic status, geographical areas, and environment, although a deeper understanding of these factors may explain the relative sparing of some ethnicities with respect to the development of severe ROP versus others that seem more susceptible. Although a connection between racial variation and the incidence and severity of diseases such as ROP^{8 9 10} and age-related macular degeneration (ARMD)^{79 80} have been documented, it is important to take into consideration that very little is understood regarding the functional mechanisms of differences in pigmentation⁸¹ and that current studies are under way that are aimed at better understanding the molecular composition of melanosomes, along with their structure and function in the eye.^{82 83} Other studies have shown that melanin may not act alone as the substance responsible for the protective effect,^{84 85} but may instead be a confounding variable that is associated with certain genes that are more or less prevalent in certain populations. Genetic influences in the pathogenesis of these diseases may play an important role in the heterogeneity in risk of their progression.

The underlying genetic differences that could explain the differential susceptibility between rat models of OIR as well as humans of different ethnicities remain to be explored.

In conclusion, whereas some clinical studies suggest that the presence of increased melanin content confers added protection to infants that are postnatally exposed to hyperoxia as observed in African-Americans that appear to be protected against the development of severe ROP,^{8 10} other studies have concluded that fundus pigmentation may play only a limited role in this protection.^{11 12}

Present animal findings suggest an increased susceptibility of pigmented LE rats to postnatal hyperoxia in comparison with the albino SD rat. Given the discrepancy between findings, future studies warrant careful investigation of other factors, including the contribution of genetics, which may help to elucidate the differences between LE and SD rats (as well as others) and to further our understanding of the pathogenesis of OIR and ROP, to potentially facilitate the derivation of therapeutic modalities.

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9 Captions to tables and figures

Table 1: Group data obtained in scotopic and photopic conditions from LE rats raised in normoxia and hyperoxia

Table 2: Group data obtained in scotopic and photopic conditions from SD rats raised in normoxia and hyperoxia

Figure 1: Representative retinal vascular flatmounts stained with ADPase from LE (*top*) and SD (*bottom*) rats, raised in room air and euthanatized at P6 (**A, E**, respectively) or P14 (**C, G**, respectively), or raised in hyperoxia and euthanatized immediately after 6 days (P0–P6; **B, F**, respectively) or P14 (P0–P14; **D, H**, respectively) of oxygen exposure. Magnification, x40.

Figure 2: Graphic representation of retinal vasculature extent (**A, B**) and vascular density (**C, D**) (ordinate) as a function of the oxygen exposure regimen (days, abscissa) in the normoxic and hyperoxic LE and SD cohorts at P6 (SD, $n = 3$; LE, $n = 3$) or after 6 days of hyperoxia (P0–P6; **A, C**, respectively; SD, $n = 3$; LE, $n = 3$) and at P14 or after 14 days of hyperoxia (P0–P14; **B, D**, respectively; SD, $n = 3$; LE, $n = 3$). Results are expressed as a percentage of vascular extent or vascular density of control retinas at P6 and P14, respectively, ± 1 SD. *Statistically significant differences from the control at a given age found with the Student's t -test ($P < 0.05$); •, significant differences from SD rats for the same exposure regimen ($P < 0.05$).

Figure 3: Representative scotopic (mixed rod-cone, flash intensity: -6.3 through $0.60 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$; **A–E**) and photopic (flash intensity: $0.9 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$, background: $30 \text{cd} \cdot \text{m}^{-2}$; **G**) ERGs along with scotopic (**F**) and photopic (**H**) oscillatory potentials obtained at P60 from animals raised in normoxia (21% O_2) and hyperoxia (80% O_2) from P0 to P6, P0 to P14, P6 to P14, and P12 to P14. Calibrations: horizontal, 20 ms; vertical, 400 μV for scotopic mixed rod-cone ERG responses, 200 μV for photopic ERG responses and 40 μV for scotopic and photopic oscillatory potentials, respectively. All tracings include a 20-ms prestimulus baseline; *arrows*, flash onset.

Figure 4: Graphic representation of a-wave (**A, G**), rod $V_{\max}$ (**B, H**), rod-cone b-wave (**C, I**), photopic b-wave (**D, J**), scotopic SOP (**E, K**), and photopic SOP (**F, L**) amplitudes (ordinate in microvolts) obtained at P30 and P60, respectively (abscissa in days of exposure), from LE and SD rats. The hyperoxic cohort of each strain was divided at birth into seven different exposure protocols: from birth through postnatal day 6 (P0–P6: LE, $n = 24$ [P30], $n = 5$ [P60]; SD, $n = 6$ [P30], $n = 6$ [P60]); birth to postnatal day 9 (P0–P9: LE, $n = 11$ [P30], $n = 5$ [P60]); birth to postnatal day 12 (P0–P12: LE, $n = 7$ [P30], $n = 6$ [P60]); and birth to postnatal day 14 (P0–P14, LE, $n = 21$ [P30], $n = 6$ [P60]; SD, $n = 11$ [P30], $n = 7$ [P60]) or from P6, P9, or P12 through P14 (P6–P14: LE, $n = 22$ [P30], $n = 6$ [P60]; SD, $n = 8$ [P30], $n = 8$ [P60]; P9–P14: LE, $n = 17$ [P30], $n = 9$ [P60]; P12–P14: LE, $n = 15$ [P30], $n = 7$ [P60]). Data were compared with results for respective age-matched control litters raised in normoxia (LE, $n = 21$; SD, $n = 22$).

Figure 5: Representative retinal histologic sections (central retina) from LE rats obtained at P60 from the normoxic cohort (**A**), and from hyperoxic cohorts including P0 to P6 (**B**), P0 to P14 (**C**), P6 to P14 (**D**), and P12 to P14 (**E**). Retinal sections obtained from SD rats raised in normoxia (**F**) and maximum hyperoxia (P0–P14, 80%; **G**) are also shown for comparative purposes. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment. Scale bar, 50 μm .

Figure 6: Graphic representation of the mean retinal layer thicknesses (in micrometers) and total retinal thickness from control (P0–P14, 21%) and from hyperoxic LE cohorts, including P0 to P6, P0 to P14, P6 to P14, and P12 to P14 ($n = 3$ per group). Measurements were taken at P60. Significant differences within normoxic or hyperoxic cohorts were identified with a one-way ANOVA followed by the Dunnett multiple comparison test comparing data obtained from each regimen with control; *significantly different from control ($P < 0.05$). Results are given as the mean thickness ± 1 SD.

Figure 7: Western blot analysis was used to detect FGF-2 and CNTF protein levels in LE (**A, C**, respectively) and SD (**B, D**, respectively) rats. Retinas were collected from normoxia-raised rats at P6 and P14 and from rats exposed to

hyperoxia from P0 to P6, P6 to P14, and P0 to P14 ($n = 3-4$ per group). Neurotrophic factor levels were normalized to β -actin (ordinate: ratio/ β -actin, mean $\pm$ 1 SD) for quantification. *Right*: molecular mass (in kilodaltons) of each band (18 kDa for FGF-2, and 19.4 kDa for CNTF). *Statistically significant differences ($P < 0.05$) obtained with a Student's t -test comparing exposed animals from the P0 to P6, P6 to P14, and P0 to P14 groups to age-matched control rats.

Figure 8: Retinal cellular localization of FGF-2 (**A, B**) and CNTF (**C, D**) in representative control retinas at P6 and P14 and in exposed retinas after hyperoxia from P0 to P6, P6 to P14, and P0 to P14 in LE (**A, C**) and SD (**B, D**) rats. Upregulation of FGF-2 and CNTF staining was observed in the INL and GCL by fluorescence microscopy after hyperoxia regimens for both strains. Scale bars, 100 μ m.

Figure 9: Colocalization of CNTF immunostaining (**A**) with anti-GFAP labeling (**B**) specific for active Müller glial cells, revealed that Müller cells actively participate in upregulating CNTF levels after hyperoxia (shown in retinas of LE rats after maximum exposure from P0 to P14 collected at P60; **C**). Scale bar, 100 μ m.

10 Tables

Scotopic Parameters	Age	Control	P0-6	P0-9	P0-12	P0-14	P6-14	P9-14	P12-14
Rm (μV)	30d	264.34±87.25	256.41±85.60	223.34±77.82	166.76±67.68*	137.18±44.29*	167.65±64.47*	223.42±89.78	282.08±130.
	60d	220.29±58.84	185.80±59.51	182.15±56.72	175.00±100.36	100.49±27.73	94.57±61.72*†	217.62±121.44	202.48±153.
Rod Vmax (μV)	30d	497.58±124.50	422.54±123.10	202.82±96.03*	79.90±74.54*	81.63±59.71*	90.32±58.76*	173.61±105.87*	341.6±96.6
	60d	544.61±178.97	405.44±109.12	209.85±138.10*	186.29±127.92*	148.79±98.49*	70.99±42.08*	135.44±119.43*	308.85±101.
Rod-Cone b-wave (μV)	30d	864.19±193.40	593.61±200.20*	277.20±137.83*	133.00±91.77*	139.84±58.11*	187.31±68.79*	362.09±269.4*	680.97±304.
	60d	806.76±257.07	483.50±217.97	281.00±204.02*	184.08±153.75*	200.32±122.22*	142.08±46.91*	468.23±350.81*	598.27±423.
SOPs (μV)	30d	388.82±100.64	229.23±91.31*	99.52±48.02*	51.05±40.36*	24.28±20.95*	51.28±45.03*	93.99±88.76*	277.80±177.
	60d	395.69±94.98	271.81±142.43	155.79±121.60*	81.75±78.20*	50.14±47.45*	44.91±37.73*	139.43±108.68*	233.38±185.
Photopic Parameters									
b-wave	30d	209.61±72.44	111.19±40.21*	49.06±35.97*	31.41±13.51*	25.02±10.79*	42.87±27.22*	67.41±53.26*	126.59±71.1
	60d	190.29±52.24	98.22±40.65*	63.65±58.33*	42.42±23.21*	38.68±21.13*†	35.50±23.65*	89.44±64.71*	147.44±102.
SOPs (μV)	30d	55.88±21.24	42.89±16.92	19.42±17.35*	7.71±7.99*	4.20±6.28*	11.11±14.34*	28.97±32.93*	43.67±25.4
	60d	62.93±17.79	36.51±20.08*	34.08±20.46*	22.8±11.96*†	13.37±10.57*†	9.87±6.67*	25.67±15.67*	38.91±30.7

Table 1

Scotopic Parameters	Age	Control	P0-6	P0-14	P6-14
Rm (μV)	30d	361.07 $\pm$ 109.65‡	315.30 $\pm$ 50.70	287.65 $\pm$ 69.38‡	305.27 $\pm$ 70.41‡
	60d	326.35 $\pm$ 57.68‡	337.68 $\pm$ 85.41‡	214.32 $\pm$ 82.09‡	357.36 $\pm$ 96.43‡
Rod Vmax (μV)	30d	496.25 $\pm$ 146.35	366.03 $\pm$ 66.63*	123.15 $\pm$ 92.98*	201.19 $\pm$ 138.68*‡
	60d	529.24 $\pm$ 85.92	392.08 $\pm$ 90.25	138.08 $\pm$ 137.25*	232.96 $\pm$ 114.33*‡
Rod-Cone b-wave (μV)	30d	879.35 $\pm$ 226.06	656.83 $\pm$ 122.92*	324.79 $\pm$ 116.96*‡	430.05 $\pm$ 164.67*‡
	60d	766.74 $\pm$ 107.27	661.67 $\pm$ 160.81	309.18 $\pm$ 248.22*	472.25 $\pm$ 102.94*‡
SOPs (μV)	30d	574.01 $\pm$ 297.05‡	353.63 $\pm$ 81.25‡	104.86 $\pm$ 69.00*‡	225.95 $\pm$ 133.51*‡
	60d	470.19 $\pm$ 237.24	329.01 $\pm$ 92.99	96.44 $\pm$ 89.80*	244.39 $\pm$ 54.7*‡
Photopic Parameters	Age	Control	P0-6	P0-14	P6-14
b-wave	30d	209.25 $\pm$ 72.92	128.05 $\pm$ 33.09*	57.18 $\pm$ 34.09*‡	87.99 $\pm$ 42.61*‡
	60d	160.49 $\pm$ 30.08	143.67 $\pm$ 38.34	52.43 $\pm$ 44.79*	91.72 $\pm$ 19.58*‡
SOPs (μV)	30d	73.05 $\pm$ 18.91‡	51.91 $\pm$ 7.57	19.15 $\pm$ 25.35*‡	34.81 $\pm$ 25.96*
	60d	59.47 $\pm$ 40.31	55.94 $\pm$ 18.96	9.64 $\pm$ 7.66*	41.28 $\pm$ 23.73‡

Table 2

11 Figures

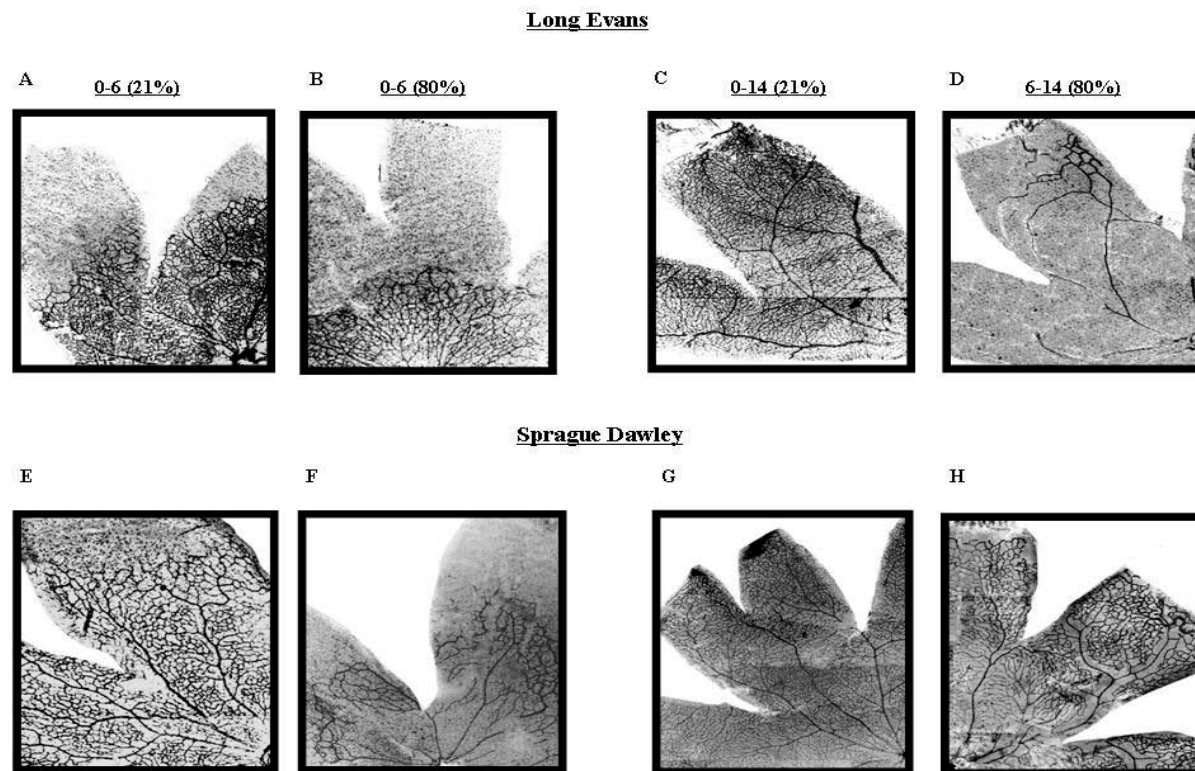


Figure 1

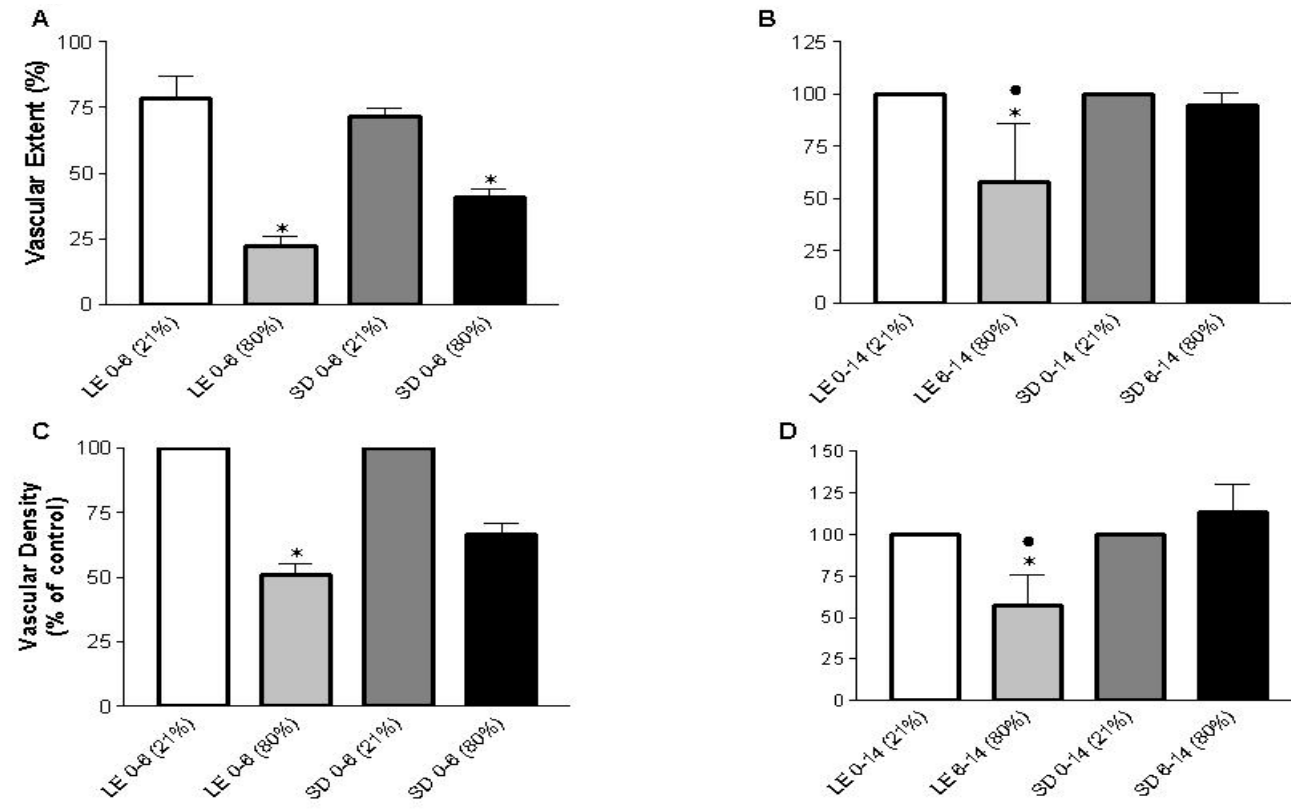


Figure 2

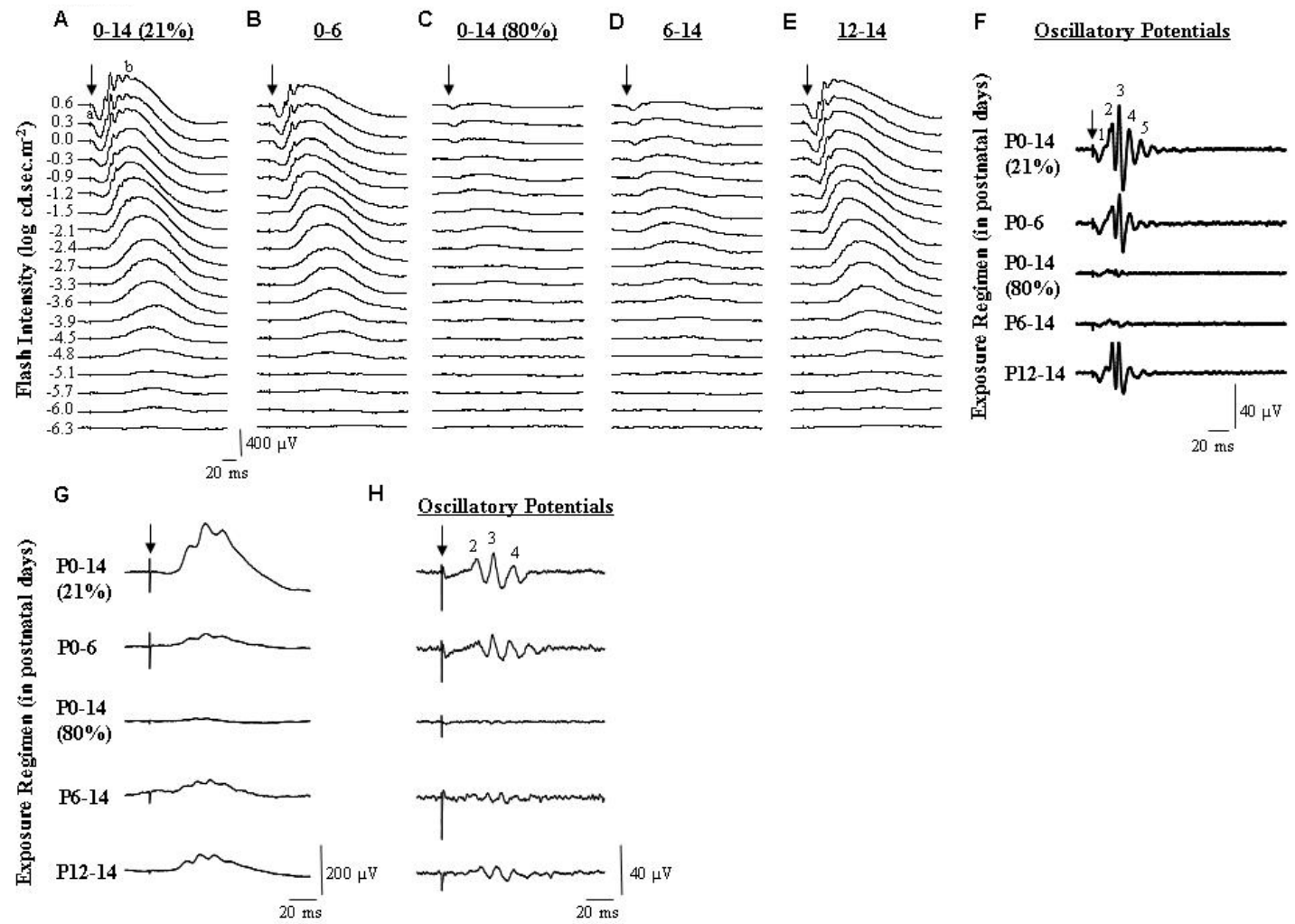


Figure 3

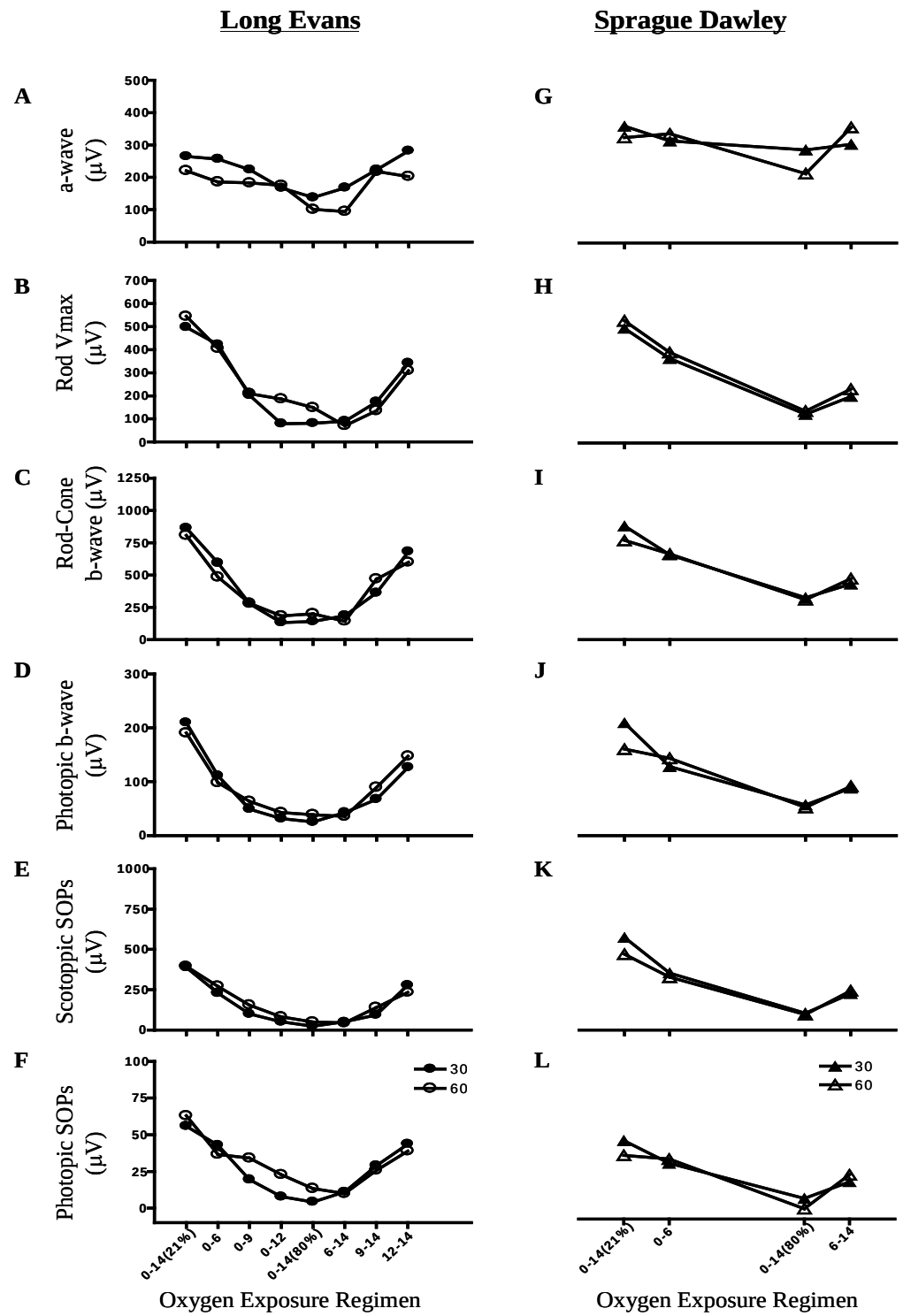
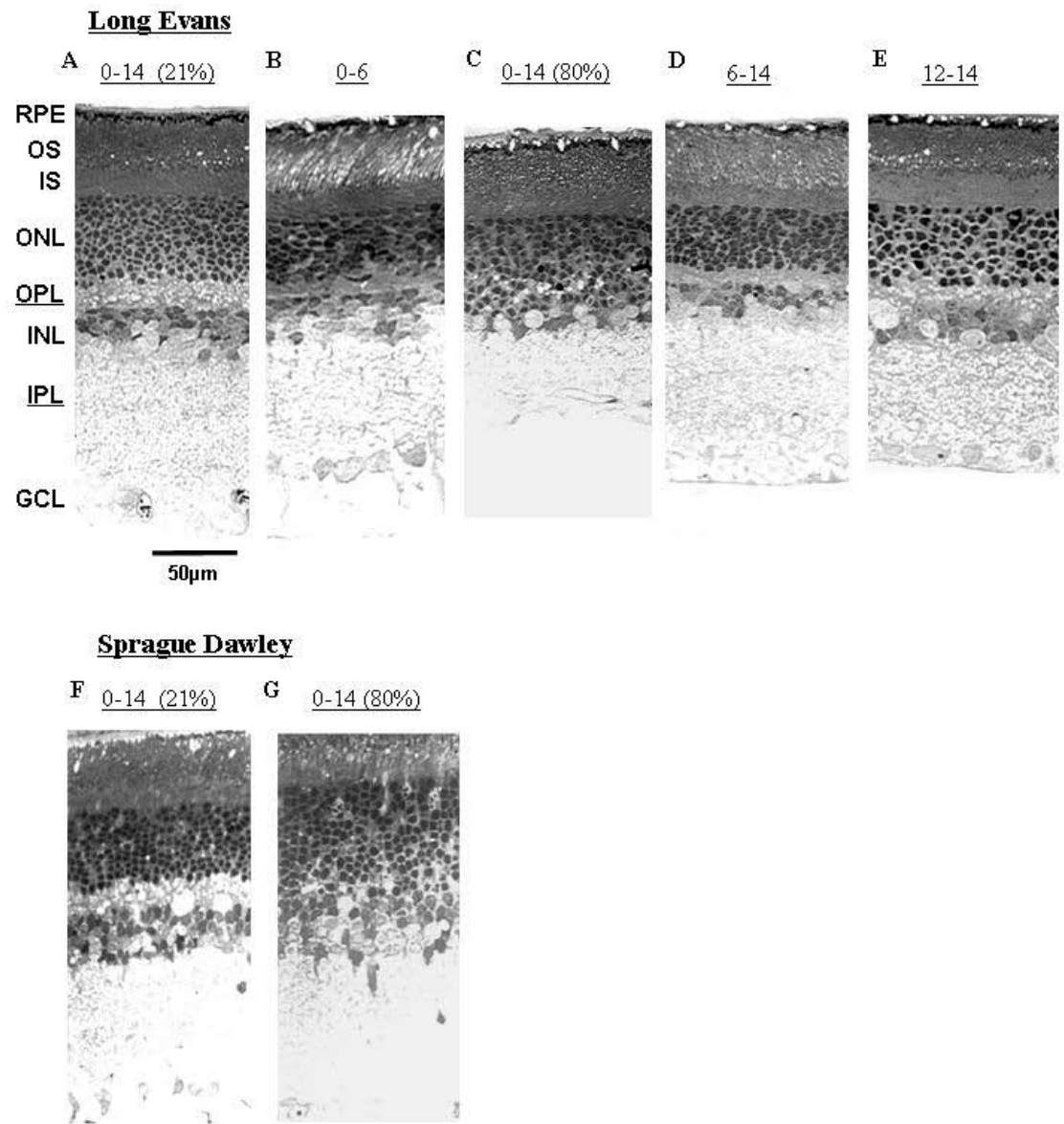


Figure 4

**Figure 5**

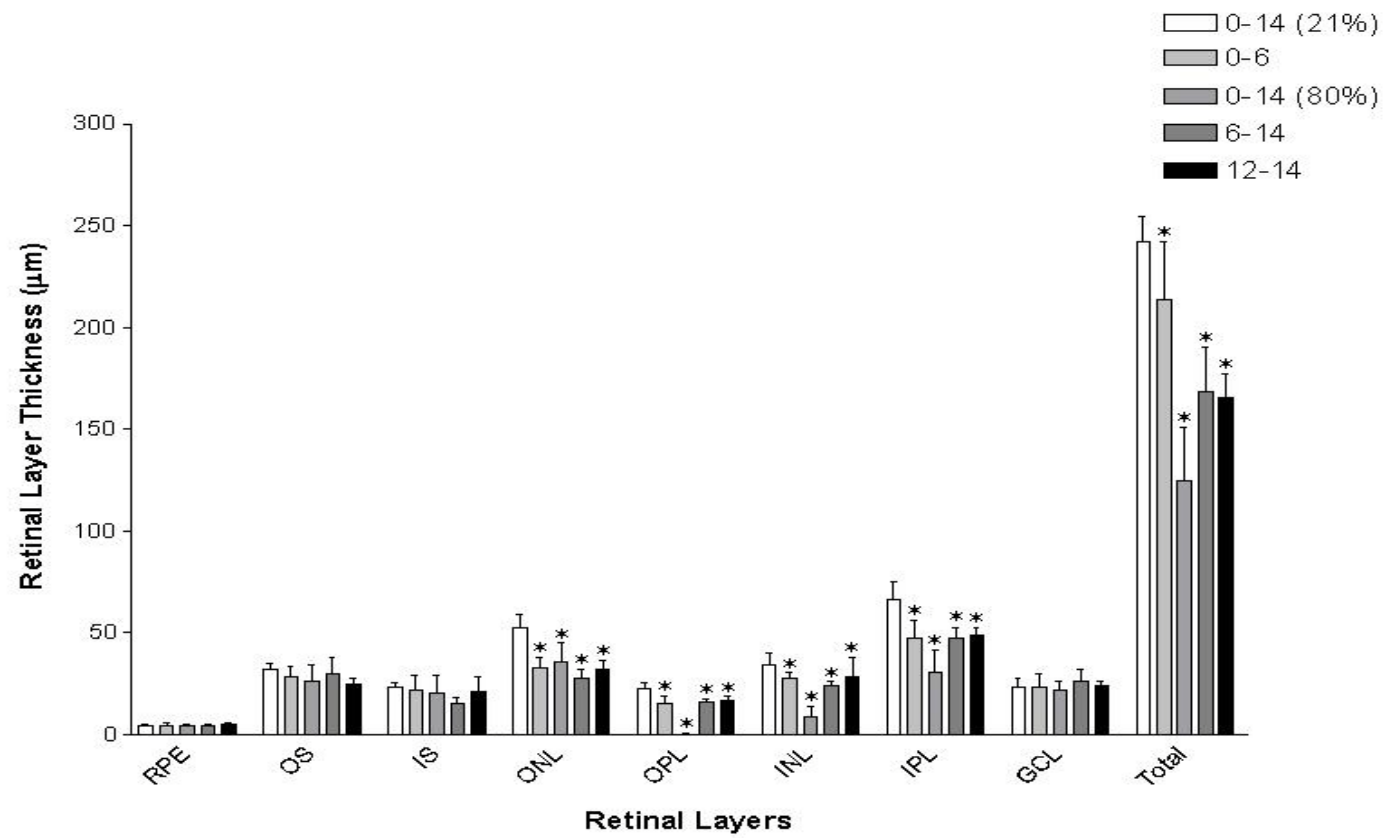


Figure 6

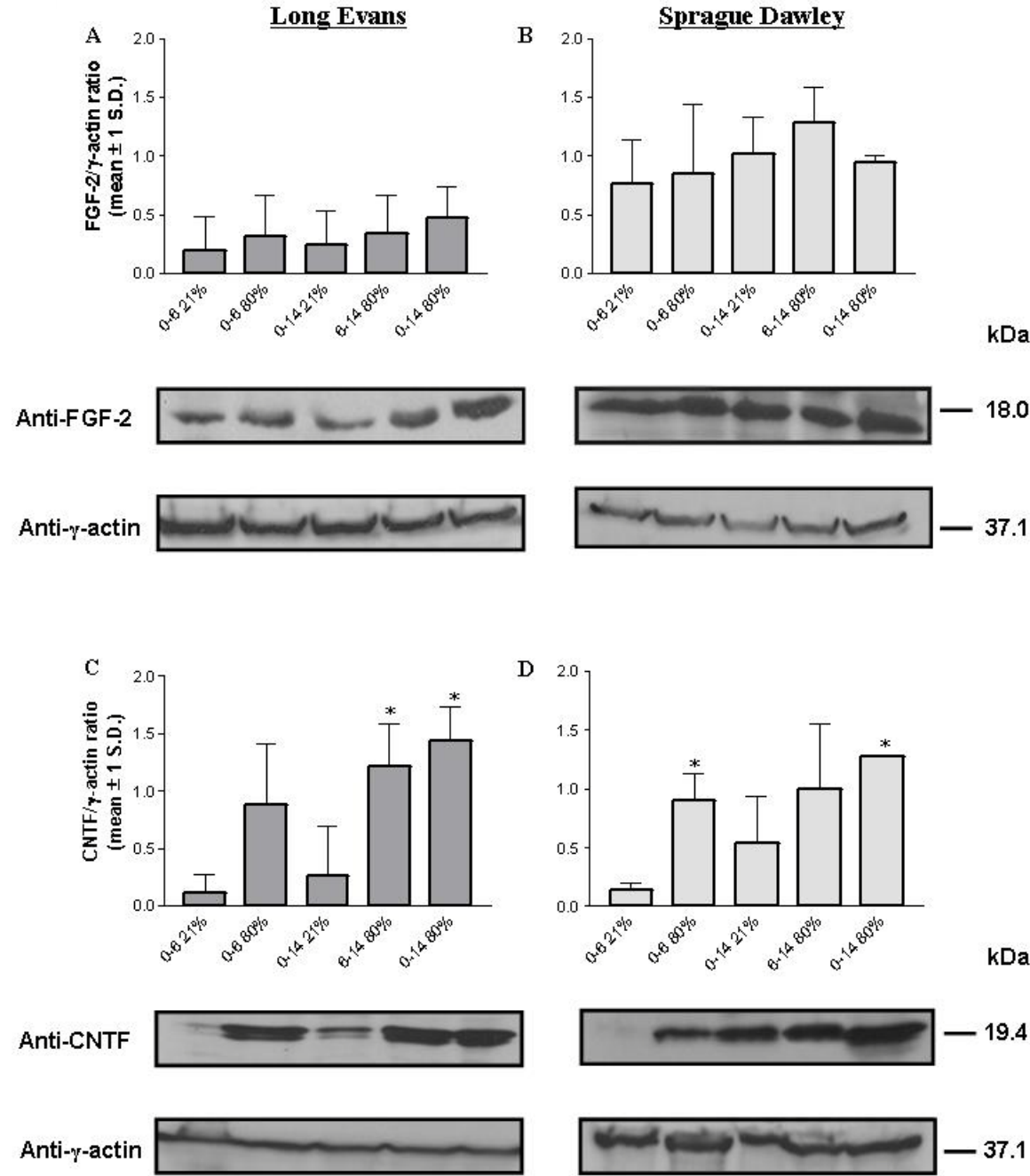


Figure 7

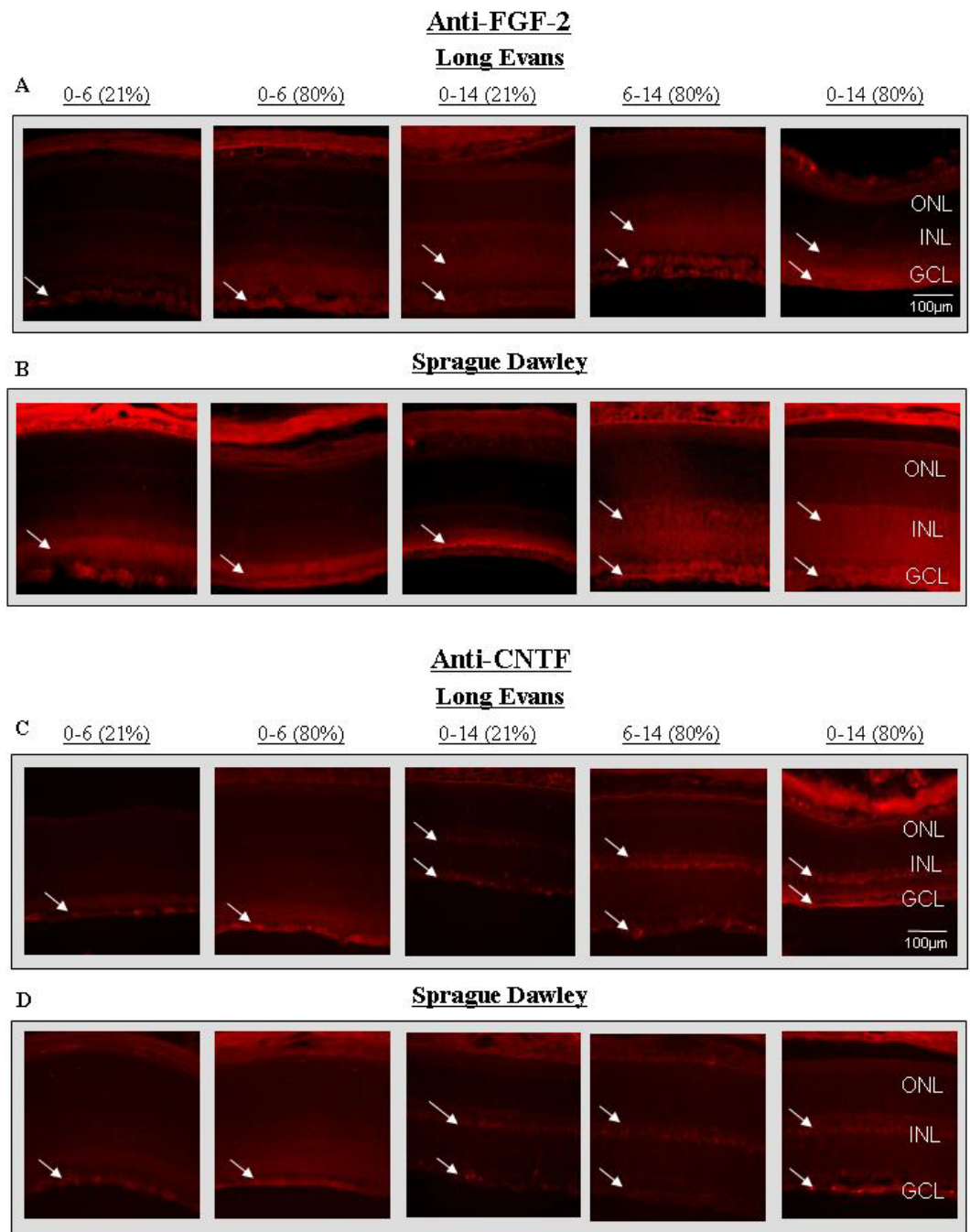


Figure 8

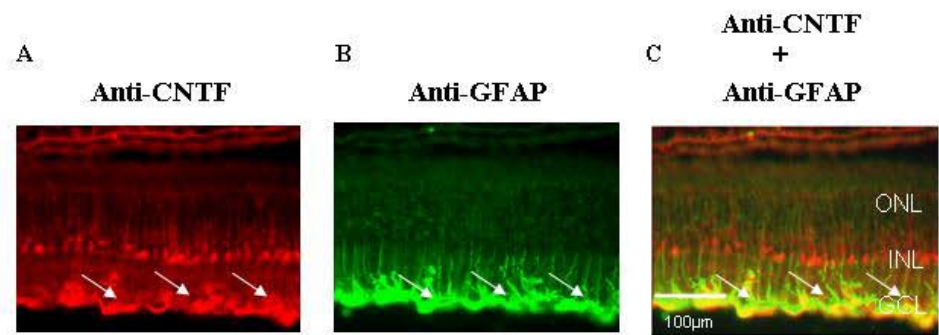


Figure 9

CHAPTER V: MANUSCRIPT IV

Dorfman AL, Cuenca N, Pinilla I, Chemtob S, Lachapelle P.

Cytoarchitectural Manifestations of OIR in the Neonatal Rat. *In preparation for submission to IOVS.*

1 Preface to chapter V

Findings described in chapters I-III above have suggested that postnatal hyperoxia results in significant alterations in retinal morphology, namely destruction of the OPL in LE and SD rats, the INL and IPL in LE rats, in addition to reduced horizontal cell counts and significant electroretinographic anomalies. The purpose of this study was to identify the mechanisms at the origin of this loss, using confocal microscopy and immunohistochemical techniques to visualize retinal neurons in order to study synaptic contact, cell morphology and cell death, which might be at the origin of strain specific differences.

2 Abstract

Purpose: We have previously shown that postnatal hyperoxia causes destruction of the plexiform layers in rats along with significant electroretinographic anomalies. The purpose of this study was to identify the mechanisms at the origin of this loss. **Methods:** Sprague Dawley (SD) and Long Evans (LE) rats were exposed to hyperoxia (n=24 control, 42 exposed) as previously described from birth to postnatal (P) 6 or P14 and from P6 to P14, following which the rats were euthanized, either at time of removal from 80% O₂ (at P6 or P14), or at P60. 10µm thick cryosections were collected for analysis. **Results:** At P60, synaptophysin (presynaptic marker) staining confirms the lack of functional synaptic terminals in the OPL of SD and in the OPL and IPL of LE rats. Uneven staining of ON–bipolar cell terminals with mGluR6 suggests that they contribute to OPL thinning. To further distinguish between rod and cone ON–bipolar cells, PKC (rods) and recoverin (cone) stainings were used and both showed a lack of dendritic terminals in the OPL with disorganized axonal projections in the IPL. Calbindin staining also revealed a reduction in the number of horizontal cells. Although photoreceptor cell bodies appeared intact, a decrease in bassoon staining (in rod and cone synaptic ribbon terminals) suggests limited communication to the inner retina. All of the above findings were significantly more pronounced in LE compared to SD rats. While an increase in TUNEL-positive cells was observed in the INL and ONL of LE rats following exposure from P0-14 [425.3% and 102.2% more than control, respectively (p<0.05)], a similar increase could only be seen in the INL of SD rats [146.3% more than control, respectively (p<0.05)]. Fewer TUNEL-positive cells were seen in the INL of LE and SD rats [67.5% and 55.6% less than control, respectively (p<0.05)] following the P0-6 exposure.

Conclusion: Our results suggest that cell death and synaptic retraction are at the root of outer plexiform layer thinning. The increased number of TUNEL positive cells in the nuclear layers confirms that these cells die, at least in part, due to apoptosis and that LE rats are more susceptible than SD rats. Finally, our results

point to the inner retina as the primary target of hyperoxic injury, followed by damage to the outer retina (to a lesser extent). Our findings propose a previously undescribed mechanism of cell death and synaptic retraction that are most probably at the origin of the functional consequences of hyperoxia.

3 Introduction

Retinopathy of Prematurity (ROP) remains a major cause of blindness in children despite many recent advances in neonatology, including the clinical monitoring of oxygen administration in the NICU as well as surgical treatments such as cryotherapy and laser photocoagulation (Holmstrom et al. 2007, 1993). While ROP is considered to be a very complex, multifactorial disease, gestational age and birth weight appear to be the most significant risk factors associated with its occurrence (Brown et al. 1998, Monos et al. 1996, Shohat et al. 1983). Interestingly, however, a number of studies have also provided evidence for a genetic basis for susceptibility to ROP and have revealed that sex, ethnicity and ocular pigmentation might all play a role in contributing to the development of this disease (Lang et al. 2005, Tadesee et al. 2002, Saunders et al. 1997, Arnold et al. 1994, Schaffer et al. 1993). However, socio-economical statuses as well as varying cultural approaches to different treatments have made it quite difficult to fully understand the relevance of ethnicity and genetics to the progression of ROP.

The rat continues to be one of the most studied of all animal models of oxygen induced retinopathy (OIR), as it offers the advantage of a highly immature retina at birth that is comparable to that of a 24-26 week old human embryo (Weidman and Kuwabara 1969). Similar to what is observed in humans, exposure of the rat pup to postnatal hyperoxia will cause severe vasoconstriction and vaso-obliteration that are followed by an abnormal proliferation of retinal vessels upon the return to room air (Patz and Payne 1998, Penn et al. 1994,

Reynaud and Dorey 1994, Moore 1990), all features which make it an elegant model with which to study the pathogenesis of OIR and better understand the sequelae of ROP. Of interest, previous studies comparing pigmented and albino rats have shown that the former are less susceptible to ischemic retinal injury than the latter (Safa and Osborne 2000), while others have demonstrated that certain pigmented strains developed a more severe retinal neovascularization following hyperoxia (Gao et al. 2002, and Zhang et al. 2001).

While vascular impairments are considered to be the clinical hallmark of ROP, studies in our laboratory have been focused not only on retinal vasculature throughout hyperoxic exposure (retinal flatmounts), but also on the structural (retinal histology) and functional (rod-mediated and cone-mediated electroretinograms) features that characterize this model. Previous findings of ours have revealed that while structural and functional impairments are evident in the albino Sprague Dawley rat (SD), including outer plexiform layer (OPL) thinning, reduced horizontal cell (HC) count along with attenuation in the b-wave amplitude of the electroretinogram (ERG) with relative sparing of the a-wave (Dorfman et al. 2009, 2008, 2006, Dembinska et al. 2002, 2001, Lachapelle et al. 1999), an even greater susceptibility of the Long Evans (LE) pigmented strain to hyperoxia was noted (Dorfman et al. 2009). This extreme vulnerability is characterized by higher vascular dropout throughout hyperoxic exposure, permanently altered retinal function that occurs following all exposure regimens (even that which is limited to the first week of life) and permanently altered retinal structure which is not only limited to loss of the OPL and horizontal cells as in SD rats, but which also extends into the inner retina resulting in significant thinning of the INL, IPL and GCL (Dorfman et al. 2009). Little is known, however, about the cytoarchitectural mechanisms that contribute to these findings.

The purpose of the present study was therefore to further investigate the impact of hyperoxia on retinal circuitry, using confocal microscopy and immunohistochemical techniques to visualize retinal neurons in order to study synaptic contact, cell morphology and cell death, which might be at the origin of strain specific differences.

4. Methods

4.1 Animals

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the McGill University-Montreal Children's Hospital Research Institute. Within 24 hours from birth, newborn litters of Sprague Dawley (SD) and Long Evans (LE) rats (Charles River Laboratories, St. Constant, Quebec, Canada) were placed, with their mothers, in room air (21% O₂) or in a hyperoxic (80% O₂) environment (mixture of medical grade 100% O₂ and room air measured with a MaxO₂ ceramatec oxygen meter, model OM25-ME; Medicana Inc., Montreal, Canada). A controlled cyclic luminous environment (80 lux; 12-hours light, 12-hours dark) was maintained throughout the exposure and the temperature of the animal facility was kept at 23°C. Exposure to hyperoxia lasted for 22.5 hours daily and was interrupted for three 0.5-hour periods at normoxia (21% O₂). Based on previous studies that suggested unequal susceptibilities to hyperoxia throughout different developmental timepoints (Dorfman et al. 2009, Dembinska et al. 2001, 2002), the hyperoxic cohort for each strain was divided at birth into four different exposure protocols: from birth through postnatal day 6 (P 0-6, LE: n = 8, SD: n = 8), and 14 (P 0-14, LE: n = 8, SD: n = 8) or from postnatal day 6 through 14 (P 6-14, LE: n = 8, SD: n = 8). Following hyperoxic exposure, groups of animals were either sacrificed immediately for TUNEL labeling (n = 4 SD and 4 LE per exposure regimen, respectively) or were returned to room air (21% O₂) until P60 for retinal histology (n = 4 SD and 4 LE per exposure regimen, respectively). Data was compared with respective control groups raised in normoxic conditions (LE: n = 8, SD: n = 8). Finally, mothers of the litters were alternated between normoxic and hyperoxic conditions every 24 hours so that pulmonary complications known to arise in adult rats raised in a hyperoxic environment could be avoided.

4.2 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Assay

Animals were euthanized immediately following removal from hyperoxia at P6 (following the P0-6 exposure regimen), or at P14 (following the P0-14 exposure regimen), (n = 4 SD and 4 LE per regimen) and were perfused intracardially with 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer (PB; pH 7.4). Following removal of the cornea and lens, eye cups were immersed in 4% PFA in 0.2 M PB (pH 7.4) (2 hours) and were cryoprotected in sequential sucrose solution gradients (10%, 20% and 30% in PB for 30 minutes, 1 hour and overnight, respectively) at 4°C. Eyecups were embedded the following day in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and were frozen in a bath containing 2-methylbutane/liquid nitrogen. 14 µm-thick cryosections were collected on gelatin-coated slides on which TUNEL assays were conducted using an apoptosis detection kit (ApopTag Fluorescein In Situ Apoptosis Detection; Chemicon, Temecula, CA). TUNEL-positive cells were counted under a microscope (Zeiss Axiophot, Zeiss microscope, Germany: 40X) attached to a digital camera (RR slider spot, Diagnostics instruments inc., Germany) with which images were captured. Results are expressed as mean $\pm$ S.D.

4.3 Retinal Immunohistochemistry

Following each hyperoxic regimen, animals were returned to room air until P60 at which point they were euthanized with CO₂ (n = 6-8 SD and 6-8 LE per regimen). Eyes were enucleated, a small incision was made in the cornea and the eyes were immersed in 4% PFA in 0.2 M PB (pH 7.4) (2 hours). The cornea and lens were then removed, following which eyecups were re-immersed in 4% PFA in 0.2 M PB (pH 7.4) (1 hour and 15 minutes) and washed in 0.1 M PBS

before being cryoprotected in sequential sucrose solution gradients (10%, 20% and 30% in PB for 30 minutes, 1 hour and overnight, respectively) at 4°C. Eyecups were subsequently embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and were frozen in a bath containing 2-methylbutane/liquid nitrogen. 14 µm-thick cryosections were collected on polylysine-S coated slides for immunohistochemical analysis. In order to block any nonspecific binding, retinal sections were incubated in 10% normal goat serum (Jackson Laboratories, West Grove, PA). Without washing, they were then immunostained with single primary antibodies or with specific combinations of antibodies diluted in PBS containing 0.5% Triton X-100 (Table 1).

Retinal sections obtained from each of the normoxic- and hyperoxic-cohorts were incubated overnight at room temperature. The following day, they were washed in 0.1 M PBS and then transferred to a cocktail of secondary antibodies to visualize antibody binding patterns, including either goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC), goat anti-mouse IgG coupled to tetramethyl rhodamine (TRITC), goat anti-rabbit IgG coupled to TRITC or goat anti-mouse IgG coupled to FITC.

All secondary antibodies were used at a 1:100 dilution in 0.1 M PBS 0.5% Triton X-100 for 1 h. Finally, the sections were washed in 0.1 M PBS, mounted in watermount (Vector Labs) and coverslipped for viewing by confocal microscopy (Zeiss LSM 510).

To control for non-specific staining, some sections were stained omitting the primary antibody (Table 1).

5 Results

5.1 Recoverin

All photoreceptors in addition to cone bipolar subtypes 2 and 8 (McGinnis 1999) are labeled with antibodies against recoverin. The ONL in recoverin-stained sections from normoxic-cohorts of LE and SD rats was approximately 12-14 cells thick (Figure 1A and E, respectively). While no significant changes could be identified with respect to the ONL and cone bipolar cells following exposure from P0-6 in LE rats (Figure 1B), thinning of the ONL to approximately 8-9 rows along with cone bipolar cell dropout was observed following exposures from P6-14 and P0-14 (Figure 1C and D, respectively). The photoreceptor nuclei located in the inner part of the ONL lose their alignment, and there are obvious gaps at the level of the OPL.

Similar to that observed in LE rats following exposure from P0-6, no significant morphological alterations in the ONL or in the cone bipolar cells could be noted in SD rats. Similarly, the ONL remains intact following exposure from P6-14 (Figure 1G). While a loss of alignment of the ONL could be observed in SD rats following exposure from P0-14 (Figure 1H), there was no suggestion of ONL thinning as was shown to occur in LE rats exposed to the same condition (Figure 1C,D).

Two types of cone bipolar cells with their somata located in the proximal row of the inner nuclear layer (INL) are labeled with antibodies against recoverin. Type 2 cone bipolar cells have a large somata (Figure 2A, *arrows*) and their axons end in sublamina a of the IPL (Euler & Wässle 1995). On the other hand, type 8 cone bipolar cells have small somata (Figure 2A, *arrowheads*) and their axons descend through the IPL and end in sublamina b of the IPL close to the ganglion cell layer (GCL) (Euler & Wässle 1995). A drastic reduction of both types of cone bipolar cells was observed following exposure from P6-14 and P0-14 in LE rats (Figure 2 C,D). Following P6-14 exposure, a loss of axon terminals in the a

and b sublaminae of the IPL can be observed (Figure 2C), and a dramatic reduction of cone bipolar cell terminals could be evidenced, particularly in sublamina a of the IPL, following the P0-14 regimen (Figure 2D, *arrowheads*). While recoverin immunoreactivity in the ONL remained unchanged in SD rats following the P6-14 and P0-14 regimens, there was, however, evidence of cone bipolar cell dropout, albeit not to the same extent as that observed in LE rats (Figure 2G, H). No differences in cone bipolar cell morphology could be observed between the P0-6 exposure regimen and control (Figure 2E, F).

5.2 γ -Transducin

Cones are specifically labeled by antibodies against γ -transducin. In both strains, prominent γ -transducin immunoreactivity is seen in the cone outer and inner segments located in the photoreceptor layer, cell bodies located in the outer third of the ONL, axons and pedicles (Figure 3A, E). Disorganization of the pedicles in the OPL of LE rats was already evident following oxygen exposure from P0-6 (Figure 3B). P6-14 and P0-14 regimens revealed a completely altered cone structure, with migration of the cone cell bodies to the center of the ONL in addition to synaptic retraction of cone bipolar cells in the inner retina, both of which occurred to a greater extent following maximal exposure (Figure 3C, D). Cone inner segments as well as cone pedicles appeared to be truncated and disrupted compared to control. Furthermore, the cell bodies of bipolar cells stained with transducin were closer in proximity to the ONL compared to control and were clearly disorganized (Figure 3C, D).

On the other hand, SD rats appeared to have a well maintained retinal structure following exposure from P0-6 compared to control (Figure 3E, F), and although exposures that include the second week of life disrupted the normal cone pedicle organization which leads to pedicle migration, the consequences were not as severe as those observed in LE rats (Figure 3G, H). OPL thinning was evident in both SD and LE rats, but the ONL as well as cone length were clearly less

affected in SD rats (see differences in ONL thickness between Figure 3 C, D and Figure 3G, H).

5.3 Protein kinase C α (PKC- α .)

All rod bipolar cells are labeled with antibodies against Protein kinase C (PKC)- α . In normoxic-cohorts of LE and SD rats, rod bipolar cell dendritic terminals establish connections with rod spherules in the OPL and their end-bulb axon terminals extend into the IPL (Figure 4 A, E).

While only subtle changes in morphology could be observed in LE rats exposed from P0-6 (Figure 4B), fewer bipolar cells could be identified following the P6-14 and P0-14 exposure regimens. A dramatic loss of dendritic terminals in the OPL along with fewer end-bulb terminals and lateral varicosities in the IPL were evidenced in the remaining bipolar cells (Figure 4C, D). Some of the remaining bipolar cell dendrites appeared flatter in rats that were maximally exposed to hyperoxia from P0-14, and there was a clear sprouting of the remaining dendrites into the ONL (Figure 4D arrows). These manifestations would suggest impaired contact between photoreceptors and bipolar cells (most evident following the P0-14 regimen), where bipolar cells appeared to be searching for contact with their presynaptic cell. Decreased PKC immunoreactivity was also evidenced in the axons and axon terminals of bipolar cells. The decreased length of the bipolar cells which occurs as a result of the manifestations described above contributes to an overall decrease in retinal thickness (Figure 4C, D).

Similar to LE rats, rod bipolar cell processes of SD rats exposed to hyperoxia from P0-6 maintained normal extension into the OPL and IPL (Figure 4F). Following exposure from P6-14 and P0-14, there was a clear sprouting of the bipolar cell dendrites into the ONL (Figure 4G, H). In addition, a reduction in length between the cell bodies and axon terminals of the rod bipolar cells could be

observed, although PKC immunoreactivity in the axon terminals was relatively well preserved (Figure 4G, H).

