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Progesterone-treatment in a model of Retinitis Pigmentosa: the rd1 mice.

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-ABBREVIATIONS

ALLO: 3α, 5α- tetrahydroprogesterone

AMD: age-related macular degeneration

AMPA: amino-3-hydroxy-5-methyl-4-isoxazolepropionate

ApoE: apolipoprotein E

ARVO: Association for Research in Vision and Ophthalmology

BBB: blood-brain barrier

BSA: bovine serum albumin

BRB: blood-retina barrier

Ca^{2+}: calcium ion

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

Cl^-: atomic chlorine

CNG: cation nucleotide-gated

CNS: central nervous system

CNTF: cilliary neurotrophic factor

CO_2: Carbon dioxide

Cys: Cysteine

CysS: Cystine

DAPI: 4',6-diamidino-2-phenylindole

DHEA: dehydroepiandrosterone

DHEAS: dehydroepiandrosterone sulfate

DHP: 5α- dihydroprogesterone

DNFB: 1-fluoro-2, 4-dinitrobenzene

DPX: mounting xilene free

ERG: electroretinogram

Et-OH: ethanol

GABA: γ-aminobutyric acid

GAD-65: glutamic acid decarboxylase

GCL: ganglion cell layer

GDNF: glial cell line-derived neurotrophic factor

GFAP: Glial fibrilary acidic protein

GLAST: glial L-glutamate/ L-aspartate transporter

GMP: guanosine monophosphate

GPx: glutathione peroxidase

GR: glutathione reductase

GS: glutamine synthetase

GSH: glutathione

GSSG: glutathione disulfide

GTP: guanosine triphosphate

HPLC: High-performance liquid chromatography

IAA: iodoacetic acid

IL-1β: interleukin-1β

ILM: inner limiting membrane

INL: inner nuclear layer

iNOS: inducible nitric oxide synthase

IPL: inner plexiform layer

i.p.: intraperitoneal

IS: inner segment

LCA: Leber's congenital amaurosis

mGluR: metabotropic glutamate receptors

MS: multiple sclerosis

Na^+: sodium ion

NADPH: Nicotinamide adenine dinucleotide phosphate

NFkB: Nuclear factor Kappa B

NMDA: N-methyl-D-aspartate

$^{\cdot}NO$: nitric oxide

NO^{2-}: nitric dioxide

$O_2^{\cdot -}$: superoxide anion

OCT: optical coherence tomography

OGD: oxygen-glucose deprivation

$^{\cdot}OH$: hidroxyl radical

OLM: outer limiting membrane

ONL: outer nuclear layer

OPL: outer plexiform layer

OS: outer segment

P4: progesterone

PB: phosphate buffer

PBS: phosphate buffered saline

PCA: perclhoric acid

PDE: phosphodiesterase

PDE6: phosphodiesterase 6

PFA: paraformaldehyde

PN: post-natal day

PREG: pregnenolone

RCS: reactive chlorine species

rd1: retinal degeneration 1

rd10: retinal degeneration 10

rds: retinal degeneration slow

RHO: rhodopsin

ROS: reactive oxygen species

RNS: reactive nitrogen species

RP: retinitis pigmentosa

RPE: retinal pigment epithelium

RT: room temperature

SOD: superoxide dismutase

TBI: traumatic brain injury

TGF-β: Transforming growth factor beta

TNF-α: tumor necrosis factor

TPx: peroxiredoxins

TUDCA: tauroursodeoxycholic acid

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

wt: wild-type

-INTRODUCTION

The visual sense is the most precious of all human senses and blindness is the most feared sensory health threat in our society. This is particularly true when one realizes life habits in Spain and developed countries, where visual communication dominates, playing a fundamental role in social integration.

The eye (Figure 1) is the organ that facilitates vision in vertebrates and most of the invertebrate organisms. It forms, together with the optic nerve and the primary visual cortex among other structures and circuits, the visual system.

In some eye diseases the retina becomes damaged or compromised and degenerative changes eventually lead to death of the retinal neurons and, as a consequence, to vision loss (Krumpaszky & Klauss, 1996). Major retinal pathologies include glaucoma, where elevated pressure in the eye leads to retinal ganglion cell death; diabetic retinopathy, where blood vessels in the retina become leaky with serious consequences; age related macular degeneration (AMD) and others less common like retinitis pigmentosa (RP) Stargardt disease, and Usher syndrome.