5.4 Synaptophysin

Figure 5 reveals an almost continuous layer of synaptophysin immunoreactivity in the OPL and IPL of both control LE and SD rats raised under normoxic conditions (Figures 5A and 5E, respectively). Following exposure to hyperoxia within the first week of life (P0-6), LE rats reveal decreased synaptophysin staining in the OPL and IPL (Figure 5B). Exposure from P6-14 resulted in a more punctuate and discontinuous staining pattern in the OPL, and a thinning of the IPL as evidenced with decreased levels of synaptophysin immunostaining (Figure 5C). The most extreme damage to the OPL and IPL, however, was observed following maximal exposure to hyperoxia from P0-14, where the most limited amount of synaptophysin staining could be evidenced (Figure 5D).

Similar to LE rats, synaptophysin immunostaining progressively decreased in the OPL of SD rats beginning with early exposure that persists throughout the first week of life (Figure 5F). This continued to an even greater extent following exposure within the second week of life (Figure 5G), and maximally to a completely thinned out OPL where very weak immunostaining could be observed following exposure from P0-14 (Figure 5H). In sharp contrast to LE rats, however, there was no evidence of any IPL changes in the SD hyperoxic-cohorts compared to control, irrespective of the regimen (Figure 5E-H).

5.5 Calbindin and Bassoon

In the rat, dendrites of β -type horizontal cells contact with cone terminals, while their axons synapse with rod terminals. Antibodies against calbindin are used for horizontal cell labeling, and double immuno-labeling with the antibody against the bassoon protein (a presynaptic protein which labels photoreceptor synaptic ribbon terminals in rod spherules and cone pedicles in the OPL) allows for their correlation with photoreceptor axon terminal distribution. This is best exemplified in figure 6A, where paired structures between the synaptic ribbons of LE photoreceptor axon terminals labeled with antibodies against bassoon (red) and the terminal tips of horizontal cells labeled with calbindin antibodies (green) can be observed (see Figure 6A inset).

In LE and SD rats raised in a normoxic environment, horizontal cell processes were all associated with horse shoe-like bassoon immunoreactivity (Figure 6A, E), which is suggestive of a normal pattern of contact between horizontal cell dendrites and photoreceptor synaptic ribbon terminals. While no evidence of change in this regular pattern of staining could be observed in LE or SD rats following exposure for the P0-6 regimen (Figure 6B, F), a decrease in calbindin immunoreactivity following the P6-14 and P0-14 exposure regimens in both LE and SD rats (Figure 6C, D and Fig.6G, H, respectively) was indicative of a loss of horizontal cells and their processes which inevitably contributes to reduced calbindin and bassoon pairing. It is of interest to note that despite the obvious decrease in horizontal cell terminals for the latter regimens, bassoon immunoreactivity, though clearly diminished, could be observed in the absence of any association with calbindin immunoreactivity (Figure 6 D, H, *arrows*). Sprouting in the ONL can be observed following P0-14 exposure LE rats (Figure 6D, *arrowheads*).

5.6 Bassoon and metabotropic glutamate receptor 6 (mGluR6)

Antibodies against mGluR6 label the postsynaptic receptors on the dendritic tips of rod and cone ON-bipolar cells, and pairing can be observed between ON-bipolar cells and the synaptic ribbons of cone and rod terminals using double immuno-labelling with mGluR6 and bassoon antibodies (Figure 7). LE and SD control rats each revealed two types of pairing patterns between presynaptic and postsynaptic elements which are morphologically distinguishable, but which were both present irrespective of strain. For example, Figure 7A reveals regions of pairing between mGluR6 stained (green) rod ON-bipolar cell dendrites and either bassoon labeled (red) presynaptic rod spherules that present with a horseshoe-like appearance (Figure 7A, *arrows*), or bassoon labeled presynaptic cone pedicles that take on more of a disc-like appearance (Figure 7A, *arrowheads*). Presynaptic and postsynaptic pairing of rod onto rod bipolar cells was found in the outermost OPL, while that of cone onto cone bipolar cells was found in the innermost OPL of control LE and SD rats (Figure 7A, E). While evidence of some pairing could still be observed following exposure to hyperoxia from P0-6 in LE rats, rods synapsed less frequently with rod ON-bipolar cells, as shown by the presence of bassoon stained rod terminals in the absence of association with mGluR6 in the outer OPL (Figure 7B). On the other hand, cone onto cone bipolar cell pairing took on more of a normal appearance and distribution in the inner OPL (Figure 7B, *arrowheads*). mGluR6 immunoreactivity was distributed around cells bodies and axons of bipolar cells (Figure 7B). Very infrequent pairing of bassoon and mGluR6 profiles were found in LE rats exposed to the P6-14 and P0-14 regimens, where the OPL appeared to be completely disrupted (Figure 7C, D). The bassoon staining that remained, however, was mostly limited to cone pedicles. Exposure of SD rats from P0-6 resulted in a reduction of immunoreactivity in the dendritic terminals of ON bipolar cells, where bassoon staining was observed in the absence of pairing with mGluR6 labelled profiles (Figure 7F), similar to that in LE rats, albeit to a lesser

extent. P6-14 and P0-14 exposure regimens revealed a complete disruption of the OPL with a near disappearance of synaptic terminals stained with mGluR6 and bassoon (Figure 7G, H). Consequently, very limited pairing between photoreceptor terminals and bipolar cells could be seen.

5.7 PKC- α and Parvalbumin

In SD and LE rats, AII amacrine cells can be identified using an antibody to parvalbumin, a calcium binding protein (Figure 8A, E) (Endo et al., 1986; Hamano et al., 1990; Cuenca et al 2002). In normal rats, the AII amacrine cell bodies were located in the proximal part of the INL, adjacent to the IPL, and their processes extended to the different sublaminae of the IPL (Figure 8A, E). Double-labeling of parvalbumin with PKC- α reveals that while the characteristic lobular appendages of AII amacrine cells are found in sublamina a of the IPL (Figure 8A, *arrowheads*), their dendrites extend into sublamina b to contact the axons of rod ON-bipolar cells (Figure 8A, *arrows*).

Following exposure of LE rats from P0-6, the dendritic processes of AII amacrine cells in the IPL became thinner and less numerous, losing their normal pattern of stratification. A reduction in contact between amacrine cell dendrites and the axon terminals of rod bipolar cells in sublamina b of the IPL was also noted (Figure 8B, *arrows*). A dropout of AII amacrine cells and a loss of normal morphology of the remaining cells can be observed in LE rats exposed from P6-14 and P0-14. Finally, no areas of contact between AII amacrine cells and the rod bipolar cell axons could be identified (Figure 8 C,D).

In the SD rat, the normal contact between AII amacrine cells and the axons of rod ON-bipolar cells (Figure 8E, *arrows*) is maintained following exposure from P0-6 (Figure 8F, *arrows*). On the other hand, the P6-14 and P0-14 exposure regimens resulted in sprouting of rod ON-bipolar cells and limited contact between AII amacrine cell dendrites and rod ON-bipolar cell axons. Furthermore,

their morphology in SD rats following these last regimens was abnormal and characterized by the loss of lobular appendages and dendrites in sublamina b of the IPL, but to a lesser extent than what was observed in LE rats, as some areas of contact could still be identified between amacrine cells and rod ON-bipolar cells following these regimens (Figure 8G, H, *arrows*).

5.8 Comparison between the central and peripheral retina

Irrespective of the oxygen exposure regimen used in LE rats, an overall preservation of the peripheral retina close to the ora serrata could be observed compared to the central retina which was more susceptible to the insult. Double immuno-labeling with γ -transducin and recoverin reveals several morphological changes in cones located in the central and peripheral retina. For example, well preserved cone outer segments can be identified in the peripheral retina, whereas those in the central retina are more disorganized. Moreover, their cell bodies are maintained in the outer layer of the ONL (as observed in control rats) and their axon terminals are also better preserved in the peripheral versus central retina. Finally, the inner plexiform layer in the peripheral retina is thicker than that of the central retina (Figure 9A, B).

Double-labeling with PKC- α and parvalbumin reveals that rod bipolar cells and AII amacrine cells are better preserved close to the ora serrata (Figure 9D), maintaining their normal number and morphology of dendrites and axons compared to the central retina (Figure 9C). Bassoon and calbindin immunostaining reveals that while few HCs can be identified in the central retina, more of them could be observed closer to the ora serrata, similar to control retinas (Figure 9E and F). Furthermore, pairing of HCs with bassoon is also well preserved and more frequently observed in the peripheral compared to central retina (Figure 9E, F). Finally, the relationship between bassoon in the synaptic ribbon of the photoreceptor axon terminals and the postsynaptic receptor mGluR6 located on ON-bipolar cells can only be identified in the area close to the ora

serrata (Figure 9G, *arrows*), while only very limited pairing can be observed elsewhere in the central retina (Figure 9G). We could not identify any of the above discrepancies between the central and peripheral retina in SD rats following exposure to hyperoxia.

5.9 TUNEL

TUNEL staining was carried out in young rats immediately following hyperoxia in order to better understand the cellular death process occurring in the rat model of OIR. Staining was performed in LE and SD rats exposed to hyperoxia for 6 days (P0-6) (Figure 10C and G, respectively) and 14 days (P0-14) (Figure 10D and H, respectively). Data was compared with control LE and SD retinas obtained at P6 (Figure 10A and B, respectively) and P14 (Figure 10E and F, respectively). Quantification of TUNEL-positive cells is shown in Figure 11. While there was evidence of TUNEL positive cells in both the ONL and INL of LE rats following exposure to hyperoxia at P14, only the INL of SD rats revealed TUNEL positive staining. Interestingly, a significant decrease in the number of TUNEL positive cells could be observed following exposure to hyperoxia from P0-6 compared to respective normoxic-cohorts in both LE (13.75 ± 3.40 compared to 42.25 ± 4.92 , respectively; $p < 0.05$) and SD (13.33 ± 2.42 compared to 30.00 ± 2.00 , respectively; $p < 0.05$) strains. These TUNEL positive cells were not confined to any particular layer at P6 as they were at P14 following oxygen exposure. In contrast, while TUNEL staining in the ONL of LE rats only tended to increase following maximal exposure from P0-14 compared to control (24.67 ± 3.22 compared to 12.20 ± 2.43 , respectively; $p > 0.05$), a significantly higher number of TUNEL positive cells were observed in the INL of LE rats compared to control (142.52 ± 29.61 compared to 27.13 ± 2.77 , respectively; $p < 0.05$). Similarly, TUNEL-positive cells were significantly more numerous in the INL of SD rats exposed to hyperoxia (73.90 ± 3.39 compared to 30.00 ± 7.07 , respectively; $p < 0.05$) following exposure from P0-14.

6 Discussion

Previous studies of ours showed that postnatal exposure to hyperoxia results in vascular dropout, a permanently altered retinal structure as evidenced with histology and permanently attenuated retinal function as evidenced with the ERG (Dorfman et al. 2009, 2008, 2006, Dembinska et al. 2002, 2001, Lachapelle et al. 1999). Our results suggested an even greater susceptibility of the pigmented LE rat to postnatal hyperoxia compared to the albino SD rat (Dorfman et al. 2009). The purpose of the present study was to identify the mechanisms at the origin of these manifestations.

Our current study suggests that the inner retina is the primary target that is most significantly impacted by hyperoxic events, while damage to the outer retina can also be evidenced, but to a lesser extent. Furthermore, our findings confirm our previous report of increased susceptibility of LE rats to postnatal hyperoxia, where irrespective of the exposure regimen, cytoarchitectural remodeling always occurred to a greater extent in this strain when compared to SD rats. Finally, the increased level of TUNEL positive cells in the INL, particularly in LE rats, suggests that cells there die, at least in part, through apoptosis.

As mentioned above, one of the most striking ultrastructural consequences of postnatal hyperoxia involves the thinning to disappearance of the OPL, evidenced with retinal histology (Dorfman et al. 2009, 2008, 2006, Dembinska et al. 2002, Lachapelle et al. 1999). This suggested to us that a loss of synaptic contact between the photoreceptor layer and the inner retina would likely be responsible for the functional changes obtained with the ERG, namely attenuation of the b-wave amplitude with increasing oxygen exposure. Therefore, we used antibodies such as bassoon and synaptophysin (as presynaptic markers) and mGluR6 (a postsynaptic marker for ON-bipolar dendrites), for example, in order to further study synaptic contact and cell morphology in this model. Our initial impressions were confirmed when we found a progressive decrease in synaptophysin staining, an integral membrane protein of synaptic vesicles that is

expressed in all types of neurons and synapses in adult vertebrate retina (Wiedenmann and Franke 1985), in the OPL of SD and LE rats, as well as in the INL of LE rats following exposure regimens that involved the second week of life, thus suggesting the lack of any functional synapses.

The synaptic markers associated with photoreceptors and processes of bipolar and horizontal cells show that significant abnormalities occur in the inner retina prior to photoreceptor damage, where bassoon staining can be seen in the absence of contact with MgluR6 stained ON-bipolar cells or calbindin stained horizontal cells, for example (Figures 6 and 7). Furthermore, recoverin staining of the ONL and cone bipolar cells reveals that thinning of the ONL (in LE rats) along with loss of normal ONL alignment (LE and SD rats) also occurs only once damage to the bipolar cells are evident, seemingly in response to a lack of cells to synapse with in the inner retina (Figure 1). Finally, while the number of TUNEL-positive cells is significantly increased in the INL of both LE and SD rats following exposure from P0-14, they only tend to increase in the ONL of LE rats, suggesting limited cell death in this layer. In contrast, fewer TUNEL positive cells were found in both LE and SD rat strains following exposure to hyperoxia from P0-6. Interestingly, apoptosis, or programmed cell death, has been suggested to play an important role in regulating the morphology and size of the developing retina. In fact, several apoptotic factors are expressed in high levels early in the retinal development of mice, for example (O'Driscoll et al. 2006, Wallace et al. 2006), and downregulated with time. Consequently, it is not surprising that staining in control animals at P6 revealed the presence of a significant number of TUNEL positive cells. While it is difficult to delineate the different retinal layers of groups exposed to hyperoxia early on (P0-6), one wonders whether at this at this young age the normal early elimination of retinal cells is halted following exposure to hyperoxia, resulting in a significantly lower level of apoptotic cell death as part of an endogenous mechanism of cell survival and rescue. Of interest, previous findings in our laboratory using another model of environmental stress, namely exposure to bright light with the light induced retinopathy model (LIR), revealed that while thinning of the ONL could be

evidenced in juvenile rats (similar to adults, albeit to a lesser extent), a concomitant thickening of the INL was also observed (Joly et al. 2006). It was suggested that this might occur as a compensatory mechanism to maintain normal retinal output in juvenile rats only, since adult rats did not reveal similar findings. Reflecting the above are our findings of only minimal retinal function impairment for the same regimen of hyperoxic exposure (Dorfman et al. 2009, Dembinska et al. 2002, 2001), whereas a window of increased susceptibility of retinal structure and function to postnatal hyperoxia exists for regimens that incorporate the second week of life (Dorfman et al. 2009, Dembinska et al. 2001). Consequently, it is not surprising that significantly higher levels of cell death following maximal exposure (P0-14) are observed. While our current findings suggest that apoptosis plays a role in the cell death in the LE and SD models of OIR, one cannot rule out the possible contribution of necrotic cell death. Consequently, future studies aimed at understanding the role of necrosis are warranted in order to fully elucidate the mechanisms of cell death in this model. Collectively, the above findings of altered cell morphology would suggest that the pathogenesis of OIR is initiated in the inner retina through bipolar cell dendrite retraction, and is followed by damage (albeit less severe) to the outer retina.

Inner retina remodeling has previously been documented in other models of retinal degeneration, where morphological changes can be observed in second-order neurons as a consequence of abnormal development or degeneration of the photoreceptors (Pignatelli et al. 2004). In contrast, our current findings suggest that the inner retina is the most important site of impact of postnatal hyperoxia. A further challenge in the photoreceptors of LE rats exposed to hyperoxia is the presence of melanin in the RPE, which although believed to have a protective antioxidant effect in the retina (Monos et al. 1996, Shohat et al. 1983), can also take on the role of a pro-oxidant under conditions of environmental stress, thereby leading to cytotoxic activity such as photoreceptor damage (Sarna et al. 2003, 1992).

Interestingly, our findings of altered retinal morphology correlate with our previous documentation of functional impairment as evidenced with the ERG.

The alterations in retinal cytoarchitecture that we are reporting are likely at the root of the functional anomalies that we had previously documented, such as the severe attenuation of the ERG b-wave, while the a-wave was always attenuated to a lesser extent. Furthermore, AII amacrine cells which normally receive excitatory inputs from ON-rod bipolar cells in S5 of the IPL and transfer the rod signal to the cone pathway by means of conventional, chemical synapses with OFF-cone bipolar cells and gap junction-mediated electrical synapses with ON-type cone bipolar cells (Kolb 2003), also reveal significant morphological changes and decreased contact with rod ON-bipolar cells, thus further contributing to the above.

Finally, the extreme susceptibility of LE retinal cytoarchitecture that we are currently reporting has also been reflected in our findings of greater functional impairment in this strain, as evidenced with the a- and b-wave parameters of the ERG. We have previously reported that the involvement of CNTF and FGF-2 may explain the preservation of photoreceptor function in SD rats. For example, FGF-2 levels were found to be approximately twice as high in SD compared to LE rats, and while CNTF levels were only significantly higher after exposure regimens that included the second week of life in LE rats, they were already significantly greater in SD rats after exposure within the first week (e.g., from P0–P6). The diffusion of various neurotrophic factors to the outer retina has been shown to occur following Muller cell gliosis in another model of environmental stress, namely exposure to a bright luminous environment (Valter et al. 2005, Wen et al. 1995, Faktorovitch et al. 1992). Given that the INL in SD rats remains relatively more intact following hyperoxia than the severely compromised LE rat, one wonders whether a similar protective mechanism of photoreceptors might also occur in SD rats. Collectively, while structural and functional findings reveal that the inner retina is the most important target site of postnatal hyperoxia; our results suggest that the SD rat is better able to cope when faced with a hyperoxic challenge.

While our current study was aimed at investigating the long-term consequences of OIR on retinal structure (at P60), previous findings of ours in the

SD rat suggested that the pathophysiological sequence of cytoarchitectural events was initiated while they were still in the hyperoxic regimen (Dorfman et al. 2008), given that OPL thinning as well as a reduced HC count could be evidenced at the time of eye-opening. Consequently, it is not surprising that there is no evidence of significant functional deficits, as determined with the ERG a-wave in the first 2 days or so after the opening of the eyes, while scotopic and photopic b-waves already tend to be attenuated in amplitude (Dorfman et al. 2008). Our findings obtained at P60 suggest that the functional and structural impairments that ensue are permanent features of OIR and that OIR is initiated at a postreceptoral level, where both outer retinal structure and function remain intact, whereas synaptic impairment at the level of the OPL prevents signal transmission to the inner retina. Future studies aimed at understanding the short-term cytoarchitectural manifestations in addition to the time course of the events in LE and SD rats with the use of techniques described herein will help to further elucidate the progression of the disease for each exposure regimen.

Given the important association that has been suggested to exist between post-receptoral neurons and retinal vasculature (Fulton et al. 2009), it is important to consider that impaired retinal vasculature might act as a potential contributing factor thus influencing the integrity of the inner retinal neurons. Normally, retinal vascularization begins in the superficial inner retina by vasculogenesis, starting at the optic nerve and radiating outward from the posterior to the anterior retina, following which the vessels then invade the retina, thus forming intermediate and the deep capillary beds (Gariano and Gardner 2005, Gogat et al. 2004). The inability of the retinal vasculature to adequately extend from the inner retina, place the OPL and HCs, which are two of the most compromised structures in OIR, in the most vulnerable location furthest away from the “source” of retinal vasculature, thus offering another plausible explanation for their sensitivity to hyperoxia. The even greater susceptibility of the retinal vasculature in LE rats to hyperoxia might therefore also explain part of the mechanism by which their INL is more severely compromised.

Interestingly, the extent of structural damage following hyperoxia was not found to be uniformly distributed in the LE rat retina. For example, while no discrepancies between the central and peripheral retina could be identified in SD rats following exposure to hyperoxia, the greatest consequences in LE rats were found in the central retina, whereas regions of normal synaptic contact and cell morphology could be identified in the peripheral retina. How do we reconcile these differences? Until recently, we have used the ERG, a pan-retinal response, to understand the functional manifestations of OIR. Preliminary studies of ours using the multifocal ERG (mfERG) which involves the simultaneous recording of electrical potentials from a number of central retinal regions, have enabled us to more easily find regional differences in retinal susceptibility to hyperoxia (Dorfman AL et al. 2005). Results obtained thus far have shown that while the flash ERG of LE and SD rats behave similarly (though LE rats are affected earlier on and more severely following exposure to hyperoxia), their mfERGs do not; recordings in SD rats are relatively unaffected compared to the attenuated responses obtained from LE rats. These findings would suggest that the central retina (particularly in LE rats) is more susceptible to hyperoxic insult compared to the periphery, and would further support our previous demonstration of impaired central retinal vasculature along with differences in the regional susceptibility of retinal structure following hyperoxia (where central retinal damage is more extensive than peripheral retinal damage in LE rats).

In conclusion, not only do present findings corroborate our previous demonstration of increased susceptibility of pigmented LE rats to postnatal hyperoxia in comparison with albino SD rats, but they reveal previously undescribed mechanisms of synaptic retraction and cell death that are at the root of plexiform layer thinning. The increased number of TUNEL positive cells in the nuclear layers following maximal exposure confirms that these cells die, at least in part, due to apoptosis and that LE rats are more susceptible than SD rats. Finally, our results point to the inner retina as the primary target of hyperoxic injury, following which damage to the outer retina occurs, albeit to a lesser extent. The mechanisms of cell death and synaptic retraction described herein are most

probably at the origin of the functional consequences of hyperoxia. Future studies aimed at defining the time course of these events in both LE and SD rats, both throughout hyperoxic exposure and thereafter are warranted. Finally, careful investigation of other factors, namely genetics as well as the role of melanin, might help to further elucidate the discrepancy between albino and pigmented strains, thereby furthering our understanding of the pathogenesis of OIR and ROP and the derivation of therapeutic modalities.

7 Acknowledgements

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8 References

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9 Captions to table and figures

Table 1: Antibodies used for immunostaining of retinal cells.

Figure 1: Anti-recoverin immunostaining of photoreceptors and type 2 and 8 cone bipolar cells in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. Scale bars, 100 μ m.

Figure 2: Two types of cone bipolar cells with their somata located in the proximal row of the inner nuclear layer (INL) are labeled with antibodies against recoverin in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. Type 2 cone bipolar cells have a large somata (A, *arrows*) and their axons end in sublamina a of the IPL (Euler & Wässle 1995). Type 8 cone bipolar cells have small somata (A, *arrowheads*) and their axons descend through the IPL and end in sublamina b of the IPL close to the GCL. *Arrowheads* (D) point to the few identifiable cone bipolar cell terminals remaining in the IPL of LE rats. Scale bars, 100 μ m.

Figure 3: Cones are specifically labeled by antibodies against γ -transducin in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. In both strains, prominent γ -transducin immunoreactivity is seen in the cone outer and inner segments located in the photoreceptor layer, cell bodies located in the outer third of the ONL, axons and pedicles. Scale bars, 100 μ m.

Figure 4: All rod bipolar cells are labeled with antibodies against Protein kinase C (PKC)- α which can be observed in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. In normoxic-cohorts of LE and SD rats, rod bipolar cell dendritic terminals establish connections with rod spherules in the OPL and their end-bulb axon terminals extend into the IPL (A, E). Sprouting of the remaining bipolar cell dendrites into the ONL in LE rats is observed following exposure from P0-14 (D, *arrows*). Scale bars, 100 μ m.

Figure 5: Anti-synaptophysin staining (presynaptic marker) located in the outer plexiform layer (OPL) and inner plexiform layer (IPL) of pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. Rats raised in normoxia (21% O₂) (5A, E) show intact synaptic terminals seen with continuous patterns of staining throughout both the OPL and IPL. Rats exposed to hyperoxia show progressively more of a punctate staining in the OPL of LE and SD rats, while decreased synaptophysin staining in the IPL with increased exposure to hyperoxia can also be evidenced in LE rats. Scale bars, 100 μ m.

Figure 6: In the rat, dendrites of B-type horizontal cells contact with cone terminals, while their axons synapse with rod terminals. Antibodies against calbindin are used for horizontal cell labeling, and double immuno-labelling with calbindin (green) and bassoon (red) label B-type horizontal cells and photoreceptor synaptic ribbon terminals in rod spherules and cone pedicles in the OPL, respectively, in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. Paired structures between the synaptic ribbons of LE photoreceptor axon terminals

labeled with antibodies against bassoon and the terminal tips of horizontal cells labeled with calbindin can be observed (A, *inset*). Scale bars, 100 μ m.

Figure 7: Double immuno-labelling with mGluR6 (green) and bassoon (red) antibodies reveals pairing between ON-bipolar cells and the photoreceptor synaptic ribbon terminals in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. Two types of pairing patterns between presynaptic and postsynaptic elements could be observed, namely pairing between mGluR6 stained rod ON-bipolar cell dendrites and either bassoon labeled presynaptic rod spherules that present with a horseshoe-like appearance (A, *arrows*), or bassoon labeled presynaptic cone pedicles that take on more of a disc-like appearance (A, *arrowheads*). Scale bars, 100 μ m.

Figure 8: Double immuno-labeling with parvalbumin (red) and PKC- α (green), reveals AII amacrine cells and rod bipolar cells, respectively, in pigmented LE rats raised in normoxia (A), or hyperoxia from P0-6 (B), P6-14 (C) and P0-14 (D) and albino SD rats raised in normoxia (E), or hyperoxia from P0-6 (F), P6-14 (G) and P0-14 (H) at P60. Characteristic lobular appendages of AII amacrine cells in sublamina a of the IPL (A, *arrowheads*), and dendrites of AII amacrine cells that extend into sublamina b of the IPL to contact the axons of rod ON-bipolar cells (A, B, E-H *arrows*) are observed. Scale bars, 100 μ m.

Figure 9: Comparison between central and peripheral retina in LE rats exposed to hyperoxia from P6-14. Double immuno-labeling with Transducin (green) and Recoverin (red) (A, B), PKC- α (green) and Parvalbumin (red) (C, D), Bassoon (red) and Calbindin (green) (E, F) and Basson (red) and MgluR6 (green) (G), was used, respectively, in order to appreciate the differences in cell morphology in the central and peripheral retina. *Arrows* (G) identify areas of normal contact

between Bassoon labeled photoreceptor synaptic ribbon terminals and MgluR6 labeled ON-bipolar cells near the ora serrata. Scale bars, 100 μ m.

Figure 10: TUNEL staining was performed in LE and SD rats exposed to hyperoxia for 6 days (P0-6) (C and G, respectively) and 14 days (P0-14) (D and H, respectively). Data was compared with control LE and SD retinas obtained at P6 (and B, respectively) and P14 (E and F, respectively). Scale bars, 100 μ m.

Figure 11: Graphic representation of the mean number of TUNEL-positive retinal cells in LE and SD rats exposed to hyperoxia for 6 days (P0-6) and 14 days (P0-14) compared with control LE and SD retinas obtained at P6 and P14. Statistically significant differences between age-matched normoxic and hyperoxic cohorts were found with Student's *t*-test ($P < 0.05$); *asterisks* (*) indicate values that are significantly different from control ($P < 0.05$). Results are given as mean number of nuclei $\pm$ 1 S.D.

10 Table

Molecular marker	Antibody (reference)	Source	Working dilution
Metabotropic glutamate receptor 6 (rod and cone ON-bipolar cells)	Rabbit anti-mGluR6 (Nakajima et al, 1993; Cagiano et al, 2007)	Neuromics	1:3000
Calbindin D-28K (horizontal cells)	Rabbit polyclonal (Oh et al, 2007)	Swant	1:500
Protein kinase C, α isoform (rod ON-bipolar cells)	Mouse, clone MC5 (Johnson et al, 2003; Brand et al, 2005)	Santa Cruz Biotechnology	1:100
Protein kinase C, α isoform (rod ON-bipolar cells)	Rabbit polyclonal (Gong et al, 2007)	Santa Cruz Biotechnology	1:100
Parvalbumin (AII amacrine cells)	Rabbit polyclonal (Cuenca et al 2004)	Swant	1:300
Recoverin (photoreceptor nuclei and cone bipolar cells)	Mouse monoclonal (McGinnis et al, 1999; Chen et al, 1999)	J.F. McGinnis, University of Oklahoma (Oklahoma City, OK,USA)	1:2000
Synaptophysin (presynaptic marker of rod spherules and cone pedicles)	Mouse, clone SY38 (Zhang et al, 2006; Martínez-Navarrete et al, 2007)	Chemicon	1:500
Transducin, Gαc subunit (cones)	Rabbit polyclonal (Kraft et al, 2005; Zhang et al, 2007)	Cytosignal	1:200
Bassoon (ribbon synapses)	Mouse monoclonal (Pinilla et al 2007)	Stressgen	1:1000

Table 1

11 Figures

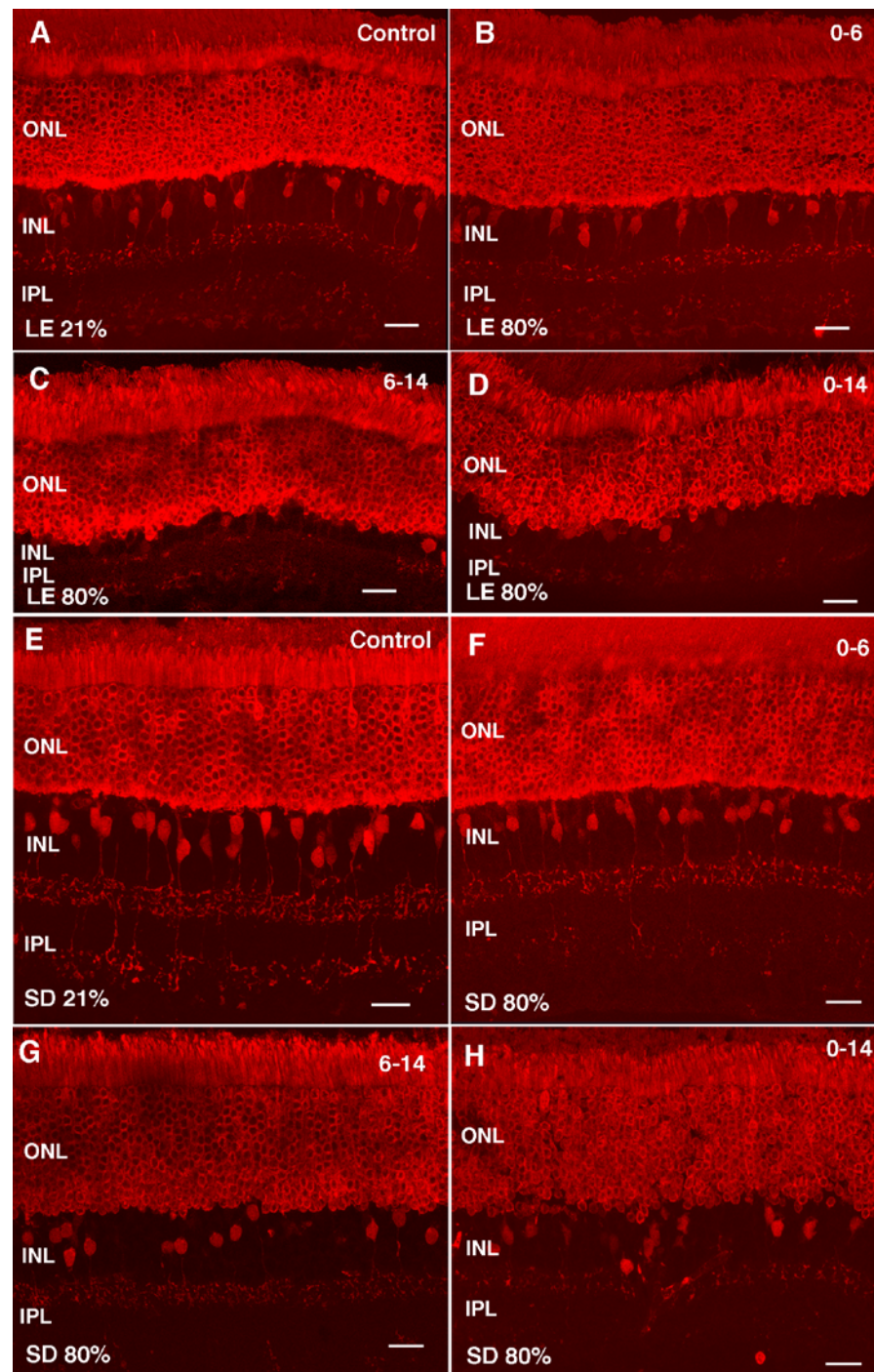


Figure 1

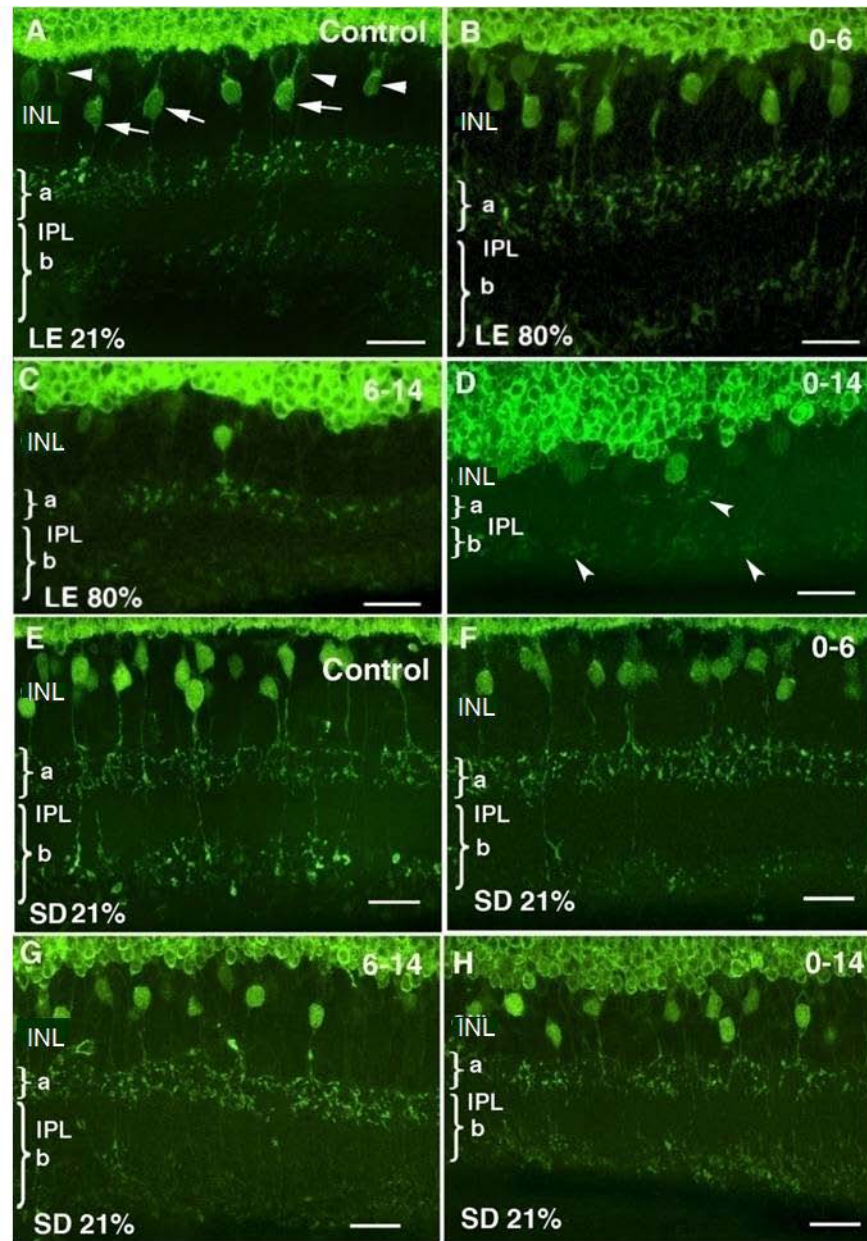


Figure 2

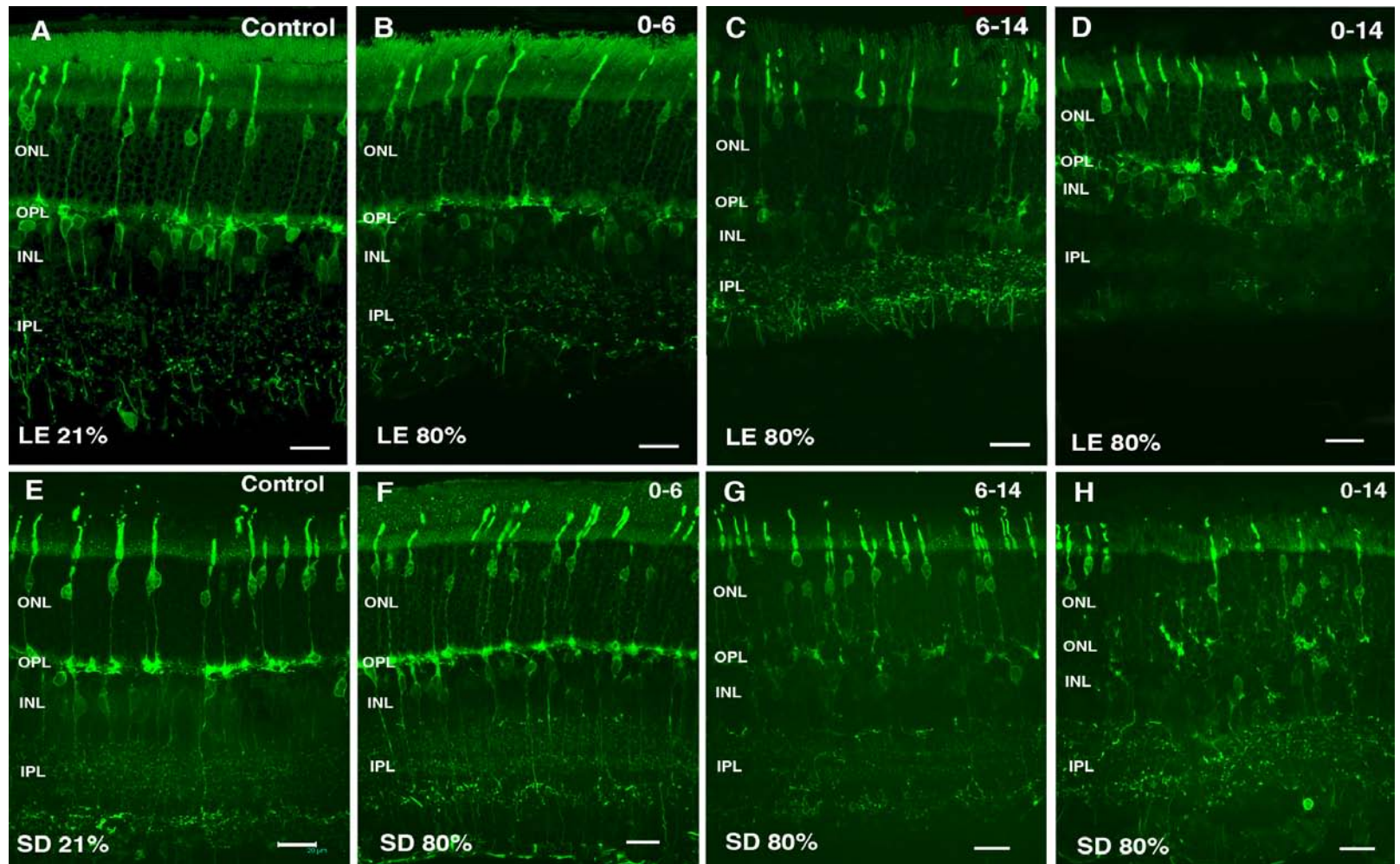
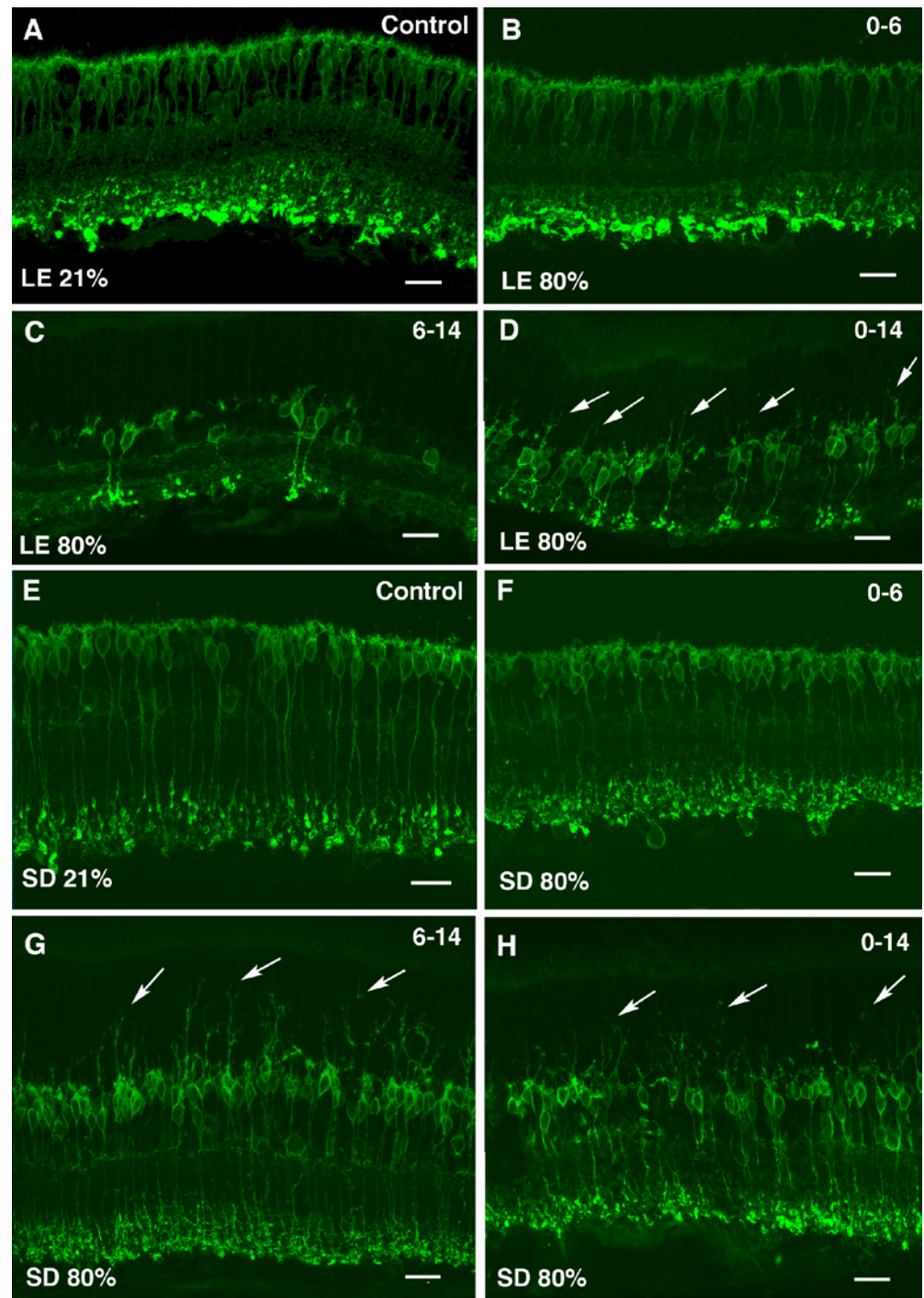


Figure 3

**Figure 4**

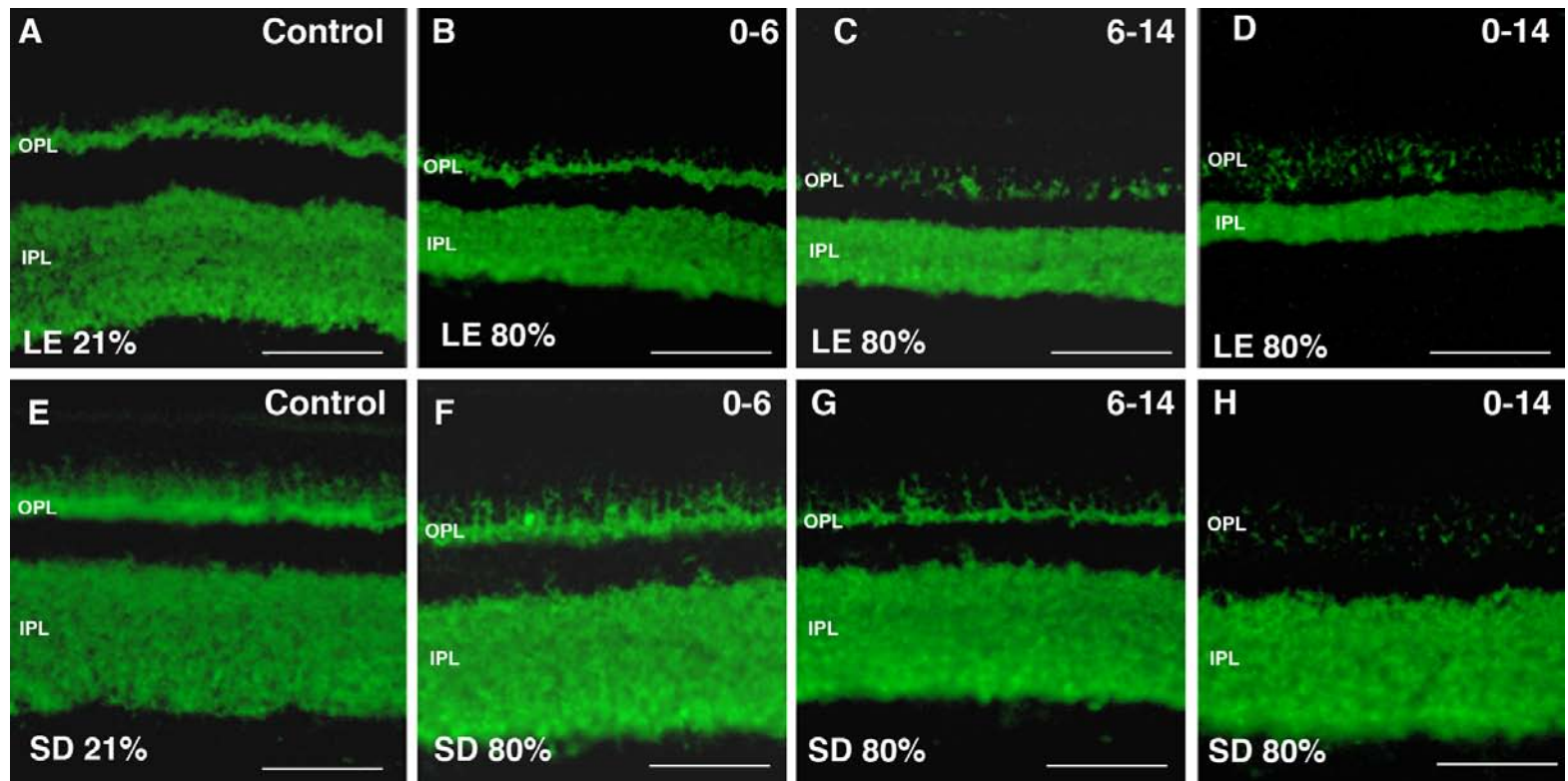


Figure 5

Figure 6

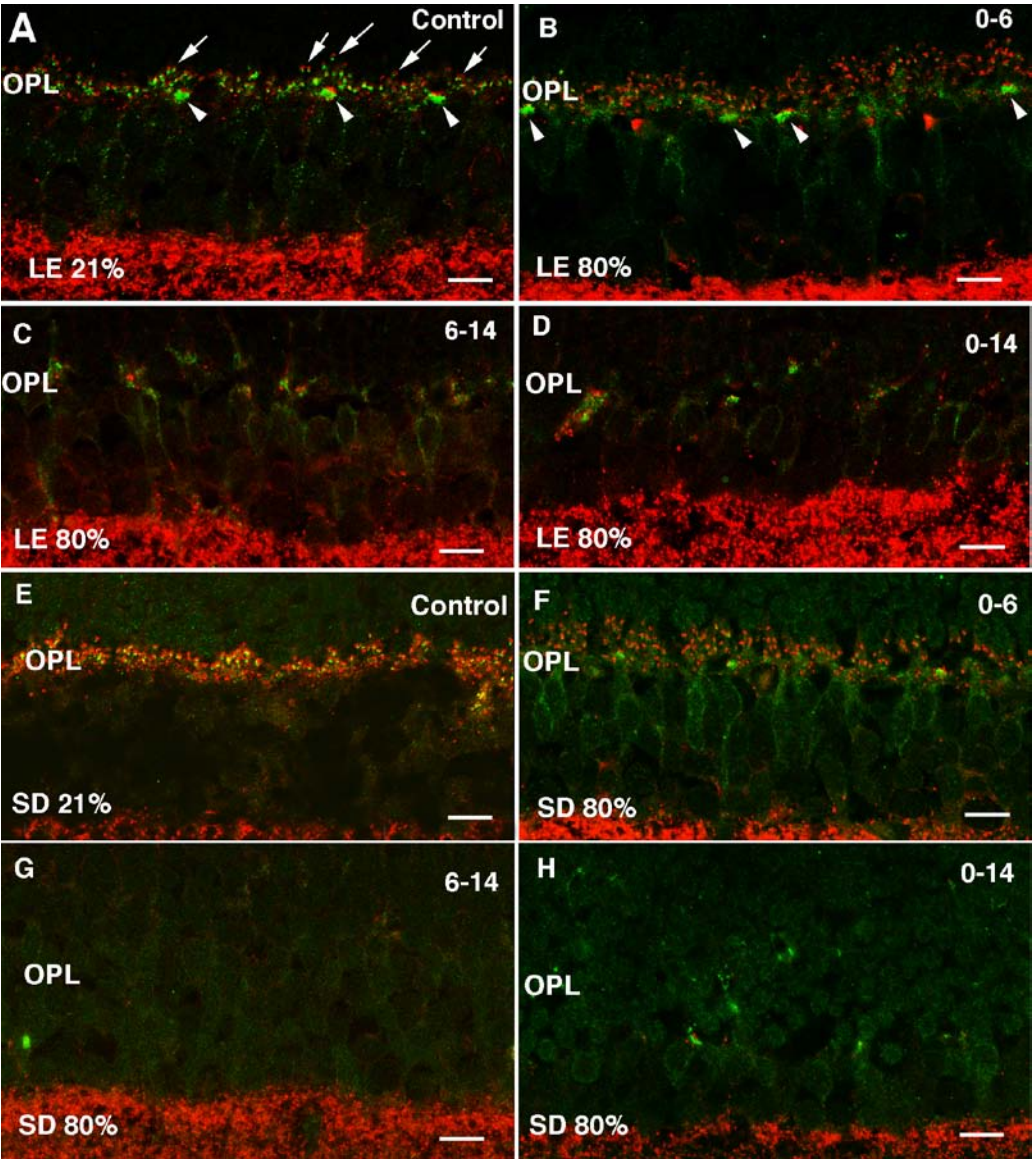


Figure 7

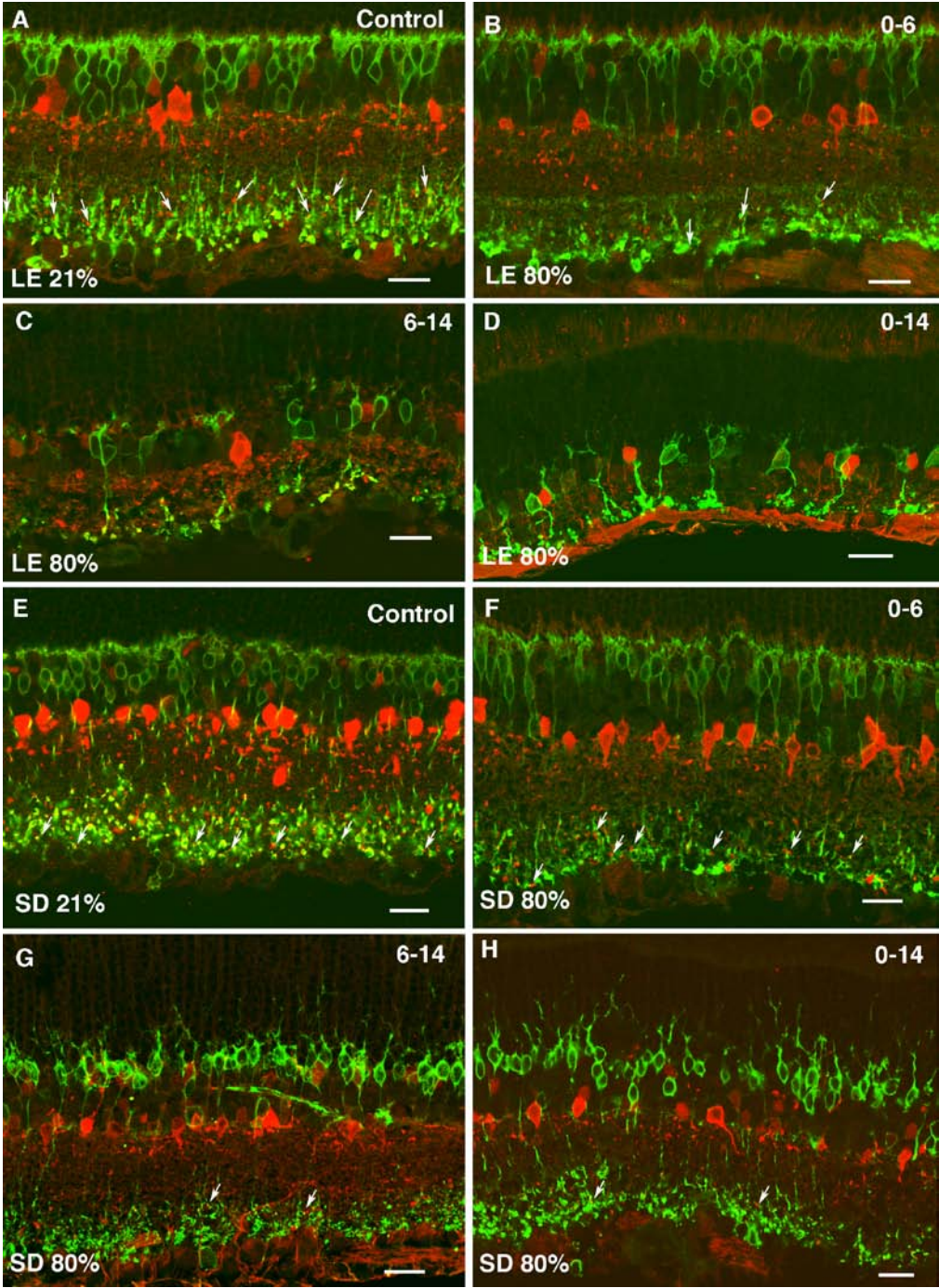


Figure 8

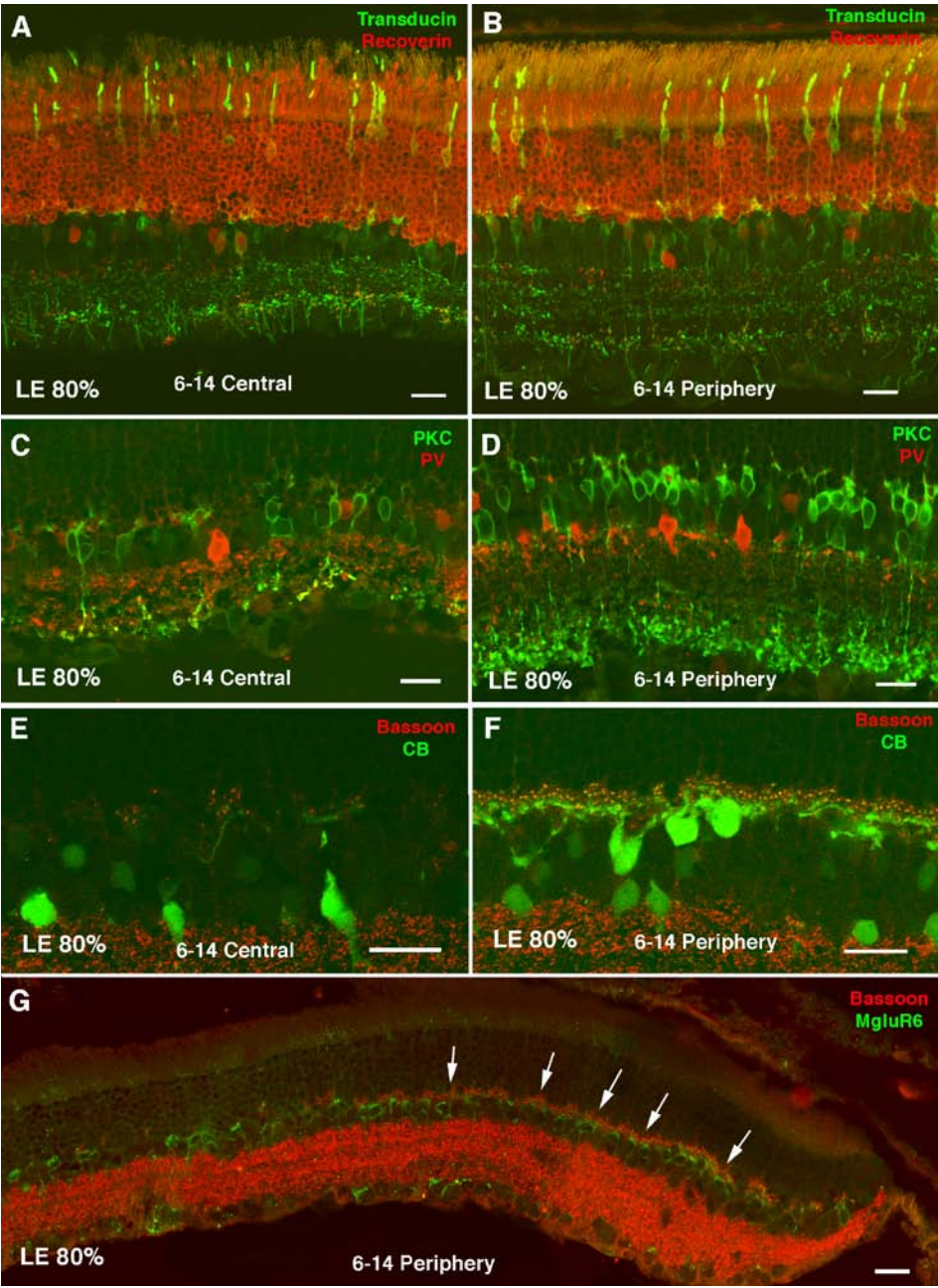


Figure 9

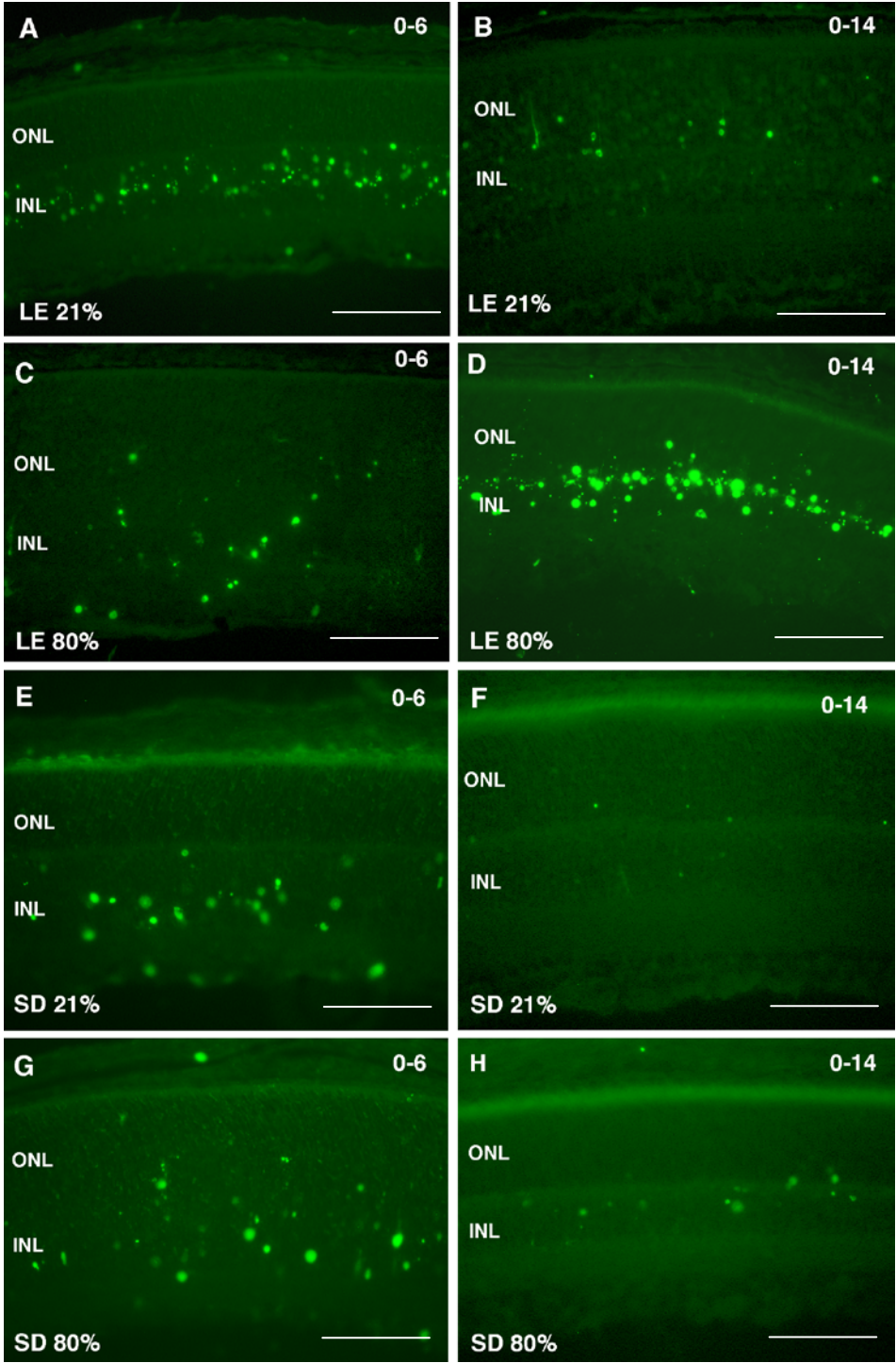
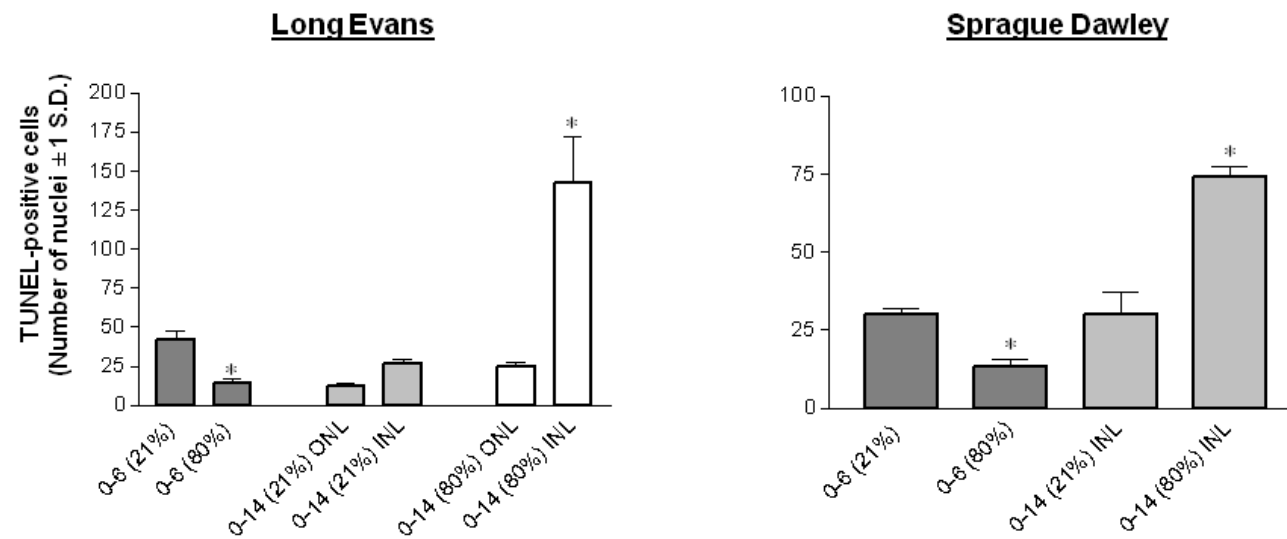


Figure 10

**Figure 11**

CHAPTER VI: MANUSCRIPT V

With kind permission from Springer Science+Business Media:

Dorfman AL, Joly S, Hardy P, Chemtob S, Lachapelle P. The effect of oxygen and light on the structure and function of the neonatal rat retina. Doc Ophthalmol. 2009; 118:37-54.

1 Preface to chapter VI

Given the immaturity of its retina at birth, the neonatal rat model represents a unique model in which to study the normal and abnormal maturation of the primary visual pathways that normally occurs in utero in human subjects. In addition to the rat model of OIR which has been discussed in the preceeding chapters, our laboratory has also developed a rat model of Light-Induced Retinopathy (LIR) which is used to better understand some forms of Retinitis Pigmentosa (RP) and even age related macular degeneration (AMD). This review highlights various studies that have explored the pathophysiological processes taking place in these models as well as various therapeutic avenues that could eventually prevent the degenerative processes.

2 Abstract

The neonatal rat is born with its eyes closed and an immature visual system, that some say is equivalent to that of a human fetus at 26 weeks of gestation. From birth, the visual system of the newborn rat will gradually mature, the first manifestation of that being the opening of the eye which usually take place at postnatal day 14. Complete maturation of the retina and visual pathways is normally reached at the end of the first month of life. The neonatal rat model thus represents a unique paradigm to study the normal and abnormal maturation of the primary visual pathways that normally occurs in utero in human subjects. Our laboratory has, over the past decade, developed two animal models of postnatally induced retinopathy, namely the Oxygen-Induced Retinopathy (OIR) that share several common features with the human Retinopathy of Prematurity (ROP) and the Light-Induced Retinopathy that is viewed by some as a valid model of some forms of Retinitis Pigmentosa (RP). The following pages review what is known of the pathophysiological processes taking place and suggest possible therapeutic avenues that could be explored in order to halt the degenerative process.

3 Introduction

The neonatal rat is born with its eyes closed and an immature visual system, that some say is equivalent to that of a human fetus at 26 weeks of gestation. From birth, the visual system of the newborn rat will gradually mature, the first manifestation of that being the opening of the eyes that usually take place at postnatal day 14 and we have recently shown that a near complete maturation of the rat's retinal structure and function is reached by the end of the first month of life [1]. The neonatal rat model thus represents a unique paradigm to study the normal and abnormal maturation of the primary visual pathways that normally

occurs in utero in human subjects. Our laboratory has, over the past decade, developed two animal models of postnatally induced retinopathy, namely the Oxygen-Induced Retinopathy (OIR) that share several common features with the human Retinopathy of Prematurity (ROP) and the Light-Induced Retinopathy that is viewed by some as a valid model of some forms of Retinitis Pigmentosa (RP). The following pages review what is known of the pathophysiological processes taking place and suggest possible therapeutic avenues that could be explored in order to halt the degenerative process.

4 The effect of postnatal hyperoxia on the developing retina

Retinopathy of Prematurity (ROP) is a condition observed in human infants that are born prematurely and receive high levels of oxygen postnatally to compensate for their unstable pulmonary status. ROP is a vasoproliferative disorder that, in its most severe form, can result in retinal detachment and possibly blindness. It is characterized by two phases, first initial vasoconstriction following which vessels are obliterated in response to higher than normal levels of oxygen which prevents normal vascular growth towards the retinal periphery, and secondary neovascularization upon the return to a normoxic environment which is the result of an inadequate blood supply [2, 3].

The rat is one of the most studied of all animal models of oxygen-induced retinopathy (OIR). Contrasting with other animal models that have been used to study the pathophysiology of this disease [4–7], the neonatal rat offers the advantage of a highly immature retina at birth that is comparable to that of a 24–26 week old human fetus [8]. As in humans, exposure of the rat pup to postnatal hyperoxia will cause severe vasoconstriction and vaso-obliteration that are followed by an abnormal proliferation of retinal vessels upon the return to room air [9–13]. Other features that are reminiscent of the human form of ROP have also been observed in that rat model of OIR, including retinal hemorrhages, retinal

folds and rosettes, though retinal detachment has not been documented in this model [14]. Some other advantages of the rat include the large litter sizes (approximately 15 pups per dam dependant upon the strain), low costs associated with developing the model, and the knowledge that has already been acquired from the rat with respect to various antioxidant, antiangiogenic, and anti-inflammatory therapeutics [11], which all make it a very attractive model to study the pathogenesis of OIR and consequently better understand the sequelae of ROP.

Given that vascular impairments are considered to be the clinical hallmark of ROP, it is not surprising that many previous efforts have been largely focused on the vasculopathy that is associated with OIR (vasoconstriction, vaso-obliteration, and neovascularisation) [5, 14–17]. Our goals, however, have not only included studying retinal vasculature throughout hyperoxic exposure (retinal flatmounts), but also to better understand the structural (retinal histology) and functional (rod mediated- and cone-mediated electroretinograms) features that characterize this model. Furthermore, given that free radicals have been thought to play a role in the pathogenesis of ROP [16–20] and that the retinal vascular anomalies found in OIR rats were shown to be partly prevented by free radical scavengers, we also examined whether they could also exert a protective effect on a structural and functional level.

Multiple attempts have been made by investigators to establish an animal model that closely mimics human-like vascular fluctuations given that the arterial oxygen level of preterm infants has been shown to fluctuate significantly. Studies have shown that severe ROP is associated with variability in transcutaneous oxygen within the first 2 weeks of life, suggesting that these variations in oxygen might interrupt the normal vascularisation process which responds to hypoxic and hyperoxic triggers [21]. Approximately, ten years after discovering the link between oxygen and human ROP, researchers began to focus on developing an experimental animal lesion that could be used to better understand the pathophysiology of the disease. Logically, the most appropriate model of ROP would be an animal model that has a similar pattern of vascular development as the human, and one that is also premature [11]. While the animals used as models

for ROP are born with an immature visual system, it is important to take into consideration that they do not experience any other consequences that are associated with prematurity, including respiratory distress syndrome, intracranial hemorrhage, necrotizing enterocolitis, and bronchopulmonary dysplasia, for example [22]. A number of different oxygen exposure regimens have been used based on variations of oxygen concentration and duration of exposure in order to more closely represent what is observed in human neonates [6, 10, 23, 24], though this is only one aspect of what these premature infants experience. Experiments in our laboratory were conducted in newborn litters of Sprague-Dawley rats that were exposed to 80% oxygen immediately after birth (mixture of medical grade 100% O₂ and room air measured with a MaxO₂ ceramtec oxygen meter, model OM25-ME, Medicana Inc., Montreal, Canada) as previously described [1, 25–28], interrupted by three intervals of 30 min' duration under normoxic conditions (21% O₂). A correlation between the duration of hypoxic episodes and neovascularization had previously been shown to occur following a similar regimen [10] as well as others including oxygen exposure of 50/10% and 40/10% O₂ [24].

The retina is extremely vulnerable to oxygen-mediated damage for a number of reasons. The retina has a very high oxygen consumption rate, and oxygen radicals are normally formed in the mitochondria as oxygen is reduced along the mitochondrial electron transport chain. Under hyperoxic conditions, the number of oxygen-derived radicals is amplified and can disturb various cellular components such as lipids, nucleic acids, and proteins, causing cytotoxicity [12]. The retina is also composed of lipids that contain high levels of polyunsaturated fatty acids such as docosahexaenoic acid (DHA; 22:6 ω -3), arachidonic acid (AA; 20:4 ω -6), and choline phosphoglyceride which are all targets for peroxidation. Finally, the retina is also the site of phototransduction and photic excitation is known to initiate oxygen radical formation [29]. Of interest, retinal endothelial cells seem particularly susceptible to peroxidation-induced injury, whereas perivascular astrocytes, pericytes, and smooth muscle cells appear to be relatively more resistant [30–32]. While previous studies have evidenced significant

reductions in vascular coverage following postnatal hyperoxia in rats [9, 10, 14–17], few have examined the susceptibility of retinal vasculature to postnatal hyperoxia as a function of the degree of vascular maturity at the time of exposure. For example, despite the attenuation in vascular growth that is apparent within the first week of life, the vascular growth process, is nonetheless, able to overcome this oxidative stress thereby achieving full coverage while under hyperoxic exposure throughout the first 2 weeks of life (Fig 1a and b). This is reminiscent of the reversible vasculopathy that is observed in the human form of ROP. The phenomenon of early vascular susceptibility is further supported by the window of blood vessel plasticity that has been described [33] where early postnatal exposure to hyperoxia (P4–P6) similarly resulted in vaso-obliteration. This was explained by the differential susceptibility of immature vessels that have not yet acquired pericytes versus more mature vessels that appear to be more capable of withstanding a similar stress. VEGF has also been implicated in the development of retinal vasculature and retinal coverage, and hyperoxia is thought to counteract its expression, which eventually leads to an underdeveloped retinal vasculature [34]. Paradoxically, however, while exogenous VEGF administration has been shown to act as a vascular survival factor, thus rescuing vasculature from hyperoxia-induced destruction [35], the revascularization process has also been shown to be accelerated throughout prolonged hyperoxia (when VEGF levels are suppressed), thereby placing less emphasis on a role for VEGF involvement [17, 36].

5 Free radical mediators

As pointed out above, isolated free radical scavengers may not be sufficient in preventing the genesis or progression of ROP. Moreover, potent antioxidants may exert adverse effects since normal cell physiology requires a redox potential. Moreover, it is often very difficult to intervene using free radical scavengers other than in a preventive manner. Accordingly, inhibiting the action of mediators of peroxidation may be more suitable for therapeutic purposes. Over

the years, we embarked in identifying major bioactive products of peroxidation and nitrooxidation. Notably, we will discuss the function of isoprostanes, thromboxane (TXA₂), platelet-activating factor (PAF), and trans-arachidonic acids (TAA).

5.1 Isoprostanes

Isoprostanes are a family of compounds produced from arachidonic acid (AA) via a free-radical-catalyzed mechanism [69]. At present, 4 series of AA-derived isoprostanes have been discovered: F₂-, E₂-, D₂-, and thromboxane-isoprostanes. An abundantly produced isoprostane in vivo is 8-iso-prostaglandin F₂ (8-iso-PGF_{2α}) (or 15-F_{2t}-isoprostane) and in view of its commercial availability the one most studied has been 8-iso-PGF_{2α} [70]. In contrast to prostaglandins synthesized by cyclooxygenase (COX), the isoprostanes are formed in situ on esterified phospholipids and are released in free form, presumably by phospholipases. In oxidant stresses, formation of isoprostanes exceeds that of COX-derived prostaglandins. Numerous forms of isoprostanes are concomitantly generated during oxidative stress. The preferential increase of one form over another has been reported for isoprostanes containing different prostane rings. For instance, D₂/E₂ isoprostanes levels have been found to augment to a greater extent during hyperoxic stress than F₂- isoprostanes [71].

In the mammalian retina, 15-F_{2t}- isoprostanes and its metabolite (2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP) evoke vasoconstriction by stimulating TXA₂ formation from neurovascular cells [72]. In addition, isoprostanes not only compromise retinal circulation by diminishing blood flow, but also seem to contribute to the oxidant stress-induced vascular injury as seen in ischemic retinopathies. Indeed, a potential role for 8-iso-PGF_{2α} in retinal microvascular cell death has also been proposed. 8-Iso-PGF_{2α} was found to cause cell death of isolated retinal microvasculature (≤25 μm). A similar vascular degeneration was

also detected by injecting in vivo into the preretinal vitreous 8-iso-PGF_{2α} in young rat pups. The mechanism of this cell death seem largely mediated by TXA₂ [16]. Since isoprostanes reproduce many effects of oxidant stress, they may serve as mediators in peroxidation-induced actions.

5.2 Platelet activating factor (PAF) in retinovascular degeneration

PAF is abundantly generated after oxidant stress and contributes to retinal neurovascular injury [73–78]. PAF also has a potentiating effect on oxidant stress by increasing the generation of oxygen radicals [79, 80]. An important role for PAF in retinal vaso-obliteration in OIR was recently suggested by the relative preservation of microvasculature by molecularly distinct PAF receptor antagonists including BN52021, CV-3988, PCA-4248 and THG315 [20]. A major finding was the direct and relative selectivity of PAF-induced cytotoxicity to retinovascular endothelial cells; umbilical, dermal, and aortic endothelial cells, and retinal pericytes were hardly affected. PAF evoked-toxicity was not only observed in vitro on cultured endothelial cells but also ex vivo on freshly isolated retinal microvessels (<30 μm, mostly endothelial cells) of distinct species. The nature of cell death induced by PAF appears to be necrapoptotic [81, 82]. PAF elicits its vasomotor and endothelial cytotoxic effects by inducing formation of TXA₂; inhibition of TXA₂ synthase and/or receptor prevents PAF effects [20, 83]. Thus PAF, which is generated concomitantly with TXA₂ during an oxidant stress, and also amplifies formation of the latter, seems to directly contribute to the retinal vaso-obliteration in ischemic retinopathies.

5.3 Role of thromboxane (TXA₂) in retinal microvascular degeneration

COX catalyzes formation of prostaglandins, thromboxane, and prostacyclin. Peroxides favor TXA₂ production over that of other COX-generated prostanoids [84, 85] because of inherent characteristics of TXA₂ synthase and reductive cofactor requirements (glutathione and NADPH) for PGI₂, PGE₂, PGD₂, and PGF_{2α} synthases. TXA₂ exerts known functions that may play a role in vaso-obliteration, especially platelet aggregation and vasoconstriction [86, 87]. Platelet aggregation is involved in other forms of ischemic retinopathies [88]. Nevertheless, studies of oxidant stress on impaired ocular hemodynamics and on OIR reveal an early endothelial cytotoxicity that is independent of platelet aggregation, although this can be detected later [6, 85, 89–93]. Effects of TXA₂ on endothelial cytotoxicity independent of platelet aggregation and vasoconstriction likely contribute to the retinal vaso-obliteration associated with oxygen-induced retinopathy (OIR) model of ROP. Indeed, TXA₂ generation causes a time- and concentration-dependent cell death of retinovascular endothelial cells while other primary prostanoids did not cause cell death [16]; and inhibition of TXA₂ formation or action preserves retinal microvascular integrity in the OIR model [16]. This TXA₂-dependent retinovascular endothelial cell death was found to be mostly by necrosis and to a lesser extent by apoptosis.

The mechanisms for TXA₂-induced cytotoxicity of retinal vascular endothelial cells have recently been explored [94]. TXA₂ induces m-calpain (but not caspase) activation, which in turn causes translocation of Bax to the mitochondria, loss of polarization of the latter and in turn release of cytochrome c into the cytosol and depletion of cellular ATP; disrupting the mitochondrial permeability transition pores and arrest of ATP production can stimulate the generation of ROS and in turn sustain a self-destructive cycle [95–97]. Specific m-calpain inhibitors prevent neurovascular endothelial cell death *ex vivo*. Collectively, findings support a robust role for TXA₂ as a major down-stream

mediator of oxidant stress involved in microvascular endothelial degeneration associated with ROP and likely other ischemic retinopathies.

5.4 Trans-arachidonic acids (TAA)

Reactive oxygen species are intimately interrelated with reactive nitrogen species. Several studies have uncovered a crucial role for $\text{NO}^\bullet$ and ensuing nitrative stress in microvascular degeneration leading to ischemia in conditions such as ROP. In particular, it has been shown that retinal microvascular degeneration is associated with increased expression of endothelial nitric oxide synthase (eNOS) [17, 98], increased generation of nitrites, nitrates and peroxynitrite, and protein tyrosine nitration; inhibition of these processes prevents development of OIR [17, 99, 100]. A novel nitroxidation process mediated by $\text{NO}_2^\bullet$, which results in cis to trans isomerization of arachidonic acid, was recently described. This reaction produces four stable trans-arachidonic acid mono-isomers, 5E-AA, 8E-AA, 11E-AA and 14E-AA [101], two of them (5E and 8E-AA) being endogenous and not found in diet [102]. A significant increase in the concentration of free (unesterified to phospholipids) as well as total (free + phospholipid-bound) TAA was shown in an experimental model of O_2 -induced microvascular degeneration upon exposure of 7 day old (P7) rat and mouse pups to 75–80% O_2 or room-air for 24 h; estimated concentrations of free and total TAA reached 1.65 μM and 4.72 μM , respectively [103]. Intravitreal injection of the specific NOS inhibitor L-NAME prior to exposure to oxygen prevents the increase in TAA levels in rats, indicating that their formation depends on $\text{NO}^\bullet$ and ensuing nitrative stress in this model. The contribution of eNOS on TAA generation in this model of ROP is corroborated in C57/BL6 mice with disrupted eNOS gene, in which retinal TAA levels do not increase in contrast to their wild-type counterparts [103].

TAA concentrations similar to those found in OIR cause a significant decrease in retinal vascular density both in vivo in the developing rat retina and ex vivo on newborn pig retinal explants. TAAs cause a concentration- and time-dependent neuroretinal microvascular EC death (EC_{50} at 24 h: 3 to 8 μ M, consistent with in vivo concentrations); in contrast, smooth muscle cells, human umbilical vein endothelial cells (HUVEC) and astrocytes are unaffected by TAA exposure [103], suggesting that TAA specifically leads to neuroretinal microvascular EC death. The mechanism by which TAA function appears to be via the generation of the pro-apoptotic and anti-angiogenic protein thrombospondin-1 (TSP-1) in cultured ECs [103]. TSP-1 acts via the transmembrane receptor CD36 [104, 105]. TSP-1 and CD36 knockout animals are insensitive to TAA. These observations unravel a novel mechanism by which TAAs, generated endogenously specifically in response to eNOS activity, can reproduce and thus mediate effects of reactive nitrogen species by participating in the microvascular degeneration observed in ischemic retinopathies. Since nitritative stress is dependent upon the redox potential [17], formation of TAA would also be subject to the same conditional state. Accordingly, the immature subject would be more vulnerable as a result of its decreased antioxidant capacity [59, 106, 107]. This inference is supported by recent findings which show that nitration, which leads to TAA generation [101], occurs when the redox state of the developing tissue is shifted towards an oxidative environment [17]. However, to date the primary receptor site on which TAA act remains elusive.

In summary, a number of bioactive products of nitrooxidation have been identified in OIR/ROP, which exerts not only vasoactive properties but also cytotoxic ones specifically on neuroretinal endothelium. These findings provide more specific targets on which one can interfere with to prevent the critical vaso-obliterative phase of ROP.

6 The effect of a bright luminous environment on the developing retina

The retina is divided into distinct cellular layers that constitute the outer retina (photoreceptor layer) and the inner retina (inner nuclear and plexiform layers and the ganglion cell layer). In normal lightening conditions, photons cross the entire retina before being captured by the photoreceptors that will transform this chemical energy into an electrical signal. As photoreceptor membranes are naturally rich in polyunsaturated fatty acids, essentially in docosahexaenoic acid, the outer segments are highly sensitive to lipidic peroxidation [108–111]. Moreover, the outer retina, completely deprived of blood vessels, receives nutrients and oxygen via the choroidal vasculature. This strengthens again the retinal susceptibility to oxidative stress [111–114].

In a luminous environment where the intensity does not exceed 1,000 lux the outer segments of the photoreceptors will modulate their length and the concentration of rhodopsin in their disks that suggest a form of cellular adaptation triggered to avoid light damage [115]. This adaptative mechanism, called photostasis, allows for gradual changes in the retinal structure. Therefore, the sensitivity of the retina is slowly modified to lead to a constant number of adsorbed photons per day independent of the luminous intensity of the environment [116–119]. However, when the amount of photons captured exceeds this daily average, photoreceptors are no longer able to repair cellular damage and, consequently, they will degenerate and die by apoptosis [115, 120, 121]. Therefore, the retina can be easily damaged when the environmental light intensity exceeds 1,000 lux [115]. As originally shown by Noell, the extent of cellular damage corresponds to the absorption spectrum of the rhodopsin pigment [120]. Deleterious effects of light on the retina lead to electrophysiological alterations [122] and to ultrastructural modifications visible at electron microscopy [123].

These irreversible retinal damages have been very well documented in adult animals [109, 112, 120, 124–130]. We know that the severity of retinal damages depends on four factors: the duration of exposure, the intensity, and the wavelength of the light, and the mode of exposure (cyclic light or constant light). As early as one hour after light exposure, photoreceptors already display the first signs of cellular degeneration that are only visible at electron microscopy [123, 131, 132]. The most distal part of the photoreceptor outer segments appears disorganized; the regular lamellar structure of the saccules is disrupted leading to the fusion of the disks and the formation of vacuoles and tubules. Cellular changes also occur in the inner segments of the photoreceptors where the mitochondrias swell and exhibit autophagic vacuoles [133, 134]. In the outer nuclear layer, photoreceptors show pycnotic nuclei with condensation of the chromatin while an increase in the number of lysosomes can be observed in the retinal pigment epithelium. In the most severe cases, the photoreceptor layer degenerates completely, whereas the more internal retinal layers (INL, IPL and GCL) are not affected [123, 130, 132, 135, 136]. These structural alterations can be easily correlated with a loss of retinal function as evaluated with the electroretinogram [125–127, 137].

Although seldom investigated, the age of the animal was also pointed out as another factor that could contribute to the severity and the extent of retinal damage following bright light exposure [124]. Indeed, previous data suggested that the retina of an adult rat suffered significantly more of light exposure compared to that of a younger rat. However, the intensity of light used in some studies may not have been sufficient enough to pass through the closed eyelids of the neonatal pups and still retain sufficient energy to be retinotoxic [124, 138, 139]. In addition, rats may have been placed in darkness after light exposure allowing then a recovery of photoreceptors from light damage. More recently, our group published a series of studies specifically addressing this issue.

Neonatal albino Sprague Dawley rats were placed in a luminous environment (10,000 lux; 12 hours light: 12 hours light) from birth to postnatal day (P) 14. After light exposure, young rats were replaced in an 80-lux luminous

environment corresponding to the normal conditions in our animal center. Electroretinograms (ERGs) were recorded at P30 and at P60 (Fig.1A–G) and histology was performed at P60 (Fig.1H–M). Our work demonstrated for the first time that, neonatal pups exposed to bright light during the first two weeks of life, when eyelids are still closed, displayed delayed retinal damages [51]. Indeed, ERGs in 30-days old rats did not show any difference after exposure compared to non-exposed rats (Fig.1A, B). In contrast at P60, the amplitudes of the mixed rod-cone a- and b-waves were slightly decreased (Fig.2). Our work suggested that despite closed eyelids, exposure to a bright luminous environment might trigger a progressive retinal degeneration (Fig.3).

In order to better characterize the window of retinal susceptibility during youth, juvenile albino rats were exposed to 10,000 lux (12 hours light: 12 hours dark) for different intervals of time during the third and the fourth week of life [140]. Results were compared with adults exposed to the same conditions. Adult retinas already showed significant decreases in the ERG parameters as well as severe morphological defects in their outermost part after 1 day of light. After 6 days of light, ERG was entirely abolished (Fig.1F) and the outer retina was completely destroyed (Fig.1M). In contrast, young rats exposed to light, for the same duration of time, from P14 to P20 still presented a recordable ERG (Fig.1C) and the morphology of the photoreceptor layer was remarkably preserved compared to adults (Fig.1J). Light exposure performed at the end of the first month of life (P28–P34) resulted in severe structural and morphological defects similar to those observed in adults exposed for 6 days (Fig.1E, L), suggesting that the window of retinal resistance gradually weakens until 1 month of age. On note, in all regimens of exposure and in juvenile rats as well as in adults, the superior retina was always more affected than the inferior one, a finding previously reported by others [120, 123, 141–144]. Our study demonstrated that juvenile rat retinas were better preserved than adult retinas from the deleterious effects of bright light, suggesting that young retinas possess an endogenous resistance to light damage.

In adult rats, intravitreal injections of neurotrophic factors are known to protect photoreceptors from light-induced degeneration [145–150]. Moreover, it has been already demonstrated that endogenous FGF-2 (Fibroblast Growth Factor-2) or CNTF (Ciliary Neurotrophic Factor) levels are upregulated in adult rat retinas in response to a moderate light intensity exposure [151–153]. We speculated that enhanced production of FGF-2 and/or CNTF factor might contribute to the resistance of the juvenile animal to light-induced degeneration. Endogenous protein levels of FGF-2 and CNTF were analyzed by Western blot immediately after 6 and 14 days of exposure [154]. In our light model, none of these neurotrophic factors was upregulated in adult retinas. Interestingly, Müller cells of juvenile retinas overexpressed FGF-2 and CNTF protein levels after 14 days of light, suggesting that upregulation of survival factors like FGF-2 and CNTF may lead to the protection of the photoreceptors from the noxious effects of light. Further experiments should confirm whether FGF-2 and CNTF delivery could promote neuroprotection of photoreceptors in the adult retina as well.

7 Conclusion

Postnatal exposure to intense light or high levels of oxygen thus offers a unique opportunity to study ex-utero animal models abnormal retinal development that would normally take place in utero in human subjects. Use of the knowledge gathered will not only be instrumental in furthering our understanding of the pathophysiological processes thus triggered but also allow us to test new therapeutic strategies that could ultimately have an impact on the management of human infants born with equivalent retinal disorders. More research is however needed before we can identify the initial insult (and thus potential treatment site) that triggered the cascade of events that ultimately lead to the development of these complex retinal disorders.

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10 Captions to figures

Figure 1 (a, b): Representative retinal vascular flatmounts stained with ADPase from control animals euthanized at postnatal day 6 and animals exposed to hyperoxia from P0 to P6 (**a**) and control animals euthanized at postnatal day 14 and animals exposed to hyperoxia from P0 to P14 (**b**), respectively. Magnification, x40. (**c–h**) Representative scotopic (**c, d, e**) and photopic (**f, g, h**)

electroretinograms from albino rats collected at P15, P30 and P60, respectively, following exposure to postnatal hyperoxia from P0 to P14. Scotopic flash intensity: $0.6 \log \text{cd} \cdot \text{sec} \cdot \text{m}^{-2}$, average of 2–5 flashes, inter-stimulus interval: 9.60 s; Photopic flash intensity: $0.9 \log \text{cd} \cdot \text{sec} \cdot \text{m}^{-2}$ following more than 15 min of light adaptation to a background of $30 \text{ cd} \cdot \text{m}^{-2}$ (average: 20 flashes, inter-stimulus interval: 1 s). Horizontal and vertical calibrations-Scotopic recordings: 20 msec and $250 \mu\text{V}$; Photopic recordings: 20 msec and $250 \mu\text{V}$. A 20 msec prestimulus baseline is included in all tracings. **(i, j)** Representative retinal histologic sections (central retina) obtained at P60 from control animals **(i)** and oxygen-exposed animals from P0 to P14 **(j)**. *Oblique arrows*: HCs located within the INL. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, $50 \mu\text{m}$.

Figure 2 (A-G): Representative scotopic **(A–F)** and photopic **(g)** electroretinograms from 30–40 days-old albino rats **(A–B)** exposed to bright light (10,000 lux; 12D: 12L) at different periods during the first postnatal month of life (as indicated on the top of the tracings). Recordings were performed between P30 and P37 in all regimens of exposed juvenile rats. For adults **(F)**, albino rats were exposed to bright light for 6 days and then replaced in normal lightning conditions (80 lux) for 24 days. Flash intensity: $0.6 \log \text{cd s/m}^2$. Horizontal and vertical calibrations-Scotopic recordings: 80 msec and $500 \mu\text{V}$; Photopic recordings: 40 msec and $50 \mu\text{V}$. A 20-msec prestimulus baseline is included in all tracings. (*Vertical arrows*): Flash onset. **(H–M)** Representative retinal photomicrographs of control rat **(H)**, of rats exposed to bright light from P0 to P14 **(I)**, from P14 to P20 **(J)**, from P14 to P28 **(K)**, from P28 to P34 **(L)** and from adults exposed to light for 6 consecutive days **(M)**. Scale bar: $50 \mu\text{m}$. Abbreviations—OS: Outer segments; IS: Inner segments; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Inner plexiform layer; GCL: Ganglion cell layer.

Figure 3 (A-D): Histogram representing the amplitudes of the scotopic mixed rod-cone a- (**A**), and b-waves (**B**), the amplitude of the Rod Vmax (**C**) as well as those of the photopic b-wave (**D**) at P30 (black bars) and at P60 (white bars). Different regimens of light exposure are indicated on the X-axis of each graph. Values are reported as mean $\pm$ SEM.

10 Figures

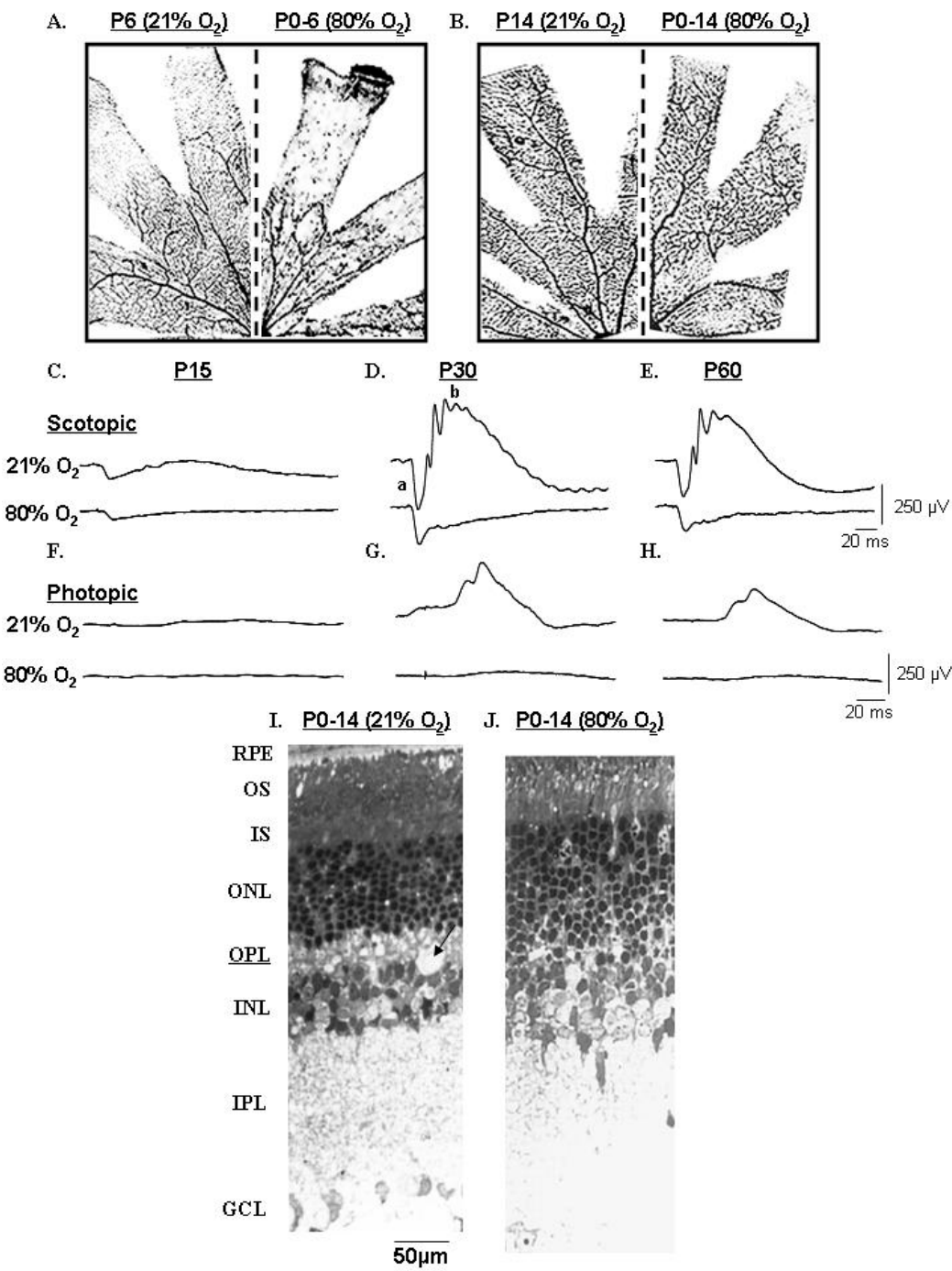


Figure 1

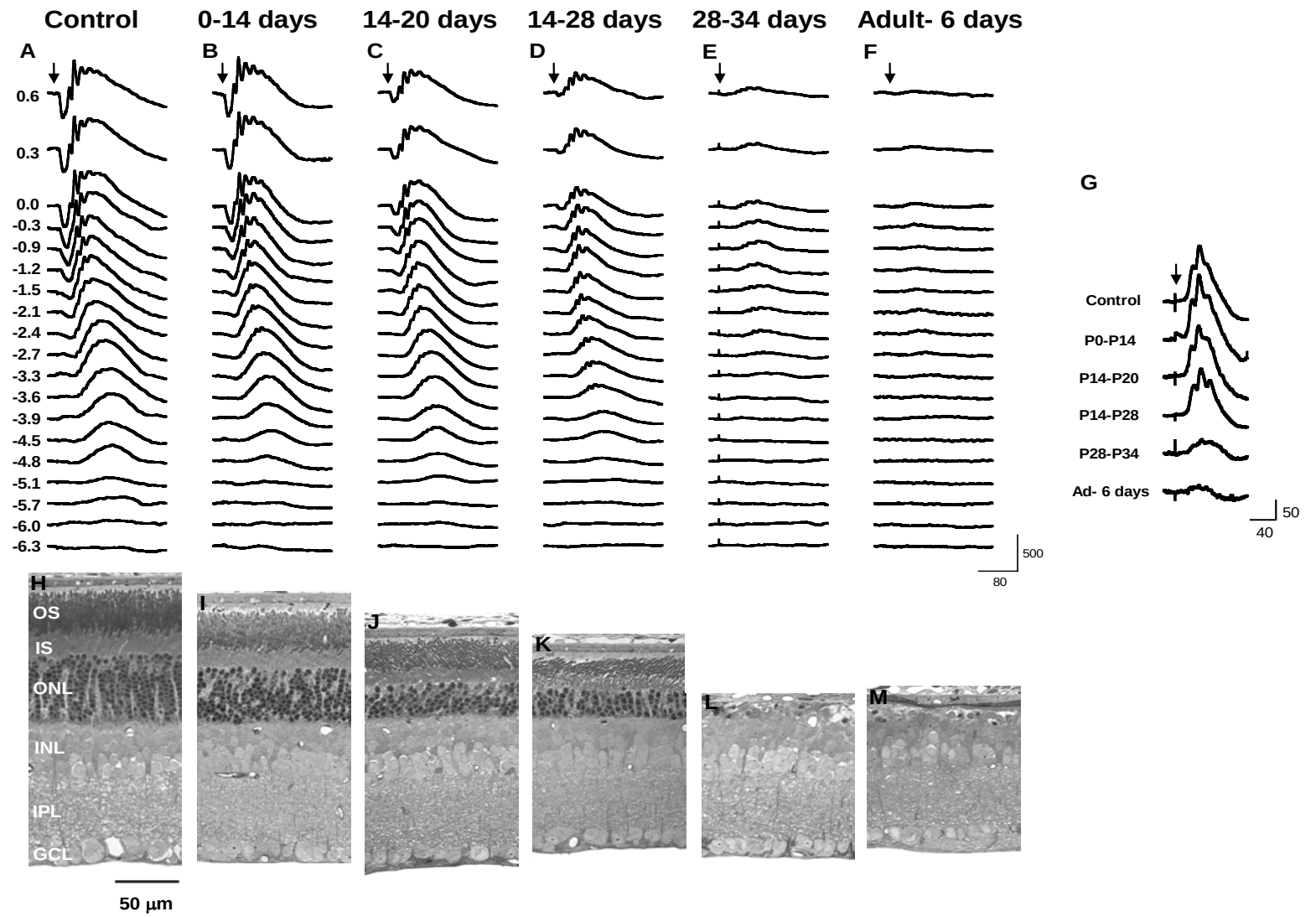


Figure 2

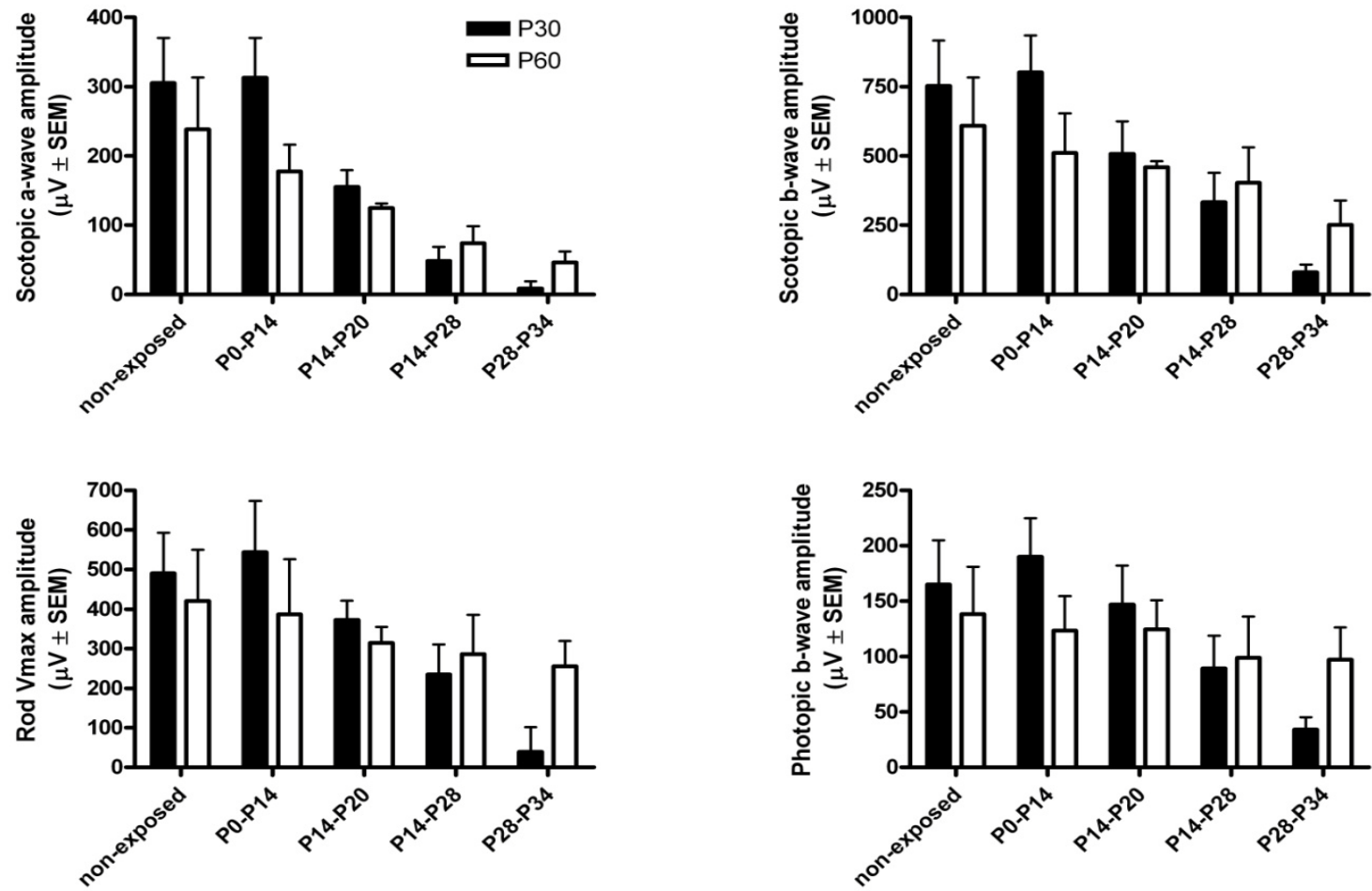


Figure 3

CHAPTER VII: MANUSCRIPT VI

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1 Preface to chapter VII

Although the albino rat model is traditionally used to better understand the pathogenesis of OIR, studies comparing the racial incidence of ROP have suggested that ocular pigmentation might offer protection against the development of severe ROP. While a great deal of emphasis has been placed on better understanding the vasculopathy that is associated with OIR (also considered to be the hallmark of ROP), our studies have also incorporated the characterization of the structural (retinal histology and immunohistochemistry) and functional (rod mediated- and cone-mediated electroretinograms) consequences of postnatal hyperoxic exposure.

This review describes what we have learned about some of the pathophysiological processes taking place in albino and pigmented models of OIR using identical exposure regimens to hyperoxia.

2 Abstract

Although a great deal of emphasis has been placed on the vasculopathy that is associated with oxygen-induced retinopathy (OIR), our studies also revealed significant and irreversible structural (retinal histology) and functional (scotopic and photopic electroretinograms) impairments that were significantly more severe in pigmented Long–Evans rats compared to the more commonly used albino Sprague Dawley rats. In the following pages, we will highlight what we have learned about the retinal pathophysiological processes of OIR taking place in strains of both rats with the hope that this will trigger investigations into new therapeutic strategies to complement those geared at preventing the vasculopathy.

3 Introduction

Previous studies of ours revealed that the structural (retinal histology) and functional (electroretinogram) deficits that resulted from the exposure of albino Sprague Dawley (SD) rats to a hyperoxic environment within the first postnatal days were permanent [1–5]. This contrasts with the damage to the retinal vasculature which can, as previously documented by others as well, overcome the hyperoxic challenge thereby achieving full coverage while still under oxygen throughout the first 2 weeks of life [1]. The latter is reminiscent of the reversible vasculopathy that is often reported in human ROP [6]. Recent studies of ours also showed that compared to the albino SD rats, pigmented Long-Evans (LE) rats developed a more severe oxygen-induced retinopathy (OIR) [7] as evidenced functionally and structurally. In the following pages, we will briefly review what we have learned from the two models of OIR with the hope that our findings will contribute to a better understanding of the retinal pathophysiological processes that are triggered following exposure of an immature retina to a high level of

oxygen. For the sake of clarity, the results reported were obtained following an exposure to 80% oxygen for 22.5 h per day from P0 to P14.

4 Functional consequences of OIR

Functional impairments resulting from postnatal exposure to high oxygen are best summarized in Fig. 1 with representative examples of the saturated rod b-wave (a–c), the mixed rod-cone b-wave (d–f) and the photopic b-wave (g–i) parameters of the ERG obtained from control (a, d, g) and oxygen-exposed SD (b, e, h) and LE (c, f, i) rats. While, compared to control rats, the a-wave of the mixed rod-cone b-wave in SD rats was minimally affected following hyperoxic exposure (Fig. 1d, e), significant amplitude reduction in the scotopic (Fig. 1a, b, d, e) and photopic (Fig. 1g, h) ERG b-waves was observed.

It is interesting to note that the functional consequences of OIR in the SD rat, which is still to date the most commonly used strain, were significantly less severe than those observed in pigmented LE rats [7]. Briefly, while the a-wave remained relatively intact in SD rats, in LE rats it was found to be significantly compromised early on in the pathophysiology of OIR (Fig. 1f). Interestingly, as with the a-wave, the attenuation in the saturated rod b-wave response was notably more pronounced in LE rats (Fig. 1c). A comparable finding was also observed for the rod-cone b-wave of LE rats, where a significant attenuation in amplitude was observed (Fig. 1f). Similarly, attenuation of the photopic b-wave was also found to be significantly more pronounced in LE rats (Fig. 1i), suggesting again that the retinal function of LE rats is more prone to hyperoxic insult compared to that of SD rats.

5 Cytoarchitectural consequences of OIR

In SD rats, the only cytoarchitectural consequence of early postnatal hyperoxia found by our group was a dose-dependant thinning of the outer plexiform layer (OPL) along with a significant reduction in the horizontal cell count (Fig. 1k) [1–3]. In comparison, analysis of LE rat retinas revealed significantly more extensive damage to retinal cytoarchitecture compared to SD rats (Fig. 1l). In addition to the thinning of the OPL, we also noted a marked thinning of the inner nuclear layer (INL), the inner plexiform layer (IPL) and the outer nuclear layer (ONL; Fig. 1l), features of OIR never observed in SD rats that again point to the significantly more severe presentation of the OIR manifestations in LE rats.

6 Managing ROP: lessons from OIR

Our previous studies have shown that in albino SD rats, the pathophysiological consequences of OIR combine retinal cytoarchitectural damage (e.g. OPL thinning, reduced horizontal cell count) with a permanent deficit of retinal function (significantly attenuated scotopic and photopic ERGs) [1–5]. In comparison, postnatal hyperoxia exerts an even greater loss of retinal structure and function in the pigmented LE rat (greater vascular dropout, generalized deficit to retinal cytoarchitecture, more severely attenuated retinal function) [7]. Moreover, the damage observed in LE rats occurred earlier compared to SD rats, following exposure to hyperoxia that was limited to the first week of life (P0–P6).

Given that ROP is considered a disease of the retinal vasculature, it is not surprising to find that most studies on OIR have focused on the effect of postnatal

hyperoxia on the development of the retinal vasculature. Although previous studies have also observed significant reductions in vascular coverage following postnatal hyperoxia in rats [8–14], few studies have examined the time course of this effect. We showed that despite the attenuation in vascular growth that is readily measurable after the first week of exposure (P0–P6) in SD rats, the vascular growth process is nonetheless able to overcome this hyperoxic insult thereby achieving full coverage while still under hyperoxic exposure (in P0–P14 exposures). This is reminiscent of the reversible vasculopathy that is observed in the human form of ROP [6]. In contrast, following oxygen exposure, the vascular supply is so severely compromised in pigmented LE rats that they never achieve a normal appearance, thus further suggesting unequal susceptibility among strains [7].

It is interesting to note that while the abnormal retinal vasculature has been documented to resolve itself, the functional sequelae that are associated with this disease have been shown to persist. Evidence of this has been published in several studies where, for example, significant alterations in the a- and b-waves of the ERG in patients with a history of ROP have been shown to continue into adulthood [15, 16]. Furthermore, scotopic visual threshold elevation has been documented in children with a history of resolved, mild ROP, thus suggesting the involvement of photoreceptors [17–19]. Finally, significant deficits in amplitude and implicit time of the multifocal ERG (mfERG) response, which stems from the central retina, have also been found in subjects who revealed complete resolution of clinical ROP more than 10–20 years earlier [20]. This would suggest that a history of ROP can alter the development of the central retina and, consequently, the mfERG response that it generates. Given that ON- and OFF-bipolar cells contribute to this central response, the attenuation in amplitude might therefore occur, should ROP specifically target these cells and thereby cause for an altered summation of depolarization and hyperpolarization in these subjects [20]. Therefore, despite the resolution of vascular abnormalities, which is a common occurrence in ROP patients, it is necessary to consider that neural dysfunction of the retina may still carry on throughout adulthood. Of course, this is not to say that

the vasculopathy that results from postnatal hyperoxia does not contribute to the structural and functional consequences that we have reported. In a previous study by Penn's et al. [12], it was shown that trolox-c (a water-soluble analogue of vitamin E) successfully limited the vascular consequences of postnatal hyperoxia, suggesting that hyperoxia was the main factor that triggered the vascular dropout observed in OIR. However, the same dose of trolox-c failed to offer an equivalent degree of protection to the retinal cytoarchitecture and function [5], suggesting again that some aspects of the vascular, structural and functional consequences of OIR may develop independently.

Our findings of impaired retinal function in our models of OIR support previous findings, where exposure of neonatal rats to 60% O₂ from birth through P14 caused inner retinal dysfunction as evidenced by b-wave attenuation [21]. Similar findings also reported that cyclical (50/10% O₂) exposure from P0 to P14 or constant exposure to 75% O₂ from P7 to P14 led to postreceptor functional deficits combined with retinal arteriole tortuosity [22]. Moreover, anomalies of the ERG oscillatory potentials (OPs) have also been shown to persist, despite the resolution of vascular abnormalities [3, 4, 23], which would further suggest the persistence of retinal functional deficits despite normalization of the retinal vasculature.

Evidence of a postreceptor effect following postnatal hyperoxia were also reported by Penn et al. [21] where the amplitude of the ERG a-wave obtained from hyperoxic- and normoxic-cohorts were identical as of P21 and beyond, compared to severely attenuated b-waves. These results are also in line with those obtained by Reynaud et al. [9] who demonstrated that following postnatal hyperoxia from P0–P11, the amplitude of the a-wave gradually improved between P13 and P18, compared to that of the b-wave which remained significantly depressed at P18. Clearly, the above would suggest that the photoreceptors are significantly less susceptible to postnatal hyperoxia. One wonders whether this results from the relative immaturity of their outer segments at the time of oxygen exposure [24], or from the relatively intact choroidal vasculature that supplies

them. The above would also further confirm that the more permanent sequelae of OIR occur in the inner retina. On the other hand, while the pathophysiological consequences of OIR are largely postreceptoral in the retina of albino rats, photoreceptor dysfunction may in fact also occur following exposure to hyperoxia, as suggested by different parameters of oxygen exposure including alternating between 50 and 10% oxygen from postnatal day P0–14, or exposure to 75% oxygen from P7 to P14 [22]. These findings would further support our observations of impaired photoreceptor function in the pigmented LE rat and would suggest that not all experimental models may react equally to an equivalent hyperoxic stress. Findings such as these warrant the evaluation of retinal structure, and studies of ours have also been geared towards better understanding of the impact of postnatal hyperoxia on retinal cytoarchitecture, which is often overlooked.

The cytoarchitectural manifestations of OIR occur, at least in the SD rat, during the period that the rats are still submitted to the hyperoxic regimen, as evidence of OPL thinning and HC count are already observed directly following exposure [1]. Furthermore, given that the most notable changes occur in the OPL and HCs, the relatively intact a-wave that can be recorded within the first few days following the cessation of hyperoxic exposure comes as little surprise, while as expected from histological findings, scotopic and photopic b-waves already tend to be attenuated in amplitude. Finally, whether the lower count of horizontal cells results from their susceptibility to oxygen (or to the relative hypoxia that follows hyperoxia) or from another degenerative process that might impact the establishment of proper synaptic connections remains to be fully understood.

Our recent findings have revealed that the pigmented Long–Evans rat is also a good working model of OIR, demonstrating a more exaggerated form of the disease with a more severe pathophysiological course, involving greater damage to retinal vasculature, structure and function. In both models, findings would suggest that OIR is initiated at a postreceptoral level, where synaptic impairment at the level of the OPL prevents signal transmission to the inner retina. The

particular susceptibility of the scotopic and photopic b-waves would further support this inference [1–5, 7]. Involvement of other inner retinal structures such as the INL, IPL and GCL as well as the impaired a-wave in LE rats suggests that the pathophysiological consequences in this model are indeed more severe. Interestingly, studies have revealed that not only can an environmental stress such as exposure to hyperoxia result in structural damage to the retina, but other mechanisms that may involve the generation of reactive oxygen species, such as the reaction of iron with oxygen, can also result in such an effect. For example, intravitreal injection with FeSO_4 has been shown to cause selective thinning of the ONL [25] as well as impaired retinal function. Similarly, intravitreal injection of paraquat (an herbicide known to generate reactive oxygen species) was shown to cause thinning of the ONL and INL along with vacuolization, folds in the ONL and functional impairment [26]. These findings in addition to those which we have previously reported [1–5, 7] would collectively suggest the importance not only of retinal vasculature and functional assessment in models of retinal diseases, namely OIR, but also of retinal cytoarchitecture which can give further insight and help to explain the pathophysiological processes that characterize OIR. Future investigations are certainly warranted in order to clarify the disparities between oxygen exposure regimens and the results obtained among studies in order to not only further our understanding of the disease processes but also to enable testing of new therapeutic modalities that could eventually help to manage human infants born with retinal disorders such as ROP as well as others. In that respect, if we interpret our results as evidence that not all premature infants will react similarly to the same hyperoxic shock, experimental approaches that would make use of the SD and LE rats would be instrumental in the design of new therapeutic strategies aimed at better protecting the developing retina from postnatal hyperoxia, irrespective of the degree of maturity reached at the time of oxygen exposure or the severity of the underlying retinopathy.