The present study focuses mostly on an animal model that mimics the RP condition, while the broader aim is to better understand retinal photoreceptor degeneration in an animal model of RP (rd1) in order to try to find a possible treatment.

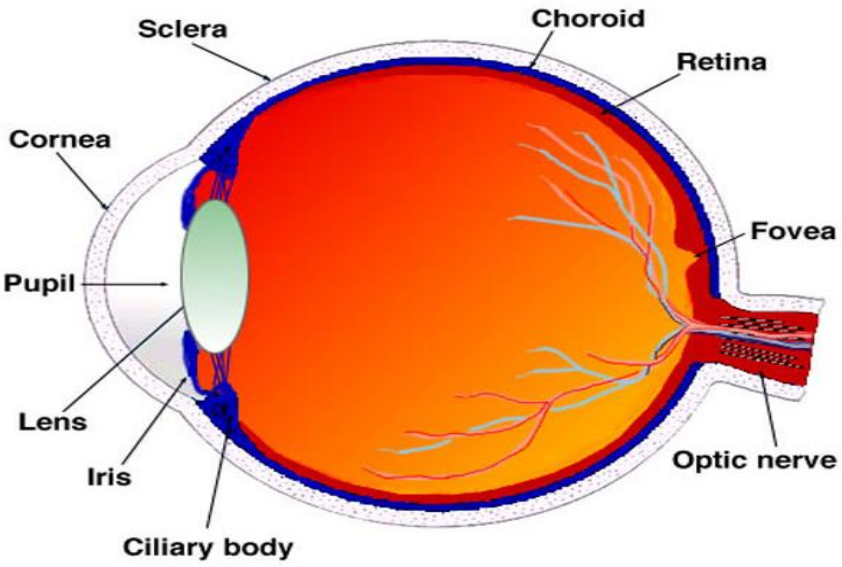

Figure1. Vertical sagital section of the adult human eye (Kolb, 2003).

1. THE RETINA

The retina is a specialized sensory organ capable of transforming light into electric signals that are transmitted via the optic nerve to the visual centers of the brain. It is composed of a thin sheet of neural tissue covering 2/3 of the inner surface of the posterior part of the eye (Lamba *et al.,* 2009). It is essentially a portion of the forebrain projected towards frontal or anterior surface of the organism, and consists of highly specialized neurons with the exclusive function of light perception and first order signal processing.

The retina and optic nerve are formed during the last stages of embryonic development (Duke-Elder, 1963; Mann, 1964; Sadler, 1990; Kolb, 2003). The retina derives during embryogenesis from the neuroectoderm, a part of the ectoderm that gives rise to the central nervous system formation (CNS) (Sadler, 1990)**.** The mature mammalian retina consists of two distinct tissues: the neural retina, composed of neurons and glial cells, and the retinal pigmented epithelium (RPE), a single epithelial cell layer (Figure 2). The cells of the neural retina derive from multipotent progenitor cells and their differentiation follows a precise chronological order that is found in many species (Turner & Cepko, 1987; Holt *et al.,* 1988; Wetts & Fraser, 1988; Cepko, 1993).