7 References

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8 Captions to figures

Figure 1: Representative scotopic [saturated rod b-wave, flash intensity: $2.4 \log \text{cd.s.m}^{-2}$ (a–c); and mixed rod-cone b-wave, flash intensity: $0.60 \log \text{cd.s.m}^{-2}$ (d–f)] and photopic [b-wave, flash intensity: $0.9 \log \text{cd.s.m}^{-2}$, background:

30 cd.m^{-2} (**g–i**) ERGs obtained at P60 from LE rats raised in normoxia (21% O_2) and LE and SD rats raised in hyperoxia (P0–P14, 80% O_2), respectively. Calibrations: *horizontal*, 20 ms; *vertical*, 450 μV for scotopic saturated rod b-wave and mixed rod-cone b-wave ERG responses, 150 μV for photopic ERG responses. All tracings include a 20-ms prestimulus baseline; *vertical arrows* indicate flash onset. Representative retinal histological sections obtained at P60 from SD and LE rats raised in normoxic (**j**) and maximal hyperoxic conditions (**k, l**; *SD* and *LE*, respectively). *RPE* retinal pigment epithelium, *OS* outer segment, *IS* inner segment, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer. Calibration *bar*: 50 μm

9 Figures

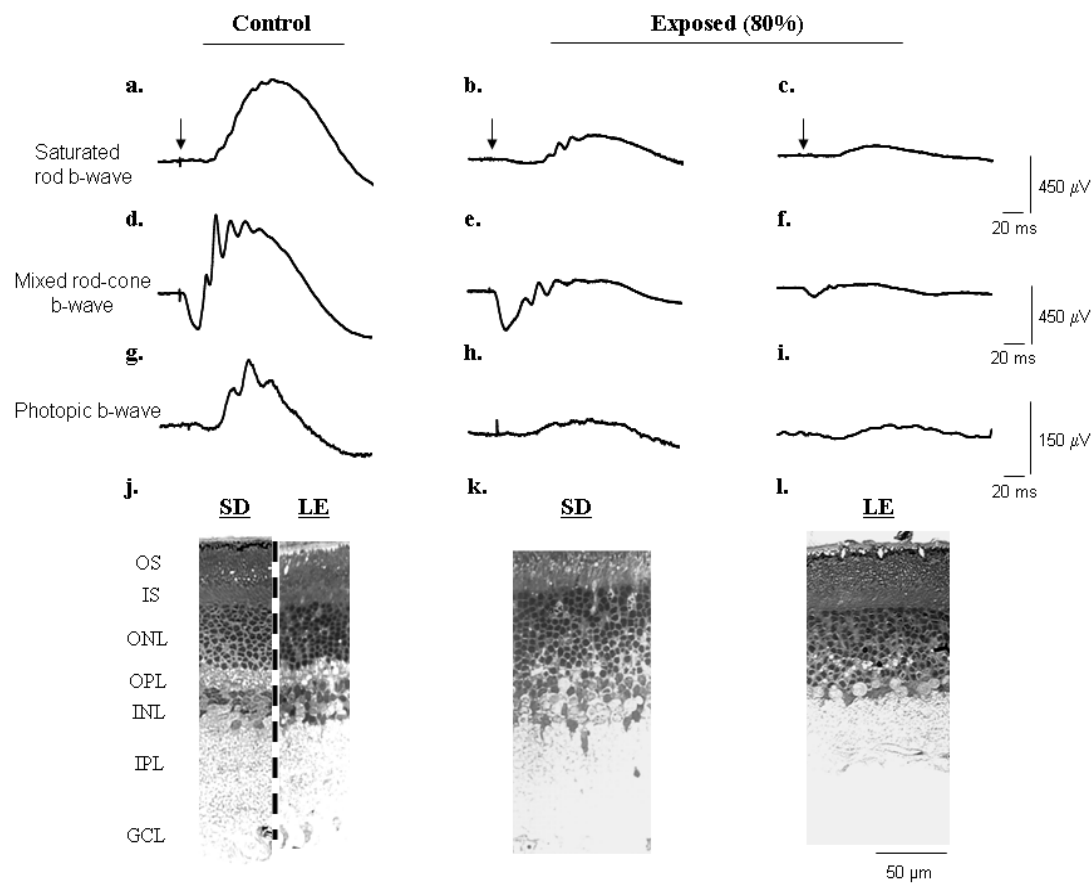


Figure 1

CHAPTER VIII: GENERAL DISCUSSION AND

CONCLUSION

1 General Discussion

Research performed throughout my doctoral studies revealed for the first time that:

- 1) The earliest manifestations of OIR, including damage to retinal cytoarchitecture and function, can be documented immediately following the cessation of hyperoxia at the time of eye-opening.
- 2) OIR is primarily an inner retinal disorder, characterized by cell death and synaptic retraction that result in the thinning of the OPL.
- 3) Free radicals cannot fully explain the pathogenesis of OIR, as evidenced with the limited role of free radical scavengers, whether endogenous (melanin) or exogenously administered (trolox C), in preventing the deleterious effects of hyperoxia.

The novel findings presented herein have enabled us to unveil important features of the rat model of OIR that could apply to human ROP. The following sections will address various aspects of these results which will undoubtedly be useful in the management of clinical ROP as well as other ischemic retinopathies, and in the derivation of new therapeutic avenues to protect the developing visual system during this critical period.

1.1 The earliest manifestations of OIR

Altricial animals such as the rat are born with their eyes closed and a relatively immature retina (Braekevelt and Hollenberg 1970, Weidman and Kuwabara 1969). Therefore, hyperoxic exposure occurs at a time when the retina is highly underdeveloped and undergoing vigorous maturation. Consequently, permanent functional and structural sequelae have been shown to persist following exposure to hyperoxia, despite the resolution of vascular abnormalities that occurs, at least in SD rats. Previous studies conducted in our laboratory have suggested that not only are these permanent features of the OIR model, but that they reveal a dose-effect relationship that is dependent on the degree of retinal immaturity (Dorfman et al. 2009a, Dorfman et al. 2008, Dembinska et al. 2001). The purpose of this thesis was not only to investigate the vasculopathy that is associated with OIR, but to study beyond it in order to further characterize this retinal disorder.

Findings reported herein reveal for the first time that despite the ability of the retinal vasculature to repair itself while maintained in a hyperoxic environment, irreversible cytoarchitectural damage in addition to attenuation in retinal function occurs, the consequences of which are documented immediately following the cessation of hyperoxia (Manuscript 1, Chapter 2). Previous studies that had been performed at intervals further removed from the oxidative insult and at a time when the retina was considered to be mature (e.g., at postnatal days 30 and/or later) (Dorfman et al. 2006, Dembinska et al. 2002, Dembinska et al. 2001, Lachapelle et al. 1999) had made it difficult to understand this progression. Given that these permanent cytoarchitectural anomalies are limited to postreceptoral regions, it is not surprising that there is no evidence of significant functional deficits as determined with the ERG a-wave in the first 2 days or so after the opening of the eyes, while scotopic and photopic b-waves already tend to be attenuated in amplitude. One wonders whether the susceptibility of the different retinal elements to excess oxygen might be a function of the level of maturity reached at the time of exposure and/or the duration of exposure (dependent on

their birth date), given that altricial animals such as the rat are born with their eyes closed and a relatively immature retina (Braekevelt and Hollenberg 1970, Weidman and Kuwabara 1969). Previous studies in mice have shown that cones, GCs, ACs, and HCs are the first identifiable retinal cells at approximately E10, whereas rods and Müller cells appear at birth (Cepko et al. 1996). Normally, this results in the well-organized maturation of retinal function along with the development of saturated rod a- and b-wave amplitudes (related to rod outer segment length and rhodopsin content levels) that occurs between P12 and P30 (Fulton et al. 1995b). Photopic OPs have also been shown to reach maturity later on at P30 compared with the earlier maturation of scotopic OPs at P17 (el-Azazi and Wachtmeister 1990). Our results are in accordance with these findings, given that we observed a peak in the normal rod $V_{\max}$ amplitude at P24, whereas that of the photopic b-wave occurs at P30. Surprisingly, however, the peak for both parameters after hyperoxic exposure is found at P24, suggesting that despite the attenuation in both rod and cone responses, the rod $V_{\max}$ still follows a normal developmental time course after hyperoxia, in contrast to the cone and rod-cone b-waves whose maturation peaks tend to fall short of normal. Furthermore, the attenuation in the cone b-wave amplitude at the height of maturation (as a function of normal, as per Manuscript 1, Chapter 2 Figure 5) is significantly more pronounced than that of the rod $V_{\max}$. When taken together with the normal process of retinal cellular development, these findings suggest that the earliest existing structures (cones and HCs, for example) are those that are most susceptible to maturation-induced changes that follow postnatal hyperoxia. This concept could also explain the more severe impact of oxidative stress on HCs and the OPL (the layer of synaptic contact between photoreceptors and HCs and BCs), as the former are more developed at birth, and would consequently be exposed to the hyperoxic regimen for a longer duration.

Of interest, exposure to hyperoxia within the first two weeks of life has less of an effect on the a-wave amplitude, particularly in SD rats, which remains relatively intact in comparison to the other ERG parameters (Manuscripts 1, 2, 3, Chapters 2, 3, 4). Therefore, it would appear that the photoreceptor layer is more

resistant to this stress and that the normal cellular refinement of the outer retina that occurs over time can still progress under conditions of excess oxygen. For example, we have previously reported that an age-related decline occurs in both scotopic and photopic ERGs recorded from control SD rats between P30 and P60 (Dorfman et al. 2006, Joly et al. 2006, Dembinska et al. 2002, Dembinska et al. 2001, Lachapelle et al. 1999). Following hyperoxia, the a-wave is the only parameter that undergoes similar age-dependent amplitude attenuation from P30 to P60, while this could not be evidenced for the rod V_{max} , rod-cone b-wave, or photopic b-wave. These results have been corroborated by Penn et al. (1988), who found a similar decline in the scotopic b-wave amplitude (by ~40%) between control animals aged 5 and 9 weeks (from P35 to P63), whereas no such findings were found in the hyperoxic cohort. However, when comparing the a-waves obtained at the same time-points from animals raised in the different oxygen environments (normoxia and hyperoxia), they were both similarly attenuated (Penn et al. 1988). When taken together, these findings suggest that not only is hyperoxia of little consequence on the a-wave amplitude, but that maturation of the a-wave is not affected to the extent that it is for the other parameters studied. Collectively, these findings would suggest that OIR is initiated at a postreceptoral level, where synaptic impairment at the level of the OPL prevents signal transmission to the inner retina, while both outer retinal structure and function remain intact.

While the retinal vasculature has been studied in depth in ROP and OIR, less is known about the role of the choroidal vasculature which is instrumental in supplying oxygen to the photoreceptors. While choroidal blood flow is autoregulated in the adult over a wide range of perfusion pressure, there is almost a complete lack of autoregulation in the newborn. Similarly, choroidal hemodynamics in newborn animals are compromised following oxidative stress (Chemtob et al. 1995). Consequently, choroidal blood flow (and retinal blood flow) are unable to be maintained at constant levels in situations where the perfusion pressure is increased. This inevitably results in a harmful increase in oxygen delivery to the retina. Interestingly, however, the decreased photoreceptor

susceptibility of SD rats to regimens of hyperoxia that persisted within the first two weeks of life (P0-14, for example) along with the increased susceptibility of the more mature retina to later exposure (P14-28) would not reflect the above. In fact, one would expect that the abundant levels of reactive oxygen species (ROS) generated would more easily attack the photoreceptors which contain very high levels of PUFAs in their lipid membranes and which have high metabolic rates (Daemen 1973). Our findings might be explained by the higher endogenous levels of FGF-2 in SD rats along with the unique ability of juvenile rats to significantly upregulate CNTF under conditions of hyperoxia. While neurotrophic factors play a role in neuronal differentiation and development, they can also be upregulated, along with their receptors, in many forms of retinal degenerations as an intrinsic protective mechanism (Joly et al. 2007, Chaum 2003) and such might be the case in our model. The relative resistance of photoreceptors to hyperoxia prior to eye-opening (up to P14) could be also be a feature of the more “plastic” immature retina, which might fight to preserve the photoreceptors that were otherwise destined to die through apoptosis, a process of cell death that occurs in the normally developing retina (Maslim et al. 1995). The specific mechanisms involved in this early protection (whether through neurotrophic factor upregulation, for example, or otherwise) require further elucidation. Finally, a better understanding of the choroidal vasculature in our model could be carried out using the plastic corrosion cast technique which can help to visualize the changes in the choroidal vessels of animal models in a three-dimensional fashion (Teschner et al. 2003, Bhutto and Amemiya 2001).

Our ability to demonstrate altered retinal function (with the exception of the a-wave) immediately following the cessation of hyperoxic exposure in SD rats (Manuscript 1, Chapter 2) would put less emphasis on the contribution of neovascularization (which follows the recovery in room air) to these manifestations (Holmes and Duffner 1996, Aiello et al. 1995, Smith 1994, Reynaud and Dorey 1994, Penn et al. 1994, Gole et al. 1990). If the above scenario also applies to the humans, emphasis should be placed not only on routine fundus exams in premature infants exposed to hyperoxia, but also on the

careful assessment of the functional consequences that might persist despite the lack of significant evidence of any underlying vasculopathy, including NV. Future studies aimed at determining the earliest point at which cytoarchitectural and functional manifestations of OIR occur in other rat strains are already underway. Finally, the visual evoked potential (VEP) can provide important information regarding the functional integrity of the visual system. A better understanding of the effect of postnatal hyperoxia on retinal output would help to determine whether a neuropathy and retinopathy develop concomitantly, thus enabling us to better appreciate the characteristics of the rat model of OIR.

1.2 Mechanisms of OPL thinning

1.2.1 OIR is an inner retinal disorder

One of the most striking ultrastructural consequences of postnatal hyperoxia involves the thinning to disappearance of the OPL. This suggested to us that a loss of synaptic connectivity between the PL and the inner retina would likely be responsible for the functional changes obtained with the ERG, namely attenuation of the b-wave amplitude with increasing oxygen exposure. Therefore, we used antibodies such as bassoon and synaptophysin (as presynaptic markers) and mGluR6 (a postsynaptic marker for ON-bipolar dendrites), for example, in order to further study synaptic connectivity and cell morphology in this model. Our initial impressions were confirmed when we found a progressive decrease in synaptophysin staining in the OPL of SD and LE rats, as well as in the INL of LE rats following exposure regimens that involved the second week of life, thus suggesting the lack of any functional synapses. The synaptic markers associated with photoreceptors and processes of bipolar and horizontal cells show more extreme abnormalities than those associated with photoreceptors, thus confirming, based on previous assessment of retinal function, that the inner retina is the

primary target in the pathogenesis of OIR (**Figure 2**). Furthermore, our results using confocal microscopy and immunohistochemistry also disclose a previously undescribed mechanism of cell death and synaptic retraction that are at the root of OPL thinning. Finally, while the vascularized central retina and avascular peripheral retina that result from exposure to hyperoxia would have suggested that OIR is primarily a peripheral retinal disorder, our findings would suggest otherwise. For example, our findings surprisingly show that while the most devastating consequences of OIR are observed in the central retina, areas of intact structure and function are observed in the peripheral retina (Dorfman et al. 2007, Dorfman et al. 2005).

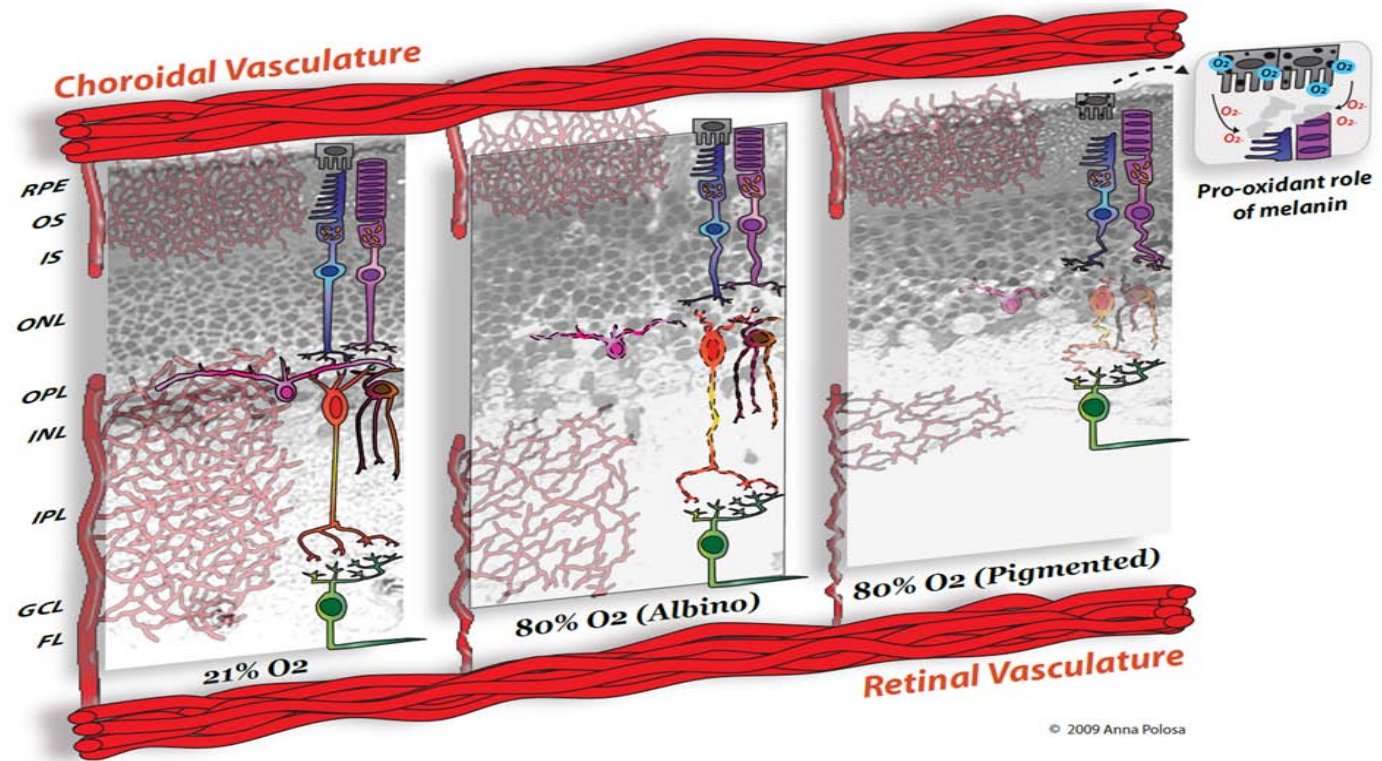


Figure 2: Schematic representation of retinal damage in albino SD and pigmented LE models of OIR. LE rats are more susceptible following postnatal exposure to hyperoxia as evidenced through greater impairment of retinal vasculature and more severe thinning of the inner retinal layers as a result of synaptic retraction and cell death. Greater damage to retinal photoreceptors was also revealed in LE rats. One wonders whether the pro-oxidant role of melanin in LE rats, which could exacerbate the level of ROS in the retina (*inset*), might contribute to these findings.

1.2.2 Retinotopic distribution of the OIR anomaly

Interestingly, the extent of structural damage following hyperoxia was not found to be uniformly distributed in the retina. For example, while the greatest consequences were found in the central retina, particularly in LE rats, regions of normal synaptic contact and cell morphology could be identified in the peripheral retina (Manuscript 4, Chapter 5). How do we reconcile the difference in the susceptibility of different loci to postnatal hyperoxia? Until recently, we have used the ERG, a pan-retinal response, in order to understand the functional manifestations of OIR. Our preliminary studies using the multifocal ERG (mfERG) which involves the simultaneous recording of electrical potentials from a number of central retinal regions, have enabled us to more easily find regional differences in retinal susceptibility to hyperoxia (Dorfman et al. 2005). Results obtained thus far have shown that similar to the flash ERG of LE and SD rats which behave alike (though LE rats are affected earlier on and more severely following exposure to hyperoxia), so to do their mfERGs, where SD rats are attenuated to a lesser extent than those obtained from LE rats. For example, while the most centrally located responses reveal the greatest degree of amplitude attenuation in LE rats, regions that were more functionally intact could be revealed peripherally (**Figure 3**). These results further corroborate our demonstration of differences in regional susceptibility of retinal structure following exposure to hyperoxia, where central retinal damage, as evidenced with cellular dropout and synaptic retraction, is more extensive than peripheral retinal damage in LE rats (Manuscript 4, Chapter 5). Finally, it is also of interest to mention that throughout hyperoxic exposure, the central retina is that which is vascularized to a greater extent than the peripheral retina (Manuscript 1, Chapter 2 and Manuscript 3, Chapter 4). Consequently, one would expect that the peripheral retina would be most compromised following the hyperoxic insult, should OIR be considered predominantly a vascular disease. Our findings suggest otherwise, however, where the significant structural and functional damage that ensues following hyperoxia occurs in the central retina where vaso-attenuation and

microvascular degeneration occur, as revealed with retinal flatmounts. Our results suggest that despite the pathological growth of some of these new vessels into the vitreous, the re-establishment of the superficial retinal vasculature, which also paves the way for inner retinal vasculature, could play a role in maintaining peripheral regions that are both functionally and structurally intact. Consequently, while NV is often a therapeutic target in OIR (Zhang et al. 2009, Barnett et al. 2007, Aiello et al. 1995), one wonders whether eliminating it might actually compromise the peripheral areas of normalcy that we have identified, with respect to retinal structure and function.

Impaired retinal vasculature is another factor to consider which might influence the integrity of the inner retinal neurons, as it has been suggested that post-receptoral neurons and retinal vasculature are intimately related (Fulton et al. 2009). Normally, retinal vascularization begins in the inner retina spreading to the outer retina and from the posterior pole spreading anteriorly. For example, vasculogenesis is initiated in the inner retina which is followed by angiogenic sprouting that progresses towards the outer retina where intermediate and the deep capillary beds are formed (Gariano and Gardner 2005, Gogat et al. 2004). The inability of the retinal vasculature to adequately extend from the inner retina, place the OPL and HCs, which are two of the most compromised structures in OIR, in the most vulnerable location at the limit of both the choroidal and retinal circulation (**Figure 2**), respectively, thus offering another plausible explanation for their sensitivity to hyperoxia. In fact, vaso-attenuation and microvascular degeneration which occurs initially in a select number of retinal capillaries and then on larger groups of capillaries, are key features of OIR that occur prior to pathological NV (Reynaud et al. 1995, Reynaud and Dorey 1994, Chan-Ling et al. 1992). The initial vessel regression that occurs is characteristic of ischemic retinopathies, including OIR, and reflects the normal response of the retinal tissue to an excessively rich oxygen environment in order to meet its metabolic needs. Retinal flatmount preparations such as those presented in manuscripts 1 and 3 (chapters 2 and 4, respectively) reveal superficial vascular changes with respect to the central and peripheral retina. Future studies aimed at better understanding the

intraretinal blood supply in OIR with the use oxygen-sensitive microelectrodes, could be instrumental in determining the intraretinal oxygen distribution and oxygen consumption in this model. Recent findings by Cringle et al. (2006) have suggested that the oxygen environment and oxygen consumption rates change significantly during retinal development. For example, the intra retinal oxygen distribution and consumption is significantly lower in rats at P15 compared to mature adult rats at increasing levels of systemic oxygenation (20%, 40%, 60%, 80% and 100%). One would expect that the vaso-attenuation and vaso-oblivation associated with exposure to hyperoxia would result in lower levels of oxygen supply to the inner retina of animals raised in that environment, particularly in the central retina which reveals the greatest extent of damage, while the revascularization phase (following the return to room air), might result in increased levels of oxygen supplied to the periphery as suggested by areas of intact structure and function. In addition, techniques such as the plastic corrosion cast of retinal vessels which enables specific changes in the retinal blood vessel supply to be clearly revealed in a three-dimensional fashion could also help to better visualize the consequences of hyperoxia on inner retinal vasculature (Teschner et al. 2003, Bhutto and Amemiya 2001). As described above, one might expect to find a limited availability of central retinal vessels in the inner retina as a result of the microvascular degeneration phase, while the revascularization phase could reveal significant vasculature in the peripheral retina, sufficient to maintain regions of normal structure and function.

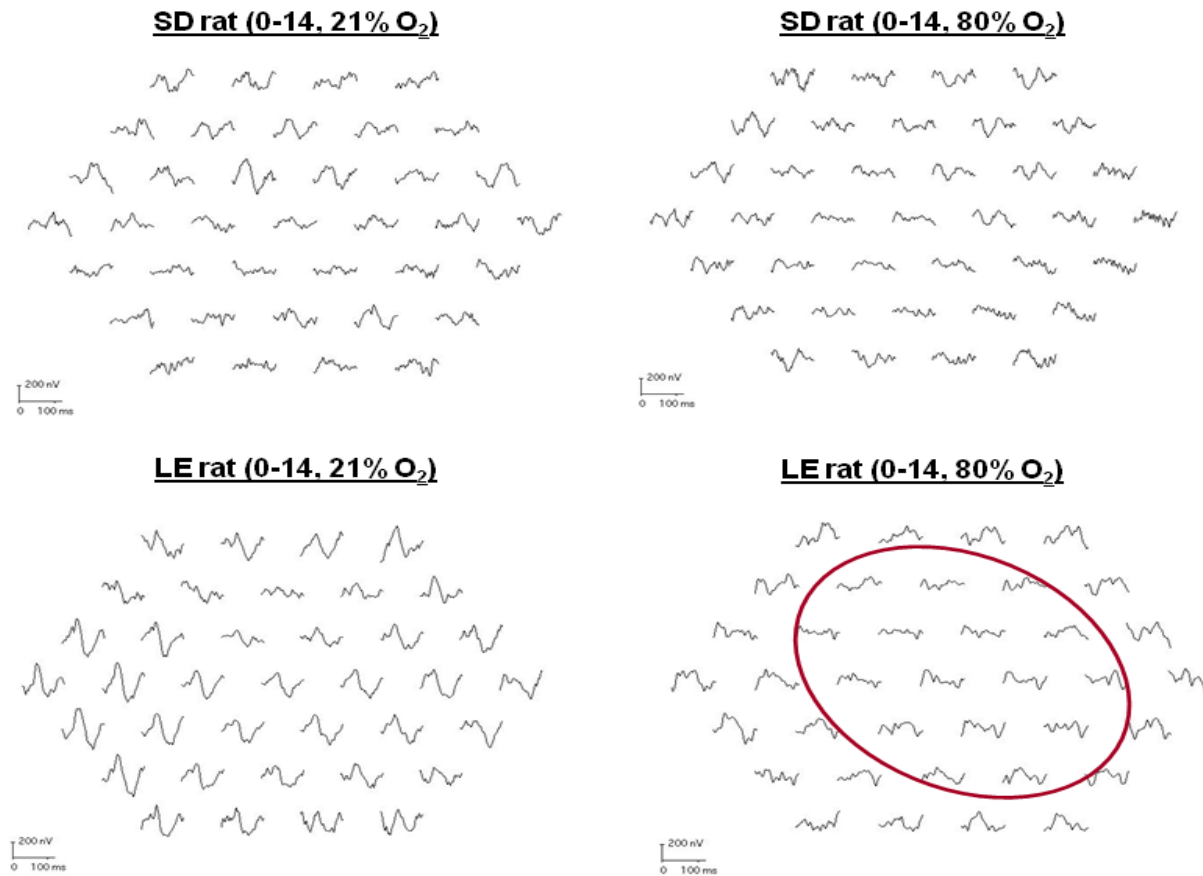


Figure 3: mfERG tracings obtained from SD rats raised in room air and hyperoxia (P0-14, 80%), respectively (Top panels), and from LE rats raised in room air and hyperoxia (P0-14, 80%), respectively (Bottom panels). The susceptibility of the central retinal responses in LE rats is observed in tracings that are circled in red, while more peripheral tracings are relatively intact.

1.3 Therapeutic avenues in OIR

1.3.1 Free radical scavengers

The underdeveloped antioxidant systems in the neonate, which normally include heme oxygenase-1, Cu–Zn superoxide dismutase, catalase and vitamins C and E, for example, render these infants particularly susceptible to the effects of an excessively high oxygen environment (Flynn et al. 1992, Nielson et al. 1988). Given that free radicals do, at least in part, appear to play a role in the pathogenesis of ROP, it is not surprising that clinicians and investigators have explored the possibility of antioxidant administration in preterm neonates in order to protect the retina from their deleterious effects. It was suggested that vitamin E supplementation might be beneficial to this end, especially when considering that plasma levels of this antioxidant are low in prematurely born infants (Katz and Robison 1988, Nielson et al. 1988, DeVito et al. 1986). Furthermore, since retinal vascular endothelial cell membranes were thought to be affected by free radicals, it was suggested that vitamin E could terminate lipid peroxidation reactions given its ability to integrate into the lipid bilayer where they occur (Flower et al. 1984). However, the effectiveness of vitamin E supplementation in reducing the severity of ROP remains quite controversial (McColm and Fleck 2001). For example, intravenous treatment with vitamin E resulted in increased retinal and intraventricular hemorrhage without any beneficial effect on the development of ROP (Phelps et al. 1987), while in other studies, a favorable effect of vitamin E on the incidence of ROP was reported (Liu et al. 2005, Johnson et al. 1995). It has also been suggested that conflicting results may stem from the difficulty of delivering a lipid soluble molecule such as vitamin E to the immature retina (Nielson et al. 1988).

1.3.1.1 Trolox C

Trolox C, a water soluble analogue of vitamin E with potent antioxidant activity, was used by Penn et al. (1997) in the rat model of OIR in order to determine its effect on the retinal vasculopathy. Interestingly, retinas of rats treated with trolox C throughout oxygen exposure had greater vascular coverage and higher capillary density than those that were untreated. While these findings suggest that trolox C could help prevent the vascular consequences of OIR, previous studies showed a lack of correlation between the retinal vascular and retinal structural and functional consequences of OIR. For example, vasculature often resumed normalcy shortly after the return to normoxia, whereas the electroretinographic and cytoarchitectural anomalies were permanent features of the hyperoxic cohorts (Dorfman et al. 2009, Dembinska et al. 2001, Lachapelle et al. 1999, Fulton et al. 1999, Reynaud et al. 1995). We therefore attempted to determine whether the same free radical scavenger administered throughout exposure to hyperoxia could also protect the structure and function of the retina. Our findings reveal a beneficial therapeutic effect of Trolox C on retinal function, namely on the OPs and the photopic mediated responses which are thought to be most susceptible to hyperoxia-mediated damage (Dorfman et al. 2006, Dembinska et al. 2002). However, its protective effect on retinal cytoarchitecture was limited to prevention of OPL thinning without any significant impact on the horizontal cell count.

Trolox C has been reported to have a dual mode of action, namely as an antioxidant and as a prooxidant, depending on the method by which peroxidation is induced. For example, trolox C acts preferentially as a toxic prooxidant and is converted to an α -tocopheroxyl radical as cellular functions deteriorate when the oxidative stress is induced by Cu^{2+} or Fe^{3+} (Dyatlov 1998). Transition metals such as the above can act as catalysts for reactions of O_2^- with H_2O_2 to produce the very highly reactive hydroxyl radical (Hardy et al. 2000) and might therefore encourage the prooxidant rather than antioxidant state of trolox C. Furthermore, it is important to take this into account when considering that higher levels of free

iron are found in newborn neural tissue in contrast to adults (Moncada et al. 1997, Stamler et al. 1992). The inability of trolox C to fully prevent oxygen induced retinal damage from occurring could suggest, as previously proposed (Dembinska et al. 2001) that free radicals are likely not solely responsible for the structural and functional damage that is observed in OIR. Alternatively, a significant proportion of the retinal damage induced by ROS might occur in hydrophobic domains where trolox C would lose its efficacy as a result of its water solubility. Delivering a combination of liposoluble and hydrosoluble free radical scavengers might be worthwhile in order to further our understanding of the role that free radicals play in OIR.

Finally, it has recently been suggested that the administration of exogenous antioxidants might be of limited use to counteract oxidative stress, given the fast pace at which ROS are generated and the importance of appropriate levels of the anti-oxidant required in specific cellular compartments (Dong et al. 2006), which may in fact have been a limitation of our study using trolox C. In a study by Dong et al. (2006), two models of oxidative stress, namely hyperoxia and paraquat, were used in order to evaluate the effects of superoxide dismutase 1 (SOD-1) over-expression in mice. Transgenic mice that over-expressed SOD-1 were spared structural and functional damage in both the hyperoxia and paraquat models, suggesting its potential usefulness as a candidate gene for gene therapy in diseases where oxidative stress is involved (Dong et al. 2006). However, similar to the mechanism by which trolox C can take on the role of a pro-oxidant or anti-oxidant, SOD-1 can provide a protective effect in some instances, while increasing the vulnerability to oxidative stress in others (Rader and Lanthorn 1989). While strategies aimed at limiting oxidative stress can be helpful for a wide range of retinal diseases, including ROP, AMD, DR and retinitis pigmentosa (RP), the therapeutic usefulness of antioxidants should therefore be assessed with caution, and the benefits carefully weighed against the potential harmful effects. Future studies aimed at exploring the potential benefits of other free radical scavengers on ROS, or rather the effect of targeting various steps of the cascade involved in generating them in the rat model of OIR are warranted. For example,

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been shown to increase the expression of VEGF, to cause increased migration of endothelial cells (Ushio-Fukai and Alexander 2004) and to activate proapoptotic signaling proteins (Bedard and Krause 2007). Apocynin, an NADPH oxidase inhibitor, has been shown to reduce the superoxide radical accumulation that occurs in rd1 mice as a result of NADPH oxidase overactivation (Komeima et al. 2008), as well as avascularity and apoptosis (Saito et al. 2007) and neovascularization (Al-Shabrawey et al. 2005) in the rat model of OIR. One wonders whether therapeutic avenues such as this that either directly target ROS or the steps that are involved in generating ROS might have a beneficial protective effect on the structural and functional sequelae that are associated with OIR.

1.3.1.2 Melanin

Previous studies have suggested that ocular pigmentation might offer protection against the development of severe ROP, resulting in differences in racial incidence of the disease (Tadesse et al. 2002, Saunders et al. 1997, Schaffer et al. 1993). We therefore hypothesized that the melanin pigment found in the pigmented LE rat might protect the retina from the consequences of OIR, particularly given its free radical scavenging role. To our surprise, not only did we show that melanin was unable to effectively protect the developing retina of LE rats subjected to postnatal hyperoxia, but our findings suggested an increased susceptibility of LE rats to postnatal hyperoxia in comparison to SD rats, evidenced with higher vascular dropout throughout exposure, more severely depressed retinal function and permanently altered retinal structure that extended beyond the loss of the OPL and horizontal cells as in SD rats, but also involved the inner retina, resulting in significant loss of the INL and IPL. Furthermore, the tendency for the shortening of inner segments of the photoreceptors which is accompanied by a significantly attenuated a-wave in LE rats after hyperoxia was also indicative photoreceptor involvement as well, a feature of OIR that was not

observed in SD rats (Manuscript 3, Chapter 4). The disparity in findings obtained between LE and SD rats which were subjected to identical exposure regimens, along with the previously described window of increased susceptibility to the same duration of oxygen exposure within the second week of life compared to the first, would collectively place even less emphasis on a role for free radicals in the pathophysiology of OIR and further support the contribution of genetic differences between strains along with the degree of retinal maturation at the time of exposure to these processes. Furthermore, as discussed above, our findings could be explained by the higher endogenous levels of FGF-2 in SD rats along with the unique ability of juvenile LE and SD rats to significantly upregulate CNTF under conditions of hyperoxia. In LE rats, however, low levels of endogenous FGF-2 along with significant CNTF upregulation that occurs only following later exposures might be insufficient in rescuing photoreceptors from damage in this strain. In addition, the proximity of the photoreceptor layer to the RPE could be a contributing factor to the increased susceptibility of LE rats should melanin indeed take on the role of a pro-oxidant under conditions of extreme hyperoxia, thus facilitating free radical attack of the photoreceptors.

Strain-related differences in retinal vasculature between albino and pigmented models of OIR have been previously reported. For example, although there were no differences with respect to the time course of retinal vascular development between normal pigmented Brown Norway (BN) and SD rats in a study by Gao et al. (2001), those raised in hyperoxia showed significant differences in vascular permeability and retinal NV. This was characterized by retinal artery constriction along with large avascular regions in the retina of BN rats at P12, 1 day after their removal from the hyperoxic environment, while SD rats studied under the same conditions revealed a lower degree of retinal artery constriction and larger areas of retinal vascular perfusion (Gao et al. 2001). Furthermore, increased vascular permeability followed by a more severe NV could also be observed in the BN strain (Zhang et al. 2005, Floyd et al. 2005, Gao et al. 2001), characterized by tortuosity and dilatation of retinal vessels, neovascular tufts, and hemorrhage, while SD rats were lacking neovascular tufts

and evidence of microaneurysms (Gao et al. 2001). Moreover, the BN model of OIR revealed higher retinal VEGF levels and more severe retinal vascular leakage than did the SD strain (Lu et al. 2007). Taken together with our results, these findings suggest that retinal vascular development along with the NV phase that ensues is significantly more affected by hyperoxia in pigmented compared to albino rats. Variations in the regulation of the antiangiogenic pigment epithelium-derived factor (PEDF) and the angiogenic VEGF among strains are thought to contribute to these discrepancies, which may be genetically regulated (Gao et al. 2001). Similarly, other strain-dependant factors involved in regulating the effects of oxygen tension on retinal angiogenesis, such as glial cell sensitivity to hyperoxia, for example, might also play a role (van Wijngaarden et al. 2007). The link that appears to exist between ocular pigmentation and the susceptibility to NV also requires further elucidation.

Finally, while our studies show that significant strain differences exist between the albino SD and pigmented LE rat, future studies making use of different albino and pigmented rat models of OIR, for example albino Fischer 344 (F344), albino Wistar, pigmented Dark Agouti (DA) and pigmented Brown Norway (BN), respectively, would help to determine whether the discrepancies among strains is characteristic of all albino and pigmented rats. Previous studies have shown that melanin may not act alone as the substance responsible for the protective effect (Gorin et al. 1999, Cruickshanks et al. 1997), but may instead be a confounding variable that is associated with certain genes that are more or less prevalent in certain populations. Genetic influences in the pathogenesis of these diseases may play an important role in the heterogeneity in risk of their progression. Interestingly, casualties reported in our lab were more frequently observed for pigmented LE and Brown Norway rats exposed to hyperoxia (data not shown), findings that might be explained by inter-strain differences in the rat genome that contribute to variations in ventilation, metabolism, and ventilatory responses to inspired levels of O₂ and CO₂ (Hodges et al. 2002, Strohl et al. 1997), all of which are areas that require further elucidation. Of interest, tyrosinase is an enzyme involved in the synthesis of melanin and is often mutated

in albinos. For example, albinism in the Wistar rat was recently attributed to a tyrosinase gene missense mutation (Blaszczyk et al. 2005). Melanin synthesis has been associated with the generation of ROS, and Dihydroxyphenylalanine (DOPA), a product of tyrosinase catalysis, has been shown to be particularly damaging to endothelial cells (Akeo et al. 1994, 1989) which might explain the susceptibility of pigmented rats in OIR. One wonders whether transgenic expression of functional tyrosinase (the rate limiting enzyme in melanin synthesis) in the RPE of albino rats or mice, essentially rendering them pigmented, could help to further elucidate whether a causal link exists between ocular pigmentation and increased susceptibility of pigmented rats.

1.3.2 Neurotrophic factors

Neurotrophic factors play a role in neuronal differentiation and development, and they can also be upregulated, along with their receptors, in many forms of retinal degenerations as an intrinsic protective mechanism (Joly et al. 2007, Chaum 2003). We hypothesized that the expression of similar survival factors might explain the relative resistance of SD rats compared to LE rats with respect to (1) photoreceptor damage (as evidenced by the relatively intact a-wave) and to (2) less severe thinning of the inner retinal layers. Of note, our results suggest that although only limited variations in FGF-2 occur in both LE and SD models after hyperoxic exposure, higher levels (approximately twofold) are observed for each exposure regimen in SD rats. Furthermore, although CNTF levels are only significantly higher after exposure regimens that include the second week of life in LE rats, they are already significantly greater in SD rats after exposure within the first week (e.g., from P0–P6). These results would therefore suggest that SD rats are better equipped with survival factors in the face of a hyperoxic challenge. The involvement of CNTF and FGF-2 may also explain the preservation of photoreceptor function in SD rats as well as the relative resistance to functional and structural damage that follows hyperoxia from P0 to P6.

Overall, our findings using the pigmented LE would suggest that, similar to the limited role of trolox C in protecting the developing retina from oxidative stress, melanin is insufficient in carrying out a beneficial protective effect under the same conditions, and that it might also potentiate the effect of ROS as a pro-oxidant. In humans, the pro-oxidant role of melanin can lead to cytotoxic activity such as photoreceptor damage and even AMD (Sarna et al. 2003, 1992). Given that previous studies in animal models of retinal degeneration have revealed a delay in photoreceptor cell death following intravitreal injection of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Okoye et al. 2003), ciliary neurotrophic factor (CNTF) (LaVail et al. 1998, Cayouette et al. 1998), fibroblast growth factor (FGF)-2 (LaVail et al. 1998, Gao and Hollyfield 1996, Faktorovitch et al. 1990) or glia-derived neurotrophic factor (GDNF) (McGee Sanftner et al. 2001), future studies including the concomitant administration of neurotrophic factors throughout hyperoxic exposure are warranted, particularly in the highly susceptible pigmented model.

1.4 Comparing OIR with other animal models of retinal disorders

Retinopathy of Prematurity (ROP) is a condition observed in human infants that are born prematurely and receive high levels of oxygen postnatally to compensate for their unstable pulmonary status. In the extrauterine environment, the immature retinal tissue is particularly susceptible to injury (Chen and Smith 2007, Saugstad 2006) and consequently, the earlier the preterm birth, the greater the risk for retinal damage.

ROP is a vasoproliferative disorder that can result in retinal detachment and blindness. Vasoconstriction is the first phase that characterizes ROP, following which vaso-obliteration occurs in response to excess oxygen, preventing normal vascular growth towards the retinal periphery. The initial vasoconstriction and vaso-obliteration that occur are characteristic of ischemic retinopathies and

reflects the normal response of the retinal tissue to an excessively rich oxygen environment. The secondary phase of NV occurs upon the return to a normoxic environment as a result of an inadequate blood supply (Patz and Payne 1998, Moore 1990). Given that vascular impairments are considered to be the clinical hallmark of ROP, it is not surprising that many previous efforts have been largely focused on the vasculopathy that is associated with OIR (Beauchamp et al. 2004, 2001, Penn et al. 1997, Smith et al. 1994, Penn et al. 1992).

However, despite the fact that ROP is considered a disease of the developing retinal vasculature and that retinal vascular repair indeed occurs in some cases, marked functional impairment has been shown to persist nonetheless. For example, in patients with a history of ROP, significant abnormalities in the a- and b-waves of the ERG have been shown to carry on into adulthood (Fulton et al. 2001, 1996, 1995a). Moreover, elevations of the scotopic visual thresholds in children with a history of resolved, mild ROP are also suggestive of these lasting effects (Barnaby et al. 2007, Hansen and Fulton 2000, Reisner et al. 1997). Finally, significant deficits in amplitude and implicit time of the mfERG response has been shown in subjects whose clinical ROP had completely resolved more than 10 to 20 years earlier (Fulton et al. 2005). Consequently, while abnormal retinal vasculature in ROP patients can resolve itself, studies aimed at further elucidating the neural dysfunction of the retina that seemingly continues on through adulthood are warranted.

As in ROP, retinal NV is considered to be the hallmark of other retinal diseases, including AMD and DR, which can eventually result in blindness (Feigl 2009, Paulus and Gariano 2009). Angiogenic factors can be upregulated following periods of ischemia or hypoxia, which can ultimately result in pathological angiogenesis referred to as NV. Vascular endothelial growth factor (VEGF) has been implicated in this process, and its overexpression in transgenic mice results in retinal NV (Tobe et al. 1998). In contrast, VEGF antagonists have also been shown to attenuate the development of NV (Aiello et al. 1995). Studies aimed at unraveling the molecular pathogenesis behind ischemic retinopathies can provide the basis for identifying novel therapeutic targets for their treatment.

1.4.1 Streptozotocin-induced diabetic retinopathy

The Streptozotocin (STZ)-induced diabetic rat model shows many of the changes in retinal structure and function that are associated with human DR, including capillary dropout, pericyte loss, microaneurysms and eventually NV (Yu et al. 1998, Cringle et al. 1993), and therefore also bears some similarities with both ROP and OIR. It is thought that these changes in retinal vasculature precede the detection of any structural consequences of DR (Mizutani et al. 1996). Apoptosis has been shown to play a role in the loss of retinal microvascular cells, MCs and GCs in this model (Marc et al. 2003). Moreover, even once good glycemic control is established in diabetic rats, retinal vascular cells still continue to undergo apoptosis as evidenced through the upregulation of apoptosis-related genes that include the tumor necrosis factor (TNF) ligand and receptor, caspase and BCL-2, for example (Kowluru and Chan 2009). It is thought that specifically targeting specific steps leading to apoptosis might help to slow or stop the progression of this disease (Kowluru and Chan 2009). More recently, the STZ mouse model, which reveals GC death via apoptosis, has also been suggested as a valuable model in which to study DR (Martin et al. 2004). Finally, while DR has also long been considered to be primarily a vascular disease, it has also been suggested that neural loss may occur in DR prior to vascular damage. In fact, while apoptosis, loss of GC bodies, and glial reactivity in the retina have been shown in animal and human forms of DR (Abu-El-Asrar et al. 2004, Martin et al. 2004, Barber 2003, Lieth et al. 2000, Zeng et al. 2000, Barber et al. 1998, Lieth et al. 1998), a selective thinning of the inner retinal layers in diabetic patients was recently evidenced (van Dijk et al. 2009).

Indeed, many of the features of the DR model described above are reminiscent of the pathophysiology of ROP and OIR. In fact, given that the core pathology of OIR and DR are thought to be similar in nature with respect to their VEGF-mediated mechanisms (Yoo et al. 2007), it is not surprising that similar strategies have been explored in the rat model of OIR, including the use of anti-

VEGF therapies in order to control the NV phase (Jardeleza and Miller 2009, Kinose et al. 2005). It is important to mention, however, that irrespective of the similarities between them, the STZ model of DR and the rat model of OIR are fundamentally different from one another. For example, mature adult rats are used to produce the STZ model, while young rats with a developing retina are used to produce the OIR model. Consequently, whereas treatments aimed at controlling NV might be deemed beneficial in DR rats, the findings presented herein would suggest that they would be of very limited use (and potentially even destructive) in the developing retina of OIR rats, and even in controlling ROP if our findings in the rat resemble those in humans that present with the disease.

1.4.2 Light induced retinopathy (LIR)

Similar to the rat model of OIR which enables us to further elucidate the pathogenesis of human ROP, different models of oxidative damage have also been used in order to better understand the progression of various other retinal diseases. It is of interest to note that despite the fact that these models all involve the generation of ROS, they do not necessarily result in the same structural and functional consequences. For example, exposure to another environmental stress, namely bright light, has been suggested to result in 1) the generation of free radicals, 2) alterations of retinal metabolism, and 3) cellular damage as a result of phototoxicity (Glass 1990). Premature infants are routinely exposed to bright continuous light in the neonatal intensive care unit. In addition to ambient light, other light sources that the premature infants are subjected to include heat lamps, indirect ophthalmoscopes for frequent eye exams and phototherapy for neonatal jaundice which consists of a bank of 8-10 fluorescent tubes that surround the incubator and deliver approximately 3000 to 4000 lux of light. While the infant's eyes are often patched, they do not always receive adequate protection (Glass 1990). Consequently it has been suggested that light might be implicated in the development of ROP (Glass et al. 1985, Lawson et al. 1977), and more recent efforts have been made to reduce ambient light exposure in neonatal intensive care

units (Reynold et al. 1998). In combination with hyperoxia, the impact of light was of minimal consequence on the development of ROP in the kitten model (Kremer et al. 1992), while it generated a more severe retinopathy in the rat model, including significant photoreceptor damage (Ricci 1990).

The rat model of LIR reveals outer retinal damage that is characterized by severe thinning of the ONL and OS layer along with almost complete abolition of the ERG in exposed adult rats. It has been suggested that this retinal degeneration initially involves damage to rod photoreceptors following which the gradual death of cones occurs (Shen et al. 2005). Of interest, however, studies carried out in our laboratory revealed, for the first time, that juvenile rats exposed to the same intensity of bright light for 14 days were significantly less affected when ERGs and retinal histology were evaluated at P30 and P60, compared to the more severe findings obtained in exposed adult rats (Joly et al. 2006). Moreover, while thinning of the ONL could still be evidenced (albeit not to the same extent as that observed in exposed adult rats), a concomitant thickening of the INL was also observed. It was suggested that this might occur as a compensatory mechanism to maintain normal retinal output in juvenile rats only, since adult rats did not reveal similar results (Joly et al. 2006). Findings with the OIR and LIR models would suggest the importance of considering the impact of retinal maturity in the interpretation of results which are instrumental in better understanding the pathogenesis of many retinal diseases.

1.4.3 FeSO₄ and paraquat-induced oxidative stress

When comparing two different environmental stresses described above, namely hyperoxia and light, a differential susceptibility of the retinal elements becomes evident. For example, while the rat model of OIR reveals damage that is largely limited to the postreceptor retina following early exposure to hyperoxia, the LIR model generates an outer retinal pathology, leaving the inner retina

relatively intact. Iron toxicity has been shown to be a potential factor in AMD and RP, and consequently this model can be used to better understand the progression of these diseases. Intravitreal injection of FeSO_4 caused increased generation of superoxide radicals and oxidative damage in photoreceptors in 6 to 8-week-old C57BL/6 mice. This was associated with attenuated ERG responses, particularly those obtained in photopic conditions, photoreceptor cell death as evidenced with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for apoptosis and thinning of the ONL with a relatively intact INL (Rogers et al. 2007), features which are reminiscent of the LIR model (in adult rats), which is used to study the progression of similar diseases. In contrast, paraquat (1,1'-dimethyl-4,4'-bipyridinium) is a herbicide that reacts with molecular oxygen to form the highly reactive superoxide anion (Dinis-Oliviera et al. 2006) which results in progressive retinal damage when administered to mice intra-vitreally (Dong et al. 2006), namely functional attenuation as observed with the ERG as well as structural damage that involved both the ONL and INL (Dong et al. 2006). These findings are reminiscent of the damage observed in the LE rat model of OIR, which reveals both outer and inner retinal consequences. Interestingly, it has been suggested that endogenous antioxidant defense systems may differ between rods and cones, rendering the latter system more susceptible in the face of reactive oxygen species induced by hyperoxia (Dembinska et al. 2002), or FeSO_4 (Rogers et al. 2007), for example. The above supports the hypothesis whereby devastation of the cone pathway (which begins its development in utero) occurs to a greater extent than that of the rod pathway (which begins its development postnatally). Our previous demonstration of HC susceptibility following exposure to hyperoxia further support the increased susceptibility of the cone pathway, given that the negative feedback from HCs to cones has been suggested to be of great importance in maintaining the cone systems balanced (Kamermans and Spekreijse 1999). Collectively, these findings would suggest that endogenous antioxidant defense systems differ among retinal photoreceptor and neuronal cells, and that the efficacy of these systems can change depending on the type of oxidative stress that is experienced.

The latter two models of oxidative stress (FeSO_4 and paraquat) were carried out in adult mice, and given our findings of different susceptibility of juvenile versus mature rats in the OIR and LIR models, future studies are warranted to determine whether or not similar findings could be documented in immature animals.

2 Conclusion

Our results obtained with the more commonly used albino rat model of OIR not only confirm previous reports of reduced retinal vascular coverage that accompanies the earliest manifestation of postnatal hyperoxia in rats, but suggest that while retinal vasculature can repair itself, irreversible cytoarchitectural and functional changes occur that are documented immediately after the cessation of hyperoxia. Our ability to demonstrate altered retinal function (with the exception of the a-wave) immediately after cessation of hyperoxic exposure would put less emphasis on the contribution of NV to these manifestations, since this aggravated angiogenic process is known to be triggered after recovery in room air (Holmes and Duffner 1996, Aiello et al. 1995, Reynaud and Dorey 1994, Penn et al. 1994, Smith et al. 1994, Gole 1990). In fact, our findings suggest that the initial phase of microvascular degeneration and vaso-obliteration of the central retinal vessels (while the peripheral retina remains relatively avascular) culminates in the structural and functional damage that ensues following hyperoxia. Moreover, the ability of retinal vasculature to repair itself while still subjected to the hyperoxic regimen, along with the NV phase that occurs following the return to room air, likely plays a role in maintaining areas of relatively normal retinal structure and function in the peripheral retina, as evidenced with immunohistochemistry and mfERGs in the more susceptible LE rat model of OIR. While the initial loss of vessels that occurs in the first phase of OIR reflects the normal reaction of blood vessels to a hyperoxic environment, it would seem that a better understanding of

the various steps in this cascade of events could lead to the prevention of the most devastating consequences of OIR and ROP that occur thereafter.

While studies have begun to uncover some of the mechanisms responsible for microvascular degeneration in OIR, they remain, for the most part, poorly understood. Recently, however, the role of PAF as well as TxA_2 in retinal vasoobliteration in OIR have been explored (Beauchamp et al. 2002, 2001), as both are generated in high levels following oxidative stress and have been shown to contribute to neurovascular injury (Akisu et al. 1998, Liu et al. 1996, Chemtob et al. 1995, Alloati et al. 1994). Findings revealed that PAF indeed plays a role in the vasoobliterative phase of OIR as suggested, for example, by the relative preservation of the microvasculature following administration of PAF receptor blockers (Beauchamp et al. 2002). Similarly, TxA_2 receptor blockers also successfully preserved retinal microvasculature, as evidenced with ADPase staining of the retinal endothelial cells (Beauchamp et al. 2001), suggesting that it too plays a role in microvascular degeneration. While using the above as targets to reduce the initial vasoobliterative phase of OIR could successfully be of use in dilatation of the vessels throughout hyperoxia and thus prevent the latter neovascular phase, it is important to consider the extremely high levels of oxygen that the retinal tissue and vasculature would have to face as a result. As previously mentioned, free radicals have been shown to play a role in generating the pathogenesis of OIR (Hardy et al. 2005a, 2005b, Dorfman et al. 2006, Penn et al. 1997, Niesman et al. 1997, Penn et al. 1992, Penn 1990). We therefore attempted to administer trolox C, a water soluble analogue of vitamin E, to neonatal albino rats throughout exposure to hyperoxia, especially given its previously described ability to facilitate retinal vasculogenesis under similar conditions. Our findings revealed that trolox C was unable to fully prevent the structural and functional sequelae that characterize this retinopathy. Similarly, our findings of an increased susceptibility of LE rats to hyperoxia, despite their higher levels of ocular melanin which has been suggested to exert a protective free radical scavenging effect (as observed in ROP), suggest that free radicals cannot alone explain the devastating effects of hyperoxia on the retina. Given that the

oxygen rich environment that premature infants are exposed to is only a transient means of compensating for their immature lung function, one wonders whether concomitant administration of therapeutics aimed at targeting the initial vaso-obliterative phase in addition to antioxidants might be of use in limiting the damage to retinal vasculature, structure and function following hyperoxia. Further evaluation of the above in both albino and pigmented models of OIR will undoubtedly be instrumental in the clinical management of ROP as well as other ischemic retinal disorders and in the derivation of therapeutic modalities used to target the various steps in their pathogenesis.

CHAPTER IX: REFERENCES

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CHAPTER X: APPENDIX

Early Manifestations of Postnatal Hyperoxia on the Retinal Structure and Function of the Neonatal Rat

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PURPOSE. Postnatal hyperoxia in rats causes an arrest in growth of retinal blood vessels, along with severe changes in retinal ultrastructure and function. Previous studies focused on consequences of postnatal hyperoxia at time points substantially removed from the hyperoxic insult. In this study, the earliest manifestations of this retinopathy were examined.

METHODS Newborn rats were exposed to 80% O₂ from birth to postnatal day 14. The retinas were collected for vascular assessment at postnatal days 6, 9, 12, and 14, and electroretinograms were recorded at postnatal days 15, 16, 17, 19, 24, 30, and 60, after which retinal histology was performed.

RESULTS Hyperoxia significantly attenuated vascular development, especially after 6 and 9 days of exposure which resulted in 64% and 72% of normal coverage, respectively. Vascular growth continued despite hyperoxic exposure, reaching 87% of normal by postnatal day 14. Electroretinograms of hyperoxic rats retained very immature features throughout with nearly abolished b-waves and relatively preserved a-waves. Finally, while retinal structure was virtually complete in the control animals by postnatal day 15, hyperoxic rats always showed a significantly thinner outer plexiform layer (OPL) and lower horizontal cell count ($P < 0.05$), irrespective of the duration of exposure.

CONCLUSION The findings confirm previous reports of reduced retinal vascular coverage that accompanies the earliest manifestation of postnatal hyperoxia in rats and suggest increased retinal susceptibility to hyperoxia within the first week of life. However, despite the fact that vasculature appears to repair itself, irreversible cytoarchitectural and functional changes occur, the consequences of which are documented immediately after the cessation of hyperoxia. (*Invest Ophthalmol Vis Sci*. 2008;49:458–466) DOI:10.1167/iovs.07-0916

The rat model of oxygen-induced retinopathy (OIR) has been widely used as an animal model of the human form of this disease, retinopathy of prematurity (ROP). ROP is a vasoproliferative disease that, in its most severe form, can result in childhood blindness.^{1–3} In contrast with other animal models

that have been used to study the pathophysiology of this disease, the neonatal rat offers the advantage of a highly immature retina at birth that is comparable to that of a 24- to 26-week-old human embryo.⁴ As in humans, exposure of the rat pup to postnatal hyperoxia causes severe vasoconstriction and vaso-obliteration, followed by an abnormal proliferation of retinal vessels on the return to room air.^{5–9} Accompanying this vasculopathy are permanent structural and functional abnormalities, as documented with retinal histology and electroretinography, respectively.^{10–18} Of interest, however, in only a few of these studies did the researchers try to identify the earliest structural and functional manifestations of the disease process triggered by the postnatal hyperoxic shock,^{10,11,16–18} where retinal function was evaluated either at predetermined time points (at postnatal days [P]16 and P29,¹¹ P31,¹⁰ or P13 and P18,^{11,16} for example) or on a weekly basis, beginning immediately after the exposure regimen.¹⁸ These and other studies that were performed at intervals that were further removed from the oxidative insult and at a time when the retina was considered to be mature (e.g., at postnatal days 30 and/or later)^{12–15} have made it difficult to understand the pathophysiological progression of OIR. Therefore, the purpose of the present study was to examine first the effects of postnatal hyperoxia on vascular development throughout hyperoxic exposure and, second, to determine how early after the cessation of hyperoxia we observe changes in retinal structure and function that are known to characterize adult rats that have been exposed to the same conditions during the first 2 weeks of life.

METHODS

All experimental procedures were approved by the McGill University/Montreal Children's Hospital Research Institute Animal Care committee according to the guidelines of the Canadian Council on Animal Care and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Newborn litters of Sprague-Dawley rats (Charles River Laboratories, St-Constant, Quebec, Canada) were exposed to 80% oxygen immediately after birth (mixture of medical grade 100% O₂ and room air measured with an oxygen meter; MaxO₂ Ceramtec, model OM25-ME; Medica Inc., Montreal, Quebec, Canada), as previously described.^{12–15} Briefly, exposure to hyperoxia (80% O₂) persisted from birth until postnatal day 14 for 22.5 hours daily, interrupted with three intervals of 30 minutes' duration under normoxic conditions (21% O₂). Animals were euthanatized for vascular assessment at P6 ($n = 3$), P9 ($n = 3$), P12 ($n = 3$), and P14 ($n = 3$) throughout exposure to 80% O₂ and compared with that in animals raised simultaneously in room air (21% O₂, $n = 3$). After hyperoxic exposure, animals were assigned to one of seven experimental groups to be studied at age P15 ($n = 6$), P16 ($n = 6$), P17 ($n = 6$), P19 ($n = 6$), P24 ($n = 6$), P30 ($n = 8$), and P60 ($n = 7$). Data were compared with that obtained from control age-matched animals raised simultaneously in normoxic conditions (21% O₂) on P15 ($n = 6$), P16 ($n = 6$), P17 ($n = 6$), P19 ($n = 6$), P24 ($n = 6$), P30 ($n = 14$), and P60 ($n = 14$). The rats were maintained in a cyclic lighting environment (80 lux; 12 hours dark/12 hours light). Finally, mothers of the litters were alternated between normoxic and hyperoxic conditions every 24 hours so that pulmonary complications known to arise in adult rats raised in a hyperoxic environment could be avoided.

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Retinal Flatmounts

After the rats were euthanatized, the eyes were enucleated, the anterior segments dissected, and the eyecups fixed overnight in 4% formalin. The retinas were subsequently isolated, and flatmounts were prepared for staining with adenosine diphosphatase (ADPase), as previously described.^{15,19,20} Specimens were mounted and photographed (40 $\times$, Axiophot microscope; Carl Zeiss Meditec, GmbH, Oberkochen, Germany). To quantify the extent of vascular coverage (in normoxia- and hyperoxia-raised animals), the ratio of total area of vascularized retina over the total retinal area was calculated (Image-Pro Plus 4.1 software; Media Cybernetics, Silver Spring, MD).

Electroretinography

Electroretinograms (ERGs) and oscillatory potentials (OPs) were recorded with a data acquisition system (Acknowledge; Biopac MP 100WS; BioPac Systems, Inc., Goleta, CA), according to a technique previously reported by us,^{12–15} on rats aged 15, 16, 17, 19, 24, 30, and 60 days. After a period of 12 hours of dark-adaptation, the rats were anesthetized, under dim red light, with an intramuscular injection of ketamine hydrochloride (85 mg/kg) and xylazine (6 mg/kg). The pupils were dilated with a 1% cyclopentolate hydrochloride solution (Mydracyl; Alcon Laboratories, Fort Worth, TX) and a DTL silver-coated conductive nylon yarn (27/7 X-Static; Sauquoit Industries, Scranton, PA) electrode was placed on the cornea to serve as the active electrode. The latter was maintained in place on the cornea with 2% hydroxymethylcellulose (Gonioscopic solution; Alcon Laboratories) which also prevented corneal desiccation. Reference and ground electrodes were placed in the mouth (model E5 disc electrode; Grass Technologies, Quincy, MA) and tail (model E2 subdermal needle electrode, Grass Technologies), respectively. The rats were then placed in a light-proof recording chamber of our design that included the light stimulus and background light.^{12–15} Full-field ERGs and OPs were recorded simultaneously (ERG: 10,000 $\times$, 1–1,000-Hz bandwidth, 6 dB of attenuation and OPs: 50,000 $\times$, 100–1,000 Hz, 6 dB of attenuation) using analog preamplifiers (model P511; Grass Technologies). Scotopic responses were evoked by flashes of white light spanning a 6.0-log-unit range in 0.3-log increments (maximum intensity: 0.6 log $\cdot$ cd $\cdot$ s $\cdot$ m⁻²; average, two to five flashes; interstimulus interval, 10.240 seconds). Photopic ERGs (average, 20 flashes, interstimulus interval: 1 second) were evoked by flashes of white light of 0.9 log $\cdot$ cd $\cdot$ sec $\cdot$ m⁻² delivered after more than 15 minutes of light adaptation (after the dark-adaptation period) to a background of 30 cd $\cdot$ m⁻² to avoid the light adaptation effect.^{21,22}

Retinal Histology

After the P15, P17, P19, and P24 ERG recording sessions, three to four animals per group were killed, and their retinas were collected for histology. Sections of the central retina were embedded in epoxy resin (Epon 812; Mecalab, Montreal, Quebec, Canada) and ultrathin sections (0.7 μ m) were stained with toluidine blue, according to a method previously described.^{12,15} Measurements of retinal thickness were obtained on 250- μ m wide sections with the use of an ocular with a calibrated grating. Three retinas per group were analyzed and an average of 10 different retinal measurements was obtained from each. Pictures were taken with a microscope (40 $\times$, Axiophot; Carl Zeiss Meditec, GmbH).

Data Analysis

ERG components were measured, as previously described by us.^{12–14} Briefly, the amplitude of the a-wave was measured from the baseline to the most negative trough and the amplitude of the b-wave was measured from the trough of the a-wave to the most positive peak of the response. The amplitude of the b-wave was plotted against the corresponding flash intensity to generate the scotopic luminance-response function curve that was fitted to a sigmoidal regression curve (Prism, ver. 3.00 for Windows; GraphPad Software, San Diego, CA) from which

the rod $V_{\max}$ (maximum pure rod response) was derived. Also, the ERG response evoked by the brightest flash delivered in scotopic conditions (the so-called mixed rod-cone response) was also included in the analysis. Finally, since no a-wave can be identified in the photopic responses of rats (Fonteille VL, et al. *IOVS* 2005;46:ARVO E-Abstract 2246),^{12–14,23,24} the photopic b-wave was measured from baseline to the peak of the wave. A one-way ANOVA ($P < 0.05$) followed by the Tukey honestly significant difference (HSD) or a Student's *t*-test was used to compare the effect of postnatal hyperoxia on retinal structure (vasculature and histology) and function. The data are presented as the mean $\pm$ 1 SD.

RESULTS

Retinal Vasculature

Figure 1 shows representative examples of retinal flatmounts obtained at P6 (Fig. 1A), P9 (Fig. 1B), P12 (Fig. 1C), and P14 (Fig. 1D) from animals raised in normoxia (top) and hyperoxia (bottom) from P0 to P6 (Fig. 1E), P0 to P9 (Fig. 1F), P0 to P12 (Fig. 1G), and P0 to P14 (Fig. 1H). The extent of retinal vasculature (percent of total retinal surface) is represented graphically in Figure 2. In normal rats, vascular growth gradually progressed from the center to the periphery as the rat pup aged from P6 (71.56% $\pm$ 3.91% of coverage as per Fig. 2) to P12 (98.93% $\pm$ 1.05%). By P12, vascular coverage was complete, and no further changes were observed at P14 (99.07% $\pm$ 0.95%). In comparison, hyperoxia significantly hampered the growth of the retinal vasculature. Hyperoxic exposure between P0 and P6 (Fig. 1E) significantly limited the vascular coverage to 46.50% $\pm$ 2.95% (Fig. 2), representing 64.9% of age-matched normal values ($P < 0.05$), which were not yet completely vascularized, as just mentioned. However, despite the hyperoxia regimen, the retinal blood vessels continued to grow and reached 87.90% $\pm$ 2.07% of coverage at P14, a value that was still significantly less than in the control rats ($P < 0.05$).

Maturation of the Scotopic ERG

Figure 3 shows representative scotopic and photopic ERG waveforms obtained at P15, P16, P17, P19, P24, P30, and P60 from control (Fig. 3A) and hyperoxic (Fig. 3B) rats, respectively. Group data are graphically reported in Figure 4.

In normal rats, the scotopic ERG obtained on eye opening (P15) was of low voltage with a negative morphology, due to a prominent a-wave and underdeveloped b-wave. The ascending limb of the b-wave was also devoid of well-demarcated OPs. As the rat aged (P16–P17), the a-wave continued to dominate the response, despite the growth in amplitude of the b-wave and the appearance of easily identifiable OPs. Maximum a-wave amplitude was reached at P19, whereas the b-wave and OPs continued to grow until P30. Responses obtained at P60 suggest that a decline in ERG amplitude takes place during the second month of life. The latter findings are further supported by the group data illustrated in Figures 4A–C, which show a rapid and significant growth in a-wave amplitude to a maximum reached at P19 (P15: 175.45 $\pm$ 52.20 μ V; P19: 485.88 $\pm$ 44.45 μ V; $P < 0.05$), representing a 176.9% increase. At P60, the amplitude of the a-wave (366.16 $\pm$ 55.18 μ V) was 75.4% of that measured at P19. Similarly, the amplitude of the rod $V_{\max}$ increased from 160.81 $\pm$ 58.86 μ V measured at P15 to a maximum of 684.12 $\pm$ 136.64 μ V reached at P24, representing a significant 325.4% increase ($P < 0.05$), whereas the rod-cone b-wave was augmented by 428.3% ($P < 0.05$) from 212.42 $\pm$ 97.52 μ V at P15 to a maximum of 1122.11 $\pm$ 174.52 μ V at P30, suggesting a delayed maturation compared with the rod $V_{\max}$. At P60, the amplitudes of the rod $V_{\max}$ (609.74 $\pm$ 73.64 μ V) and of the rod-cone b-wave (919.77 $\pm$ 103.65 μ V)

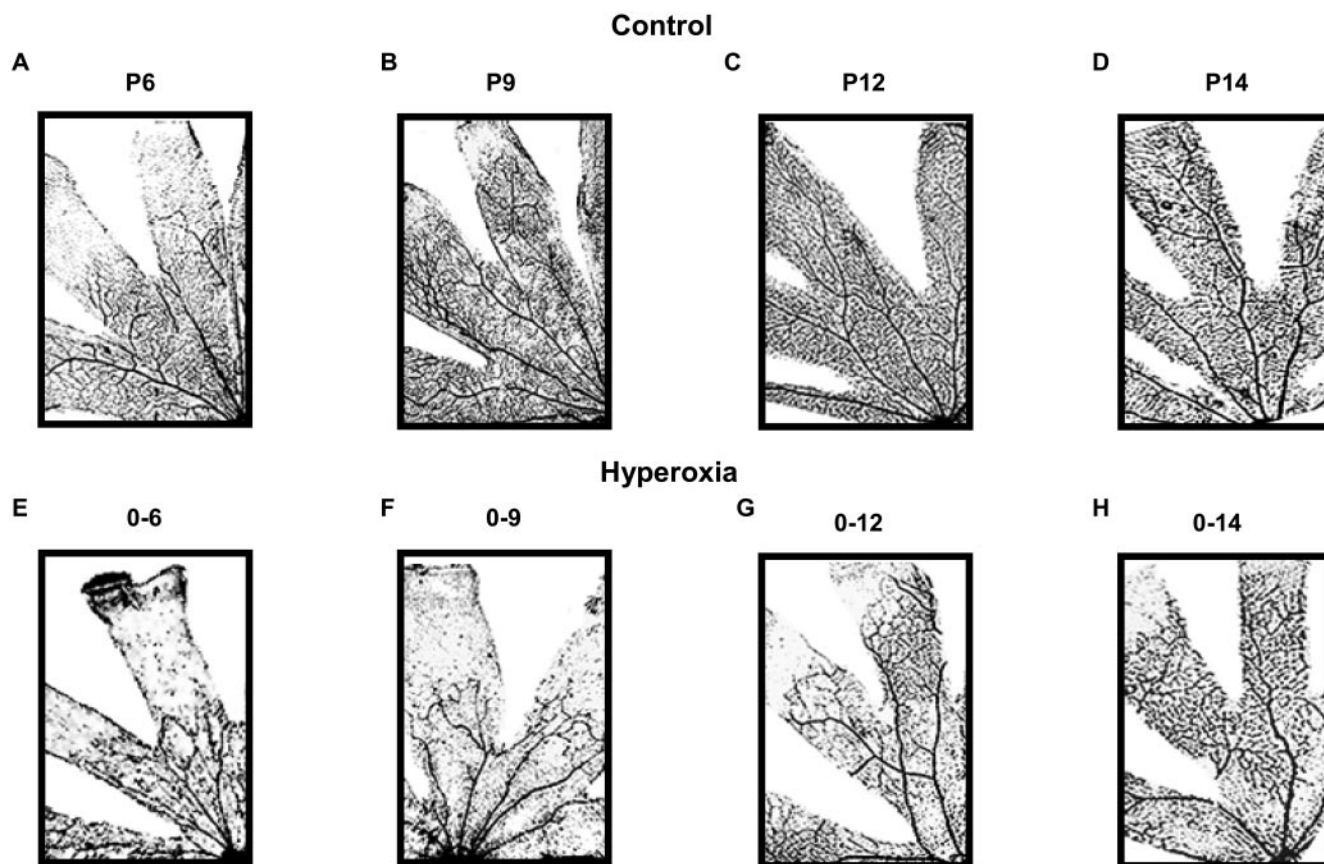


FIGURE 1. Representative retinal vascular flatmounts stained with ADPase from control animals (*top*) euthanatized at postnatal day 6 (A), 9 (B), 12 (C) and 14 (D), respectively, and from animals (*bottom*) that were euthanatized immediately after 6 (P0–6, E), 9 (P0–9, F), 12 (P0–12, G), and 14 (P0–14, H) days of oxygen exposure. Magnification, $\times 40$.

were 89.1% and 82.0% of maximum, respectively, most probably as a result of the normal aging process, as is documented elsewhere.^{10,12–14,25}

Postnatal hyperoxia markedly hampered the normal maturation process of the scotopic ERG signal, as exemplified in Figure 3B. As in normal rats, responses obtained from hyperoxic rats on eye opening (P15) were of negative morphology due to a prominent a-wave and a nearly absent b-wave. Again, the rising phase of the remnant b-wave was devoid of OPs. With time, there was a gradual growth in amplitude of the ERG components to reach maximum amplitude at P24, where the morphology of the resulting waveform was reminiscent of the ERG recorded at P17 in control rats. The increase was then followed by a gradual decline in ERG amplitude, which was most pronounced for the b-wave and OPs. Group data analysis (Figs. 4A–C) confirmed the relative resistance of the a-wave maturation process to postnatal hyperoxia. Results indicate a rapid and significant growth in a-wave amplitude to a maximum reached at P24 (P15: $166.42 \pm 49.81 \mu\text{V}$; P24: $445.23 \pm 57.40 \mu\text{V}$, $P < 0.05$) representing a 167.5% increase ($P < 0.05$). It is worth mentioning that, although delayed by 5 days, the maximum a-wave amplitude reached in hyperoxic rats is not significantly different from that reached in normal rats ($445.23 \pm 57.40 \mu\text{V}$ and $485.88 \pm 44.45 \mu\text{V}$, respectively; $P > 0.05$). At P60, the amplitude of the a-wave ($314.21 \pm 47.29 \mu\text{V}$) was 70.6% of that measured at P24 and 85.8% of that measured in age-matched control animals. Similarly, the amplitude of the rod V_{max} increased from nonrecordable at P15 to a maximum of $290.70 \pm 99.52 \mu\text{V}$ at P24 (42.5% of age-matched control amplitude; $P < 0.05$), whereas the rod-cone b-wave was

augmented by 295.2% ($P < 0.05$) from $121.35 \pm 52.41 \mu\text{V}$ at P15 (57.1% of control amplitude) to a maximum of $479.55 \pm 101.32 \mu\text{V}$ at P24 (47.2% of age-matched control amplitude; $P < 0.05$).

Maturation of the Photopic ERG

Similar to the results reported for the scotopic ERG parameters, the course of maturation of the photopic b-wave did not occur at the same rate among animals raised in the different oxygen environments (Fig. 3).

In normal rats, the photopic ERG obtained on eye opening (P15) was identifiable, though it was of low voltage and lacking well-demarcated OPs. As the rat aged, the amplitude of the photopic b-wave and OPs grew gradually to reach a peak at P30, after which it declined. As shown in Figure 4D, there was a significant (529.7%) increase in amplitude of the photopic b-wave from eye opening to age P30 (P15: $41.50 \pm 21.18 \mu\text{V}$; P30: $261.34 \pm 31.77 \mu\text{V}$, $P < 0.05$). At P60, the amplitude of the photopic b-wave ($230.82 \pm 19.50 \mu\text{V}$) declined to 88.3% of the maximum.

Again, postnatal hyperoxia markedly halted the normal maturation process of the photopic ERG signal as shown in Figure 3B. From nearly undetectable at eye opening (P15), the photopic b-wave grew gradually to reach maximum amplitude at P24, at which point the morphology of the resulting waveform was reminiscent of that recorded at P17 in control rats (Fig. 3A). As with the scotopic responses, the photopic ERG recorded at the end of the second month of life (P60) was smaller than that obtained 1 month earlier (P30). Group data analysis

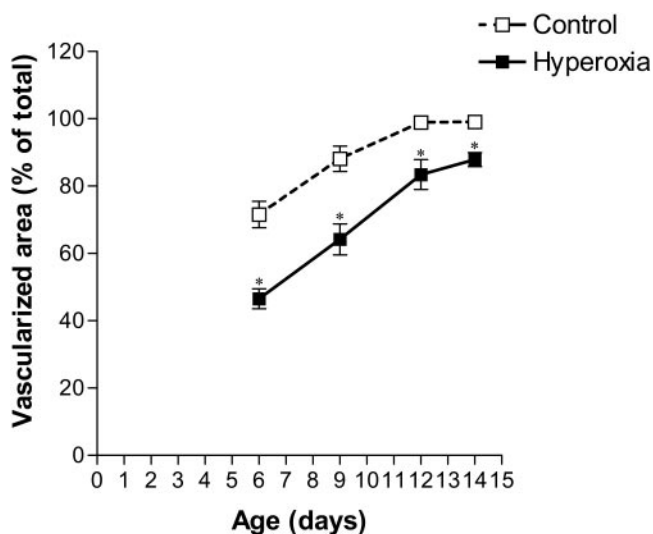


FIGURE 2. Graphic representation of retinal vascularization coverage (ordinate) as a function of age (days, abscissa) in the normoxic-cohort (open symbols, dashed line) compared with that observed in the hyperoxic-cohort (shaded symbols, solid line). Results are expressed as a percentage of completely vascularized retinas at P14 (control) $\pm$ 1 SD. *Significant differences from control at a given age; the Student's *t*-test ($P < 0.05$).

(Fig. 4D) confirms the high susceptibility of the photopic b-wave maturation process to postnatal hyperoxia. Results indicate a gradual growth in photopic b-wave amplitude to a maximum reached at P24 ($78.82 \pm 31.36 \mu\text{V}$), a value that is 35.4% of age-matched control subjects ($P < 0.05$). Of interest, this peak in amplitude occurred 6 days earlier than that observed in the normoxic cohort (P30). Finally, at P60, the amplitude of the photopic b-wave ($77.44 \pm 35.95 \mu\text{V}$) is 98.2% of that measured at P24 ($P > 0.05$) and 33.5% of that measured in age-matched control rats ($P < 0.05$).

It is noteworthy that in control rats the reduction in amplitude noted between P30 and P60 is significant for the a-wave, the rod-cone b-wave, and the cone b-wave, whereas it was not for the rod V_{max} . In contrast, in exposed rats, only the a-wave's amplitude was reduced significantly between P30 and P60,

whereas the other ERG parameters remained basically the same.

Finally, to identify which of the ERG components was the most affected by the postnatal hyperoxic regimen, a ratio comparing the maximum amplitude in exposed rats with the maximum amplitude in normal rats was used. Results obtained are graphically represented in Figure 5. Calculations were performed on a-wave and rod V_{max} data generated at P19 and P24, respectively, and on rod-cone b-wave and cone b-wave data at P30, since these time points correspond to each respective peak in the normal maturation process, as described earlier. The resultant a-wave ratio of $75.06\% \pm 7.06\%$ at P19 confirms our initial impression that the a-wave is only minimally susceptible to postnatal hyperoxia. This ratio further increased to $91.50\% \pm 11.85\%$ when re-evaluated at P24, suggesting that although the peak in a-wave maturation after hyperoxia was delayed by 5 days (occurring at P24 instead of P19, as in normal eyes), it nonetheless almost completely developed, as observed in Figure 1. In comparison, the development of the scotopic b-wave, including the rod V_{max} and mixed rod-cone response, was compromised to a greater extent, as evidenced with ratios of $42.50\% \pm 14.52\%$ and $39.69\% \pm 13.85\%$, respectively. Of interest, however, despite its attenuated amplitude, the rod V_{max} ratio is significantly greater ($P < 0.05$) than that of the cone b-wave ($25.40\% \pm 9.70\%$), suggesting an overall greater susceptibility of cone function to postnatal hyperoxia compared with pure rod function, a finding that supports previous claims of ours.^{12,13}

Effect of Hyperoxia on Retinal Cytoarchitecture

The maturation-induced changes in retinal function described earlier were also accompanied by changes in retinal structure. Representative retinal cross sections obtained at postnatal days 15, 17, 19, and 24 from control and oxygen-exposed animals are shown in Figure 6. Given that our previous studies^{12,14,15} showed that postnatal hyperoxia affects only the thickness of the outer plexiform layer (OPL) and the horizontal cell (HC) count, data analysis was limited to these two cytoarchitectural features. Group data are graphically reported in Figure 7. In control rats, the thickness of the OPL grew slightly, but not significantly, from eye-opening to P24 (P15: $6.82 \pm 2.09 \mu\text{m}$, P24: $9.62 \pm 1.06 \mu\text{m}$; $P > 0.05$). In contrast, the OPL of the

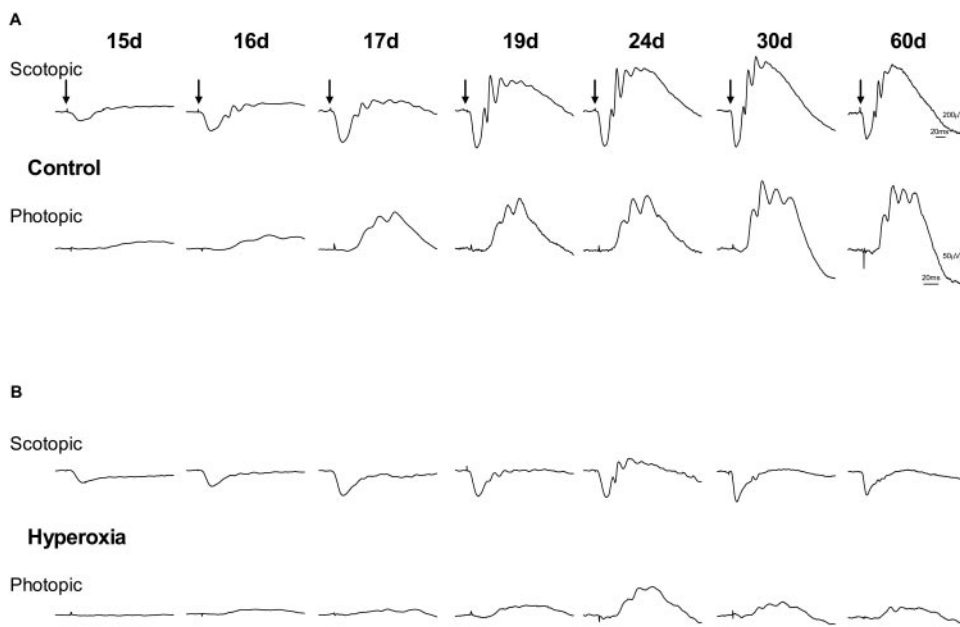


FIGURE 3. Representative scotopic (mixed rod-cone, flash intensity: $0.60 \log \cdot \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$) and photopic (flash intensity: $0.9 \log \cdot \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$, background: $30 \text{ cd} \cdot \text{m}^{-2}$) ERGs obtained from animals raised in normoxia (21% O_2 , top tracings, A) and hyperoxia (80% O_2 , bottom tracings, B) from postnatal day 0 through 14. Recordings were obtained at postnatal days 15, 16, 17, 19, 24, 30, and 60, respectively. Calibrations: horizontal, 20 ms; vertical, 200 μV for scotopic mixed rod-cone responses and 50 μV for photopic responses. All tracings include a 20-ms prestimulus baseline; vertical arrows: flash onset.

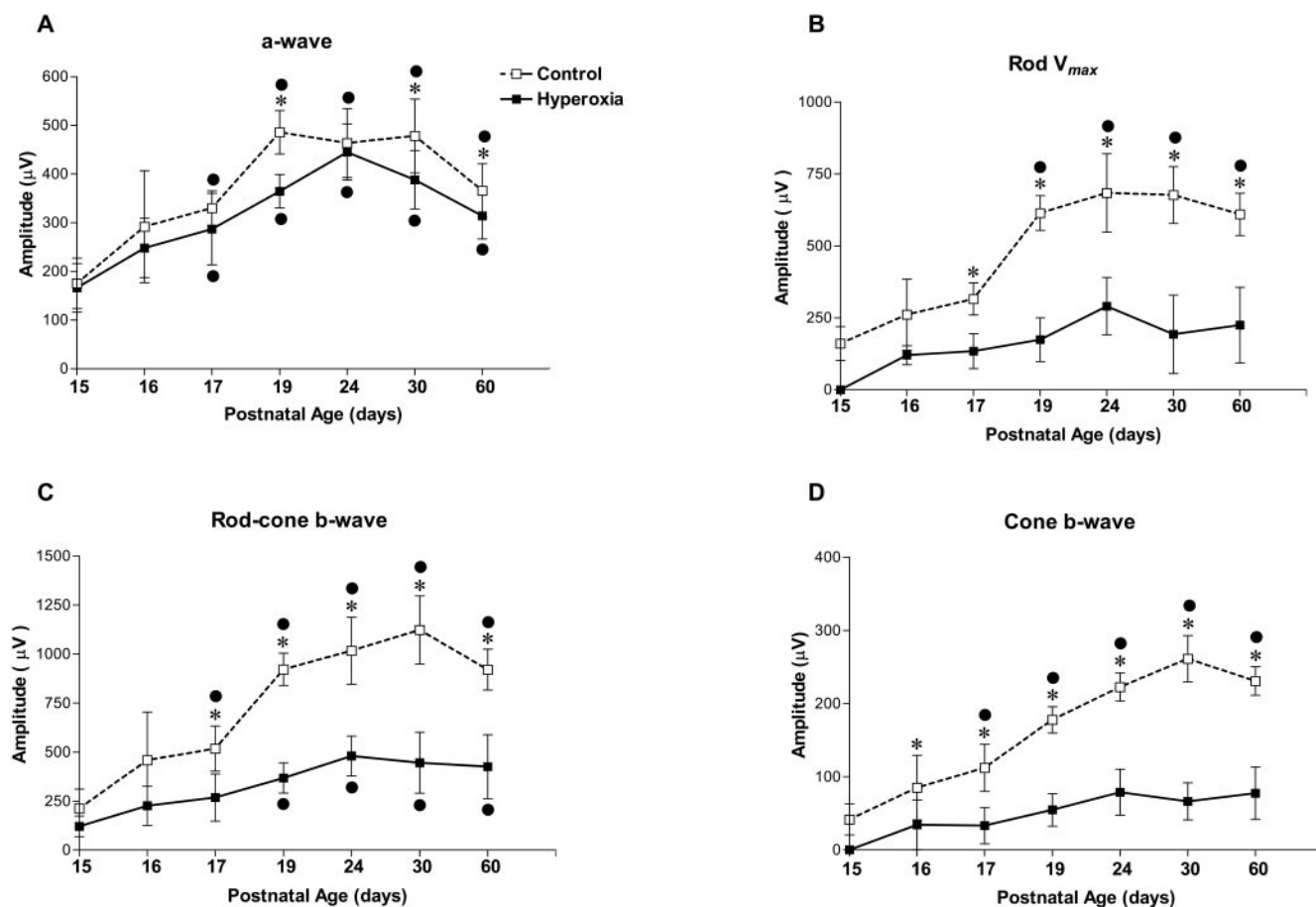


FIGURE 4. Graphic representation of a-wave (A), rod V_{max} (B), rod-cone b-wave (C), and photopic b-wave (D) amplitudes (ordinate in microvolts) obtained at postnatal days 15, 16, 17, 19, 24, 30, and 60, respectively (abscissa in days). Amplitudes from control animals raised in normoxia (open symbols, dashed line) are compared with those obtained from animals raised in hyperoxia (shaded symbols, solid line). *Significant differences between normoxia- and hyperoxia-reared groups for each given age; Student's *t*-test ($P < 0.05$). Significant differences within normoxic or hyperoxic cohorts were identified with a one-way ANOVA followed by a post hoc Tukey test. (●) Values found to be significantly different from those obtained at P15, or the time at which the earliest recordable response was obtained ($P < 0.05$). Results are given as the mean amplitude ± 1 SD.

hyperoxic-cohort reached maximum thickness at P16, after which a gradual and significant thinning occurred (P16: $5.42 \pm 0.84 \mu\text{m}$, P24: $2.39 \pm 1.44 \mu\text{m}$, $P < 0.05$). Furthermore, animals that were raised in 80% O_2 always showed a thinner OPL than that in age-matched control rats, which reached significance at P16 (68.1% of control at P15 to 24.8% of control at P24; $P < 0.05$), confirming our previous demonstration of OPL susceptibility to postnatal hyperoxia.^{12,14,15} This hyperoxia-induced OPL thinning was also accompanied by a significant reduction in the total number of HCs, as reported in Figure 6. In control rats, we noticed a slight but significant attrition of the HC count with age (P15: 3.53 ± 0.12 cells, P24: 2.40 ± 0.35 cells; 68.0% of P15, $P < 0.05$). The above contrasts with the severe reduction in HC count noted in the hyperoxic cohort from P15: 0.93 ± 0.23 cells (26.3% of age-matched control HC count, $P < 0.05$) to P24: 0.28 ± 0.25 cells (11.7% of age-matched control HC count, $P < 0.05$).

DISCUSSION

Previous studies have shown that postnatal exposure to hyperoxia severely damages retinal structure (e.g., significant thinning of the OPL and attrition of the HC count) and function (significant reduction in amplitude of photopic and scotopic ERGs).^{10–18} These irreversible sequelae are preceded by a typical and reversible vasculopathy reminiscent of that known to

characterize the human form of this retinal disorder, retinopathy of prematurity (ROP). Although previous studies have also evidenced significant reductions in vascular coverage after postnatal hyperoxia in rats,^{5,8,9,19,26–28} few studies have examined the differential susceptibility of retinal vasculature to postnatal hyperoxia as a function of the degree of vascular maturity reached at the time of hyperoxic exposure. To our knowledge, however, our study is the first to show that despite the damage that is observed within the first week or so of exposure (e.g., P0–P6 and P0–P9 in Figs. 1 and 2), the retinal vascular growth process appeared to fight this oxidative stress, to achieve nearly full coverage while remaining under hyperoxic exposure throughout the first 2 weeks of life. The window of blood vessel plasticity described by Benjamin et al.²⁹ would further support our findings, in which early postnatal exposure to hyperoxia (P4–P6) similarly resulted in vaso-obliteration. This phenomenon is explained by the increased susceptibility of immature vessels that have not yet acquired pericytes at such an early age, whereas vessels that are more mature appear to better withstand an equivalent stress that occurs later on.²⁹ Furthermore, VEGF has been shown to play a pivotal role in vascular development and retinal coverage, and hyperoxia counteracts its expression, which inevitably results in underdeveloped retinal vasculature.³⁰ In fact, exogenous VEGF administration has been shown to act as a vascular survival factor, rescuing vasculature from hyperoxia-induced

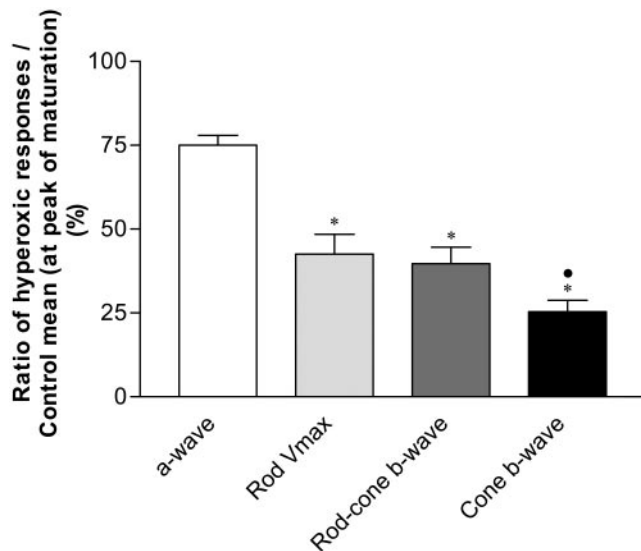


FIGURE 5. The mean ratios of individual ERG measures from exposed groups/control mean (at maximum value) were calculated for each parameter and are represented graphically. Results were obtained based on a-wave and rod $V_{\max}$ data generated at P19 and P24, respectively, and on rod-cone b-wave and cone b-wave data at P30, which are the times corresponding to each of their respective peaks in maturation. A one-way ANOVA followed by a Tukey post hoc test comparing data obtained from each group was used to detect statistically significant differences between ratios. *Significantly different from the a-wave ratio ($P < 0.05$); (●) significantly different from the rod $V_{\max}$ ratio ($P < 0.05$). Results are given as the mean ratio ± 1 SD.

destruction.³¹ Of interest, however, other studies have also shown that prolonged hyperoxia (during which VEGF levels are suppressed) paradoxically results in an enhanced revascularization process,^{19,32} thus placing less of an emphasis on a role for VEGF. For example, vaso-obliteration occurred to a greater extent in rats exposed to hyperoxia from P7 to P9 than in those exposed from P7 to P12.¹⁹ Similarly, mice that are maintained in a continuous hyperoxic environment undergo more rapid revascularization than those that are returned to room air after exposure, thought perhaps to result from the preservation of astrocytes and Müller glia in these conditions.³² Concomitant increases in NO levels via increased eNOS expression with early hyperoxia-induced vaso-obliteration and with subsequent revascularization after prolonged hyperoxia have also suggested a cytotoxic and cytoprotective role for NO, respectively, in each of these conditions.^{19,33} Whether similar mechanisms are responsible for enabling the retina to overcome vascular deficits that are initiated early on in our model of continuous exposure, however, requires further elucidation. Nevertheless, previous studies, in addition to our findings reported herein, have shown that despite full vascular coverage, permanent structural and functional damage inevitably occurs.^{10–15} It is important to keep in mind, however, that many of the results referred to from previous studies were obtained from mature or nearly mature animals (ranging from P18 to P60) and at a time interval remote from the cessation of the oxidative insult. With this study, we confirm that the pathophysiological sequence of events that ultimately yields the clinical picture described earlier was initiated while the rats were still in the hyperoxic regimen, at least when the retinal cytoarchitecture is considered. Given that these cytoarchitectural anomalies appear to be limited to OPL thinning and reduced HC counts, it is not surprising that there is no evidence of significant functional deficits, as determined with the ERG a-wave in the first 2 days or so after the opening of the

eyes (and consequently after the cessation of the hyperoxic regimen, as per our protocol), while scotopic and photopic b-waves already tend to be attenuated in amplitude. These findings would therefore suggest that OIR is initiated at a postreceptor level, where both outer retinal structure and function remain intact, whereas synaptic impairment at the level of the OPL prevents signal transmission to the inner retina. Our results are in accord with previous studies that documented early differences between control and hyperoxia-exposed animals on eye opening at P13 and P14, 2 days after and immediately after the cessation of hyperoxic exposure, respectively.^{17,18} While hyperoxia resulted in attenuated b-wave amplitudes compared with those in control rats raised in room air, the a-wave appeared to be either similarly¹⁷ or less¹⁸ affected. Evaluation at further time points revealed even greater scotopic b-wave attenuations in hyperoxic rats compared with control as evidenced at P17,¹¹ P18,¹⁷ and at P21,¹⁸ whereas the a-wave amplitude improved with age, reaching values virtually identical with the control at P18,¹⁷ at P21, and beyond.¹⁸ These findings of impaired postreceptor function that occur shortly after the hyperoxic episode may therefore be explained by the thinning of the OPL, which we have identified as early as eye opening (P15).

Previous studies of ours also suggested a normal age-related decline in amplitude of scotopic and photopic ERG parameters between P30 and P60,^{12–15,25} findings that are corroborated with results reported herein (Fig. 4). Of interest, only the a-wave showed a similar age-dependent amplitude attenuation after exposure to hyperoxia, whereas the rod $V_{\max}$, rod-cone b-wave, and photopic b-wave did not. A study by Penn et al.¹⁸ revealed a similar decline in the scotopic b-wave amplitude (by ~40%) between control animals aged 5 and 9 weeks (from P35 to P63, time points that resemble those used in the present study), whereas no such findings were evident in the hyperoxic cohort. No other significant gain or decline in amplitude was observed in recordings obtained throughout 16 weeks of age (P112), thereby excluding the likelihood of any delay in this age-related decline. On the other hand, the a-waves obtained from animals raised in the different oxygen environments within that age-range were both similarly attenuated, and when taken together with the results just reported, further support our current findings. These results collectively suggest that whereas normal developing animals are subjected to a decline in retinal function [which is also accompanied by a normal thinning of the retinal layers between the time of eye opening (approximately P15) through adulthood],³⁴ a different picture emerges after exposure to hyperoxia. The thinning of the retina, which is likely to be at the root of the functional attenuation that takes place with age, most probably occurs through apoptotic mechanisms or otherwise, as a part of the normal aging process. As described earlier, hyperoxia that occurs from birth throughout P14 is of only little consequence on the a-wave, suggesting that the photoreceptor layer is resistant to this stress. Consequently, the normal cellular refinement of the outer retina that occurs with time (between P30 and P60) can still progress under conditions of excess oxygen. In contrast, hyperoxic exposure culminated in a compromised b-wave along with considerable damage to the generating cells, and of note, the irreversible reorganization of retinal structure and/or cell death that occurred in the postreceptor retinal layers of exposed animals appeared to be stationary once maximum amplitude was attained. This finding suggests that the damage triggered by hyperoxia precludes further deterioration of the retina, such as that which occurs in the normal aging process. The steps involved in this “protective” mechanism remain to be fully understood.

Unlike precocial species (such as humans, for example) that are born with eyes opened and relatively mature retinal struc-

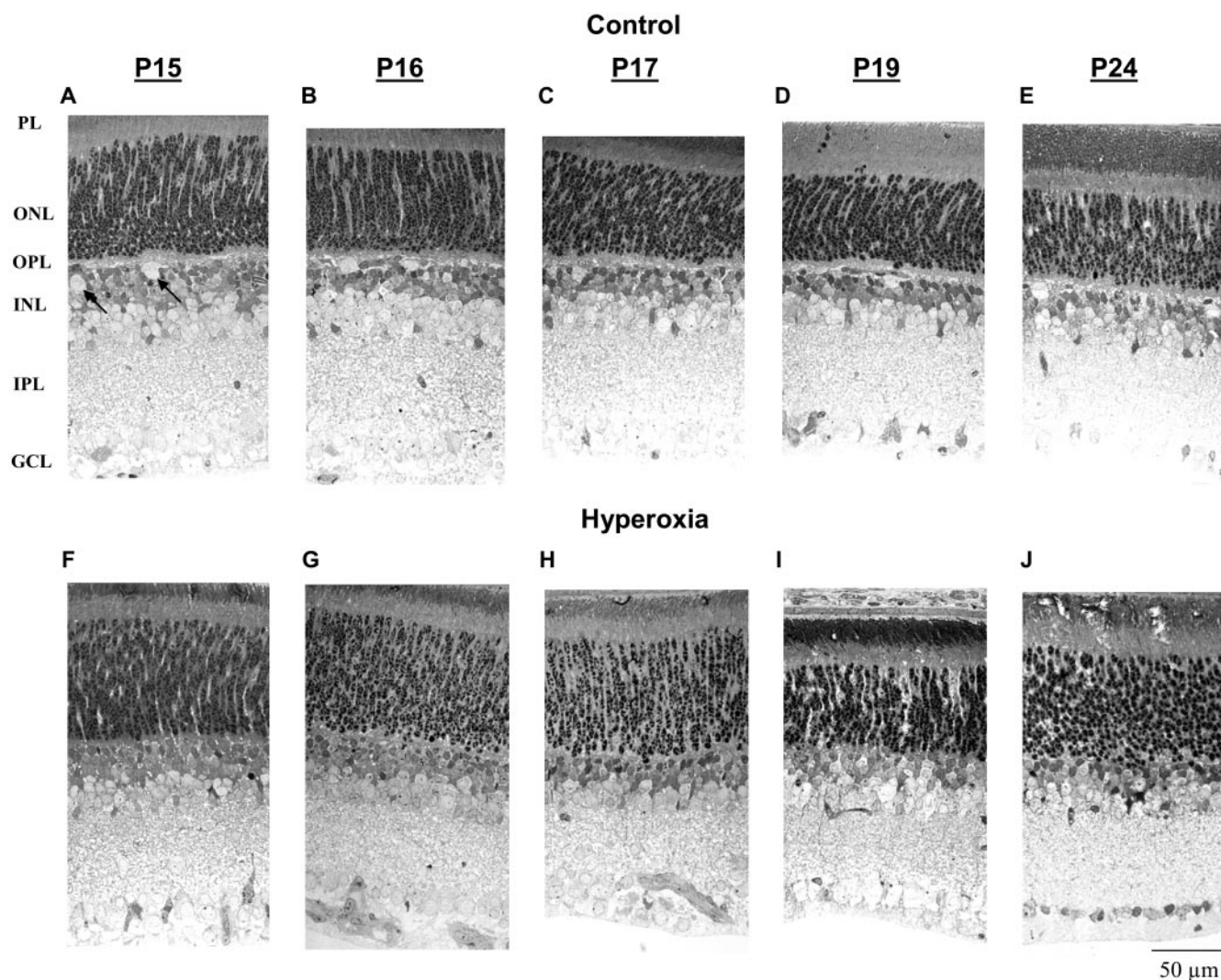


FIGURE 6. Representative retinal histologic sections (central retina) obtained from animals at P15 (control, A; oxygen-exposed, F), P16 (control, B; oxygen-exposed, G), P17 (control, C; oxygen-exposed, H), P19 (control, D; oxygen-exposed, I), and P24 (control, E; oxygen-exposed, J). *Oblique arrows:* HCs located within the INL. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 50 μ m.

ture and function,^{35–38} altricial animals (such as the rat and mouse) are born with their eyes closed and a relatively immature retina,^{39,40} comparable to a prematurely born human infant at 24 to 26 weeks of gestation.⁴ Consequently, the susceptibility of the different retinal elements to the oxidative insult could depend on the level of maturity that they reached at the time of the hyperoxic exposure and/or the duration of the hyperoxic exposure that they suffered (also dependent on their birth date). For example, it has been shown in mice that cones, ganglion cells, amacrine cells, and HCs are the first identifiable retinal cells at E14, whereas rods and Müller cells appear at birth.⁴¹ In a normal environment, this results in a well-organized maturation of retinal function, where development of saturated rod a- and b-wave amplitudes has been shown to occur between P12 and P30, which is related to rod outer segment (ROS) length and rhodopsin content levels.⁴² Similarly, photopic OPs have also been shown to reach maturity much later (P30) compared with the earlier maturation of scotopic OPs at P17.⁴³ Results obtained in our present study are in accordance with these findings, given that we observed a peak in the normal rod V_{max} amplitude at P24, whereas that of the photopic b-wave occurs at P30. Surprisingly, however,

the peak for both parameters after hyperoxic exposure is found at P24, suggesting that despite the attenuation in both rod and cone responses, the rod V_{max} still follows a normal developmental time course after hyperoxia, in contrast to the cone and rod-cone b-waves whose maturation peaks tend to fall short of normal. Furthermore, the attenuation in the cone b-wave amplitude at the height of maturation (as a function of normal, as per Fig. 5) is significantly more pronounced than that of the rod V_{max} . When taken together with the normal process of retinal cellular development, these findings suggest that the earliest existing structures (cones and HCs, for example) are those that are most susceptible to maturation-induced changes that follow postnatal hyperoxia. This concept could also explain the more severe impact of oxidative stress on HCs and the OPL (the layer of synaptic contact between photoreceptors and HCs and bipolar cells), as the former are more developed at birth, and would consequently be exposed to the hyperoxic regimen for a longer duration. Of interest, previous studies have described a role for free radicals in generating the pathogenesis of OIR.^{5,6,12,27,28,44,45} It has also been suggested that endogenous antioxidant defense systems may differ between rods and cones, rendering the latter system more sus-

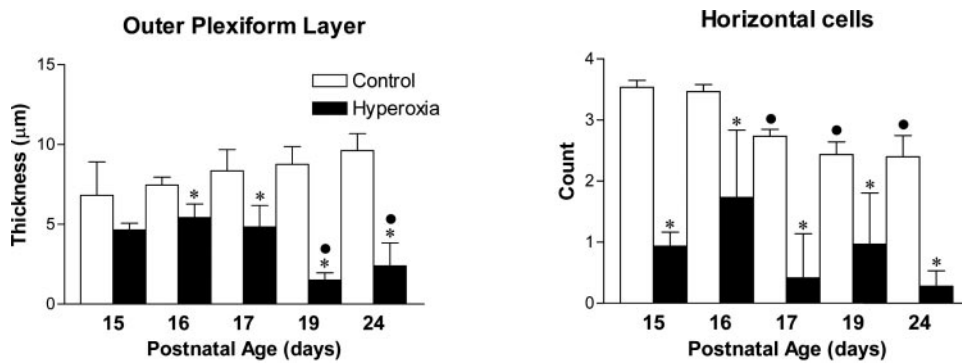


FIGURE 7. The mean thickness (in micrometers) of the OPL (A) and the mean HC count (B) in animals raised in normoxic and hyperoxic environments. Measurements were taken at P15, P16, P17, P19, and P24, respectively. *Significant differences between normoxia- and hyperoxia-reared groups at each given age found using the Student's *t*-test ($P < 0.05$). Significant differences within normoxic or hyperoxic cohorts were identified with a one-way ANOVA followed by Tukey post hoc test comparing data obtained from all age groups. (●) Significantly different values from those obtained at P15 ($P < 0.05$). Results are given as the mean thickness or cell count $\pm$ 1 SD.

ceptible in the face of reactive oxygen species induced by hyperoxia,¹³ or FeSO₄,⁴⁶ for example. Furthermore, our finding of a more potent effect of Trolox C, a vitamin E analogue with antioxidant capacity, on cone-mediated function in the hyperoxic retina, suggests to us that treatment is targeted to the areas that need it most.¹² Collectively, these results support the hypothesis whereby devastation of the cone pathway (which begins its development in utero) occurs to a greater extent than that of the rod pathway (which begins its development postnatally).

Our ability to demonstrate altered retinal function (with the exception of the a-wave) immediately after cessation of hyperoxic exposure would put less emphasis on the contribution of neovascularization to these manifestations, since this aggravated angiogenic process is known to be triggered after recovery in room air.^{8,9,47–50} Although in the present study we have not tried to quantify the level of neovascularization, our findings suggest that it may play a negligible role in the initial impairment of retinal function (at birth) compared with the devastating structural consequences (e.g., retinal detachment) that it could cause, should it fully manifest itself. If the above scenario also applies to the human counterpart, care will have to be taken when evaluating the functional consequences of hyperoxic exposure in human premature infants, despite the lack of significant evidence of underlying neovascularization.

In conclusion, posthyperoxia events, including the return to normoxia (e.g., relative hypoxia), and the resulting neovascularization have been deemed largely responsible for the pathogenesis of OIR and its human form, ROP. The results obtained with the present study suggest that alterations in retinal cytoarchitecture concurrent with hyperoxic exposure in addition to the arrest in functional maturation that directly follows the exposure regimen in neonatal rats may play an equally important role. Whether the above are induced as a result of the vasculopathy or occur independently remains to be further elucidated.

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Structural and Functional Consequences of Trolox C Treatment in the Rat Model of Postnatal Hyperoxia

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PURPOSE. Previous studies have shown that newborn rats exposed to hyperoxia within the first 2 weeks of life develop vasculopathy in addition to permanent changes in retinal structure and function. It has also been suggested that free radicals may be the source of these pathologic effects. Trolox C, a water-soluble analogue of vitamin E, was previously shown to limit the vascular consequences of exposure to postnatal hyperoxia. The aim of this study was to investigate whether trolox C could also help prevent the functional (electroretinography) and structural (retinal histology) consequences associated with oxygen-induced retinopathy (OIR).

METHODS. Newborn albino Sprague-Dawley rats exposed or not exposed to hyperoxia received daily injections of trolox C in doses of 300, 600, and 900 $\mu\text{g/kg}$ (total volume, 50 μL). The effect of treatment was evaluated through electroretinography and retinal histology.

RESULTS. Although trolox C tended to have a retinoactive effect on the normal retina, normalization of the hyperoxia-treated group to hyperoxic control and of the normoxia-treated group to normoxic control revealed that the a-wave remained relatively unaffected by hyperoxia exposure and by treatment with trolox C, the efficacy of trolox C at doses of 600 and 900 $\mu\text{g/kg}$ largely outweighed the retinoactive effect, and the oscillatory potentials (OPs) benefited to the greatest extent from trolox C treatment. Furthermore, trolox C was able to limit the reduction in outer plexiform layer thickness but not the concomitant reduction of the horizontal cell count, each of which is associated with OIR.

CONCLUSIONS. These results show that, as had been previously demonstrated with retinal vasculature, trolox C limited the retinal functional and structural damages inherent in the rat model of OIR. However, despite treatment, there were still signs (albeit less severe) indicative of OIR. This suggests, as previously advanced, that the pathophysiology of OIR is not solely caused by the action of free radicals or that trolox C is inadequate in treating all aspects of OIR. (*Invest Ophthalmol Vis Sci.* 2006;47:1101-1108) DOI:10.1167/iovs.05-0727

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Retinopathy of prematurity (ROP) is a potentially blinding retinal disorder that affects small prematurely born infants.¹ Supplemental oxygen is often delivered to these infants to maintain adequate blood levels and stable pulmonary status.² It has been suggested that the formation of free radicals in the retina may result from the exposure of these premature infants to excessive oxygen, given that they are deficient in antioxidant defenses.³ Some consequences associated with the formation of free radicals include vasoconstriction and vasoobliteration of the developing retinal vessels, which are often followed by neovascularization on the return to normoxia.^{4,5} These clinical signs are used to identify and grade the severity of the human and animal forms of this retinopathy.⁵⁻¹⁵ It is the later stages that cause the most severe consequences of postnatal hyperoxia.

Previous studies have shown that vitamin E therapy results in decreased vasoobliteration of retinal blood vessels in newborn rats exposed to hyperoxic conditions.^{15,16} However, high doses of vitamin E treatment were necessary to produce a measurable protective effect against free radicals, a factor potentially leading to toxicity. It thus appears that to be effective, the antioxidant of choice must not only be able to enter the cells and subcellular compartments in which reactive oxygen species (ROS) exist¹⁷ but, more important, must be safe to use.

Trolox C is a water-soluble vitamin E analogue previously used for antioxidant therapy in various models in which ROS are formed, including myocardial injury¹⁸ and diabetic retinopathy.¹⁹ This was also the antioxidant used by Penn et al.²⁰ in the rat model of oxygen-induced retinopathy (OIR), by which they showed that the retinas of newborn rats exposed to oxygen while receiving trolox C had greater vascular coverage and higher capillary density, suggesting that trolox C could help prevent the vascular consequences of OIR. However, apart from vasculopathy, OIR was also shown to include cytoarchitectural and functional anomalies of the retina. Newborn Sprague-Dawley rats exposed postnatally to hyperoxia were shown to develop severe electroretinographic anomalies characterized by marked attenuation of the b-wave and oscillatory potentials of the electroretinogram (ERG) and relative sparing of the a-wave, suggesting that the functional deficit in OIR was located at a postreceptor site.²¹⁻²⁶ This finding correlated well with the reported decrease in the number of horizontal cells and the reduction in thickness of the outer plexiform layer (OPL) along with the sparing of the photoreceptor layer, which was also shown to characterize this type of retinopathy.^{21,22} Of interest, previous studies also showed a lack of correlation between the vascular and the retinal structural-functional consequences of OIR, in which the retinal vasculature often resumed normalcy shortly after the return to normoxia, whereas the electroretinographic and cytoarchitectural anomalies became permanent features of the hyperoxic rats.²¹⁻²⁴ Therefore, although the retinal vascular anomalies of OIR rats were shown to be partly prevented by free radical scavengers,²⁰ it is not yet established whether they can also exert a protective effect on both structural and functional levels. Because of these discrepancies, this study was con-

ducted to determine whether trolox C could also protect the structure and function of the retina after postnatal hyperoxia.

METHODS

The experimental protocol was approved by the McGill University/Montreal Children's Hospital Research Institute Animal Care committee according to the guidelines of the Canadian Council on Animal Care. Experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Newborn litters of Sprague-Dawley (SD) rats (Charles River Laboratories, St. Constant, Quebec, Canada) were exposed to 80% oxygen (mixture of medical grade 100% O₂ and room air measured with a MaxO₂ ceramtec oxygen meter, model OM25-ME; Medicana Inc., Montreal, Canada) from postnatal day 0 through postnatal day 14 for 22.5 hours daily, interrupted three times for a 30-minute period of normoxia (21% oxygen).^{21,26} There were eight experimental groups in total. The hyperoxic cohort (postnatal exposure to 80% O₂) was subdivided into four experimental groups: one that received oxygen only (i.e., control hyperoxic group, $n = 16$) and three that were exposed to oxygen (80%) while receiving trolox C (hyperoxia-treated group) at concentrations of 300 ($n = 8$), 600 ($n = 11$), and 900 ($n = 8$) $\mu\text{g/kg}$, respectively. The normoxic cohort (postnatal exposure to 21% O₂) consisted of one group that was raised under normoxic conditions without receiving the antioxidant treatment (i.e., control normoxic group, $n = 16$) and three groups also raised in normoxic conditions while receiving trolox C (normoxia-treated group) at concentrations of 300 ($n = 6$), 600 ($n = 5$), and 900 ($n = 5$) $\mu\text{g/kg}$, respectively. Because it was previously shown that postnatal hyperoxia occurring within the first week of life had a negligible effect on retinal structure and function^{21,26} and to minimize trauma to the newborn pups, intraperitoneal injections of trolox C (300, 600, and 900 $\mu\text{g/kg}$, respectively), dissolved in 10% EtOH, were administered starting at postnatal day 5 and continuing through postnatal day 14. Finally, to avoid pulmonary complications that are known to develop in adult rats raised in a hyperoxic environment, mothers of the litters were alternated between normoxic and hyperoxic conditions every 24 hours.

Electroretinography

ERGs and oscillatory potentials (OPs) were recorded with a data acquisition system (Biopac MP 100WS; Biopac Systems Inc., Goleta, CA) using a method that was previously reported.^{21,22,26} Briefly, after a period of 12 hours of dark adaptation, rats were anesthetized (under dim red light illumination) with intramuscular injections of ketamine hydrochloride (85 mg/kg) and xylazine (6 mg/kg). Pupils were dilated with 1% cyclopentolate hydrochloride (Mydracil solution; Alcon Laboratories, Fort Worth, TX), and full-field ERGs were recorded (10,000 $\times$, 1- to 1000-Hz bandwidth, 6-dB attenuation) along with OPs (50,000 $\times$, 100- to 1000-Hz bandwidth, 6-dB attenuation) using analogue preamplifiers (P511; Grass Instruments, Quincy, MA). A fiber electrode (DTL; 27/7 X-Static silver-coated conductive nylon yarn; Sauquoit Industries, Scranton, PA) was placed on the cornea and was used as the active electrode. Hydroxymethylcellulose (2%; Gonioscopic solution; Alcon Laboratories) was placed on the cornea thereafter to prevent corneal desiccation and to hold the DTL fiber in place. A disc electrode (model E6GH; Grass Instruments) was placed in the mouth, and a needle electrode (model E2; Grass Instruments) was inserted into the tail to serve as the reference and to ground electrodes, respectively. The rats were then placed in a light-proof recording chamber of our design that included the light stimulus and background light.^{21,26} Scotopic responses were evoked to flashes of white light spanning a 7.2-log unit range in 0.3-log increments (maximal intensity, 0.6 log candelas/m² [log cd·s/m²]; average, 2–5 flashes; interstimulus interval, 10.24 seconds), whereas photopic responses were evoked to flashes of white light of 0.9 log cd·s/m² delivered after more than 15 minutes of light adaptation to a background of 30 cd/m² (average, 20

flashes; interstimulus interval, 1 second) to avoid a light adaptation effect.²⁷ A first set of recordings was obtained at 30 days of age, at which point rod and cone functions are known to be fully mature.^{28–30} Furthermore, to determine whether the functional abnormality that was created was permanent, a second set of recordings was obtained at postnatal day 60.

Histology

Structural changes were assessed on specimens collected at 60 days of age. Histologic sections (2- μm cross-sections) of whole-eye mounts were embedded in epoxy resin (Epon; Resolution Performance Products, Houston, TX) and stained with toluidine blue according to a method previously described.²² Measurements of OPL thickness and horizontal cell counts were performed on 250- μm -wide fields of the central retina. Three retinas per group were analyzed, and an average of 10 measurements from each was obtained. Pictures were obtained using a photograph microscope (Acti; Zeiss, Oberkochen, Germany).

Data Analysis

Amplitudes of ERG components were measured according to a method previously described.^{21,26} Briefly, the amplitude of the a-wave was measured from baseline to trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Oscillatory potentials were measured in a similar fashion from the preceding trough to the peak of the OP evaluated and were reported individually or as the sum of OPs ($\text{SOP} = \text{OP}_2 + \text{OP}_3 + \text{OP}_4 + \text{OP}_5$).

For each animal, scotopic luminance-response function curves were derived by plotting b-wave amplitudes against flash intensities. A sigmoidal-intensity response regression curve was then used to fit the data (Prism 3.00 software; GraphPad, San Diego, CA), from which the rod V_{max} (maximal rod response) was calculated. In addition, the ERG response evoked to the brightest flash delivered in scotopic conditions (the mixed rod-cone response) was also included in the analysis. Two-way repeated measures ANOVA ($P < 0.05$) with Bonferroni post-tests were used to determine the effect of hyperoxia and trolox C treatment on the different parameters of the ERG and on retinal histologic structures, with maturation (30 and 60 days) as the repeated factor and treatment group as the independent factor. Data are presented as the mean $\pm$ 1 SD.