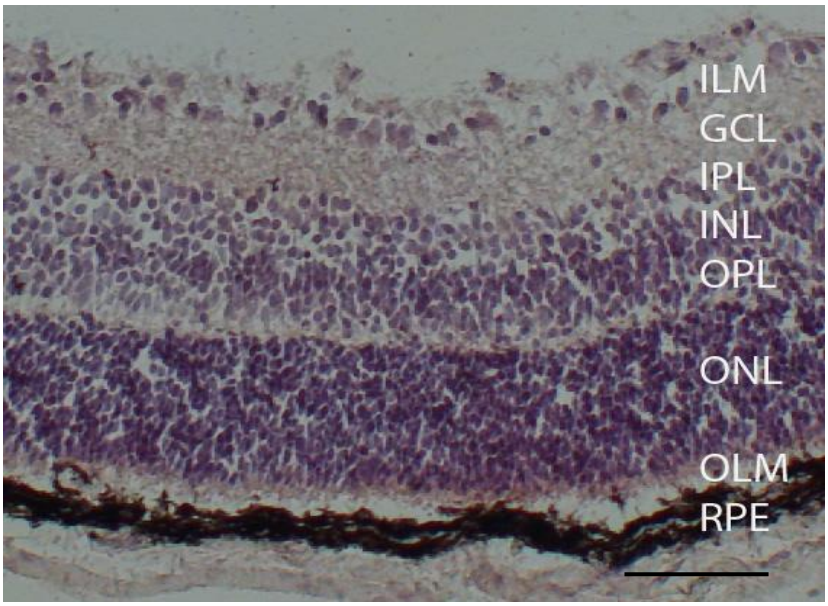

Figure 2. Cellular structure of normal retina from a control mouse. RPE: retinal pigment epithelium; OLM: outer limiting membrane; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; ILM: inner limiting membrane (Scale bar = 50 µm).

The retina has numerous similarities with the brain: the principal cells are neurons, such as the photoreceptors; the blood retina barrier (BRB) is analogous to the blood-brain barrier (BBB) and has similar functionality; both employ glial cells (micro- and macroglia) to maintain the health and function of the neurons (Dowling, 1987).

The retina is a seven-layer structure, with light entering the ganglion cell layer (GCL) first. From there light must penetrate each layer with its cells (bipolar, amacrine and horizontal cells) before reaching the rods and cones, the two types of photoreceptors that absorb light and transform it into electrical signals that initiate downstream events that are essential for the sense of sight (Dowling, 1987).

The signal is transmitted to bipolar cells, responsible for processing and transmitting the signal. Amacrine and horizontal cells are also involved in perpetuating the signal from the photoreceptors. Ganglion cells receive the signal from the bipolar cells and connect, via the optic nerve, directly to the brain for further processing and visual perception (Dowling, 1987; Kolb, 2003; Sancho-Pelluz *et al.,* 2008).

Metabolic supply is partially provided by the retinal pigment epithelium cells.

1.1. STRUCTURE OF THE RETINA

The mature neural retina shows a highly organized structure (Duke-Elder & Shimkin, 1926) composed of three cellular layers (Figure 2 and Table 1):

- the outer nuclear layer (ONL), composed of photoreceptors,
- the inner nuclear layer (INL), containing neurons (horizontal, bipolar, amacrine and interplexiform cells) and retinal Müller glial cells, and
- the ganglion cell layer (GCL), that contains in addition to ganglion cells also displaced amacrine cells and astrocytes (Ogden, 1978; Boycott & Hopkins, 1981).

And two synaptic layers separate these nuclear layers:

- the outer plexiform layer (OPL) and
- the inner plexiform layer (IPL).

The axons of the ganglion cells converge to the exit of the optic nerve, forming the nerve fiber layer.

Retinal pigment epithelium	RPE	Monolayer of cells located behind the retina, which supports the neuroretina, among other responsabilities.
Outer limiting membrane	OLM	Barrier between the subretinal space and the outer nuclear layer.
Outer nuclear layer	ONL	Where the photoreceptor nuclei and cell bodies are located.
Outer plexiform layer	OPL	The synapses between rods and cones and the INL neuronal cells are established in this layer.
Inner nuclear layer	INL	Include cell bodies of bipolar, horizontal, and amacrine cells. Müller cell somas are located here.
Inner plexiform layer	IPL	Synapses between the INL cells and the ganglion cells.
Ganglion cell layer	GCL	Monolayer composed of ganglion cells.
Optic nerve fiber layer		Formed by the axons of ganglion cells on their way to the brain.
Inner limiting membrane	ILM	Separate the retina from the intravitreal space.

Table 1. Layers of the retina.

1.2. RETINAL CELL TYPES

The retina is constituted by the pigment epithelium (non-neuronal layer) and the neuronal retina (neuronal cell layers).