Finally, because ERG measurements were repeated at 30 and 60 days of age, we devised the retinal maturation index as a means to determine the change in retinal function resulting from the normal aging process. This index was calculated by taking the average of the percentage difference between ERGs obtained at 30 and 60 days for each individual rat. One-way ANOVA followed by Tukey's honest significance difference (HSD) test was used to detect significant differences. Data are presented as mean percentage change in amplitude $\pm$ 1 SD.

RESULTS

Figure 1 shows representative scotopic (rod V_{max} , mixed rod-cone ERGs, and mixed rod-cone OPs) and photopic (cone ERG and cone OPs) retinal responses obtained from four of the eight study groups. The first group illustrated in Figure 1 consists of rats raised in room air (normoxic control). They are compared with rats raised in room air while receiving 600 $\mu\text{g/kg}$ trolox C from postnatal day 5 through postnatal day 14 (normoxia-treated), with rats raised in a hyperoxic environment from birth through postnatal day 14 (hyperoxic control), and with rats exposed to postnatal hyperoxia for 14 days from birth while receiving 600 $\mu\text{g/kg}$ trolox C (hyperoxia-treated) from postnatal day 5 through postnatal day 14. ERG responses were obtained twice, at 30 days (upper tracings) and at 60 days (lower tracings) of age. Amplitude measurements are reported in Table 1.

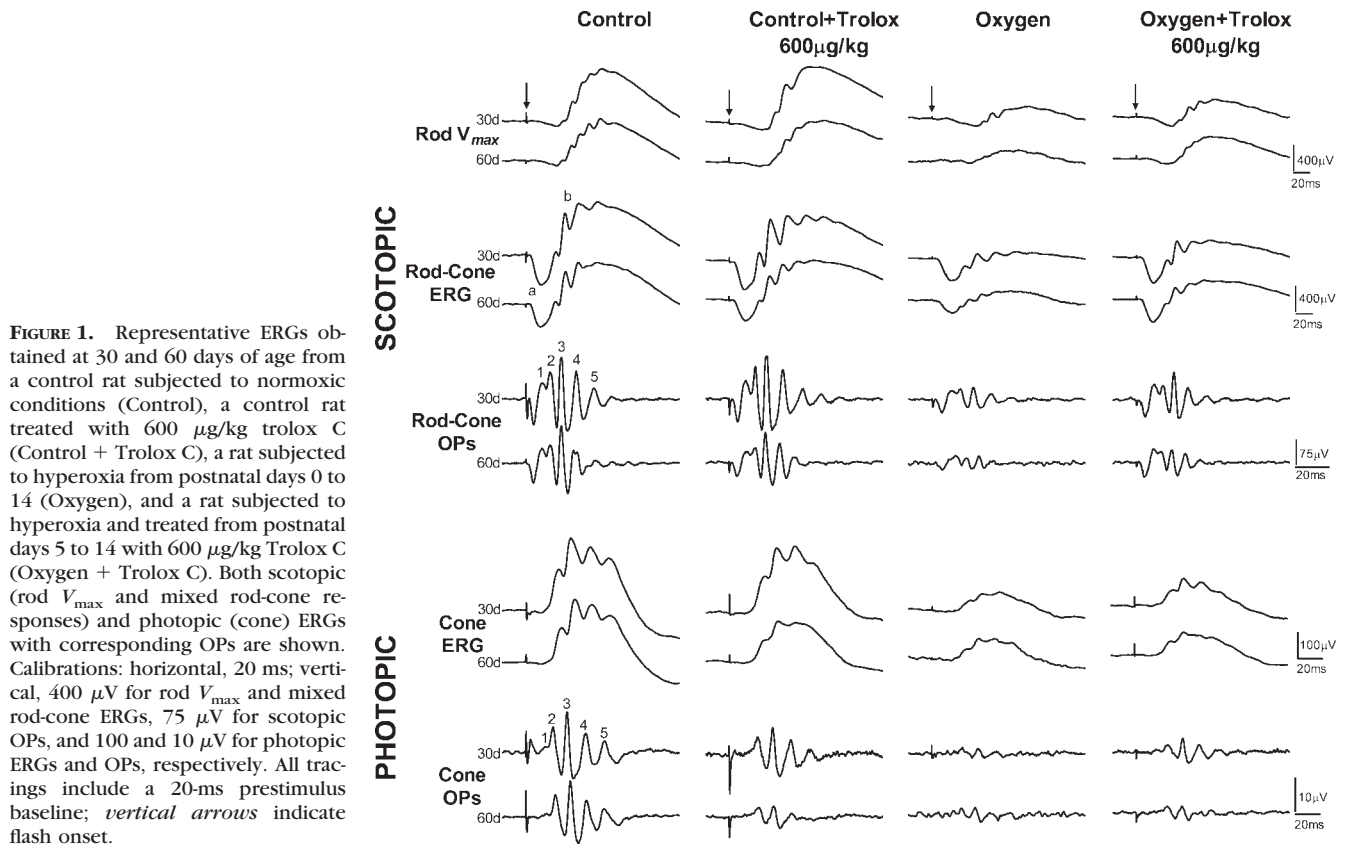


FIGURE 1. Representative ERGs obtained at 30 and 60 days of age from a control rat subjected to normoxic conditions (Control), a control rat treated with 600 µg/kg trolox C (Control + Trolox C), a rat subjected to hyperoxia from postnatal days 0 to 14 (Oxygen), and a rat subjected to hyperoxia and treated from postnatal days 5 to 14 with 600 µg/kg Trolox C (Oxygen + Trolox C). Both scotopic (rod V_{max} and mixed rod-cone responses) and photopic (cone) ERGs with corresponding OPs are shown. Calibrations: horizontal, 20 ms; vertical, 400 µV for rod V_{max} and mixed rod-cone ERGs, 75 µV for scotopic OPs, and 100 and 10 µV for photopic ERGs and OPs, respectively. All tracings include a 20-ms prestimulus baseline; vertical arrows indicate flash onset.

Amplitude attenuation of the scotopic and photopic b-waves, scotopic a-wave, and OPs can be observed in the hyperoxic control rat compared with the normoxic control rat. As shown in Table 1, amplitudes of the rod V_{max} and the scotopic and photopic SOPs measured from hyperoxic rats treated with 600 µg/kg trolox C were nearly double the size of those measured from hyperoxic control rats ($P < 0.05$). There was also a slight, but not significant, enhancement ($P > 0.05$) of the rod-cone b-wave and photopic b-wave amplitudes, whereas the amplitude of the a-wave was unaltered by trolox C treatment (Table 1).

Results presented in Table 1 also suggest that trolox C might alter the functioning of the normal (unexposed) retina. The scotopic ERG responses (rod V_{max} and rod-cone b-wave) of control treated rats were larger (but not significantly; $P > 0.05$) than those of untreated controls, whereas the photopic SOPs were significantly smaller ($P < 0.05$) than untreated controls. Consequently, to dissociate the protective effect that trolox C exerted on the retinas of newborn rats exposed to hyperoxia from its retinoactive effect observed in the normal retina, we normalized ERG amplitude measurements obtained from each normoxia-treated rat (trolox C 300-, 600-, and 900-µg/kg

TABLE 1. Group Data

Parameters	Age (d)	Control	Control+Trolox (600 µg/kg)	Oxygen	Oxygen+Trolox (600 µg/kg)
Scotopic a-wave	30	485.2 ± 78.3	468.5 ± 78.5	363.1 ± 74.4*	376.9 ± 72.0*
	60	358.4 ± 61.5†	317.8 ± 42.8†	294.1 ± 50.7†	282.8 ± 86.9*†
Rod V_{max}	30	688.0 ± 96.7	859.3 ± 80.3‡	229.2 ± 125.9*	390.6 ± 123.8*§
	60	606.7 ± 71.9	701.4 ± 117.5‡	214.4 ± 110.4*	347.7 ± 117.2*
Rod-cone b-wave	30	1142.3 ± 171.7	1147.5 ± 169.3‡	435.0 ± 134.7*	599.5 ± 144.6*
	60	913.9 ± 102.4†	922.1 ± 122.3‡	407.5 ± 137.6*	530.1 ± 162.3*
Scotopic SOPs	30	469.4 ± 60.0	456.8 ± 62.0‡	140.2 ± 87.7*	271.6 ± 92.2*§
	60	354.9 ± 66.6†	352.8 ± 68.3‡	120.8 ± 54.7*	199.3 ± 82.6*
Photopic b-wave	30	262.0 ± 30.0	241.6 ± 37.0‡	86.3 ± 31.8*	111.2 ± 26.5*
	60	229.0 ± 20.0	161.9 ± 21.9*†	78.7 ± 28.9*	110.2 ± 38.1*
Photopic SOPs	30	112.7 ± 26.0	81.0 ± 18.8*‡	27.9 ± 15.3*	43.7 ± 13.2*§
	60	100.3 ± 15.7	34.9 ± 8.1*†	32.0 ± 10.0*	39.5 ± 14.9*

Data were obtained in scotopic (rod-cone a-wave, rod V_{max} , rod-cone b-wave, rod SOPs) and photopic (b-wave and SOPs) conditions from rats raised in normoxic and hyperoxic conditions, with or without treatment with 600 µg/kg of trolox C. Amplitudes are reported in mean microvolts ± SD.

* Significant difference from the control group.

† Significant difference from 30-day results.

‡ Significant difference from the oxygen+Trolox (different from O₂+respective dose).

§ Significant difference from the oxygen-exposed group.

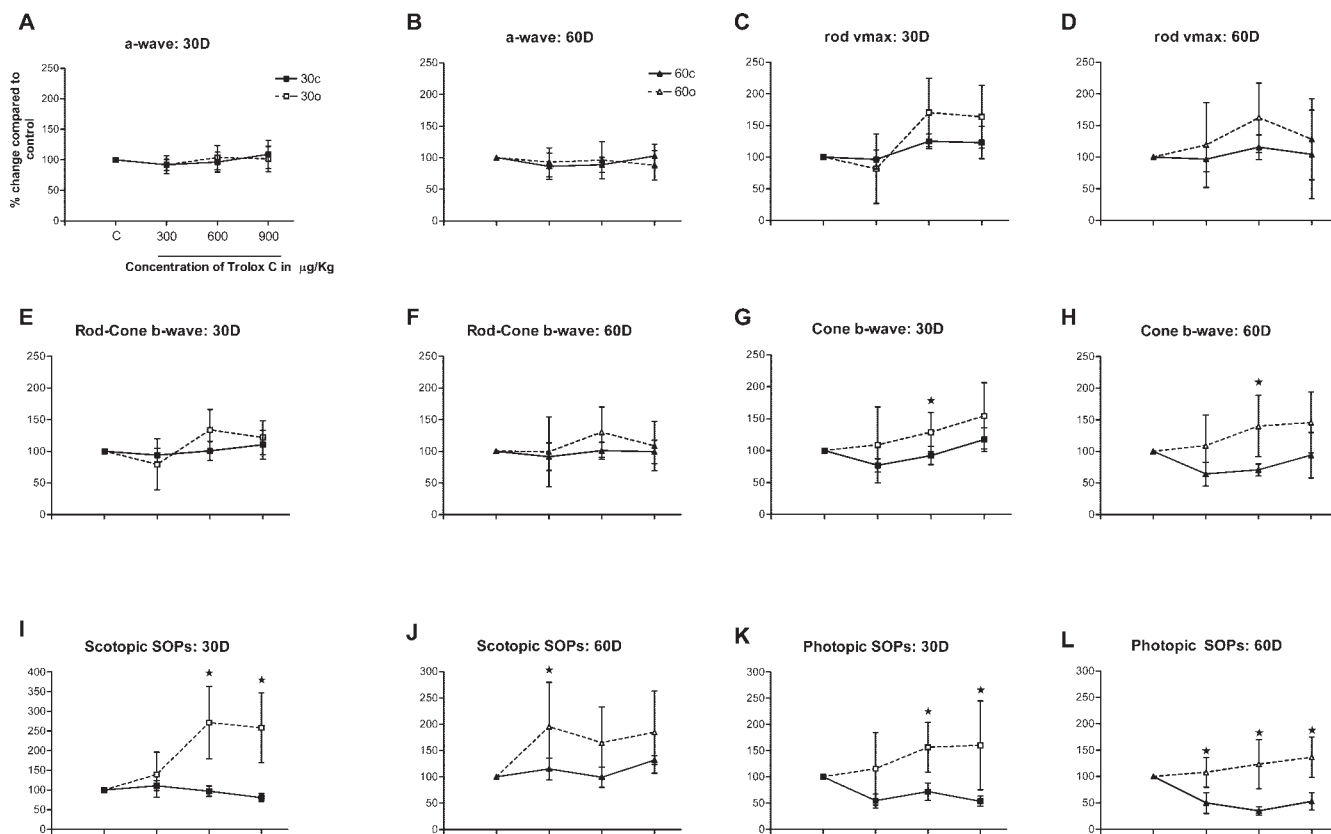


FIGURE 2. Graphic representation of the dissociation between the protective effect of Trolox C on animals reared in hyperoxia and the retinoactive effect on the normal retina. Data manipulation involved normalizing individual ERG amplitude measurements from normoxia-treated rats to the normoxia control mean, which was taken as 100%. Hyperoxia-treated animals were similarly normalized to the hyperoxia control group that was taken as 100%. Each ERG parameter is compared at 30 and 60 days, and individual graphs (A–L) compare normoxia-treated (shaded symbols, solid line) and hyperoxia-treated (open symbols, dashed line) animals (a-wave, A–B; rod $V_{\max}$, C–D; rod-cone b-wave; E–F; photopic b-wave; G–H; scotopic SOPs; I–J; photopic SOPs, K–L). Student's *t* test was used to compare the effect of trolox C on animals raised in a normoxic with those raised in a hyperoxic environment. Asterisks indicate significant differences between normoxia- and hyperoxia-treated groups. Results are given as mean amplitude change $\pm$ 1 SD.

groups, respectively) to the mean amplitude of the normoxic control group (taken as 100%). Similarly, ERG amplitude measurements obtained from each hyperoxia-treated rat (trolox C 300-, 600-, and 900- $\mu\text{g/kg}$ groups, respectively) were normalized to the mean amplitude of the hyperoxia control group (also taken as 100%). Results obtained from this data manipulation are shown in Figure 2, in which data obtained at postnatal days 30 and 60 are also compared. Our results confirm our initial impression that, despite the lack of an effect on the a-wave (Figs. 2A, 2B), treatment with trolox C does indeed have an impact on retinal function. Amplitude ratios generated from hyperoxia-treated rats always tended to be greater than those obtained from normoxia-treated rats, suggesting that the therapeutic effect of trolox C outweighed its (normal) retinoactive effect. In fact, when normoxia-treated and hyperoxia-treated rats are compared, significant differences favoring the therapeutic effect of trolox C are seen for the photopic b-wave at postnatal days 30 and 60 and for scotopic and photopic SOPs at postnatal days 30 and 60 (Figs. 2G–L). Similar tendencies can also be seen for the rod $V_{\max}$ and the rod-cone b-waves. As mentioned, OPs have been shown to be particularly susceptible to postnatal hyperoxia. The dose-dependent effect observed on the SOPs after the administration of trolox C is, therefore, not surprising. Once normalized to their respective controls, scotopic and, more important, photopic SOPs measured in the hyperoxia-treated group are significantly different

from those obtained from the normoxia-treated rats, indicating that the OPs clearly benefited from the therapeutic effect of trolox C. This holds true for groups that received as little as 300 $\mu\text{g/kg}$ (Figs. 2J–L).

In previous studies, we show that the amplitude of ERG responses decreases with age when data obtained from 30- and 60-day-old rats are compared.^{21,26} This was further exemplified in Figure 3, with its comparison of the maturation-induced ERG amplitude decrease in normoxic and hyperoxic rats regardless of whether they received trolox C treatment. To facilitate the comparison, we devised a maturation index that represented the average of the percentage difference between ERGs obtained at 30 days and those obtained at 60 days for each rat as measured with the ERG a-wave and scotopic (combined rod $V_{\max}$ and mixed rod-cone) and photopic b-waves. Figure 3A shows the index of maturation for the a-wave, which was similar for normoxic control and hyperoxic control rats ($23.19\% \pm 21.14\%$ vs. $24.18\% \pm 16.40\%$; $P > 0.05$). Furthermore, despite some variability, responses obtained from animals raised in either environment while receiving trolox C, irrespective of concentration, were not significantly different from those of respective controls, suggesting that oxygen exposure alone or with concomitant trolox C treatment did not result in maturation-induced attenuation of the a-wave that exceeded what was considered the expected normal range. This result further supported a previous claim of ours that,

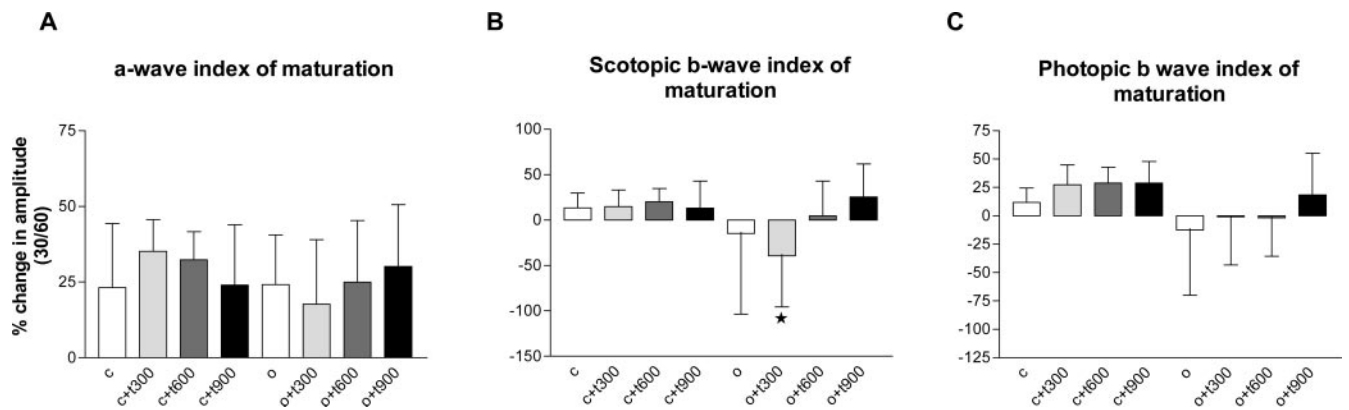


FIGURE 3. Retinal maturation indices for the a-wave (A), scotopic b-wave (including rod V_{max} and mixed rod-cone responses) (B), and photopic b-wave (C). This index represents the average of the percentage difference between ERGs obtained at 30 and 60 days [$\Delta\%$ (30/60)] for all individual rats. All values are given as mean $\pm$ 1 SD. Asterisks indicate significant difference from normoxic control.

compared with the other ERG components, the a-wave is minimally affected after postnatal hyperoxia.^{21,26} On the other hand, the scotopic b-wave (including the rod V_{max} and mixed rod-cone responses) appeared to have matured differently depending on whether the rats were exposed to the hyperoxic environment or not, as shown with the maturation indices in Figure 3B. The index of maturation obtained from the normoxic control rats ($13.49\% \pm 16.12\%$) was similar ($P > 0.05$) to that obtained from normoxia-treated rats, irrespective of concentration, indicating that trolox C did not impair the normal maturational process. In contrast, the negative ($-14.73\% \pm 88.95\%$) index of maturation found for the hyperoxic control rats suggested that the hyperoxic environment tended, albeit not significantly ($P > 0.05$), to alter the normal course of the maturation process. Interestingly, however, a positive (normoxic-like) index of maturation was reinstated if the hyperoxic rats were treated with 600 and 900 $\mu\text{g/kg}$ trolox C but not with the 300- $\mu\text{g/kg}$ dose. A similar trend was also observed with the photopic b-wave index of maturation (Fig.

3C), though in these conditions, the lowest dose, 300 $\mu\text{g/kg}$, already tended to return the index of maturation closer to normal, generating results similar to what was obtained using 600 $\mu\text{g/kg}$, whereas the 900- $\mu\text{g/kg}$ dose yielded a maturation index identical with that measured in normoxic control rats.

Effect of Trolox on the Retinal Cytoarchitecture

Illustrated in Figure 4 are representative cross-sections of retinas obtained from control (with and without 600 $\mu\text{g/kg}$ trolox C supplementation) and oxygen-exposed (with and without 600 $\mu\text{g/kg}$ trolox C supplementation) rats at 60 days of age. We had previously demonstrated that rats exposed to hyperoxic conditions undergo gradual thinning of the OPL and reduction in the number of horizontal cells.²¹ Retinal sections shown in Figure 4 confirm these findings. OPL thickness is significantly reduced in the hyperoxic control group (Fig. 4C) compared with that in the normoxic control rats (Fig. 4A). Hyperoxia-treated rats (600 $\mu\text{g/kg}$ trolox C; Fig. 4D) showed a better-

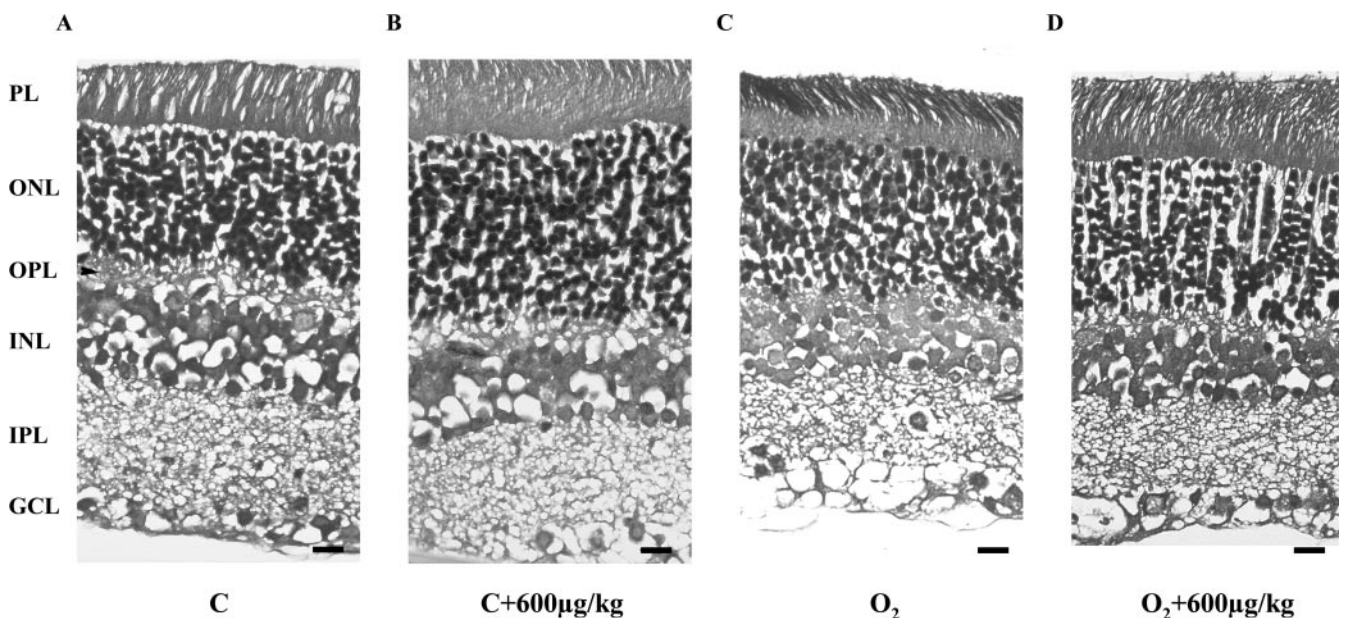


FIGURE 4. Photomicrograph of cross-sections of the central retinas from (A) 60-day-old control (C), (B) control-treated with 600 $\mu\text{g/kg}$ trolox C (C+600), (C) oxygen-exposed (O_2), and (D) oxygen-exposed treated with 600 $\mu\text{g/kg}$ trolox C (O_2 +600) rats. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 10 μm .

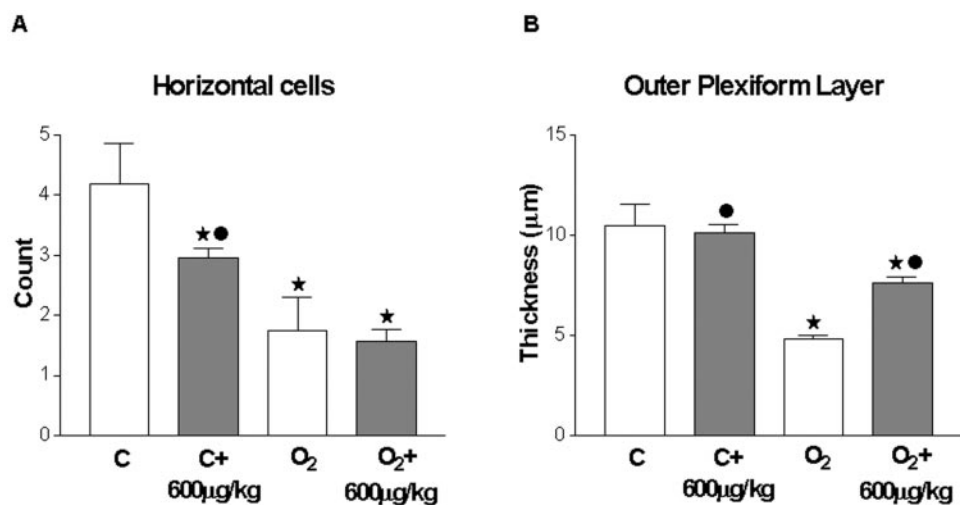


FIGURE 5. Graphic representation of the number of horizontal cells (A) and the mean thickness (in micrometers) of the OPL (B) for 60-day-old control (C), control with trolox C (C+600), oxygen-exposed (O₂), and oxygen-exposed with trolox C (O₂+600) rats. Error bars represent ± 1 SD. Asterisks indicate significant difference from normoxic control, and filled symbols indicate significant difference from hyperoxic control.

preserved OPL than was observed in the hyperoxic control rats (Fig. 4C). In contrast, no changes in OPL thickness were observed in the normoxia-treated group (Fig. 4B). Measurements of OPL thickness and horizontal cell counts are graphically presented in Figure 5. Hyperoxic control rats showed a significant ($P < 0.05$) 55% reduction in the thickness of the OPL that was accompanied by a significant ($P < 0.05$) 60% reduction in horizontal cell count. Concomitant treatment of oxygen-exposed rats with 600 µg/kg trolox C limited the reduction of the OPL thickness to 75% of control ($P < 0.05$) but had no impact on the horizontal cell count. It should be noted that trolox C alone (without hyperoxia), though not altering the thickness of the OPL layer, still significantly reduced the number of horizontal cells by 30%.

DISCUSSION

Our findings suggest that trolox C represents a valid therapeutic alternative in limiting the structural and functional damages intrinsic to the rat model of OIR. When retinal function is considered, our results (Fig. 2) show that the a-wave was not impaired by hyperoxic exposure or by trolox C treatment (irrespective of dosage), the therapeutic effect of trolox C (especially at higher doses) outweighed its retinoactive effect, and the OPs are the ERG components that benefit most from the therapeutic effects of trolox C. Furthermore, results presented in Figure 2 also suggest that the photopic (cone-mediated) function benefits significantly more than the scotopic (rod-mediated) function from the therapeutic effects of trolox C. Given that the cone-mediated function was previously shown to be relatively more impaired than the rod-mediated function after postnatal hyperoxia,²⁶ a feature also evidenced in the present study (Table 1), our finding of a more potent effect of trolox C therapy on cone-mediated signals indicates that the treatment is targeted to where it is needed. The therapeutic efficacy of trolox C is further evidenced by the fact that it never modified the ERG a-wave, a component that was also unaffected by postnatal hyperoxia. Trolox C minimized the cytoarchitectural damage inherent in postnatal hyperoxia in that it limited the reduction in OPL thickness that is associated with OIR.²¹ In contrast, its use had no significant impact on the reduction in horizontal cell count that also is associated with this retinopathy, suggesting either that trolox C might preferentially target specific cells whose processes contribute to OPL structure rather than target the horizontal cells or that the only two cytoarchitectural manifestations of the disease

process involved in OIR might proceed from a different pathophysiological path.

Interestingly, the effect that trolox C exerted on the ERG signal appeared to depend on whether ROS were present. In the hyperoxic group, trolox C treatment enhanced the cone- and rod-mediated signals (compared with the untreated hyperoxic group), whereas in the normoxia-treated group, its use enhanced the rod response but reduced the cone-mediated ERG (compared with the normoxia-untreated group). Trolox C has been reported to have a dual mode of action—antioxidant and prooxidant—depending on the method by which peroxidation is induced. For example, when the oxidative stress is induced by Cu²⁺ or Fe³⁺, trolox C acts preferentially as a toxic prooxidant and is converted to an α -tocopheroxyl radical as cellular functions deteriorate.³¹ In contrast, trolox C exerts its antioxidant role after metal-independent, peroxy radical-induced oxidation.^{32,33} This dual mode of action could apply to our model of OIR by which, in the presence of free radicals and, after exposure to postnatal hyperoxia, trolox C would carry out its antioxidant role. In the normal unexposed retina, in the absence of free radicals, trolox C could take on its more toxic role as a prooxidant. As with the mechanism by which cone function is more susceptible than rod function to postnatal hyperoxia (in the absence of any antioxidant), as previously reported by us,²⁶ the toxic effect of trolox C, as a prooxidant in the normal retina, could also be more devastating to cone function.

In addition, it has been shown that different mechanisms, namely apoptosis and necrosis, could be implicated in the pathophysiology of OIR and its human counterpart ROP.³⁴ Recent *in vitro* studies carried out on cultured bursal cells and neurons in which cell death occurred resulting from oxidative stress suggest that trolox C could have a protective effect against necrosis but none against apoptosis.^{35–37} Other studies, however, have concluded that trolox C was successful, for example, in reducing apoptosis in mouse thymocytes, rabbit myocytes, and anterior pituitary cells.^{38–40} It will be useful to investigate the presence of apoptotic and necrotic markers in OIR to better understand the predominant mode of neuronal cell death taking place in this model and, more specifically, the implications on rod and cone pathways. Future studies in this area will also help to further elucidate the role of trolox C on these mechanisms of cellular death. Finally, though the present study indicates a beneficial effect on retinal structure, retinal function was spared to a lesser degree in animals that receive trolox C, suggesting that changes in retinal vasculature, retinal

structure, and retinal function after exposure to postnatal hyperoxia might have proceeded in a cascade of events. For example, as previously described,^{4,5} postnatal exposure to hyperoxia generates a reduction in vasculature because of vasoconstriction followed by vaso-obliteration. Consequently, this inadequate blood supply could have made it difficult for the inner retina to receive adequate nourishment to carry out its proper function. It is possible that we observed loss or retraction of synapses that would lead to a thinning of the OPL only after cell function was lost. This could suggest a lag time between functional and structural impairment and perhaps an important role for vasoconstriction and vaso-obliteration as the trigger of the pathogenesis of OIR because this appeared to be the stage at which the cascade began. Given that each phase appeared to be interdependent (beginning with early changes in retinal vasculature and ending with changes in retinal cytoarchitecture and function), it seems logical to target the first step of this pathologic cascade. This would further support the use of a free radical scavenger such as trolox C. Furthermore, it is possible that though trolox C aids in facilitating vasculogenesis under hyperoxic conditions (thereby improving retinal function), its ability to take on the role of an antiapoptotic mediator prevents synaptic death that leads to a thinning of the OPL.

In summary, though we observed a therapeutic effect of trolox C, its use could not fully prevent oxygen-induced retinal damage from occurring. This could suggest, as previously proposed,²¹ that free radical formation is likely not the sole cause of the damage in retinal structure and function observed in OIR or that a significant proportion of the retinal damage induced by ROS occurs in hydrophobic domains in which trolox C would lose its efficacy because of its water solubility. For example, trolox C could act in limiting the circulating (plasma) ROS before lipid peroxidation. Should that be the case, a combination of liposoluble and hydrosoluble free radical scavengers (such as vitamin E [at a nontoxic level] + trolox C) could represent an interesting therapeutic alternative for further investigation.

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Functional and Structural Changes Resulting from Strain Differences in the Rat Model of Oxygen-Induced Retinopathy

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PURPOSE. Results of studies that compared the racial incidence of retinopathy of prematurity (ROP) suggested that ocular pigmentation might offer protection against the development of severe ROP. The structural and functional consequences of postnatal hyperoxia (oxygen-induced retinopathy; OIR) were compared in albino Sprague-Dawley (SD) and pigmented Long-Evans (LE) rats to verify this finding.

METHODS. Newborn rats were exposed to 80% O₂ during selected postnatal day intervals. The severity of the OIR was determined by examining retinal flatmounts (retinal vasculature assessment), protein level quantification and cellular localization of fibroblast growth factor (FGF)-2 and ciliary neurotrophic factor (CNTF; Western blot analysis and immunohistochemistry, respectively), retinal histology, and photopic and scotopic electroretinograms (ERGs).

RESULTS. Irrespective of the parameter considered, structural and functional deficits resulting from postnatal hyperoxia were significantly more pronounced in LE rats. Although FGF-2 levels in LE rats had a tendency to increase after hyperoxia compared with normoxic littermates, it did not reach statistical significance. A similar finding was observed in SD rats. Of interest, however, baseline levels of FGF-2 were approximately four to five times higher in SD rats than in LE rats. There was a similar, hyperoxia-induced increase in CNTF levels between SD and LE rats.

CONCLUSIONS. The findings suggest an increased susceptibility of newborn LE rats to postnatal hyperoxia in comparison with SD rats. Whether a pro-oxidant rather than antioxidant role of melanin or other genetic factors can explain these differences in oxygen susceptibility of the animal model of this retinopathy, remains to be determined. (*Invest Ophthalmol Vis Sci*. 2009;50:2436–2450) DOI:10.1167/iovs.08-2297

Despite many advances in the clinical monitoring of oxygen administration in neonatal intensive care units, retinopathy of prematurity (ROP) continues to be a major cause of morbidity

affecting prematurely born infants. Similar to other types of proliferative retinopathies, such as diabetic retinopathy and sickle cell retinopathy, ROP is characterized by retinal neovascularization.^{1–4} It is this abnormal growth of new retinal vessels that can lead to the most devastating consequences of the disease, namely retinal detachment and eventually blindness. Through years of research, it has become increasingly evident that ROP is a very complex, multifactorial disease. Some of the risk factors known to be associated with the incidence of ROP include low birthweight and low gestational age^{5–7} and, notably, relative hyperoxia (compared with oxygen tension in utero).¹ Other studies have also shown that race is a significant factor that influences the epidemiology of ROP^{8,9}; for example, a greater number of Caucasian than African-American infants have been shown to reach threshold ROP.^{8,10} These studies suggest that melanin pigmentation offers protection against the development of severe ROP, suggesting that fundus pigmentation may play an instrumental role in this protection.^{6,8} On the other hand, other studies have suggested that an even greater incidence in the onset of threshold ROP exists in another darkly pigmented race, namely native Alaskans, when compared with that in other racial groups.^{11,12} Despite the fact that several of these studies have controlled for birth weight, gestational age, length of oxygen therapy, and ethnic group in multivariate analysis, the influence of race on the rate and severity of ROP is evidently an area that requires further clarification.

The rat model of oxygen-induced retinopathy (OIR) mimics what occurs in the human form of ROP and continues to provide insight into the pathogenesis of this disease. Findings of ours have shown that in addition to the vasculopathy that develops in the rat model of OIR, permanent retinal cytoarchitectural and functional anomalies can also be documented.^{13–17} The most striking changes in retinal ultrastructure that we have evidenced in the albino SD rat model of OIR include outer plexiform layer (OPL) thinning and a decrease in horizontal cell count. Attenuation in the amplitude of the electroretinogram (ERG) b-wave with relative sparing of the a-wave are also features that are characteristic of this model.^{13–17} Previously published studies that compared pigmented and albino rats have shown that the former are less susceptible to ischemic retinal injury than are the latter,¹⁸ whereas others have demonstrated that certain pigmented strains develop more severe retinal neovascularization.^{19,20} Consequently, the purpose of this study was to determine whether similar strain differences might also extend to retinal vasculature (flatmounts), structure (histology), and function (ERG), in addition to neurotrophic factor expression. In the present study, we therefore sought to explore these factors in two rat models of OIR: the albino SD and the pigmented LE strains.

METHODS

Animals

All experiments were approved by the McGill University-Montreal Children's Hospital Research Institute according to the guidelines of

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the Canadian Council on Animal Care and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Within a 24-hour period after birth, newborn litters of Sprague-Dawley (SD) and Long Evans (LE) rats (Charles River Laboratories, St. Constant, Québec, Canada) were placed, with their mothers, in room air (21% O₂) or in a hyperoxic (80% O₂) environment (mixture of medical grade 100% O₂ and room air measured with an oxygen meter [MaxO₂ Ceramtec, model OM25-ME; Medicana Inc., Montréal, Québec, Canada]). A controlled cyclic light environment was maintained throughout the exposure (12 hours light–dark). Exposure to hyperoxia lasted for 22.5 hours daily and was interrupted for three 0.5-hour periods of normoxia (21% O₂), as per previously published protocols.^{13–17} Casualties were more frequently observed throughout hyperoxic exposure of the pigmented LE strain, a finding that might be explained by interstrain differences in the rat genome that contribute to variations in ventilation, metabolism, and ventilatory responses to inspired levels of O₂ and CO₂.^{21,22} Based on previous studies in SD rats that suggested unequal susceptibilities to hyperoxia throughout different developmental time points,^{15,16} the hyperoxic cohort of each strain was divided at birth into seven different exposure protocols: from birth through postnatal day 6 (P0–P6]: LE, *n* = 24 [P30], *n* = 5 [P60]; SD, *n* = 6 [P30], *n* = 6 [P60]; birth through postnatal day 9 (P0–P9]: LE, *n* = 11 [P30], *n* = 5 [P60]; birth through postnatal day 12 (P0–P12]: LE, *n* = 7 [P30], *n* = 6 [P60]; and birth through postnatal day 14 (P0–P14]: LE, *n* = 21 [P30], *n* = 6 [P60]; SD, *n* = 11 [P30], *n* = 7 [P60]; or from postnatal day 6, 9, or 12 through postnatal day 14 (P6–P14]: LE, *n* = 22 [P30], *n* = 6 [P60]; SD, *n* = 8 [P30], *n* = 8 [P60]; P9–P14]: LE, *n* = 17 [P30], *n* = 9 [P60]; and P12–P14]: LE, *n* = 15 [P30], *n* = 7 [P60]). Select time points within the first and second weeks of life were taken for comparing SD rats to LE rats, as we had documented the effects of postnatal hyperoxia in the former strain elsewhere.^{15–16} After hyperoxic exposure, animals were returned to room air (21% O₂) until P30 when retinal function was first assessed. Data were compared with respective age-matched control litters raised in normoxic conditions (LE, *n* = 21; SD, *n* = 22).

Malondialdehyde Assay

Malondialdehyde (MDA), a product of peroxidation, was determined as previously described.^{23,24} LE and SD rats were killed immediately after exposure to hyperoxia (P0–P6 and P6–P9, *n* = 3 to 4 per group), given that levels (relative to normoxia) have been shown to subside as the hyperoxic period is extended for longer periods.²³ Each retina was harvested less than 1 minute later, ground twice for 30 seconds each with a homogenizer (Omni International, Marietta, GA) in a buffer containing butylhydroxytoluene (BHT; 5 mM Tris, 0.02% ASA, 0.5 mM EGTA), and centrifuged at 1000g for 10 minutes. To each sample was added 0.33% 2-thiobarbituric acid (TBA) in water mixed with glacial acetic acid and then the sample was heated at 95°C for 60 minutes. HPLC-grade *n*-butanol was added, and samples were vortexed and spun down at 1000g for 10 minutes. The upper phase was read at 532 nm with a spectrophotometer.

Retinal Flatmounts

Groups of animals killed on P6 and P14 after exposure from P0 to P6 (SD, *n* = 3; LE, *n* = 3) and P6 to P14 (SD, *n* = 3; LE, *n* = 3), respectively. After enucleation and dissection of the cornea and lens, eye cups were collected and fixed overnight at 4°C in 4% vol/vol formalin. Retinas were isolated, and flatmounts were prepared for adenosine diphosphatase (ADPase) staining, according to a method that was previously described.^{17,25,26}

Sequential, overlapping images of the entire retina were subsequently collected and arranged to construct a montage of the complete retina (Photoshop, ver. 6.0; Adobe Systems Inc., San Jose, CA). Vascularized areas were manually outlined and measured as a percentage of the total retinal area to assess vascular extent. Density analysis obtained from oxygen-exposed groups was compared with that from animals

raised in normoxia, for which values were set at 100% (ImagePro Plus, ver. 4.1; Media Cybernetics, Silver Spring, MD).

Retinal Immunohistochemistry

Other groups of animals were assigned to be killed on postnatal day 6 after exposure from P0 to P6 (LE, *n* = 3; SD, *n* = 3) and on P14 and P60 after exposure from P6 to P14 (P14: LE, *n* = 3; SD, *n* = 3; P60: LE, *n* = 3), respectively, for detection of endogenous levels of FGF-2 and CNTF neurotrophic factors that were found to be upregulated in another model of oxidative stress—namely, light-induced retinopathy (LIR).²⁷ Animals were euthanatized immediately after removal from hyperoxia and were perfused intracardially with 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer (PB; pH 7.4). After removal of the cornea and lens as described, eye cups were immersed in 4% PFA in 0.2 M PB (pH 7.4; 2 hours) and were cryoprotected in sequential sucrose solution gradients (10%, 20%, and 30% in PB for 30 minutes, 1 hour, and overnight, respectively) at 4°C. Eye cups were subsequently embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and were frozen in a bath containing 2-methylbutane/liquid nitrogen. Fourteen-micrometer-thick cryosections were collected on polylysine-S-coated slides for immunohistochemical analysis. To block any nonspecific binding of the antibodies, we incubated retinal sections in 3% normal goat serum, 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in 0.1 M PBS (pH 7.4) for 30 minutes at room temperature. The sections were then incubated overnight at 4°C with the following primary antibodies: monoclonal anti-human FGF-2 (type II, clone bFM-2, 10 µg/mL; Upstate Biotechnology, Lake Placid, NY), monoclonal anti-mouse CNTF (5 µg/mL; Chemicon, Temecula, CA), and polyclonal anti-rabbit GFAP (1:100; Sigma-Aldrich). Normally, GFAP expression in Müller cells is very limited^{28,29} but increases after neuronal cell death. In fact, studies conducted in the rat model of OIR have suggested that increasingly intense GFAP reactivity is observed once animals are returned to room air after 14 days of hyperoxia, the most intense of which is seen at approximately 10 weeks of age.³⁰ Consequently, we chose to conduct our colocalization studies with GFAP in retinas from rats exposed to hyperoxia from P0 to P14, collected at P60. The next day, sections were incubated with the appropriate secondary antibody for 1 hour at room temperature, washed in PBS, and mounted with an antifade reagent (SlowFade; Invitrogen-Molecular Probes, Eugene, OR). Photographs were taken with a microscope (40× objective, Axiophot; Carl Zeiss Meditec, Oberkochen, Germany).

Western Blot

Retinas from right and left eyes of LE and SD control and oxygen-exposed rats (80% from P0 to P6, P6 to P14, and P0 to P14) were isolated (*n* = 3 to 4 per group), independently homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (0.1% sodium dodecyl sulfate, 20 mM Tris [pH 8.0], 135 mM sodium chloride, 1% NP-40, 10% glycerol supplemented with protease inhibitors), and then pooled. Retinal lysates were kept on ice for 30 minutes and then centrifuged at 14,000 rpm for 15 minutes, after which the supernatant was collected. Protein concentration was determined by the Lowry method (Bio-Rad Life Science, Mississauga, ON, Canada). For each sample, 100 µg of protein was resolved by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad Life Science), as previously described.²⁷ After nonspecific signal blocking in 10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20 (TBST), and 5% lyophilized skim milk for 1 hour at room temperature, the membranes were incubated overnight at 4°C with the primary antibodies used according to the following conditions: monoclonal anti-human FGF-2 (type II, clone bFM-2, 2.5 µg/mL; Upstate Biotechnology) and monoclonal anti-mouse CNTF (5 µg/mL; Chemicon). The membranes were subsequently washed with TBST and incubated with anti-mouse peroxidase-linked secondary antibodies (Amersham Pharmacia, Baie d'Urfé, Québec, Canada) for 1 hour at room temperature. A chemiluminescent reagent

(ECL; GE Healthcare, Piscataway, NJ) was used for detection of protein signals, followed by the exposure of the membranes to autoradiograph film (X-OMAT; Eastman Kodak, Rochester, NY).

The membranes were incubated in a stripping solution (200 mM glycine [pH 2.8], 500 mM NaCl, and 0.7% β -mercaptoethanol) at 55°C for 1 hour and then reprobed with an anti-actin polyclonal antibody (1:10,000; generous gift from J. Chloe Bulinski) followed by incubation with an anti-rabbit peroxidase-linked secondary antibody (Amersham Pharmacia Biotech).

The γ -actin protein was chosen as a reference instead of β -actin, given that the latter isoform patterns vary with age.^{31–33} Ponceau-S (Sigma-Aldrich) was used to ensure equal protein loading. Densitometry analysis was performed on scanned autoradiographic films and quantified according to pixel intensity (Quantity One 4.1.0 software; Bio-Rad Laboratories). The densitometry values obtained for each neurotrophic factor were subsequently normalized to the γ -actin level in the same blot.

Electroretinography

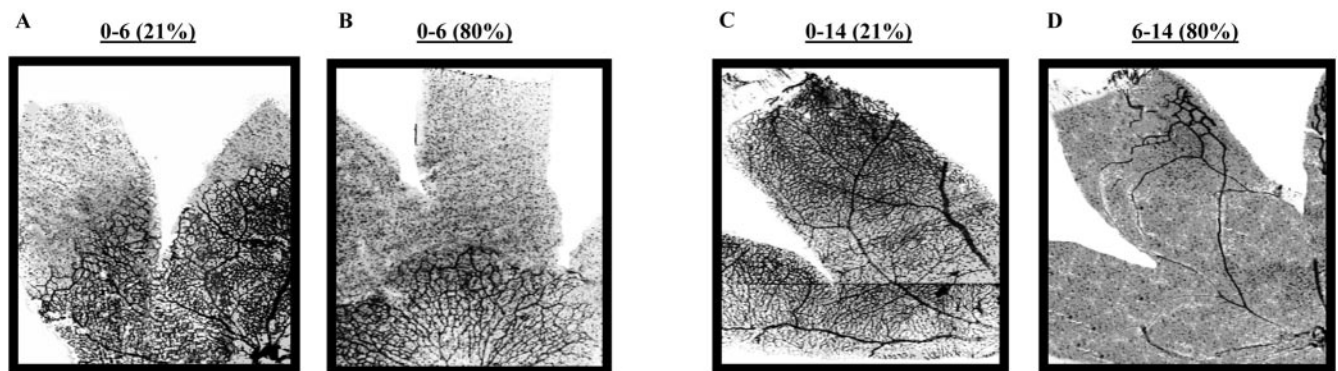
ERGs and oscillatory potentials (OPs) were recorded (AcqKnowledge Data Acquisition System, Biopac MP 100WS; BIOPAC Systems Inc., Goleta, CA) as previously described^{13–17} from control and hyperoxic cohorts at postnatal days 30 and 60. Briefly, after 12 hours of dark adaptation, rats were anesthetized, under a dim red light illumination, with an intramuscular injection of ketamine (85 mg/kg) and xylazine (5 mg/kg). The pupils were subsequently dilated with 1 to 2 drops of 1% cyclopentolate hydrochloride, and the rat was placed on its side in a recording chamber that included both the flash stimulator and back-

ground light. A DTL fiber electrode (27/7 X-Static silver-coated conductive nylon yarn; Sauquoit Industries, Scranton, PA) that served as the active electrode was placed over the corneal surface and was kept in place with 2% methylcellulose (Gonioscopic solution; Alcon Laboratories, Fort Worth, TX), which also helped to prevent corneal desiccation. Grass disc (model E6GH; Grass Instruments, Quincy, MA) and subdermal needle electrodes (model E2; Grass Instruments) were placed in the mouth as the reference and inserted in the tail as the ground, respectively. Broadband ERGs (10,000 $\times$, 1–1000 zHz bandwidth, 6 dB of attenuation; P511 analog preamplifiers; Grass Instruments) and OPs (50,000 $\times$, 100–1000 Hz, 6 dB of attenuation; P511 analog preamplifiers) were recorded simultaneously with the data acquisition system (AcqKnowledge; Biopac MP 100 WS; Biopac System Inc.). Scotopic luminance-response functions were evoked by flashes of white light spanning a 6-log-unit range in 0.3-log increments (maximal intensity, 0.6 log cd $\cdot$ s $\cdot$ m⁻², average, two to five flashes, interstimulus interval, 9.60 seconds). Photopic (cone-mediated) signals were evoked to flashes of white light of 0.9 log cd $\cdot$ s $\cdot$ m⁻² that were delivered after more than 15 minutes of light adaptation to a background of 30 cd $\cdot$ m⁻² (average, 20 flashes; interstimulus interval, 1 second) to avoid the previously described light adaptation effect.³⁴

Retinal Histology

Retinal histology was performed at P60. After the completion of ERG recordings, the rats were euthanatized with an anesthetic overdose followed by intracardiac perfusion with 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The eyes were enucleated and postfixed in 3.5% glutaraldehyde for 3 hours. The corneas were then dissected, the

Long Evans



Sprague Dawley

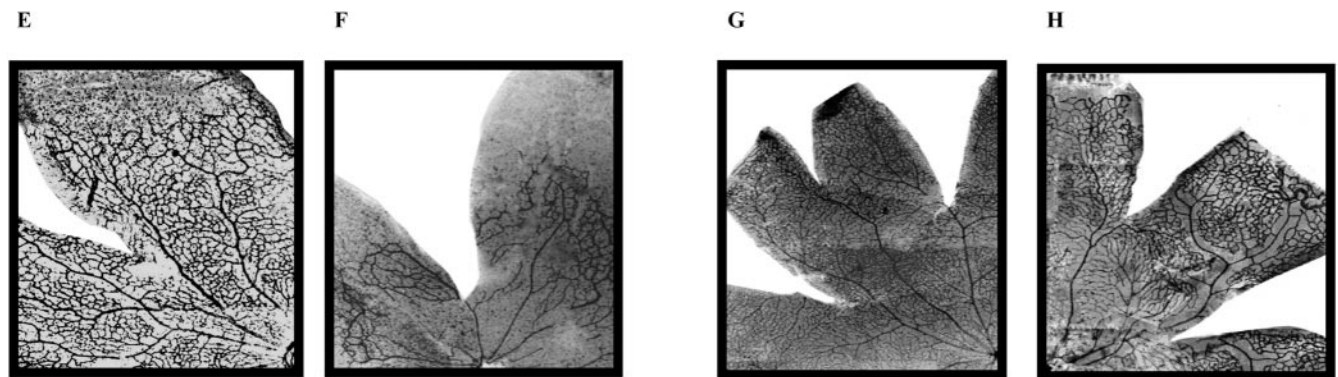


FIGURE 1. Representative retinal vascular flatmounts stained with ADPase from LE (*top*) and SD (*bottom*) rats, raised in room air and euthanatized at P6 (**A**, **E**, respectively) or P14 (**C**, **G**, respectively), or raised in hyperoxia and euthanatized immediately after 6 days (P0–P6; **B**, **F**, respectively) or P14 (P0–P14; **D**, **H**, respectively) of oxygen exposure. Magnification, $\times 40$.

lenses were removed as described earlier, and the eyes were subsequently placed in 3.5% glutaraldehyde overnight (12 hours). The eye cups were then immersed in a 4% osmium tetroxide solution with 0.1 M phosphate buffer. After sequential dehydrations in ethanol baths (50%, 70%, 90%, 95%, and 100% ethanol) and propylene oxide, the specimens were embedded in epoxy resin (Durcupan ACM Fluka epoxy resin kit; Sigma-Aldrich). Once polymerization was completed (48 hours at 58°C), the tissue was cut in 0.7- μ m ultrathin sections that were mounted on glass slides and stained with 0.1% toluidine blue. Images were obtained with a digital camera (RR slider spot; Diagnostic Instruments Inc., Sterling Heights, MI) that was mounted on a microscope (Axiophot, 40 $\times$; Carl Zeiss Meditec). Three retinas per group were analyzed, and an average of 10 measurements was obtained from each.

Data Analysis

Retinal histology and ERGs were obtained from the same group of animals, whereas retinal flatmounts, MDA assay, and neurotrophic factor analysis through Western blot analysis and immunofluorescence were all performed independently. Amplitudes of ERG components along with their peak times were measured as previously described.^{13–17} Briefly, the a-wave amplitude was measured from baseline to the most negative trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Similarly, oscillatory potentials were measured from the preceding trough to the peak of the OP under evaluation, with the exception of OP₂, which was measured from the baseline to its peak. OPs were reported as the sum of OPs (SOP = OP₂ + OP₃ + OP₄ + OP₅).

Scotopic luminance-response function curves were derived for each animal by plotting b-wave amplitudes against flash intensities. A sigmoidal intensity-response regression curve was then applied to fit the data (Prism 3.00 software; GraphPad, San Diego, CA), from which the rod $V_{\max}$ (maximal rod response) could be calculated. Furthermore, the ERG response evoked to the brightest flash delivered in scotopic conditions (the mixed rod-cone response) was also analyzed. To determine the effect of the different oxygen regimens on retinal structure and function, we used one-way analysis of variance (ANOVA; $P < 0.05$) and the Dunnett multiple comparison as a post hoc test. Functional data obtained from each exposure regimen at each recording session (P30 and P60) in addition to retinal vascular findings and

protein analysis (immunoblotting) were compared by Student's *t*-test ($P < 0.05$). Finally, Student's *t*-test ($P < 0.05$) was also used to compare values obtained at P30 and P60 from LE and SD rats from preselected oxygen exposure regimens.

RESULTS

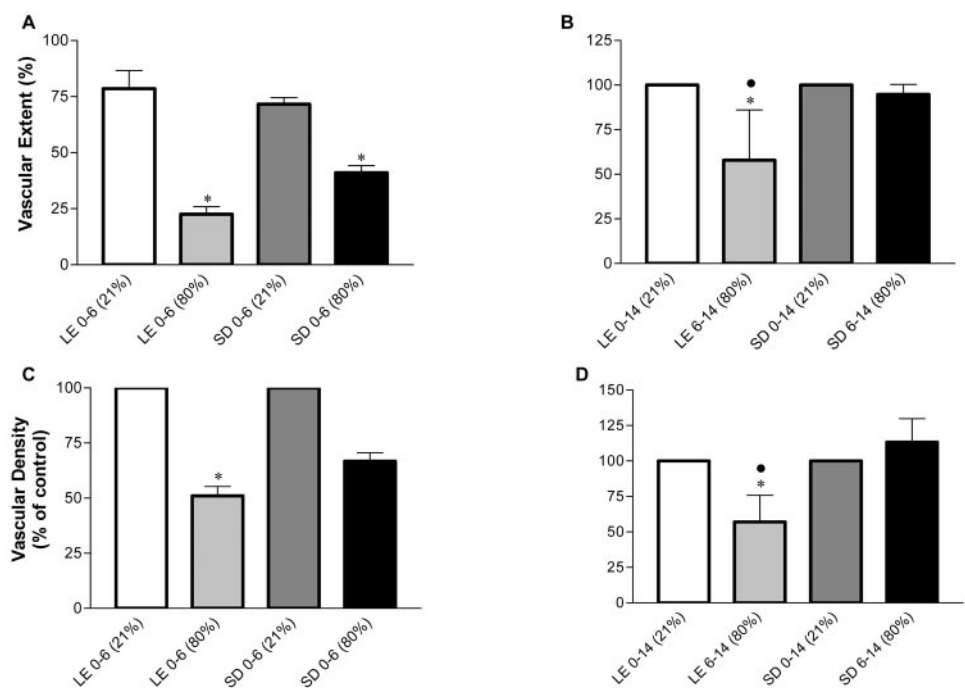
Effect of Postnatal Hyperoxia on MDA Concentration

Given the effects of the redox state on peroxidation^{35,36} we measured levels of malondialdehyde (MDA), a product of peroxidation. Although a 31.9% increase in MDA levels was observed in SD rats after early hyperoxia (P0–P6, 3.06 ± 0.34 [80%] compared with 2.32 ± 0.09 [21%]; $P < 0.05$), a strikingly greater difference in levels was found in LE rats after the same regimen, resulting in a 91.5% increase (P0–P6, 3.63 ± 0.13 [80%] compared with 1.90 ± 0.25 [21%]; $P < 0.05$). After hyperoxic exposures that extended into the second week of life (P6–P9), MDA levels were reduced to near normal levels (1.99 ± 0.35 [80%] compared with 2.50 ± 0.65 [21%] in SD rats and 2.47 ± 0.40 [80%] compared with 3.37 ± 0.69 [21%] in LE rats; $P > 0.05$).

Effect of Postnatal Hyperoxia on Retinal Vasculature

Retinal vasculature in the neonatal rat undergoes significant development from birth through P14, beginning in the central retina and moving outward to the periphery.¹³ Retinal flatmounts reveal that normoxia-raised LE and SD rats are similarly vascularized at P6 ($78.50\% \pm 8.11\%$ and $71.67\% \pm 2.89\%$, respectively; Figs. 1A, 1E), whereas vascular growth to the periphery is complete (100% vascularized) by P14 (Figs. 1C, 1G). On the other hand, hyperoxia (P0–P6 exposure) significantly attenuated the postnatal vascular growth, reducing the vascularized area to 28.7% ($P < 0.05$) and 57.2% ($P < 0.05$) of control in LE and SD rats, respectively (Figs. 1B, 1F, 2A). This attenuation in vascular growth was accompanied by a marked reduction in vascular density for the same exposure regimen, resulting in $51.03\% \pm 4.25\%$ ($P < 0.05$) and $66.70\% \pm 3.96\%$

FIGURE 2. Graphic representation of retinal vasculature extent (A, B) and vascular density (C, D) (ordinate) as a function of the oxygen exposure regimen (days, abscissa) in the normoxic and hyperoxic LE and SD cohorts at P6 (SD, $n = 3$; LE, $n = 3$) or after 6 days of hyperoxia (P0–P6; A, C, respectively; SD, $n = 3$; LE, $n = 3$) and at P14 or after 14 days of hyperoxia (P0–P14; B, D, respectively; SD, $n = 3$; LE, $n = 3$). Results are expressed as a percentage of vascular extent or vascular density of control retinas at P6 and P14, respectively, ± 1 SD. *Statistically significant differences from the control at a given age found with the Student's *t*-test ($P < 0.05$); ●, significant differences from SD rats for the same exposure regimen ($P < 0.05$).



($P > 0.05$) of normal density in LE and SD rats, respectively (Fig. 2C).

Similarly, exposure from P6 to P14 resulted in a vascularized area that was also reduced in LE rats to $57.90\% \pm 28.15\%$ of normal coverage ($P < 0.05$), whereas SD rats were not significantly affected ($94.76\% \pm 5.61\%$ of normal, $P > 0.05$; Figs. 1D, 1H, 2B). As shown in Figure 2D, this trend was also reflected in vascular density measurements ($57.03\% \pm 18.72\%$ and $113.36\% \pm 16.46\%$ of normal in LE [$P < 0.05$] and SD [$P > 0.05$] rats, respectively).

Effect of Postnatal Hyperoxia on Retinal Function

Figure 3 shows representative scotopic (Figs. 3A-F) and photopic (Figs. 3G, 3H) ERGs (Figs. 3A-E, 3G) and oscillatory potentials (Figs. 3F, 3H) recorded from control (21% O_2) and hyperoxic rats (80% O_2) at P60. Exposure durations are indicated in postnatal day intervals. Group data are graphically reported in Figure 4. Although parallel experiments for LE and SD rats were performed, retinal function characteristics of SD rats have been reported by us¹³⁻¹⁷; hence, a more elaborate description of retinal function parameters will be focused on LE rats, and comparisons concluded for strain differences.

In normal LE rats, the use of a progressively brighter flash stimulus (from bottom to top) resulted in a gradual increase in

the a- and b-wave parameters, a trend also observed in rats that were exposed postnatally to each of the hyperoxic regimens.

A comparison between the scotopic (a-wave, rod V_{max} , rod-cone b-wave, and SOPs) and photopic (b-wave and SOPs) ERG parameters of LE and SD rats obtained at P30 and P60 is graphically represented in Figure 4. Group data along with statistical analysis for LE and SD rats are shown in Tables 1 and 2, respectively.

Effect of Postnatal Hyperoxia on Scotopic Function

In all instances, there is a gradual decline in the amplitude of ERG parameters at P30 and P60, culminating in maximum attenuation after exposures to hyperoxia from P0 to P14 and P6 to P14. After hyperoxia initiated at birth, a dose-response effect was observed between the duration of hyperoxia and the amplitude of the resulting a-wave amplitude in LE rats (Fig. 4A) for measurements obtained at P30 and P60. Although a tendency for a decline in a-wave amplitude was observed on exposure to hyperoxia in the first postnatal week (Fig. 4A), a more pronounced attenuation was observed when hyperoxic exposure extended into the second week of life; this culminated in a decline to 51.9% and 45.6% of control values at P30 and P60, respectively ($P < 0.05$). Regimens that were limited

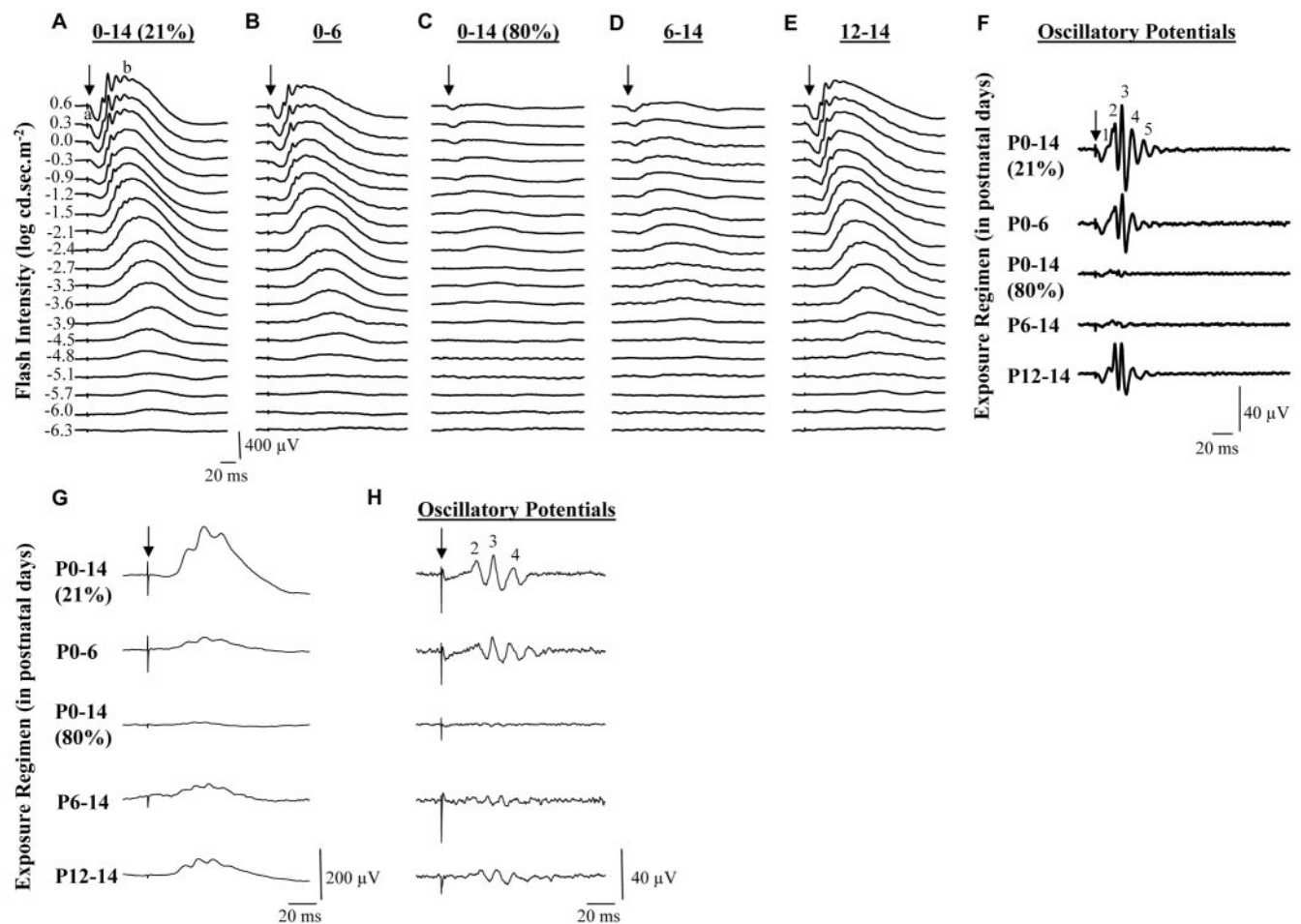


FIGURE 3. Representative scotopic (mixed rod-cone, flash intensity: -6.3 through $0.60 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$; A-E) and photopic (flash intensity: $0.9 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$, background: $30 \text{cd} \cdot \text{m}^{-2}$; G) ERGs along with scotopic (F) and photopic (H) oscillatory potentials obtained at P60 from animals raised in normoxia (21% O_2) and hyperoxia (80% O_2) from P0 to P6, P0 to P14, P6 to P14, and P12 to P14. Calibrations: horizontal, 20 ms; vertical, 400 μV for scotopic mixed rod-cone ERG responses, 200 μV for photopic ERG responses and 40 μV for scotopic and photopic oscillatory potentials, respectively. All tracings include a 20-ms prestimulus baseline; arrows, flash onset.

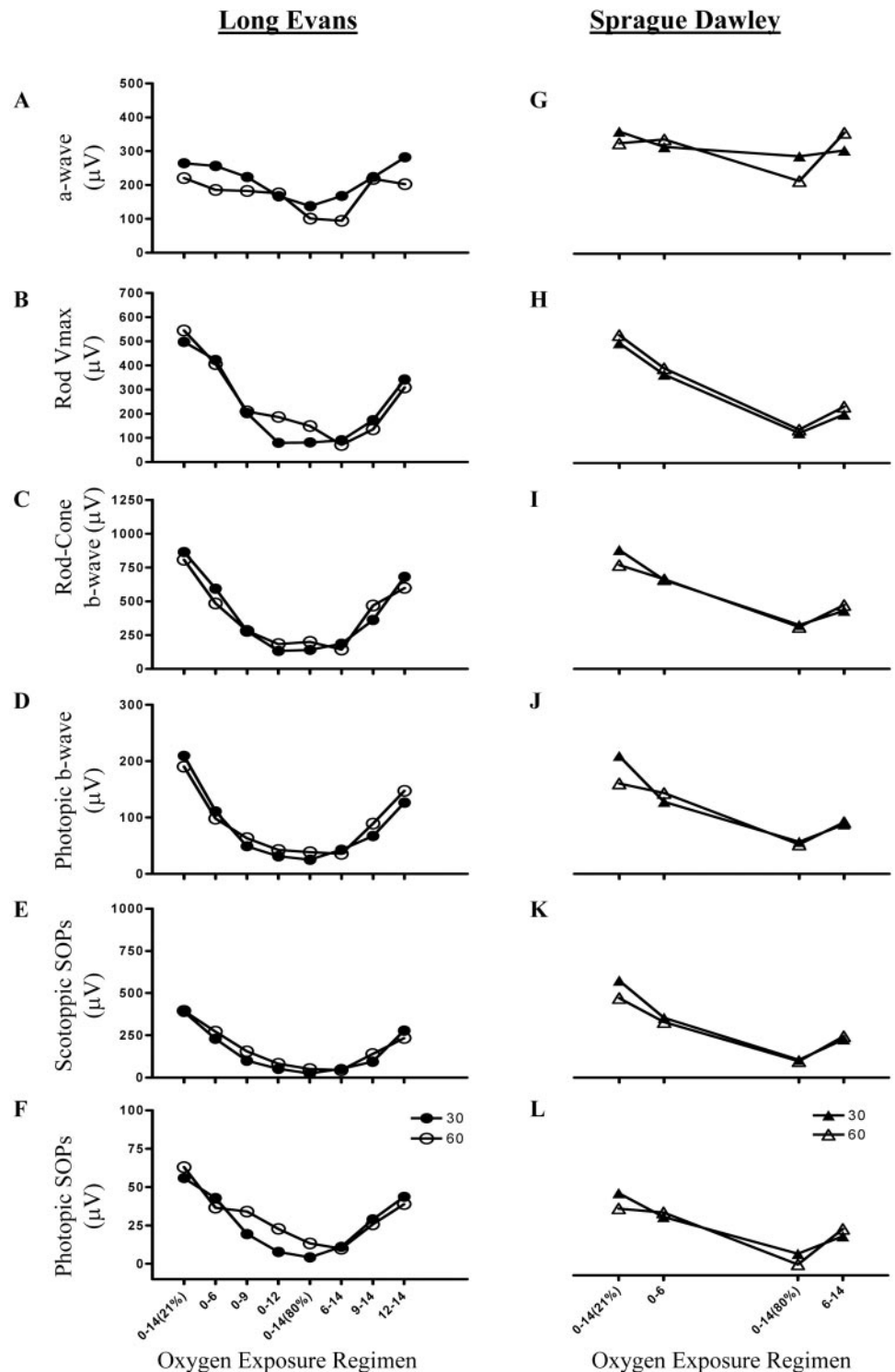


FIGURE 4. Graphic representation of a-wave (A, G), rod V_{max} (B, H), rod-cone b-wave (C, I), photopic b-wave (D, J), scotopic SOP (E, K), and photopic SOP (F, L) amplitudes (ordinate in microvolts) obtained at P30 and P60, respectively (abscissa in days of exposure), from LE and SD rats. The hyperoxic cohort of each strain was divided at birth into seven different exposure protocols: from birth through postnatal day 6 (P0–P6: LE, $n = 24$ [P30], $n = 5$ [P60]; SD, $n = 6$ [P30], $n = 6$ [P60]); birth to postnatal day 9 (P0–P9: LE, $n = 11$ [P30], $n = 5$ [P60]); birth to postnatal day 12 (P0–P12: LE, $n = 7$ [P30], $n = 6$ [P60]); and birth to postnatal day 14 (P0–14: LE, $n = 21$ [P30], $n = 6$ [P60]; SD, $n = 11$ [P30], $n = 7$ [P60]) or from P6, P9, or P12 through P14 (P6–P14: LE, $n = 22$ [P30], $n = 6$ [P60]; SD, $n = 8$ [P30], $n = 8$ [P60]; P9–P14: LE, $n = 17$ [P30], $n = 9$ [P60]; P12–14: LE, $n = 15$ [P30], $n = 7$ [P60]). Data were compared with results for respective age-matched control litters raised in normoxia (LE, $n = 21$; SD, $n = 22$).

to the second week of life also resulted in an attenuation of the a-wave amplitude to 63.4% and 42.9% of P30 and P60 control values, respectively ($P < 0.05$). Notably, the a-wave is considerably more affected in LE than SD rats exposed to hyperoxia, where a decline to 79.7% and 65.7% of control values, respectively, was observed at P30 and P60 in SD rats, compared with 51.9% and 45.6% of control values at P30 and P60, in LE rats (Fig. 4G).

A similar dose-response correlation between the duration of hyperoxia and the amplitude of the rod V_{max} in LE rats was

also observed in P30 and P60 recording sessions (Fig. 4B). The amplitude of the rod V_{max} was already attenuated by hyperoxia from P0 to P6 to 84.9% (P30) and 74.4% (P60) of control; this parameter continued to decline when oxygen exposure was extended into the second week of life. Similar findings were also observed when hyperoxia was initiated in the second week resulting in attenuations to 18.2% (P30) and 13.0% (P60) of control ($P < 0.05$). As seen for the a-wave, the rod V_{max} attenuation after maximum hyperoxia was notably less severe in SD rats (Fig. 4H) than in LE rats.

TABLE 1. Group Data Obtained in Scotopic and Photopic Conditions from LE Rats Raised in Normoxia and Hyperoxia

Age	Control	P0-P6	P0-P9	P0-P12	P0-P14	P6-P14	P9-P14	P12-P14
Scotopic parameters								
a-Wave (μ V)	264.34 $\pm$ 87.25	256.41 $\pm$ 85.60	223.34 $\pm$ 77.82	166.76 $\pm$ 67.68*	137.18 $\pm$ 44.29*	167.65 $\pm$ 64.47*	223.42 $\pm$ 89.78	282.08 $\pm$ 130.44
Rod V_{max} (μ V)	220.29 $\pm$ 58.84	185.80 $\pm$ 59.51	182.15 $\pm$ 56.72	175.00 $\pm$ 100.36	100.49 $\pm$ 27.73	94.57 $\pm$ 61.72*	217.62 $\pm$ 121.44	202.48 $\pm$ 153.34
Rod-cone b-wave (μ V)	497.58 $\pm$ 124.50	422.54 $\pm$ 123.10	202.82 $\pm$ 96.03*	79.90 $\pm$ 74.54*	81.63 $\pm$ 59.71*	90.32 $\pm$ 58.76*	173.61 $\pm$ 105.87*	341.60 $\pm$ 96.67*
	544.61 $\pm$ 178.97	405.44 $\pm$ 109.12	209.85 $\pm$ 138.10*	186.29 $\pm$ 127.92*	148.79 $\pm$ 98.49*	70.99 $\pm$ 42.08*	135.44 $\pm$ 119.43*	308.85 $\pm$ 101.74*
SOPs (μ V)	864.19 $\pm$ 193.40	593.61 $\pm$ 200.20*	277.20 $\pm$ 137.85*	133.00 $\pm$ 91.77*	139.84 $\pm$ 58.11*	187.31 $\pm$ 68.79*	362.09 $\pm$ 269.4*	680.97 $\pm$ 304.62*
	806.76 $\pm$ 257.07	483.50 $\pm$ 217.97	281.00 $\pm$ 204.02*	184.08 $\pm$ 153.75*	200.32 $\pm$ 122.22*	142.08 $\pm$ 46.91*	468.23 $\pm$ 350.81*	598.27 $\pm$ 423.08*
Photopic parameters	388.82 $\pm$ 100.64	229.23 $\pm$ 91.31*	99.52 $\pm$ 48.02*	51.05 $\pm$ 40.36*	24.28 $\pm$ 20.95*	51.28 $\pm$ 45.03*	93.99 $\pm$ 88.76*	277.80 $\pm$ 177.02*
	395.69 $\pm$ 94.98	271.81 $\pm$ 142.43	155.79 $\pm$ 121.60*	81.75 $\pm$ 78.20*	50.14 $\pm$ 47.45*	44.91 $\pm$ 37.73*	139.43 $\pm$ 108.68*	233.38 $\pm$ 185.07*
b-Wave (μ V)	209.61 $\pm$ 72.44	111.19 $\pm$ 40.21*	49.06 $\pm$ 35.97*	31.41 $\pm$ 13.51*	25.02 $\pm$ 10.79*	42.87 $\pm$ 27.22*	67.41 $\pm$ 53.26*	126.59 $\pm$ 71.18*
	190.29 $\pm$ 52.24	98.22 $\pm$ 40.65*	63.65 $\pm$ 58.33*	42.42 $\pm$ 23.21*	38.68 $\pm$ 21.13*	35.50 $\pm$ 23.65*	89.44 $\pm$ 64.71*	147.44 $\pm$ 102.81
SOPs (μ V)	55.88 $\pm$ 21.24	42.89 $\pm$ 16.92	19.42 $\pm$ 17.35*	7.71 $\pm$ 7.99*	4.20 $\pm$ 6.28*	11.11 $\pm$ 14.34*	28.97 $\pm$ 32.93*	43.67 $\pm$ 25.46
	62.93 $\pm$ 17.79	36.51 $\pm$ 20.08*	34.08 $\pm$ 20.46*	22.8 $\pm$ 11.96*	13.37 $\pm$ 10.57*	9.87 $\pm$ 6.67*	25.67 $\pm$ 15.67*	38.91 $\pm$ 30.77

Amplitudes are reported in microvolts (± 1 SD).

* Significant differences between hyperoxic cohorts and control groups identified by one-way ANOVA followed by post hoc Dunnett multiple comparison test.

† Significant differences between recordings obtained at 30 and 60 days were identified by Student's *t*-test.

A comparable trend was also observed for the rod-cone b-wave of LE rats (Fig. 4C), where significant attenuations in amplitude were observed after hyperoxic exposure from P0 to P6 to 68.7% of control at P30 ($P < 0.05$) and 59.9% of control at P60 ($P < 0.05$). Exposures from P0 to P14 further reduced the amplitude of the rod-cone b-wave. When initiated later in life, hyperoxia caused similar amplitude attenuations that were most pronounced after exposure from P6 to P14 (reductions to 21.7% of control at P30 and 17.6% of control at P60; $P < 0.05$).

Finally, given that there was no specific effect of hyperoxia on any particular oscillatory potential (OP), we are reporting them collectively as the sum of OPs (SOPs). Scotopic SOPs (Fig. 4E) in LE rats were all significantly attenuated after hyperoxia, irrespective of the exposure regimen. Attenuations in SOP amplitude were observed in exposures from P0 to P6 (59% of control [P30], and 68.7% of control [P60]; $P < 0.05$). After maximum exposure from P0 to P14, SOP amplitudes were significantly ($P < 0.05$) reduced to 6.2% and 12.7% of control amplitude at P30 and P60, respectively. Hyperoxia resulted in an almost equivalent attenuation after P6 to P14 as it did after a 2-week oxygen exposure.

Again, the rod-cone b-wave (Fig. 4I) along with the scotopic SOPs (Fig. 4K) were less attenuated after postnatal hyperoxia in SD rats where maximum rod-cone b-wave attenuations were 36.9% (P30) and 40.3% (P60) of control compared with 18.3% (P30) and 20.5% (P60) of control for the scotopic SOPs ($P < 0.05$).

Effect of Postnatal Hyperoxia on Photopic Function

The photopic b-wave and SOPs of LE rats raised in a hyperoxic environment were also markedly affected. A striking decline in the photopic b-wave amplitude (Fig. 4D), was observed after P0 to P6 exposure to 53.0% and 51.6% of control at P30 and P60, respectively ($P < 0.05$); this trend continued during extended hyperoxia (P0–P14). Oxygen exposure regimens initiated in the second week of life also resulted in significant amplitude attenuations. Photopic SOPs were similarly affected after postnatal hyperoxia (Fig. 4F). As described for the scotopic condition, the cone-mediated function of the SD rats also appeared to be less susceptible to the hyperoxic insult than was that of the LE rats. Along these lines, after hyperoxia from P0 to P14, the photopic b-wave of the SD rats (Fig. 4J) was attenuated to 27.3% and 32.7% of control at P30 and P60, respectively, compared with 11.9% and 20.3% in the LE rats.

Effect of Postnatal Hyperoxia on Retinal Cytoarchitecture

The most striking ultrastructural consequences of postnatal hyperoxia, reported in the neonatal SD rat model included the near disappearance of the OPL after maximum exposure to approximately 20.0% of control along with a significant reduction in horizontal cell count to approximately 20.0% of the control, both of which were most obvious after regimens that extended hyperoxia into the second week of life^{13,14,16,17} (Figs. 5F, 5G). Added to this, postnatal hyperoxia in LE rats also damaged other retinal structures as exemplified with representative histologic sections shown in Figures 5A–E. These are characterized by a marked thinning of the inner retina as well as outer retinal damage limited to the outer nuclear layer (ONL). Group data are graphically presented in Figure 6. As shown in Figures 5B and 6, early exposure from P0 to P6 resulted not only in a reduction in OPL thickness to 65.6% of control ($P < 0.05$), but also in a significantly thinner ONL, inner nuclear layer (INL), and inner plexiform layer (IPL); reduced to 61.5%, 80.2%, and 70.9% of the respective controls). Progressive increases in duration of oxygen exposure, partic-

TABLE 2. Group Data Obtained in Scotopic and Photopic Conditions from SD Rats Raised in Normoxia and Hyperoxia

	Age	Control	P0-P6	P0-P14	P6-P14
Scotopic parameters					
a-Wave (μV)	30 d	361.07 $\pm$ 109.65*	315.30 $\pm$ 50.70	287.65 $\pm$ 69.38*	305.27 $\pm$ 70.41*
	60 d	326.35 $\pm$ 57.68*	337.68 $\pm$ 85.41*	214.32 $\pm$ 82.09*	357.36 $\pm$ 96.43*
Rod V_{max} (μV)	30 d	496.25 $\pm$ 146.35	366.03 $\pm$ 66.63†	123.15 $\pm$ 92.98†	201.19 $\pm$ 138.68*†
	60 d	529.24 $\pm$ 85.92	392.08 $\pm$ 90.25	138.08 $\pm$ 137.25†	232.96 $\pm$ 114.33*†
Rod-cone b-wave (μV)	30 d	879.35 $\pm$ 226.06	656.83 $\pm$ 122.92†	324.79 $\pm$ 116.96*†	430.05 $\pm$ 164.67*†
	60 d	766.74 $\pm$ 107.27‡	661.67 $\pm$ 160.81	309.18 $\pm$ 248.22†	472.25 $\pm$ 102.94*†
SOPs (μV)	30 d	574.01 $\pm$ 297.05*	353.63 $\pm$ 81.25*	104.86 $\pm$ 69.00*†	225.95 $\pm$ 133.51*†
	60 d	470.19 $\pm$ 237.24	329.01 $\pm$ 92.99	96.44 $\pm$ 89.80†	244.39 $\pm$ 54.7*†
Photopic parameters					
b-Wave (μV)	30 d	209.25 $\pm$ 72.92	128.05 $\pm$ 33.09†	57.18 $\pm$ 34.09*†	87.99 $\pm$ 42.61*†
	60 d	160.49 $\pm$ 30.08‡	143.67 $\pm$ 38.34	52.43 $\pm$ 44.79†	91.72 $\pm$ 19.58*†
SOPs (μV)	30 d	73.05 $\pm$ 18.91*	51.91 $\pm$ 7.57	19.15 $\pm$ 25.35*†	34.81 $\pm$ 25.96†
	60 d	59.47 $\pm$ 40.31	55.94 $\pm$ 18.96	9.64 $\pm$ 7.66†	41.28 $\pm$ 23.73*

Amplitudes are reported in microvolts ($\pm$ 1 SD).

* Significant differences between values obtained from SD rats and LE rats in Table 1, identified using the Student's *t*-test.

† Significant differences between hyperoxic cohorts and control groups identified with a one-way ANOVA followed by the post hoc Dunnett multiple-comparison test.

‡ Significant differences between recordings obtained at 30 and 60 days identified by Student's *t*-test.

ularly when encompassing the second week of postnatal life, resulted in even greater structural anomalies. As shown in Figures 5C and 6, exposures within the first 2 weeks of life

resulted in maximum thinning of the ONL, OPL, INL, and IPL to 67.6%, 0.7%, 26.0%, and 45.8% of control, respectively ($P < 0.05$). When hyperoxia was initiated in the second postnatal

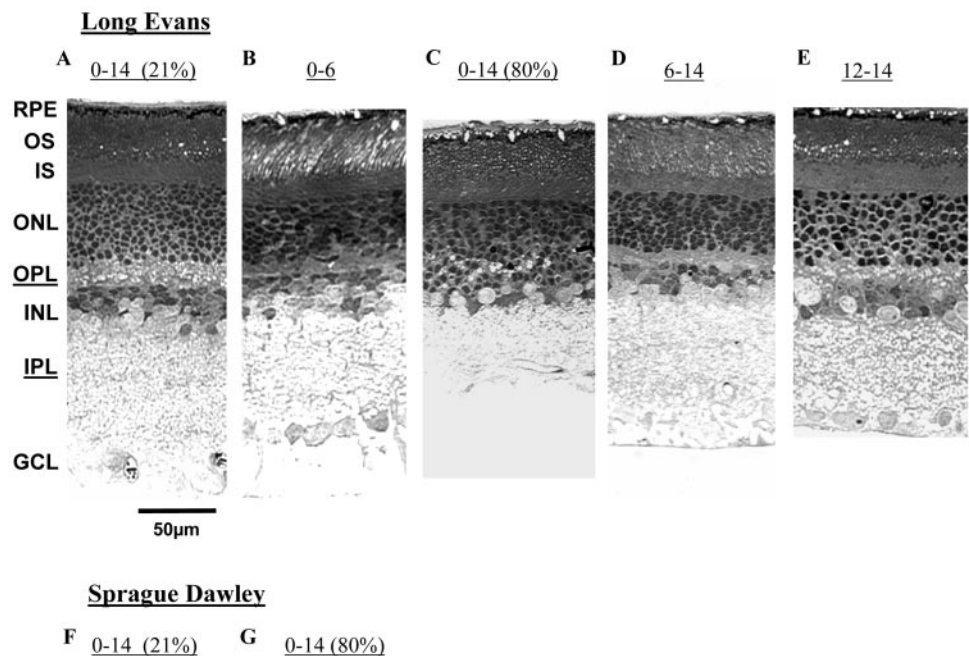


FIGURE 5. Representative retinal histologic sections (central retina) from LE rats obtained at P60 from the normoxic cohort (A), and from hyperoxic cohorts including P0 to P6 (B), P0 to P14 (C), P6 to P14 (D), and P12 to P14 (E). Retinal sections obtained from SD rats raised in normoxia (F) and maximum hyperoxia (P0-P14, 80%; G) are also shown for comparative purposes. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment. Scale bar, 50 μm .

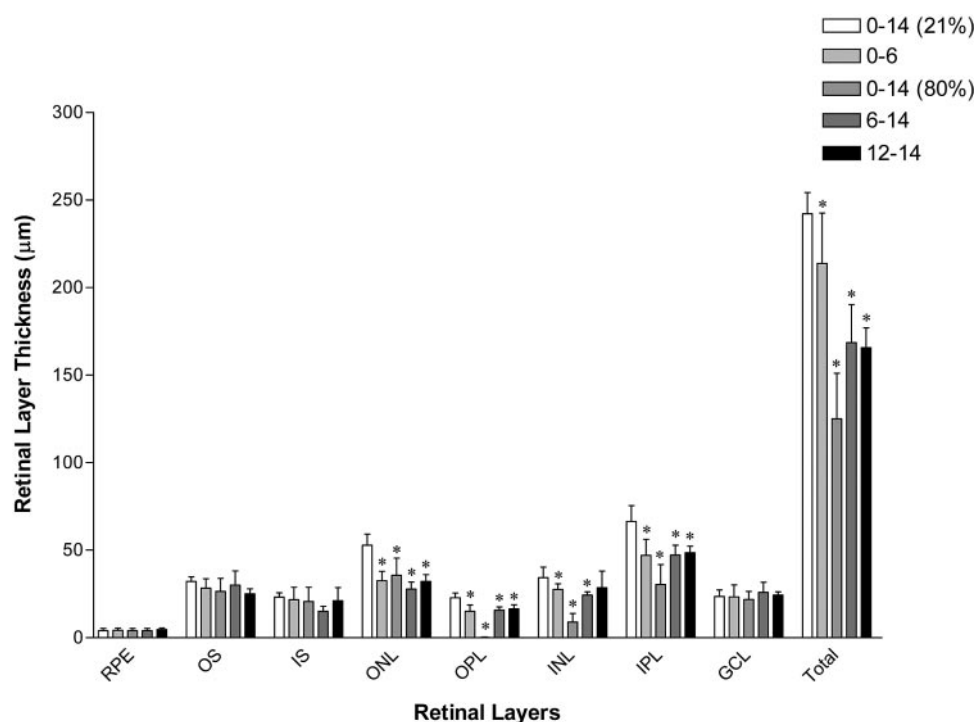


FIGURE 6. Graphic representation of the mean retinal layer thicknesses (in micrometers) and total retinal thickness from control (P0–P14, 21%) and from hyperoxic LE cohorts, including P0 to P6, P0 to P14, P6 to P14, and P12 to P14 ($n = 3$ per group). Measurements were taken at P60. Significant differences within normoxic or hyperoxic cohorts were identified with a one-way ANOVA followed by the Dunnett multiple comparison test comparing data obtained from each regimen with control; *significantly different from control ($P < 0.05$). Results are given as the mean thickness ± 1 SD.

week (P6–P14 and P12–P14; Figs. 5D, 5E, 6), the thinning of the retinal layers was more important than that observed after exposures in the first postnatal week, but not as severe as that observed during 2 weeks of hyperoxia (P0–P14).

Neurotrophic Factor Expression after Postnatal Hyperoxia

Previous studies have suggested that mechanical injury to the retina,^{37–39} in addition to light-induced damage,^{40–42} can lead to the upregulation of neurotrophic factors. Furthermore, studies conducted in our laboratory have suggested that an increase in endogenous levels of some of these factors, such as FGF-2 and CNTF for example, plays a role in the neuroprotection of juvenile rat retinas against another environmental oxidative stress—namely, light-induced damage.²⁷ To determine whether a similar protective mechanism might explain the difference between SD and LE rats, we examined the levels of retinal FGF-2 and CNTF (Fig. 7) by Western blot analysis. LE and SD rat retinas were collected immediately after early oxygen exposure (P0–P6) at P6 and late oxygen exposure (P6–P14) at P14 and were compared with retinas collected from age-matched control rats at P6 and P14, respectively. Although FGF-2 levels in LE rats had a tendency to increase after exposures from P0 to P6, P6 to P14, and P0 to 14 compared with their respective controls (Fig. 7A), results did not reach statistical significance. Similarly, FGF-2 levels also tended to increase in SD rats after postnatal hyperoxia (Fig. 7B) after each exposure regimen compared with control. It is interesting to note, however, that control SD rats appeared to have approximately four to five times higher endogenous levels of FGF-2 than did the LE rats. This tendency extended during exposure to hyperoxia.

In all hyperoxic cohorts of LE and SD rats studied, CNTF levels appeared higher compared with their corresponding controls (Figs. 7C, 7D).

Retinal immunohistochemistry was performed to determine the cellular localization of FGF-2 and CNTF proteins in LE and SD rats after exposure to hyperoxia. Figures 8A and 8B reveal that, as described above, FGF-2 immunoreactivity was more pronounced during exposure to hyperoxia compared with the

control. Immunofluorescence in both strains was limited to the INL and ganglion cell layer (GCL). Basal levels of CNTF staining were observed in the INL and GCL of the control animals and were markedly increased after exposure to hyperoxia in both LE and SD rats (Figs. 8C, 8D). The localization of CNTF suggested that these were Müller cells (GFAP positive, Fig. 9).

DISCUSSION

Previous studies showed that postnatal hyperoxia in the albino SD rat model of OIR resulted in severe structural and functional damage, including the permanent loss of the OPL, significant reduction in the horizontal cell count, and permanent attenuation in retinal function, as observed with the scotopic and photopic ERGs.^{13–17} Results from the present study suggest an even greater susceptibility of the pigmented LE rat to postnatal hyperoxia, as evidenced with higher vascular dropout throughout exposure, more severely depressed retinal function and permanently altered retinal structure that was not only limited to the loss of the OPL and horizontal cells as in SD rats, but also extended into the inner retina, resulting in significant loss of the INL and IPL.

These alterations in retinal cytoarchitecture are likely to be at the root of the functional anomalies, such as the severe attenuation of the ERG b-wave we are reporting. The most critical, and most permanent, sequelae of OIR in the SD rat model occurred in the more inner part of the retina. However, the tendency for the shortening of inner segments of the photoreceptors which is accompanied by a significantly attenuated a-wave in LE rats after hyperoxia is suggestive of a photoreceptor involvement as well. Therefore, the short- and long-term consequences of postnatal hyperoxia on the retinal structure and function were significantly more pronounced in LE rats than in SD rats.

Free radicals that are generated through lipid peroxidation have been thought to play an important role in the pathogenesis of ROP and its animal counterpart, OIR.⁴³ It has been suggested that the formation of free radicals in the retina may result from the exposure of premature infants to a hyperoxic

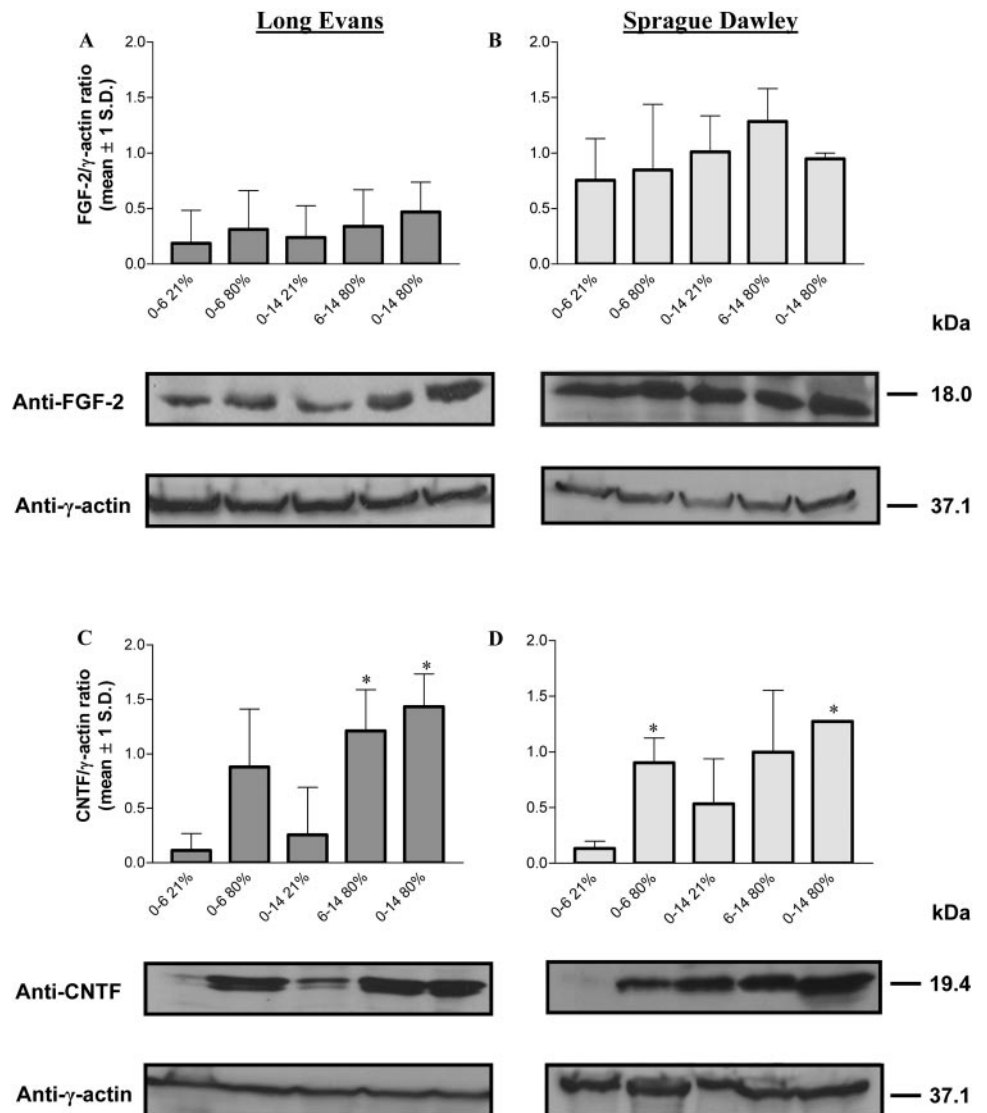


FIGURE 7. Western blot analysis was used to detect FGF-2 and CNTF protein levels in LE (A, C, respectively) and SD (B, D, respectively) rats. Retinas were collected from normoxia-raised rats at P6 and P14 and from rats exposed to hyperoxia from P0 to P6, P6 to P14, and P0 to P14 ($n = 3-4$ per group). Neurotrophic factor levels were normalized to γ -actin (ordinate: ratio/ γ -actin, mean $\pm$ 1 SD) for quantification. *Right:* molecular mass (in kilodaltons) of each band (18 kDa for FGF-2, and 19.4 kDa for CNTF). *Statistically significant differences ($P < 0.05$) obtained with a Student's *t*-test comparing exposed animals from the P0 to P6, P6 to P14, and P0 to P14 groups to age-matched control rats.

environment, particularly due to their deficiency in antioxidant defenses, such as superoxide dismutase, α -tocopherol, catalase, and glutathione peroxidase, for example.^{44,45} Reactive oxygen species (ROS) have also been shown to contribute to the vaso-obliterative phase that is associated with OIR⁴⁶⁻⁴⁹ either through direct cytotoxic effects on endothelial cells or through an initial phase of vasoconstriction which ultimately results in reduced tissue perfusion. Of interest, treatment with vitamin E, a free-radical scavenger, has been shown to protect against the loss of superoxide dismutase in cats exposed to hyperoxia⁵⁰ and has successfully attenuated the severity of vaso-obstruction in the rat model of OIR.^{49,51} A previous study of ours revealed that administration of the water-soluble antioxidant Trolox C to SD rats prevents only some of the structural and functional sequelae associated with OIR, suggesting that free radical formation may not be the sole cause of the damage.¹⁴ Similarly, our current results suggest that melanin would be insufficient in having any beneficial protective effect under similar circumstances; and moreover, it appeared as though it might even potentiate the effect of ROS. Melanin is produced and stored in the retinal pigment epithelium (RPE) cytoplasm and melanocytes, and continuous exposure of the RPE to environmental stressors such as light and oxygen have been thought to dampen its antioxidant capacity.⁵² Furthermore, melanin has been known to take on the role of a

pro-oxidant under these conditions, leading to cytotoxic activity such as photoreceptor damage and even age-related macular degeneration.^{53,54} This would explain the severity of OIR in LE rats, including a significantly injured photoreceptor layer, given its proximity with the RPE. Our findings of increased MDA levels in LE compared with SD rats (after hyperoxic exposure initiated at birth throughout the first week of life) support this inference. Recently, we showed that despite the damage to retinal vasculature that is observed within the first week or so of exposure of SD rats (e.g., P0-P6 and P0-P9), the retinal vascular growth process appears to be able to fight this oxidative stress to achieve nearly full coverage while remaining under hyperoxic exposure throughout the first 2 weeks of life,¹³ a finding that is further corroborated by the results in the present study. A different picture emerges in the pigmented rat, however, where vascular dropout and extent are so severely compromised that they never achieve a normal appearance (Fig 1). Taken together, these anatomic characteristics are inconsistent with a protective role of melanin in the retinal vasculature and inner structures. This result suggests that other mechanisms partake in the increased vulnerability of LE compared with SD rats, as observed in the present study. It is of interest to note that the complex series of events that characterize retinal development occur differently in albino and pigmented animals, where retinal maturation is delayed in the

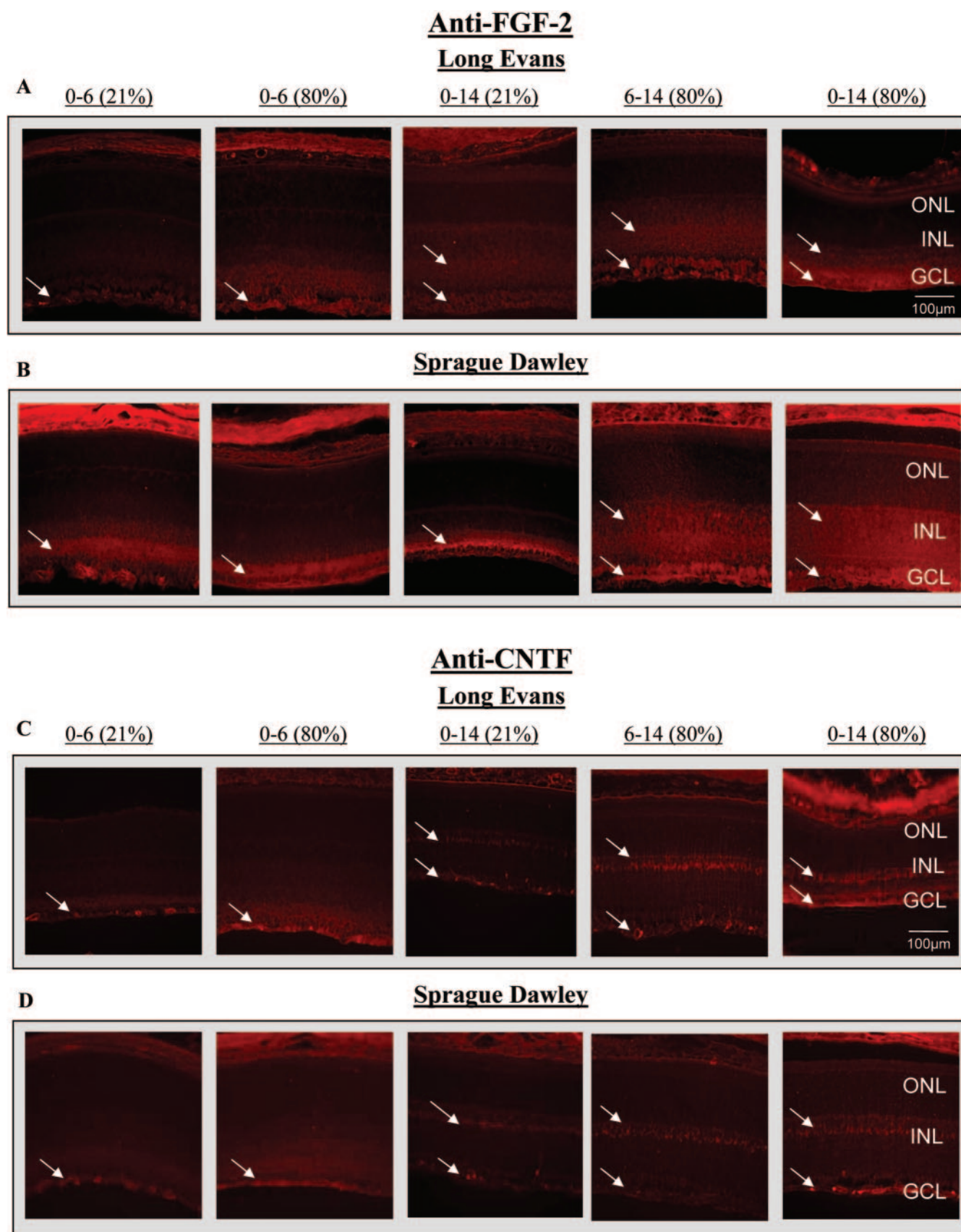
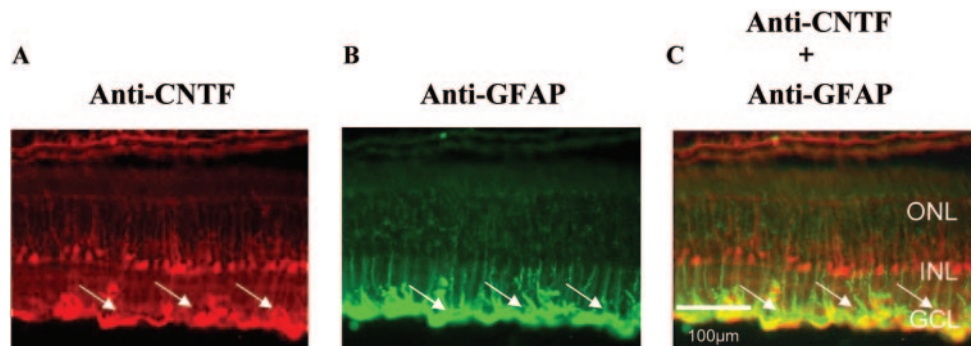


FIGURE 8. Retinal cellular localization of FGF-2 (**A, B**) and CNTF (**C, D**) in representative control retinas at P6 and P14 and in exposed retinas after hyperoxia from P0 to P16, P6 to P14, and P0 to P14 in LE (**A, C**) and SD (**B, D**) rats. Upregulation of FGF-2 and CNTF staining was observed in the INL and GCL by fluorescence microscopy after hyperoxia regimens for both strains. Scale bars, 100 μ m.

FIGURE 9. Colocalization of CNTF immunostaining (A) with anti-GFAP labeling (B) specific for active Müller glial cells, revealed that Müller cells actively participate in upregulating CNTF levels after hyperoxia (shown in retinas of LE rats after maximum exposure from P0 to P14 collected at P60; C). Scale bar, 100 μ m.



absence of melanin.⁵⁵ For example, studies have revealed a temporal delay in the central-to-peripheral pattern of cell production^{56,57} along with a delay in cell death of the INL and GCL in the albino rat retina.⁵⁷ One wonders whether the more “mature” retina of the LE rat might result in a greater difficulty in overcoming hyperoxic stress, whereas the more plastic immature retina of the albino rat is better able to overcome a similar stress. Variations in the responses of different rat strains to hyperoxia, including the increased susceptibility of the Dark Agouti and Hooded Wistar rats compared with several albino strains,⁵⁸ as well as that of the pigmented offspring of Fischer 344 X Dark Agouti compared with the albino progeny,⁵⁹ further support the association between ocular pigmentation and vulnerability in OIR. Furthermore, although no difference in the time course of retinal vascular development was observed between age-matched control Brown Norway and SD rats in a study by Gao et al.,¹⁹ those raised in hyperoxia showed significant differences in vascular permeability and retinal neovascularization. For example, retinal artery constriction along with large avascular regions in the retina were observed in Brown Norway rats at P12—that is, 1 day after their removal from the hyperoxic environment. In contrast, SD rats studied under the same conditions revealed a lower degree of retinal artery constriction and larger areas of vascular perfusion throughout the retina.¹⁹ Increased vascular permeability followed by a more severe neovascularization in the Brown Norway strain^{19,60,61} was also observed. This effect was characterized by tortuosity and dilatation of retinal vessels, neovascular tufts, and hemorrhage when compared with the SD rats, which were lacking neovascular tufts and evidence of microaneurysms.¹⁹ Moreover, the Brown Norway model of OIR revealed higher retinal VEGF levels and more severe retinal vascular leakage than did the SD strain.⁶² Taken together with our current results and considering that the secondary neovascular phase of OIR is thought to be a mirror image of the initial vasoconstriction and vaso-oblivation which takes place, these findings suggest that retinal vascular development along with the neovascularization phase that ensues is significantly more affected by hyperoxia in pigmented than albino SD rats. Variations in the regulation of the antiangiogenic pigment epithelium-derived factor (PEDF) and the angiogenic vascular endothelial growth factor (VEGF) among strains are thought to contribute to these discrepancies, which may be genetically regulated,¹⁹ not to mention other strain-dependant factors that may be involved in regulating the effects of oxygen tension on retinal angiogenesis, such as glial cell sensitivity to hyperoxia, for example.⁶³

Evidently, despite using identical oxygen exposure protocols, the pathophysiological consequences of OIR were much more severe in the pigmented LE model than in the albino SD rat model. Not only did the hypothesized free-radical-scavenging role of melanin fail to protect the LE cohorts that were exposed to hyperoxia postnatally, but significantly greater levels of the MDA product of peroxidation were also evidenced in

the LE model. We therefore sought to further understand the mechanisms by which SD rats remained significantly more resistant to a hyperoxic stress by exploring the potential involvement of specific neurotrophic factors throughout the hyperoxia. In a previous study, we showed that the relative (compared with adult) resistance of juvenile SD rats exposed after birth to a bright luminous environment (not combined with oxygen), another oxidative stress, might be explained by the upregulation of neurotrophic factors such as FGF-2 and CNTF. In adult retinas, FGF-2 and CNTF levels are also upregulated after mechanical injury. Furthermore, intravitreal injection of the above-mentioned factors in addition to brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor have all been shown to delay photoreceptor cell death in animal models of retinal degeneration.^{64–69} We therefore hypothesized that the expression of similar survival factors might explain the relative resistance of SD rats to (1) photoreceptor damage (as evidenced by the relatively intact a-wave) and to (2) more severe thinning of the inner retinal layers. Of note, our results suggest that although only limited variations in FGF-2 occur in both LE and SD models after hyperoxic exposure, higher levels (approximately twofold) are observed for each exposure regimen in SD rats. Furthermore, although CNTF levels are only significantly higher after exposure regimens that include the second week of life in LE rats, they are already significantly greater in SD rats after exposure within the first week (e.g., from P0–P6). These results would therefore suggest that SD rats are better equipped with survival factors in the face of a hyperoxic challenge. The involvement of CNTF and FGF-2 may explain the preservation of photoreceptor function in SD rats as well as the relative resistance to functional and structural damage that follows hyperoxia from P0 to P6. Moreover, previous studies have shown that, in the adult retina, light-induced damage provokes Müller cell gliosis,⁷⁰ which in turn releases several neurotrophic factors.^{58–60,71–73} Microglia have been thought to play an integral role in this process as they have been shown to secrete factors that diffuse to the outer retina.⁷⁴ Given the relatively intact inner retina that is observed in SD rats after hyperoxic exposure, the above may indeed be a plausible mechanism of photoreceptor survival in this model. The severely compromised inner retina of LE rats exposed to the same conditions, however, would make it quite difficult for a similar protective mechanism to take place and may consequently explain their increased susceptibility, particularly where photoreceptor function is concerned.

Conflicting results have been obtained using gene transfer strategy in Prph2Rd^{2/Rd2} mice receiving subretinal injections with adenoviral (Ad)-vectored CNTF that resulted in the preservation of ERG amplitudes^{75,76} and with adenoassociated virus (AAV)-vectored CNTF that resulted in accelerated deterioration of ERG amplitudes,⁷⁷ suggesting that therapeutics of this nature still require further study. More recently, however, Dong et al.⁷⁸ demonstrated that double transgenic mice with inducible glial-derived neurotrophic factor (GDNF) provides protection from loss of retinal function (a- and b-waves of the

ERG) generated by three different sources of oxidative stress, including one where mice were exposed to 75% oxygen for 3 weeks. It may therefore also be worthwhile to explore the potential antioxidant activity (or other) that GDNF exerts on pigmented and albino models of OIR and in other models of disease in which oxidative damage plays a role.⁷⁸

As described earlier, there is an apparent discrepancy between some clinical studies of human ROP which have shown, for example, that a greater number of Caucasian infants reach threshold ROP compared with African-American infants^{8,10} and the animal model, which suggests a positive correlation between pigmentation and the severity of OIR.

Of interest, however, the greater susceptibility of the SD strain to vascular attenuation after hyperoxia compared with other albino strains such as the Fischer 344, Wistar-Furth, and Lewis strains would further support a role for factors other than ocular pigmentation.⁵⁵ Given that genetic factors appear to influence the angiogenic response of different rat strains, it would not be surprising that they also play a role in the increased incidence and severity of the retinopathy observed on a functional and structural level. Of interest, studies have shown that ventilatory responses are thought to be genetically as well as environmentally regulated^{21,22} resulting in varying responses to changes in O₂ and CO₂ levels, for example.²² In fact, our findings of an increased mortality rate of Brown Norway rats after exposure to the same hyperoxic protocol used to obtain our current results may be explained by these strain-related differences, which suggests an unequal susceptibility of different rat strains. It is also of interest to mention that differences in the incidence and severity of OIR were observed among Sprague-Dawley rats obtained from independent commercial sources, suggesting an important role of genetics, even within a given strain (Kitzmann AS, et al. *IOVS* 2002;43:ARVO E-Abstract 1256). Of course, studies aimed at further elucidating the genetic and racial factors affecting the incidence of ROP in humans are even further complicated by several confounding variables including sex, socioeconomic status, geographical areas, and environment, although a deeper understanding of these factors may explain the relative sparing of some ethnicities with respect to the development of severe ROP versus others that seem more susceptible. Although a connection between racial variation and the incidence and severity of diseases such as ROP⁸⁻¹⁰ and age-related macular degeneration (ARMD)^{79,80} have been documented, it is important to take into consideration that very little is understood regarding the functional mechanisms of differences in pigmentation⁸¹ and that current studies are under way that are aimed at better understanding the molecular composition of melanosomes, along with their structure and function in the eye.^{82,83} Other studies have shown that melanin may not act alone as the substance responsible for the protective effect,^{84,85} but may instead be a confounding variable that is associated with certain genes that are more or less prevalent in certain populations. Genetic influences in the pathogenesis of these diseases may play an important role in the heterogeneity in risk of their progression.

The underlying genetic differences that could explain the differential susceptibility between rat models of OIR as well as humans of different ethnicities remain to be explored.

In conclusion, whereas some clinical studies suggest that the presence of increased melanin content confers added protection to infants that are postnatally exposed to hyperoxia as observed in African-Americans that appear to be protected against the development of severe ROP,^{8,10} other studies have concluded that fundus pigmentation may play only a limited role in this protection.^{11,12} Present animal findings suggest an increased susceptibility of pigmented LE rats to postnatal hyperoxia in comparison with the albino SD rat. Given the dis-

crepancy between findings, future studies warrant careful investigation of other factors, including the contribution of genetics, which may help to elucidate the differences between LE and SD rats (as well as others) and to further our understanding of the pathogenesis of OIR and ROP, to potentially facilitate the derivation of therapeutic modalities.

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The effect of oxygen and light on the structure and function of the neonatal rat retina

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Abstract The neonatal rat is born with its eyes closed and an immature visual system, that some say is equivalent to that of a human fetus at 26 weeks of gestation. From birth, the visual system of the newborn rat will gradually mature, the first manifestation of that being the opening of the eye which usually take place at postnatal day 14. Complete maturation of the retina and visual pathways is normally reached at the end of the first month of life. The neonatal rat model thus represents a unique paradigm to study the normal and abnormal maturation of the primary visual pathways that normally occurs in utero in human subjects. Our laboratory has, over the past decade, developed two animal models of postnatally induced retinopathy,

namely the Oxygen-Induced Retinopathy (OIR) that share several common features with the human Retinopathy of Prematurity (ROP) and the Light-Induced Retinopathy that is viewed by some as a valid model of some forms of Retinitis Pigmentosa (RP). The following pages review what is known of the pathophysiological processes taking place and suggest possible therapeutic avenues that could be explored in order to halt the degenerative process.

Keywords Retinopathy · Oxygen · Light · Neonatal rat · Electroretinogram · Histology

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Introduction

The neonatal rat is born with its eyes closed and an immature visual system, that some say is equivalent to that of a human fetus at 26 weeks of gestation. From birth, the visual system of the newborn rat will gradually mature, the first manifestation of that being the opening of the eyes that usually take place at postnatal day 14 and we have recently shown that a near complete maturation of the rat's retinal structure and function is reached by the end of the first month of life [1]. The neonatal rat model thus represents a unique paradigm to study the normal and abnormal maturation of the primary visual pathways that normally occurs in utero in human subjects. Our laboratory has, over the past decade, developed two

animal models of postnatally induced retinopathy, namely the Oxygen-Induced Retinopathy (OIR) that share several common features with the human Retinopathy of Prematurity (ROP) and the Light-Induced Retinopathy that is viewed by some as a valid model of some forms of *Retinitis Pigmentosa* (RP). The following pages review what is known of the pathophysiological processes taking place and suggest possible therapeutic avenues that could be explored in order to halt the degenerative process.

The effect of postnatal hyperoxia on the developing retina

Retinopathy of Prematurity (ROP) is a condition observed in human infants that are born prematurely and receive high levels of oxygen postnatally to compensate for their unstable pulmonary status. ROP is a vasoproliferative disorder that, in its most severe form, can result in retinal detachment and possibly blindness. It is characterized by two phases, first initial vasoconstriction following which vessels are obliterated in response to higher than normal levels of oxygen which prevents normal vascular growth towards the retinal periphery, and secondary neovascularisation upon the return to a normoxic environment which is the result of an inadequate blood supply [2, 3].

The rat is one of the most studied of all animal models of oxygen-induced retinopathy (OIR). Contrasting with other animal models that have been used to study the pathophysiology of this disease [4–7], the neonatal rat offers the advantage of a highly immature retina at birth that is comparable to that of a 24–26 week old human fetus [8]. As in humans, exposure of the rat pup to postnatal hyperoxia will cause severe vasoconstriction and vaso-obliteration that are followed by an abnormal proliferation of retinal vessels upon the return to room air [9–13]. Other features that are reminiscent of the human form of ROP have also been observed in that rat model of OIR, including retinal hemorrhages, retinal folds and rosettes, though retinal detachment has not been documented in this model [14]. Some other advantages of the rat include the large litter sizes (approximately 15 pups per dam dependant upon the strain), low costs associated with developing the model, and the knowledge that has already been acquired from the rat with respect to various antioxidant, antiangiogenic,

and anti-inflammatory therapeutics [11], which all make it a very attractive model to study the pathogenesis of OIR and consequently better understand the sequelae of ROP.

Given that vascular impairments are considered to be the clinical hallmark of ROP, it is not surprising that many previous efforts have been largely focused on the vasculopathy that is associated with OIR (vasoconstriction, vaso-obliteration, and neovascularisation) [5, 14–17]. Our goals, however, have not only included studying retinal vasculature throughout hyperoxic exposure (retinal flatmounts), but also to better understand the structural (retinal histology) and functional (rod mediated- and cone-mediated electroretinograms) features that characterize this model. Furthermore, given that free radicals have been thought to play a role in the pathogenesis of ROP [16–20] and that the retinal vascular anomalies found in OIR rats were shown to be partly prevented by free radical scavengers, we also examined whether they could also exert a protective effect on a structural and functional level.