1.2.1. Retinal Pigment epithelium

The retinal pigment epithelium (RPE) consists of a single layer of cuboid shaped epithelial cells, situated between the photoreceptors of the neural retina and the choroid, where it controls the flow of nutrients from the choroidal vascular system to the retina. This layer extends from the optic nerve until the ora serrata. The RPE serves many purposes: development and maintenance of the neural retina (Raymond & Jackson, 1995), phagocytosis of the old photoreceptor outer segments discs, formation of a barrier between the choroid and the photoreceptor layer (Cunha-Vaz, 1979), and absorption and dispersion of the light. It also harbours components of the retinoid acid cycle and is involved in the formation and continuous regeneration of visual pigments

(Young, 1978; Bok, 1985; Clark, 1986; Raymond & Jackson, 1995; Bok, 1999; Marmor, 1999; Kolb, 2003; Forrester *et al.,* 2005).

1.2.2. Neuronal retina

The neuronal retina consists of the rest of the layers mentioned above. The principal neurons of the retina are (Figure 3):

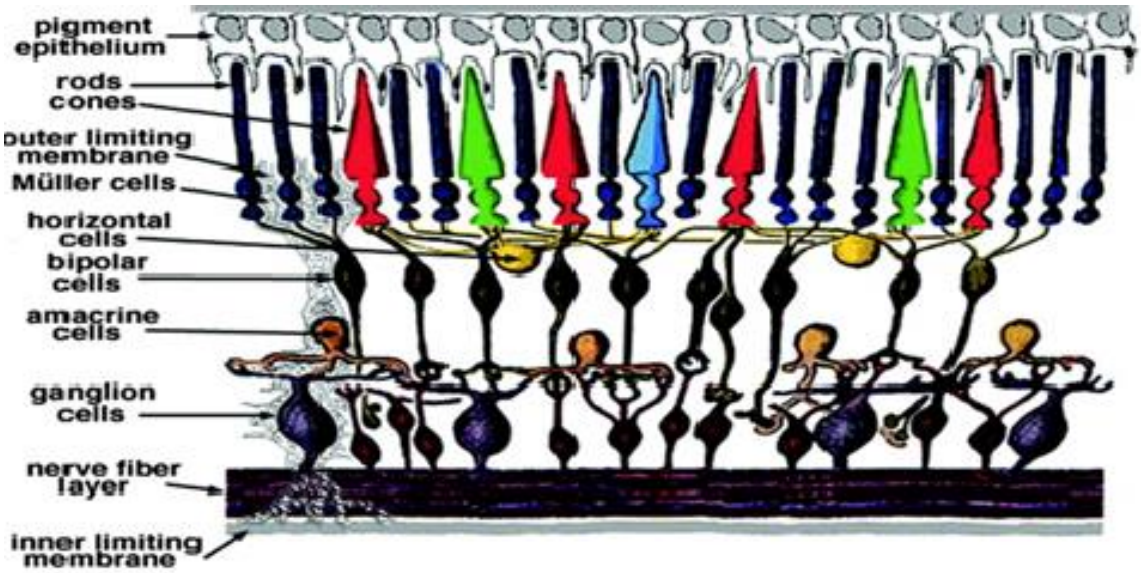

Figure 3. Simple diagram of the organization of the retina (Kolb, 2003).

1.2.2.1. Photoreceptors

Photoreceptors are polarized neurons that capture light and transform this energy into a chemical message through a process called phototransduction (Fu & Yau 2007; Wensel, 2008). Photoreceptors have a very high metabolic demand, which in normal conditions is supplied by the choroidal and intraretinal circulation (Bill & Sperber, 1990; Foulds, 1990). Their cell bodies are localized in the outer nuclear layer and are in tight contact with the RPE and retinal Müller glial cells, forming synapses with cells of the inner nuclear layer (bipolar and horizontal cells) (Figure 3).

Photoreceptors are the most abundant cell type in the retina and are divided into two types, rods and cones (Figure 4). Rods are specialized for low-light vision. They are extremely sensitive and can signal the absorption of single photons (Fu & Yau, 2007). Cones mediate daylight vision. They are much less sensitive to light than rods, but have

higher temporal resolution. The presences of typically more than one type of cones in the retina mediates color vision (Fu & Yau, 2007).

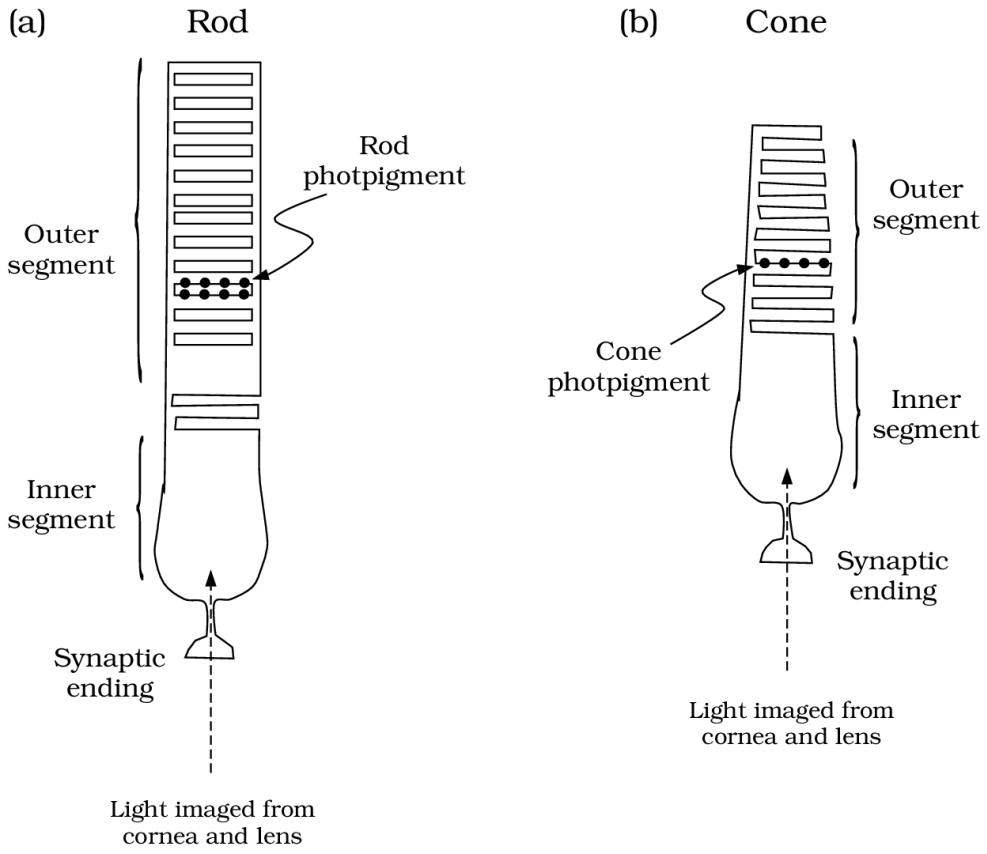

Figure 4. A mammalian rod (a) and a cone (b) photoreceptor in mammalian retina. Light enters the photoreceptors through the inner segment and is funneled to the outer segment that contains the photopigments (Baylor, 1987).

Both are composed of an outer segment (OS), inner segment (IS), nucleus, and synaptic body. Rows of membranous discs comprise the outer segment and contain the visual pigments and other proteins involved in phototransduction. These discs are constantly replaced in the base of the segment, and are phagocytosed by the pigment epithelium cells (Steinberg *et al.,* 1977). The inner segments contain the mitochondria. The segments are separated from the nucleus and the synaptic body by the barrier between the subretinal space and the outer nuclear layer (OLM). The visual pigments contained in the discs are called the opsins of which there are several different types. In rods, for instance, we find rhodopsin; while in cones there are at least four different classes of opsins, also known as photopsins (Nathans *et al.,* 1986). The synaptic

terminations of rods are called spherules (De Robertis & Franchi, 1956), while the cone synaptic terminations are known as pedicles.

Photoreceptors synapse onto the neuronal cells of the INL. They release the neurotransmitter glutamate in dark conditions, when the membrane is depolarized and cations (Na^+, Ca^{2+}) flow freely across cell membranes. When exposed to light, cyclic-nucleotide-gated (CNG) channels close, the cell goes into a hyperpolarized state, and photoreceptors stop releasing glutamate (Davson, 1980; Kolb, 2003; Forrester *et al.,* 2005).

The adult human retina contains about 96 million photoreceptors, of which approximately 5% are cones, and the remainder are rod photoreceptors (Curcio *et al.,* 1990). In mouse, only 1% of the photoreceptors are cones (Jeon *et al.,* 1998). In the human retina, photoreceptors have not a homogenous distribution. Rods are more concentrated in the periphery (or ora serrata), and cones are progressively concentrated toward the centre of the retina (Curcio *et al.,* 1990).