Multiple attempts have been made by investigators to establish an animal model that closely mimics human-like vascular fluctuations given that the arterial oxygen level of preterm infants has been shown to fluctuate significantly. Studies have shown that severe ROP is associated with variability in transcutaneous oxygen within the first 2 weeks of life, suggesting that these variations in oxygen might interrupt the normal vascularisation process which responds to hypoxic and hyperoxic triggers [21]. Approximately, ten years after discovering the link between oxygen and human ROP, researchers began to focus on developing an experimental animal lesion that could be used to better understand the pathophysiology of the disease. Logically, the most appropriate model of ROP would be an animal model that has a similar pattern of vascular development as the human, and one that is also premature [11]. While the animals used as models for ROP are born with an immature visual system, it is important to take into consideration that they do not experience any other consequences that are associated with prematurity, including respiratory distress syndrome, intracranial hemorrhage, necrotizing enterocolitis, and bronchopulmonary dysplasia, for example [22]. A number of different oxygen exposure regimens have been used based on variations of oxygen concentration and

duration of exposure in order to more closely represent what is observed in human neonates [6, 10, 23, 24], though this is only one aspect of what these premature infants experience. Experiments in our laboratory were conducted in newborn litters of Sprague-Dawley rats that were exposed to 80% oxygen immediately after birth (mixture of medical grade 100% OB_{2B} and room air measured with a MaxO₂ ceramtec oxygen meter, model OM25-ME, Medicana Inc., Montreal, Canada) as previously described [1, 25–28], interrupted by three intervals of 30 min' duration under normoxic conditions (21% O₂). A correlation between the duration of hypoxic episodes and neovascularisation had previously been shown to occur following a similar regimen [10] as well as others including oxygen exposure of 50/10% and 40/10% O₂ [24].

The retina is extremely vulnerable to oxygen-mediated damage for a number of reasons. The retina has a very high oxygen consumption rate, and oxygen radicals are normally formed in the mitochondria as oxygen is reduced along the mitochondrial electron transport chain. Under hyperoxic conditions, the number of oxygen-derived radicals is amplified and can disturb various cellular components such as lipids, nucleic acids, and proteins, causing cytotoxicity [12]. The retina is also composed of lipids that contain high levels of polyunsaturated fatty acids such as docosahexaenoic acid (DHA; 22:6 ω -3), arachidonic acid (AA; 20:4 ω -6), and choline phosphoglyceride which are all targets for peroxidation. Finally, the retina is also the site of phototransduction and photic excitation is known to initiate oxygen radical formation [29]. Of interest, retinal endothelial cells seem particularly susceptible to peroxidation-induced injury, whereas perivascular astrocytes, pericytes, and smooth muscle cells appear to be relatively more resistant [30–32]. While previous studies have evidenced significant reductions in vascular coverage following postnatal hyperoxia in rats [9, 10, 14–17], few have examined the susceptibility of retinal vasculature to postnatal hyperoxia as a function of the degree of vascular maturity at the time of exposure. For example, despite the attenuation in vascular growth that is apparent within the first week of life, the vascular growth process, is nonetheless, able to overcome this oxidative stress thereby achieving full coverage while under hyperoxic exposure throughout the first 2 weeks of life (Fig 1a and b). This is reminiscent of the

reversible vasculopathy that is observed in the human form of ROP. The phenomenon of early vascular susceptibility is further supported by the window of blood vessel plasticity that has been described [33] where early postnatal exposure to hyperoxia (P4–P6) similarly resulted in vaso-obliteration. This was explained by the differential susceptibility of immature vessels that have not yet acquired pericytes versus more mature vessels that appear to be more capable of withstanding a similar stress. VEGF has also been implicated in the development of retinal vasculature and retinal coverage, and hyperoxia is thought to counteract its expression, which eventually leads to an underdeveloped retinal vasculature [34]. Paradoxically, however, while exogenous VEGF administration has been shown to act as a vascular survival factor, thus rescuing vasculature from hyperoxia-induced destruction [35], the revascularization process has also been shown to be accelerated throughout prolonged hyperoxia (when VEGF levels are suppressed), thereby placing less emphasis on a role for VEGF involvement [17, 36].

Despite the fact that ROP is considered a disease of the developing retinal vasculature and that retinal vascular repair indeed occurs in some cases, functional sequelae have been shown to persist nonetheless. For example, in patients with a history of ROP, significant abnormalities in the a- and b-waves of the ERG have been shown to carry on into adulthood [37–39]. Furthermore, elevations of the scotopic visual thresholds in children with a history of resolved, mild ROP are also suggestive of photoreceptor involvement [40–42]. Finally, significant deficits in amplitude and implicit time of the multifocal ERG (mfERG) response, which is a central retinal response, have also been shown to occur in subjects whose clinical ROP had completely resolved more than 10 to 20 years earlier [43]. These results would suggest that a history of ROP could alter central retinal development and therefore, the most central responses of the mfERG. Given that ON- and OFF-bipolar cells contribute to this central response, the attenuation in amplitude might occur should ROP specifically target these cells and thereby cause for an altered summation of depolarization and hyperpolarization in these subjects [43]. Therefore, despite the resolution of vascular abnormalities which is a common occurrence in ROP patients, it is necessary to consider that neural dysfunction of the retina may still carry on throughout adulthood.

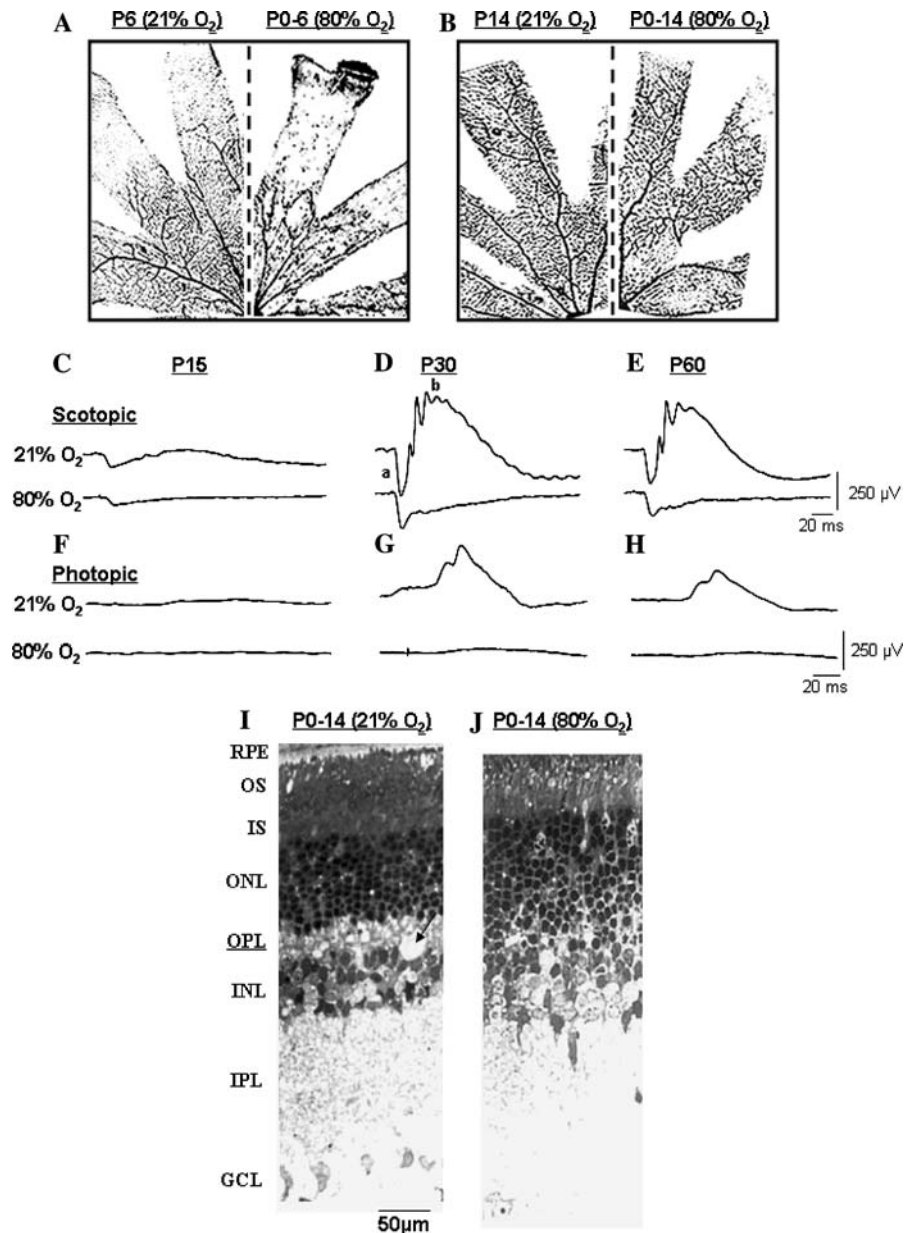


Fig. 1 (a, b) Representative retinal vascular flatmounts stained with ADPase from control animals euthanized at postnatal day 6 and animals exposed to hyperoxia from P0 to P6 (a) and control animals euthanized at postnatal day 14 and animals exposed to hyperoxia from P0 to P14 (b), respectively. Magnification, $\times 40$. (c–h) Representative scotopic (c, d, e) and photopic (f, g, h) electroretinograms from albino rats collected at P15, P30 and P60, respectively, following exposure to postnatal hyperoxia from P0 to P14. Scotopic flash intensity: $0.6 \log \text{cd sec mP}^{-2\text{P}}$, average of 2–5 flashes, inter-stimulus interval: 9.60 s; Photopic flash intensity: $0.9 \log \text{cd sec mP}^{-2\text{P}}$ following more than

15 min of light adaptation to a background of $30 \text{ cd mP}^{-2\text{P}}$ (average: 20 flashes, inter-stimulus interval: 1 s). Horizontal and vertical calibrations—Scotopic recordings: 20 msec and 250 μ V; Photopic recordings: 20 msec and 250 μ V. A 20 msec prestimulus baseline is included in all tracings. (i, j) Representative retinal histologic sections (central retina) obtained at P60 from control animals (i) and oxygen-exposed animals from P0 to P14 (j). Oblique arrows: HCs located within the INL. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 50 μ m

Similarly, notwithstanding the repair in retinal vascular coverage described above, permanent structural and functional damage occur following hyperoxic exposure in the rat model of OIR [1, 25–28, 44, 45]. Functional deficits that result from postnatal exposure to hyperoxia are best exemplified in Fig. 1d, where scotopic ERGs obtained from hyperoxia-exposed rats (P0–14 regimen) at P30 reveal an attenuated rod-cone b-wave when compared to those raised in room air. A similar b-wave specific irreversible deficit was reported by Penn et al. [46] following exposure of neonatal rats to 60% O₂ from birth through P14, which also suggested a permanent functional effect of OIR. We have also reported that the photopic b-wave (Fig. 1g) is significantly attenuated following the same exposure regimen. Other studies have also suggested that rat models exposed to cyclic oxygen (50%/10%) from P0 to P14 in addition to those exposed to 75% oxygen from P7 to P14 result in similar postreceptoral functional deficits along with increased tortuosity of retinal arterioles [47], and would therefore, further corroborate the findings described above. When taken together with the previously described oscillatory potential (OP) deficits, even at a time following the spontaneous resolution of vascular abnormalities [26, 27, 45], these findings collectively support the persistence of retinal functional deficits in spite of the reversible vasculopathy. Furthermore, the greatest pathophysiological consequences of OIR are in the post receptor retina (Fig. 1c–e) although photoreceptor dysfunction may also persist following hyperoxic exposure [26, 47, 48]. Similar results were also reported by Penn et al. [46] where a-wave amplitudes obtained from hyperoxic- and normoxic-cohorts were identical as of P21 and beyond, while the b-wave was already attenuated by ~40% and up to as much as ~70% at further time points. These results are also in line with those obtained by Reynaud et al. [10] who demonstrated that while the a-wave parameter improved with age between P13 and P18 following hyperoxic exposure within the first 11 days of life, the b-wave amplitudes obtained at P18 were significantly attenuated. Together with the above, these findings would suggest that photoreceptors appear to be significantly less susceptible to this stress, possibly resulting from the fact that the outer segments of the photoreceptors only reach maturity during the second week of life [49], and would also further confirm that the more

permanent sequelae of OIR occur in the inner retina. In order to confirm this hypothesis, ultra-thin retinal sections were collected immediately following ERG recording sessions at P60 [25–28]. The cytoarchitectural anomalies associated with hyperoxic exposure included thinning of the outer plexiform layer (OPL) (Fig. 1f, g) which was also accompanied by a significant reduction in the horizontal cell count (arrow). Consequently, it appears that exposure to hyperoxia prevents the formation of synapses in the OPL and, most probably, the inner nuclear layer (INL) and/or outer nuclear layer (ONL), as well. The disappearance of the horizontal cells could be the result of a particular hypersensitivity to oxygen (or to hypoxia following hyperoxia) or the consequence of some degenerative process triggered by their failure to establish proper synaptic connections. Both pathophysiological processes would yield a thinner OPL. Given that the outer retinal structure appears to be unaffected (also confirmed by the intact a-wave), our results suggest that the synaptic exchange between the a-wave generators (photoreceptor outer segments) and the b-wave generators (postreceptoral retinal elements, such as bipolar and Müller cells) would be seriously compromised, resulting in a significantly attenuated b-wave.

Of interest, however, only few studies have been aimed at unraveling the earliest structural and functional manifestations of the disease process triggered by the postnatal hyperoxic shock [44–46, 48, 50], as retinal function was evaluated either at predetermined time points (at approximately postnatal days [P] 18 and 31 [44, 45] or P13 and P18 [50], for example) or on a weekly basis, beginning immediately after the exposure regimen [46]. Consequently, this made it quite difficult to interpret the pathophysiological progression of OIR, given that these studies were performed at intervals that were further removed from the oxidative insult and at a time when the retina was considered to be mature (e.g., at postnatal days 30 and/or later) [26–28]. Recent studies by Dorfman et al. [1] have suggested that these alterations in retinal function (with the exception of the a-wave) can be evidenced shortly following the cessation of hyperoxic exposure at P15 (Fig. 1c, f). As in normal rats, scotopic responses obtained from hyperoxic rats at P15 were of negative morphology due to a prominent a-wave and a nearly absent b-wave that lacked the presence of well demarcated OPs

(Fig. 1c). Gradually, the various components of the ERG obtained from the hyperoxic-cohort attained greater amplitudes culminating in maximum amplitude at P24, where the morphology of the resulting waveform was reminiscent of the ERG recorded at P17 in control rats. Similarly, the course of maturation of the photopic b-wave did not occur at the same rate among animals raised in the different oxygen environments (Fig. 1d). For example, the photopic b-wave obtained from the hyperoxic-cohort was nearly undetectable at eye opening (P15) and it grew gradually to reach maximum amplitude at P24, at which point the morphology of the resulting waveform was reminiscent of that recorded at P17 in control rats. Interestingly, a normal age-related decline in amplitude of scotopic and photopic ERG parameters has been documented between P30 and P60 [25–28, 51]. In exposed rats, however, only the a-wave's amplitude was reduced significantly between P30 and P60, whereas the other ERG parameters remain relatively unchanged (Fig. 1d, e, g, h). These findings are further corroborated by those described by Penn et al. [46] which revealed a similar decline in the scotopic b-wave amplitude (by ~40%) between control animals aged 5 and 9 weeks, whereas no such findings were evident in the hyperoxic cohort. When taken together, these findings suggest that the damage triggered by hyperoxia might preclude any further deterioration of the retina, such as that which occurs in the normal aging process. The steps involved in this alleged “protective” mechanism remain to be fully elucidated. Interestingly, the maturation-induced changes in retinal function described above were also accompanied by changes in retinal structure. Cytoarchitectural anomalies, including OPL thinning and a loss of horizontal cells were evident immediately upon eye opening, suggesting that the retinal ultrastructure is compromised immediately following the cessation of hyperoxic exposure. As mentioned above, the lack of synaptic contact between the outer and inner retina is likely at the root of the b-wave impairment that we and others have reported. Interestingly, posthyperoxic events such as the return to normoxia (e.g., relative hypoxia), and the ensuing neovascularization have been deemed largely responsible for the pathogenesis of OIR and its human form, ROP. The above mentioned results would suggest that this pathological angiogenic process may play a negligible role in

the initial impairment of retinal function (at birth) given that it is known to occur following recovery in room air [5, 9, 10, 23, 52–54]. Instead, it would appear that the alterations in retinal cytoarchitecture caused by the hyperoxic exposure in addition to the arrest in functional maturation that directly follows the exposure regimen might play equally important roles.

Another important aspect to consider in the development of OIR is the status of the retina at birth. For example, the retina of a full term rat is highly immature and the fact that it normally should undergo vigorous maturation during the period of oxygen exposure (within the first two weeks of life) has been largely overlooked. Furthermore, it has been suggested that the rat retina exhibits an increased metabolic rate in the second to third week of life which is accompanied by a significant maturation phase [55, 56]. Enhanced metabolism has been associated with increased electron leaks in the mitochondria and consequently with the facilitation of free radical generation. Of interest, the severity of retinopathy also appears to be related to the degree of retinal immaturity at the time of exposure [26, 27], where early exposure to hyperoxia within the first week of life (i.e., P0–6) results in minimal scotopic (rod Vmax and rod-cone) and photopic b-wave impairments, while later exposures that include the second week of life (i.e., 6–14, 9–14) result in significantly greater amplitude attenuations [26, 27]. Not surprisingly, our results also indicate that for exposure regimens of equivalent duration (within the first 14 days of life), those that were initiated earlier appeared to be less detrimental to the retinal structure than those that began later.

Free radicals generated by lipid peroxidation have been thought to play an important role in the pathophysiology of OIR (or human ROP), because postnatal hyperoxia in kittens causes a significant reduction in retinal superoxide dismutase [57], and vitamin E (free radical scavenger) supplementation significantly attenuates the severity of OIR in rats [14]. On the other hand, when looking at the relationship between the degree of retinal immaturity and the severity of retinopathy, results indicate that a mere 2-day exposure later in life (12–14 days) alters the retinal structure and function to a similar extent as a 9-day exposure started at birth. Furthermore, exposure of a similar duration early in life (0–3 days)

did not change retinal function at all. This would suggest that free radicals might play only a limited role in the pathogenesis of OIR (and ROP), because oxygen exposure of a longer duration did not always result in a more severe form of OIR.

Given that free radicals do, at least in part, appear to play a role in the pathogenesis of ROP, it is not surprising that clinicians and investigators have explored the possibility of antioxidant administration in preterm neonates in order to protect the retina from their deleterious effects. It was suggested that vitamin E supplementation might be beneficial to this end, especially when considering that plasma levels of this antioxidant are low in prematurely born infants [58–60]. Furthermore, since retinal vascular endothelial cell membranes were thought to be affected by free radicals, it was thought that vitamin E could terminate these lipid peroxidation reactions given its ability to integrate into the lipid bilayer where they occur [61]. However, the effectiveness of vitamin E supplementation in reducing the severity of ROP remains quite controversial [62]. For example, intravenous treatment with vitamin E resulted in increased retinal and intraventricular hemorrhage without any beneficial effect on the development of ROP [63]. In addition, it has also been suggested that conflicting results may stem from the difficulty of delivering a lipid soluble molecule such as vitamin E to the immature retina [59]. In other studies, however, a favorable effect of vitamin E on the incidence of ROP was reported [64, 65].

Trolox C, a water soluble analogue of vitamin E with potent antioxidant activity, was used by Penn et al. [15] in the rat model of OIR in order to determine its effect on retinal vasculopathy that results from the exposure of neonatal rats to high oxygen concentrations. The retinas of rats treated with trolox C while exposed to oxygen had greater vascular coverage and higher capillary density than those that were untreated, suggesting that trolox C could help prevent the vascular consequences of OIR. As described above, previous studies showed a lack of correlation between the vascular and the retinal structural and functional consequences of OIR, in which the retinal vasculature often resumed normalcy shortly after the return to normoxia, whereas the electroretinographic and cytoarchitectural anomalies became permanent features of the hyperoxic rats [25, 26, 48, 50]. Therefore, while retinal vascular

anomalies of OIR rats were shown to be partly prevented by free radical scavengers [15], whether they could exert a similar protective effect on both structural and functional levels was unknown. Dorfman et al. [28] conducted a study to determine whether concomitant treatment with trolox C throughout hyperoxic exposure could also protect the structure and function of the retina. Findings suggested that trolox C could act as a valid therapeutic alternative in limiting the structural and functional damages intrinsic to the rat model of OIR. However, while it successfully benefited retinal functional alterations, particularly the OPs and the photopic mediated responses which were previously described as being most susceptible to hyperoxia-mediated damage [27, 28], its protective effect on retinal cytoarchitecture was limited to prevention of OPL thinning without any significant impact on the horizontal cell count. Whether trolox C acts to preferentially target specific cells whose processes contribute to OPL structure rather than the horizontal cells or whether the two most prominent cytoarchitectural manifestations of the disease process involved in OIR proceed from different pathophysiological paths, remains to be fully understood. Interestingly, trolox C has been reported to have a dual mode of action—antioxidant and prooxidant—depending on the method by which peroxidation is induced. For example, when the oxidative stress is induced by Cu^{2+} or Fe^{3+} , trolox C acts preferentially as a toxic prooxidant and is converted to an α -tocopheroxyl radical as cellular functions deteriorate [66]. This is particularly important to take into account when considering that higher levels of free iron are found in newborn neural tissue in contrast to adults [67, 68]. Transition metals such as these can act as catalysts for reactions of the superoxide anion, O_2^- , with H_2O_2 to produce the very highly reactive hydroxyl radical [19] and might further support the prooxidant state of trolox C.

The inability of trolox C to fully prevent oxygen-induced retinal damage from occurring could suggest, as previously proposed [26] that free radical formation is likely not the sole cause of the damage in retinal structure and function observed in OIR or that a significant proportion of the retinal damage induced by ROS occurs in hydrophobic domains in which trolox C would lose its efficacy because of its water solubility. Future studies aimed at delivering a combination of liposoluble and hydrosoluble free

radical scavengers (such as vitamin E [at a nontoxic level] + trolox C) could represent an interesting therapeutic alternative for further investigation.

Free radical mediators

As pointed out above, isolated free radical scavengers may not be sufficient in preventing the genesis or progression of ROP. Moreover, potent antioxidants may exert adverse effects since normal cell physiology requires a redox potential. Moreover, it is often very difficult to intervene using free radical scavengers other than in a preventive manner. Accordingly, inhibiting the action of mediators of peroxidation may be more suitable for therapeutic purposes. Over the years, we embarked in identifying major bioactive products of peroxidation and nitroxidation. Notably, we will discuss the function of isoprostanes, thromboxane (TXA₂), platelet-activating factor (PAF), and trans-arachidonic acids (TAA).

Isoprostanes

Isoprostanes are a family of compounds produced from arachidonic acid (AA) via a free-radical-catalyzed mechanism [69]. At present, 4 series of AA-derived isoprostanes have been discovered: F₂-, E₂-, D₂-, and thromboxane-isoprostanes. An abundantly produced isoprostane in vivo is 8-iso-prostaglandin F₂ (8-iso-PGF_{2α}) (or 15-F_{2t}-isoprostane) and in view of its commercial availability the one most studied has been 8-iso-PGF_{2α} [70]. In contrast to prostaglandins synthesized by cyclooxygenase (COX), the isoprostanes are formed in situ on esterified phospholipids and are released in free form, presumably by phospholipases. In oxidant stresses, formation of isoprostanes exceeds that of COX-derived prostaglandins. Numerous forms of isoprostanes are concomitantly generated during oxidative stress. The preferential increase of one form over another has been reported for isoprostanes containing different prostane rings. For instance, D₂/E₂ isoprostanes levels have been found to augment to a greater extent during hyperoxic stress than F₂- isoprostanes [71].

In the mammalian retina, 15-F_{2t}- isoprostanes and its metabolite (2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP) evoke vasoconstriction by stimulating TXA₂ formation from neurovascular cells [72]. In addition, isoprostanes not only compromise retinal circulation

by diminishing blood flow, but also seem to contribute to the oxidant stress-induced vascular injury as seen in ischemic retinopathies. Indeed, a potential role for 8-iso-PGF_{2α} in retinal microvascular cell death has also been proposed. 8-Iso-PGF_{2α} was found to cause cell death of isolated retinal microvasculature ($\leq 25 \mu\text{m}$). A similar vascular degeneration was also detected by injecting in vivo into the preretinal vitreous 8-iso-PGF_{2α} in young rat pups. The mechanism of this cell death seem largely mediated by TXA₂ [16]. Since isoprostanes reproduce many effects of oxidant stress, they may serve as mediators in peroxidation-induced actions.

Platelet activating factor (PAF) in retinovascular degeneration

PAF is abundantly generated after oxidant stress and contributes to retinal neurovascular injury [73–78]. PAF also has a potentiating effect on oxidant stress by increasing the generation of oxygen radicals [79, 80]. An important role for PAF in retinal vaso-obliteration in OIR was recently suggested by the relative preservation of microvasculature by molecularly distinct PAF receptor antagonists including BN52021, CV-3988, PCA-4248 and THG315 [20]. A major finding was the direct and relative selectivity of PAF-induced cytotoxicity to retinovascular endothelial cells; umbilical, dermal, and aortic endothelial cells, and retinal pericytes were hardly affected. PAF evoked-toxicity was not only observed in vitro on cultured endothelial cells but also ex vivo on freshly isolated retinal microvessels ($< 30 \mu\text{m}$, mostly endothelial cells) of distinct species. The nature of cell death induced by PAF appears to be necroptotic [81, 82]. PAF elicits its vasomotor and endothelial cytotoxic effects by inducing formation of TXA₂; inhibition of TXA₂ synthase and/or receptor prevents PAF effects [20, 83]. Thus PAF, which is generated concomitantly with TXA₂ during an oxidant stress, and also amplifies formation of the latter, seems to directly contribute to the retinal vaso-obliteration in ischemic retinopathies.

Role of thromboxane (TXA₂) in retinal microvascular degeneration

COX catalyzes formation of prostaglandins, thromboxane, and prostacyclin. Peroxides favor TXA₂

production over that of other COX-generated prostanooids [84, 85] because of inherent characteristics of TXA₂ synthase and reductive cofactor requirements (glutathione and NADPH) for PGI₂, PGE₂, PGD₂, and PGF_{2 α} synthases. TXA₂ exerts known functions that may play a role in vaso-obliteration, especially platelet aggregation and vasoconstriction [86, 87]. Platelet aggregation is involved in other forms of ischemic retinopathies [88]. Nevertheless, studies of oxidant stress on impaired ocular hemodynamics and on OIR reveal an early endothelial cytotoxicity that is independent of platelet aggregation, although this can be detected later [6, 85, 89–93]. Effects of TXA₂ on endothelial cytotoxicity independent of platelet aggregation and vasoconstriction likely contribute to the retinal vaso-obliteration associated with oxygen-induced retinopathy (OIR) model of ROP. Indeed, TXA₂ generation causes a time- and concentration-dependent cell death of retinovascular endothelial cells while other primary prostanoids did not cause cell death [16]; and inhibition of TXA₂ formation or action preserves retinal microvascular integrity in the OIR model [16]. This TXA₂-dependent retinovascular endothelial cell death was found to be mostly by necrosis and to a lesser extent by apoptosis.

The mechanisms for TXA₂-induced cytotoxicity of retinal vascular endothelial cells have recently been explored [94]. TXA₂ induces m-calpain (but not caspase) activation, which in turn causes translocation of Bax to the mitochondria, loss of polarization of the latter and in turn release of cytochrome c into the cytosol and depletion of cellular ATP; disrupting the mitochondrial permeability transition pores and arrest of ATP production can stimulate the generation of ROS and in turn sustain a self-destructive cycle [95–97]. Specific m-calpain inhibitors prevent neurovascular endothelial cell death ex vivo. Collectively, findings support a robust role for TXA₂ as a major down-stream mediator of oxidant stress involved in microvascular endothelial degeneration associated with ROP and likely other ischemic retinopathies.

Trans-arachidonic acids (TAA)

Reactive oxygen species are intimately interrelated with reactive nitrogen species. Several studies have uncovered a crucial role for NO[•] and ensuing nitrative stress in microvascular degeneration leading to ischemia in conditions such as ROP. In particular, it has

been shown that retinal microvascular degeneration is associated with increased expression of endothelial nitric oxide synthase (eNOS) [17, 98], increased generation of nitrites, nitrates and peroxynitrite, and protein tyrosine nitration; inhibition of these processes prevents development of OIR [17, 99, 100]. A novel nitroxidation process mediated by NO[•], which results in cis to trans isomerization of arachidonic acid, was recently described. This reaction produces four stable trans-arachidonic acid mono-isomers, 5E-AA, 8E-AA, 11E-AA and 14E-AA [101], two of them (5E and 8E-AA) being endogenous and not found in diet [102]. A significant increase in the concentration of free (unesterified to phospholipids) as well as total (free + phospholipid-bound) TAA was shown in an experimental model of O₂-induced microvascular degeneration upon exposure of 7 day old (P7) rat and mouse pups to 75–80% O₂ or room-air for 24 h; estimated concentrations of free and total TAA reached 1.65 μ M and 4.72 μ M, respectively [103]. Intravitreal injection of the specific NOS inhibitor L-NAME prior to exposure to oxygen prevents the increase in TAA levels in rats, indicating that their formation depends on NO[•] and ensuing nitrative stress in this model. The contribution of eNOS on TAA generation in this model of ROP is corroborated in C57/BL6 mice with disrupted *eNOS* gene, in which retinal TAA levels do not increase in contrast to their wild-type counterparts [103].

TAA concentrations similar to those found in OIR cause a significant decrease in retinal vascular density both in vivo in the developing rat retina and ex vivo on newborn pig retinal explants. TAAs cause a concentration- and time-dependent neuroretinal microvascular EC death (EC₅₀ at 24 h: 3 to 8 μ M, consistent with in vivo concentrations); in contrast, smooth muscle cells, human umbilical vein endothelial cells (HUVEC) and astrocytes are unaffected by TAA exposure [103], suggesting that TAA specifically leads to neuroretinal microvascular EC death. The mechanism by which TAA function appears to be via the generation of the pro-apoptotic and anti-angiogenic protein thrombospondin-1 (TSP-1) in cultured ECs [103]. TSP-1 acts via the trans-membrane receptor CD36 [104, 105]. TSP-1 and CD36 knockout animals are insensitive to TAA. These observations unravel a novel mechanism by which TAAs, generated endogenously specifically in response to eNOS activity, can reproduce and thus

mediate effects of reactive nitrogen species by participating in the microvascular degeneration observed in ischemic retinopathies. Since nitrative stress is dependent upon the redox potential [17], formation of TAA would also be subject to the same conditional state. Accordingly, the immature subject would be more vulnerable as a result of its decreased antioxidant capacity [59, 106, 107]. This inference is supported by recent findings which show that nitration, which leads to TAA generation [101], occurs when the redox state of the developing tissue is shifted towards an oxidative environment [17]. However, to date the primary receptor site on which TAA act remains elusive.

In summary, a number of bioactive products of nitrooxidation have been identified in OIR/ROP, which exerts not only vasoactive properties but also cytotoxic ones specifically on neuroretinal endothelium. These findings provide more specific targets on which one can interfere with to prevent the critical vaso-obliterative phase of ROP.

The effect of a bright luminous environment on the developing retina

The retina is divided into distinct cellular layers that constitute the outer retina (photoreceptor layer) and the inner retina (inner nuclear and plexiform layers and the ganglion cell layer). In normal lightening conditions, photons cross the entire retina before being captured by the photoreceptors that will transform this chemical energy into an electrical signal. As photoreceptor membranes are naturally rich in polyunsaturated fatty acids, essentially in docosahexaenoic acid, the outer segments are highly sensitive to lipidic peroxidation [108–111]. Moreover, the outer retina, completely deprived of blood vessels, receives nutrients and oxygen via the choroidal vasculature. This strengthens again the retinal susceptibility to oxidative stress [111–114].

In a luminous environment where the intensity does not exceed 1,000 lux the outer segments of the photoreceptors will modulate their length and the concentration of rhodopsin in their disks that suggest a form of cellular adaptation triggered to avoid light damage [115]. This adaptative mechanism, called photostasis, allows for gradual changes in the retinal structure. Therefore, the sensitivity of the retina is

slowly modified to lead to a constant number of adsorbed photons per day independent of the luminous intensity of the environment [116–119]. However, when the amount of photons captured exceeds this daily average, photoreceptors are no longer able to repair cellular damage and, consequently, they will degenerate and die by apoptosis [115, 120, 121]. Therefore, the retina can be easily damaged when the environmental light intensity exceeds 1,000 lux [115]. As originally shown by Noell, the extent of cellular damage corresponds to the absorption spectrum of the rhodopsin pigment [120]. Deleterious effects of light on the retina lead to electrophysiological alterations [122] and to ultrastructural modifications visible at electron microscopy [123].

These irreversible retinal damages have been very well documented in adult animals [109, 112, 120, 124–130]. We know that the severity of retinal damages depends on four factors: the duration of exposure, the intensity, and the wavelength of the light, and the mode of exposure (cyclic light or constant light). As early as one hour after light exposure, photoreceptors already display the first signs of cellular degeneration that are only visible at electron microscopy [123, 131, 132]. The most distal part of the photoreceptor outer segments appears disorganized; the regular lamellar structure of the saccules is disrupted leading to the fusion of the disks and the formation of vacuoles and tubules. Cellular changes also occur in the inner segments of the photoreceptors where the mitochondrias swell and exhibit autophagic vacuoles [133, 134]. In the outer nuclear layer, photoreceptors show pycnotic nuclei with condensation of the chromatin while an increase in the number of lysosomes can be observed in the retinal pigment epithelium. In the most severe cases, the photoreceptor layer degenerates completely, whereas the more internal retinal layers (INL, IPL and GCL) are not affected [123, 130, 132, 135, 136]. These structural alterations can be easily correlated with a loss of retinal function as evaluated with the electroretinogram [125–127, 137].

Although seldom investigated, the age of the animal was also pointed out as another factor that could contribute to the severity and the extent of retinal damage following bright light exposure [124]. Indeed, previous data suggested that the retina of an adult rat suffered significantly more of light exposure compared to that of a younger rat. However, the intensity of light

used in some studies may not have been sufficient enough to pass through the closed eyelids of the neonatal pups and still retain sufficient energy to be retinotoxic [124, 138, 139]. In addition, rats may have been placed in darkness after light exposure allowing then a recovery of photoreceptors from light damage. More recently, our group published a series of studies specifically addressing this issue.

Neonatal albino Sprague Dawley rats were placed in a luminous environment (10,000 lux; 12 hours light: 12 hours dark) from birth to postnatal day (P) 14. After light exposure, young rats were replaced in an 80-lux luminous environment corresponding to the normal conditions in our animal center. Electroretinograms (ERGs) were recorded at P30 and at P60 (Fig. 1A–G) and histology was performed at P60 (Fig. 1H–M). Our work demonstrated for the first time that, neonatal pups exposed to bright light

during the first two weeks of life, when eyelids are still closed, displayed delayed retinal damages [51]. Indeed, ERGs in 30-days old rats did not show any difference after exposure compared to non-exposed rats (Fig. 1A, B). In contrast at P60, the amplitudes of the mixed rod-cone a- and b-waves were slightly decreased (Fig. 2). Our work suggested that despite closed eyelids, exposure to a bright luminous environment might trigger a progressive retinal degeneration (Fig. 3).

In order to better characterize the window of retinal susceptibility during youth, juvenile albino rats were exposed to 10,000 lux (12 hours light: 12 hours dark) for different intervals of time during the third and the fourth week of life [140]. Results were compared with adults exposed to the same conditions. Adult retinas already showed significant decreases in the ERG parameters as well as severe

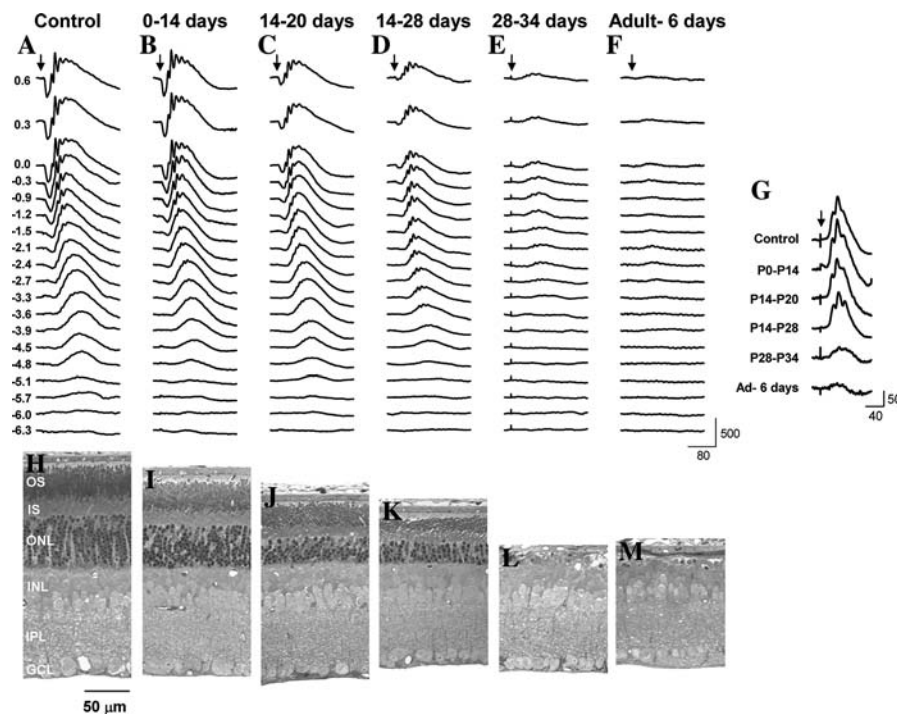
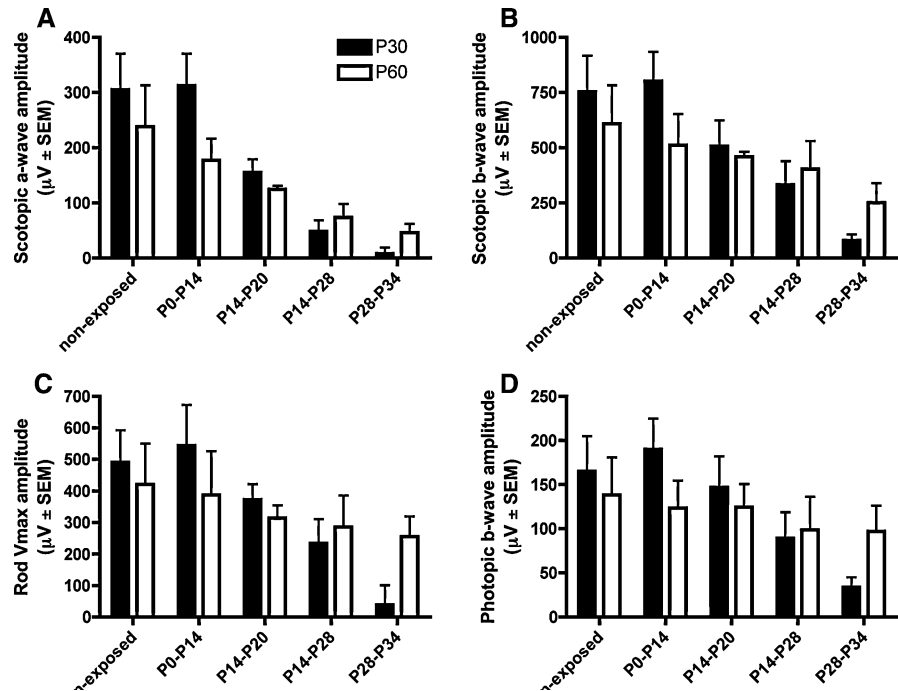


Fig. 2 (A–G) Representative scotopic (A–F) and photopic (g) electroretinograms from 30–40 days-old albino rats (A–B) exposed to bright light (10,000 lux; 12D: 12L) at different periods during the first postnatal month of life (as indicated on the top of the tracings). Recordings were performed between P30 and P37 in all regimens of exposed juvenile rats. For adults (F), albino rats were exposed to bright light for 6 days and then replaced in normal lightning conditions (80 lux) for 24 days. Flash intensity: 0.6 log cd s/m². Horizontal and vertical calibrations—Scotopic recordings: 80 msec and 500 μV;

Photopic recordings: 40 msec and 50 μV. A 20-msec prestimulus baseline is included in all tracings. (Vertical arrows): Flash onset. (H–M) Representative retinal photomicrographs of control rat (H), of rats exposed to bright light from P0 to P14 (I), from P14 to P20 (J), from P14 to P28 (K), from P28 to P34 (L) and from adults exposed to light for 6 consecutive days (M). Scale bar: 50 μm. Abbreviations—OS: Outer segments; IS: Inner segments; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Inner plexiform layer; GCL: Ganglion cell layer

Fig. 3 Histogram representing the amplitudes of the scotopic mixed rod-cone a- (A), and b-waves (B), the amplitude of the Rod Vmax (C) as well as those of the photopic b-wave (D) at P30 (black bars) and at P60 (white bars). Different regimens of light exposure are indicated on the X-axis of each graph. Values are reported as mean $\pm$ SEM



morphological defects in their outermost part after 1 day of light. After 6 days of light, ERG was entirely abolished (Fig. 1F) and the outer retina was completely destroyed (Fig. 1M). In contrast, young rats exposed to light, for the same duration of time, from P14 to P20 still presented a recordable ERG (Fig. 1C) and the morphology of the photoreceptor layer was remarkably preserved compared to adults (Fig. 1J). Light exposure performed at the end of the first month of life (P28–P34) resulted in severe structural and morphological defects similar to those observed in adults exposed for 6 days (Fig. 1E, L), suggesting that the window of retinal resistance gradually weakens until 1 month of age. On note, in all regimens of exposure and in juvenile rats as well as in adults, the superior retina was always more affected than the inferior one, a finding previously reported by others [120, 123, 141–144]. Our study demonstrated that juvenile rat retinas were better preserved than adult retinas from the deleterious effects of bright light, suggesting that young retinas possess an endogenous resistance to light damage.

In adult rats, intravitreal injections of neurotrophic factors are known to protect photoreceptors from light-induced degeneration [145–150]. Moreover, it has been already demonstrated that endogenous FGF-2 (Fibroblast Growth Factor-2) or CNTF

(Ciliary Neurotrophic Factor) levels are upregulated in adult rat retinas in response to a moderate light intensity exposure [151–153]. We speculated that enhanced production of FGF-2 and/or CNTF factor might contribute to the resistance of the juvenile animal to light-induced degeneration. Endogenous protein levels of FGF-2 and CNTF were analyzed by Western blot immediately after 6 and 14 days of exposure [154]. In our light model, none of these neurotrophic factors was upregulated in adult retinas. Interestingly, Müller cells of juvenile retinas overexpressed FGF-2 and CNTF protein levels after 14 days of light, suggesting that upregulation of survival factors like FGF-2 and CNTF may lead to the protection of the photoreceptors from the noxious effects of light. Further experiments should confirm whether FGF-2 and CNTF delivery could promote neuroprotection of photoreceptors in the adult retina as well.

Conclusion

Postnatal exposure to intense light or high levels of oxygen thus offers a unique opportunity to study ex utero animal models abnormal retinal development that would normally take place in utero in human

subjects. Use of the knowledge gathered will not only be instrumental in furthering our understanding of the pathophysiological processes thus triggered but also allow us to test new therapeutic strategies that could ultimately have an impact on the management of human infants born with equivalent retinal disorders. More research is however needed before we can identify the initial insult (and thus potential treatment site) that triggered the cascade of events that ultimately lead to the development of these complex retinal disorders.

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Postnatal hyperoxia and the developing rat retina: beyond the obvious vasculopathy

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Abstract Although a great deal of emphasis has been placed on the vasculopathy that is associated with oxygen-induced retinopathy (OIR), our studies also revealed significant and irreversible structural (retinal histology) and functional (scotopic and photopic electroretinograms) impairments that were significantly more severe in pigmented Long-Evans rats compared to the more commonly used albino Sprague Dawley rats. In the following pages, we will highlight what we have learned about the retinal pathophysiological processes of OIR taking place in strains of both rats with the hope that this will trigger investigations

into new therapeutic strategies to complement those geared at preventing the vasculopathy.

Keywords Retinopathy · Oxygen · Neonatal rat · Vasculature · Electroretinogram · Histology

Introduction

Previous studies of ours revealed that the structural (retinal histology) and functional (electroretinogram) deficits that resulted from the exposure of albino Sprague Dawley (SD) rats to a hyperoxic environment within the first postnatal days were permanent [1–5]. This contrasts with the damage to the retinal vasculature which can, as previously documented by others as well, overcome the hyperoxic challenge thereby achieving full coverage while still under oxygen throughout the first 2 weeks of life [1]. The latter is reminiscent of the reversible vasculopathy that is often reported in human ROP [6]. Recent studies of ours also showed that compared to the albino SD rats, pigmented Long-Evans (LE) rats developed a more severe oxygen-induced retinopathy (OIR) [7] as evidenced functionally and structurally. In the following pages, we will briefly review what we have learned from the two models of OIR with the hope that our findings will contribute to a better understanding of the retinal pathophysiological processes that are triggered following exposure of an

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immature retina to a high level of oxygen. For the sake of clarity, the results reported were obtained following an exposure to 80% oxygen for 22.5 h per day from P0 to P14.

Functional consequences of OIR

Functional impairments resulting from postnatal exposure to high oxygen are best summarized in Fig. 1 with representative examples of the saturated rod b-wave (a–c), the mixed rod-cone b-wave (d–f) and the photopic b-wave (g–i) parameters of the ERG obtained from control (a, d, g) and oxygen-exposed SD (b, e, h) and LE (c, f, i) rats. While, compared to control rats, the a-wave of the mixed rod-cone b-wave in SD rats was minimally affected following hyperoxic exposure (Fig. 1d, e), significant amplitude

reduction in the scotopic (Fig. 1a, b, d, e) and photopic (Fig. 1g, h) ERG b-waves was observed.

It is interesting to note that the functional consequences of OIR in the SD rat, which is still to date the most commonly used strain, were significantly less severe than those observed in pigmented LE rats [7]. Briefly, while the a-wave remained relatively intact in SD rats, in LE rats it was found to be significantly compromised early on in the pathophysiology of OIR (Fig. 1f). Interestingly, as with the a-wave, the attenuation in the saturated rod b-wave response was notably more pronounced in LE rats (Fig. 1c). A comparable finding was also observed for the rod-cone b-wave of LE rats, where a significant attenuation in amplitude was observed (Fig. 1f). Similarly, attenuation of the photopic b-wave was also found to be significantly more pronounced in LE rats (Fig. 1i), suggesting again that the retinal function of LE rats is

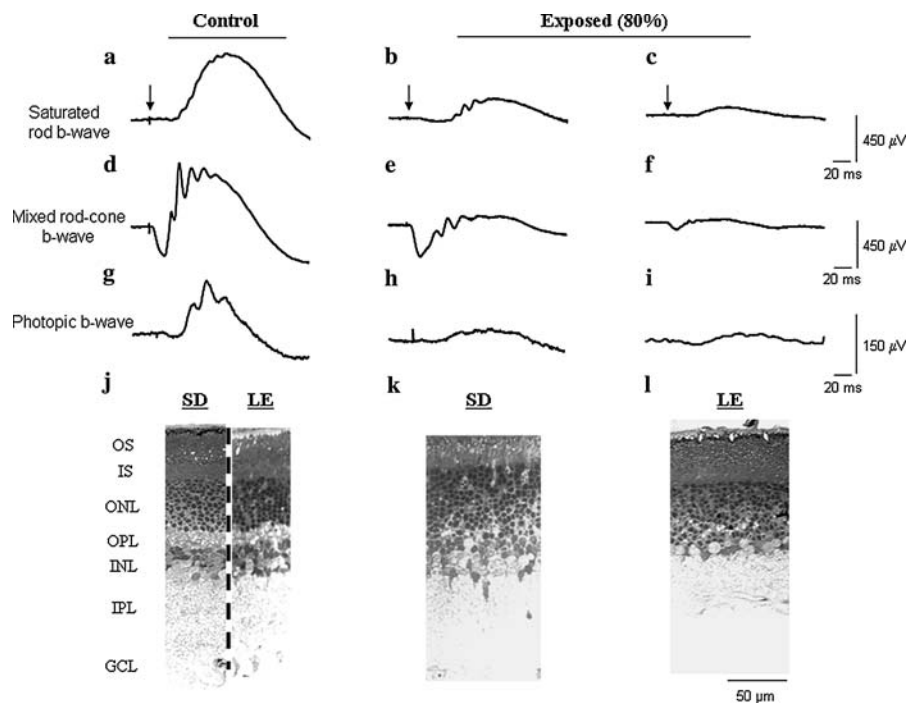


Fig. 1 Representative scotopic [saturated rod b-wave, flash intensity: $-2.4 \log \text{cd.s.m}^{-2}$ (a–c); and mixed rod-cone b-wave, flash intensity: $0.60 \log \text{cd.s.m}^{-2}$ (d–f)] and photopic [b-wave, flash intensity: $0.9 \log \text{cd.s.m}^{-2}$, background: 30 cd.m^{-2} (g–i)] ERGs obtained at P60 from LE rats raised in normoxia (21% O_2) and LE and SD rats raised in hyperoxia (P0–P14, 80% O_2), respectively. Calibrations: horizontal, 20 ms; vertical, 450 μV for scotopic saturated rod b-wave and mixed rod-cone b-wave ERG responses, 150 μV for

photopic ERG responses. All tracings include a 20-ms prestimulus baseline; vertical arrows indicate flash onset. Representative retinal histological sections obtained at P60 from SD and LE rats raised in normoxic (j) and maximal hyperoxic conditions (k, l; SD and LE, respectively). RPE retinal pigment epithelium, OS outer segment, IS inner segment, ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer, GCL ganglion cell layer. Calibration bar: 50 μm

more prone to hyperoxic insult compared to that of SD rats.

Cytoarchitectural consequences of OIR

In SD rats, the only cytoarchitectural consequence of early postnatal hyperoxia found by our group was a dose-dependant thinning of the outer plexiform layer (OPL) along with a significant reduction in the horizontal cell count (Fig. 1k) [1–3]. In comparison, analysis of LE rat retinas revealed significantly more extensive damage to retinal cytoarchitecture compared to SD rats (Fig. 1l). In addition to the thinning of the OPL, we also noted a marked thinning of the inner nuclear layer (INL), the inner plexiform layer (IPL) and the outer nuclear layer (ONL; Fig. 1l), features of OIR never observed in SD rats that again point to the significantly more severe presentation of the OIR manifestations in LE rats.

Managing ROP: lessons from OIR

Our previous studies have shown that in albino SD rats, the pathophysiological consequences of OIR combine retinal cytoarchitectural damage (e.g. OPL thinning, reduced horizontal cell count) with a permanent deficit of retinal function (significantly attenuated scotopic and photopic ERGs) [1–5]. In comparison, postnatal hyperoxia exerts an even greater loss of retinal structure and function in the pigmented LE rat (greater vascular dropout, generalized deficit to retinal cytoarchitecture, more severely attenuated retinal function) [7]. Moreover, the damage observed in LE rats occurred earlier compared to SD rats, following exposure to hyperoxia that was limited to the first week of life (P0–P6).

Given that ROP is considered a disease of the retinal vasculature, it is not surprising to find that most studies on OIR have focused on the effect of postnatal hyperoxia on the development of the retinal vasculature. Although previous studies have also observed significant reductions in vascular coverage following postnatal hyperoxia in rats [8–14], few studies have examined the time course of this effect. We showed that despite the attenuation in vascular growth that is readily measurable after the first week of exposure (P0–P6) in SD rats, the vascular growth

process is nonetheless able to overcome this hyperoxic insult thereby achieving full coverage while still under hyperoxic exposure (in P0–P14 exposures). This is reminiscent of the reversible vasculopathy that is observed in the human form of ROP [6]. In contrast, following oxygen exposure, the vascular supply is so severely compromised in pigmented LE rats that they never achieve a normal appearance, thus further suggesting unequal susceptibility among strains [7].

It is interesting to note that while the abnormal retinal vasculature has been documented to resolve itself, the functional sequelae that are associated with this disease have been shown to persist. Evidence of this has been published in several studies where, for example, significant alterations in the a- and b-waves of the ERG in patients with a history of ROP have been shown to continue into adulthood [15, 16]. Furthermore, scotopic visual threshold elevation has been documented in children with a history of resolved, mild ROP, thus suggesting the involvement of photoreceptors [17–19]. Finally, significant deficits in amplitude and implicit time of the multifocal ERG (mfERG) response, which stems from the central retina, have also been found in subjects who revealed complete resolution of clinical ROP more than 10–20 years earlier [20]. This would suggest that a history of ROP can alter the development of the central retina and, consequently, the mfERG response that it generates. Given that ON- and OFF-bipolar cells contribute to this central response, the attenuation in amplitude might therefore occur, should ROP specifically target these cells and thereby cause for an altered summation of depolarization and hyperpolarization in these subjects [20]. Therefore, despite the resolution of vascular abnormalities, which is a common occurrence in ROP patients, it is necessary to consider that neural dysfunction of the retina may still carry on throughout adulthood. Of course, this is not to say that the vasculopathy that results from postnatal hyperoxia does not contribute to the structural and functional consequences that we have reported. In a previous study by Penn's et al. [12], it was shown that trolox-c (a water-soluble analogue of vitamin E) successfully limited the vascular consequences of postnatal hyperoxia, suggesting that hyperoxia was the main factor that triggered the vascular dropout observed in OIR. However, the same dose of trolox-c failed to offer an equivalent degree of protection to the

retinal cytoarchitecture and function [5], suggesting again that some aspects of the vascular, structural and functional consequences of OIR may develop independently.

Our findings of impaired retinal function in our models of OIR support previous findings, where exposure of neonatal rats to 60% O₂ from birth through P14 caused inner retinal dysfunction as evidenced by b-wave attenuation [21]. Similar findings also reported that cyclical (50/10% O₂) exposure from P0 to P14 or constant exposure to 75% O₂ from P7 to P14 led to postreceptoral functional deficits combined with retinal arteriole tortuosity [22]. Moreover, anomalies of the ERG oscillatory potentials (OPs) have also been shown to persist, despite the resolution of vascular abnormalities [3, 4, 23], which would further suggest the persistence of retinal functional deficits despite normalization of the retinal vasculature.

Evidence of a postreceptoral effect following postnatal hyperoxia were also reported by Penn et al. [21] where the amplitude of the ERG a-wave obtained from hyperoxic- and normoxic-cohorts were identical as of P21 and beyond, compared to severely attenuated b-waves. These results are also in line with those obtained by Reynaud et al. [9] who demonstrated that following postnatal hyperoxia from P0–P11, the amplitude of the a-wave gradually improved between P13 and P18, compared to that of the b-wave which remained significantly depressed at P18. Clearly, the above would suggest that the photoreceptors are significantly less susceptible to postnatal hyperoxia. One wonders whether this results from the relative immaturity of their outer segments at the time of oxygen exposure [24], or from the relatively intact choroidal vasculature that supplies them. The above would also further confirm that the more permanent sequelae of OIR occur in the inner retina. On the other hand, while the pathophysiological consequences of OIR are largely postreceptoral in the retina of albino rats, photoreceptor dysfunction may in fact also occur following exposure to hyperoxia, as suggested by different parameters of oxygen exposure including alternating between 50 and 10% oxygen from postnatal day P0–14, or exposure to 75% oxygen from P7 to P14 [22]. These findings would further support our observations of impaired photoreceptor function in the pigmented LE rat and would suggest that not all experimental models may

react equally to an equivalent hyperoxic stress. Findings such as these warrant the evaluation of retinal structure, and studies of ours have also been geared towards better understanding of the impact of postnatal hyperoxia on retinal cytoarchitecture, which is often overlooked.

The cytoarchitectural manifestations of OIR occur, at least in the SD rat, during the period that the rats are still submitted to the hyperoxic regimen, as evidence of OPL thinning and HC count are already observed directly following exposure [1]. Furthermore, given that the most notable changes occur in the OPL and HCs, the relatively intact a-wave that can be recorded within the first few days following the cessation of hyperoxic exposure comes as little surprise, while as expected from histological findings, scotopic and photopic b-waves already tend to be attenuated in amplitude. Finally, whether the lower count of horizontal cells results from their susceptibility to oxygen (or to the relative hypoxia that follows hyperoxia) or from another degenerative process that might impact the establishment of proper synaptic connections remains to be fully understood.

Our recent findings have revealed that the pigmented Long–Evans rat is also a good working model of OIR, demonstrating a more exaggerated form of the disease with a more severe pathophysiological course, involving greater damage to retinal vasculature, structure and function. In both models, findings would suggest that OIR is initiated at a postreceptoral level, where synaptic impairment at the level of the OPL prevents signal transmission to the inner retina. The particular susceptibility of the scotopic and photopic b-waves would further support this inference [1–5, 7]. Involvement of other inner retinal structures such as the INL, IPL and GCL as well as the impaired a-wave in LE rats suggests that the pathophysiological consequences in this model are indeed more severe. Interestingly, studies have revealed that not only can an environmental stress such as exposure to hyperoxia result in structural damage to the retina, but other mechanisms that may involve the generation of reactive oxygen species, such as the reaction of iron with oxygen, can also result in such an effect. For example, intravitreal injection with FeSO₄ has been shown to cause selective thinning of the ONL [25] as well as impaired retinal function. Similarly, intravitreal injection of paraquat (an herbicide known to generate

reactive oxygen species) was shown to cause thinning of the ONL and INL along with vacuolization, folds in the ONL and functional impairment [26]. These findings in addition to those which we have previously reported [1–5, 7] would collectively suggest the importance not only of retinal vasculature and functional assessment in models of retinal diseases, namely OIR, but also of retinal cytoarchitecture which can give further insight and help to explain the pathophysiological processes that characterize OIR. Future investigations are certainly warranted in order to clarify the disparities between oxygen exposure regimens and the results obtained among studies in order to not only further our understanding of the disease processes but also to enable testing of new therapeutic modalities that could eventually help to manage human infants born with retinal disorders such as ROP as well as others. In that respect, if we interpret our results as evidence that not all premature infants will react similarly to the same hyperoxic shock, experimental approaches that would make use of the SD and LE rats would be instrumental in the design of new therapeutic strategies aimed at better protecting the developing retina from postnatal hyperoxia, irrespective of the degree of maturity reached at the time of oxygen exposure or the severity of the underlying retinopathy.

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