Humans, primates, and some birds (e.g. eagles) have a fovea, an extremely cone rich spot that perceptually facilitates highly detailed and finely focused images. In the centre of the retina lies the papilla, a portion of the inner surface of the eye without photoreceptors (blind spot), where the optic nerve leaves the retina on its way to the lateral geniculate nucleus, further perpetuating the neural information to the visual cortex. The papilla also serves as the portal through which the vasculature accesses the retina (Davson, 1980).

Rods photoreceptor cells

Rods are cylindrically shaped cells (Figure 4). Their outer segment discs contain rhodopsin.

In rods, sensitivity to light is very high, allowing them to function well even in dim light. They are largely responsible for peripheral and night vision. In the human retina, rods are more concentrated in the periphery than in the centre (Curcio *et al.,* 1990).

Cones photoreceptor cells

Cones mainly facilitate daylight vision, colour perception, and visual acuity (central vision). Cones are larger than rods but fewer in number.

There are three classes of cones in humans, each one absorbing light of different wavelengths: red cones or long wave length cones (maximum absorption at 564 nm, also referred to as L-cones), green cones or medium wave length cones (maximum absorption at 533 nm, M-cones), and blue cones or short wave length cones (maximum absorption at 437 nm, S-cones) (Wald, 1951; Marks *et al.,* 1964; Nathans *et al.,* 1986). Opposite to humans and primates, most mammals have only two types of cones: M and S. Mice are dichromats expressing an M opsin and an ultraviolet (UV)-wavelength sensitive opsin (Jacobs *et al.,* 1991).

1.2.2.2. Bipolar cells

There are more than eleven different classes of human bipolar cells. Dendrites from these cells link in the OPL with the synaptic axons of photoreceptors and horizontal cells (Figure 3). Cell bodies are located in the INL and the axons project to the IPL.

Bipolar cells are stimulated by photoreceptors and transmit their signals to the ganglion cells. Glutamate, the photoreceptor neurotransmitter is constantly released in the dark (Kolb, 2003) rendering the photoreceptor depolarized. Upon light stimulation the photoreceptor responds with a hyperpolarization, and inhibition of transmitter release. The postsynaptic bipolar cells respond with either hyperpolarization or depolarization of their membranes. The hyperpolarizing type of bipolar cell is called an OFF-center cell while the depolarizing bipolar cell is called an ON-center cell (Kolb, 2001). Rod photoreceptors transfer the signal to ON bipolar cells, cone photoreceptors signal to either ON or OFF bipolar cells (Figure 5). Signals from ON and OFF bipolar cells stimulated by cones are transmitted to ganglion cells, signals from ON bipolar cells stimulated by rods are transmitted to amacrine cells.

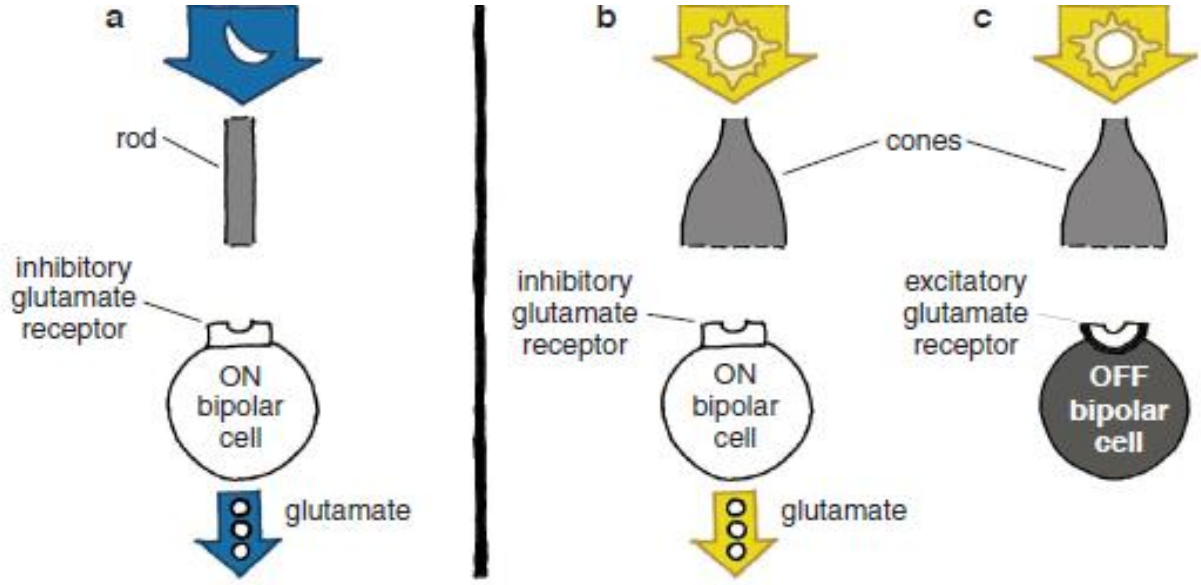

Figure 5. ON and OFF pathways along bipolar cells. Photoreceptors transmit information to bipolar cells using the molecule glutamate, but different bipolar cells respond differently to the presence of the molecule; some fire in response, whereas others cease firing, depending on the kind of glutamate receptor on their surface. ON bipolar cells have a depolarizing receptive field (a, b); OFF cells have a hyperpolarizing receptive field (c). Contrary to what one might expect, photoreceptors stop releasing glutamate when stimulated by light, in turn causing ON bipolar cells to release glutamate (from Hauck, 2005).

1.2.2.3. Horizontal cells

The horizontal cells modulate the electric signal coming from photoreceptors laterally and provide a first level of treatment of visual information. They are situated in the inner nuclear layer neighboring the outer plexiform layer where they form synapses with photoreceptors (Kolb, 2003).

Mammals have three classes of horizontal cells (HI, HII, and HIII). HI maintains connections with pedicles of the three different types of cones and HII may link mostly with pedycles from the S cones. HIII does not connect with S cones, but with the other classes (Kolb *et al.,* 1994). HII links also with the synapses of the rod spherules (Kolb, 1974). Even though their primary function is related to amplifying and transferring signals to ganglion cells, they also seem to help in depolarising the cones after being hyperpolarised by photon stimulation (Kamermans & Spekreijse, 1999).

Horizontal cells are also responsible for allowing eyes to adjust to see well under both bright and dim light conditions (Fu, 2010).

1.2.2.4. Amacrine cells

The name of these cells was first proposal by Santiago Ramon y Cajal because they are nerve cells thought to lack an axon (Ramon y Cajal, 1892). Their cellular bodies lie in the INL, while their neuritic prolongations are found in the IPL. Rather than direct synapses with the photoreceptors, amacrine cells are connected by way of bipolar and other amacrine cells (Dowling and Boycott, 1966). Currently, it is well known that certain large field amacrine cells of the vertebrate retina can have long "axon-like" processes. There are about 40 different morphological subtypes of amacrine cells. They are classified by the width of their receptive field, which layer(s) of the stratum in the IPL they are in, and by neurotransmitter type. Horizontal cells and amacrine cells directly modify the activation or inhibition of bipolar cells by releasing amino acids, catecholamines, peptides and nitric oxide (Kolb, 2003; Forrester *et al.,* 2005).

1.2.2.5. Ganglion cells

These cells are larger than the other retinal neurons and receive their information directly from cone-bipolar cells or from amacrine cells (rod-derived signals) and transmit action potentials along their axons to higher brain centers for the processing of visual information. The axons of the ganglion cells together form the optic nerve fiber layer (Forrester *et al.,* 2005).

In human retinas, two basic types of ganglion cells exist that receive signals from cone-bipolar cells (Kuffler, 1973): ON-center ganglion cells are activated when a spot of light falls in the center of their receptive field and are inactivated when light falls on the cell's periphery (see Figure 6, left panel). OFF-center ganglion cells react the opposite way (see Figure 6, left panel). In the human fovea, which contains only cone photoreceptors, a different type of ganglion cells exists: the midget ganglion cell, connected in a one-to-one ratio with midget bipolar cells (see Figure 6, right panel). The one-to-one signal transmission produces a point-to-point image from the fovea transferred to the brain (Kolb, 2001).

Interestingly, some ganglion cells are light-sensitive themselves, and appear to be involved in the regulation of the pupillary reflex and circadian rhythm (Berson, 2007).

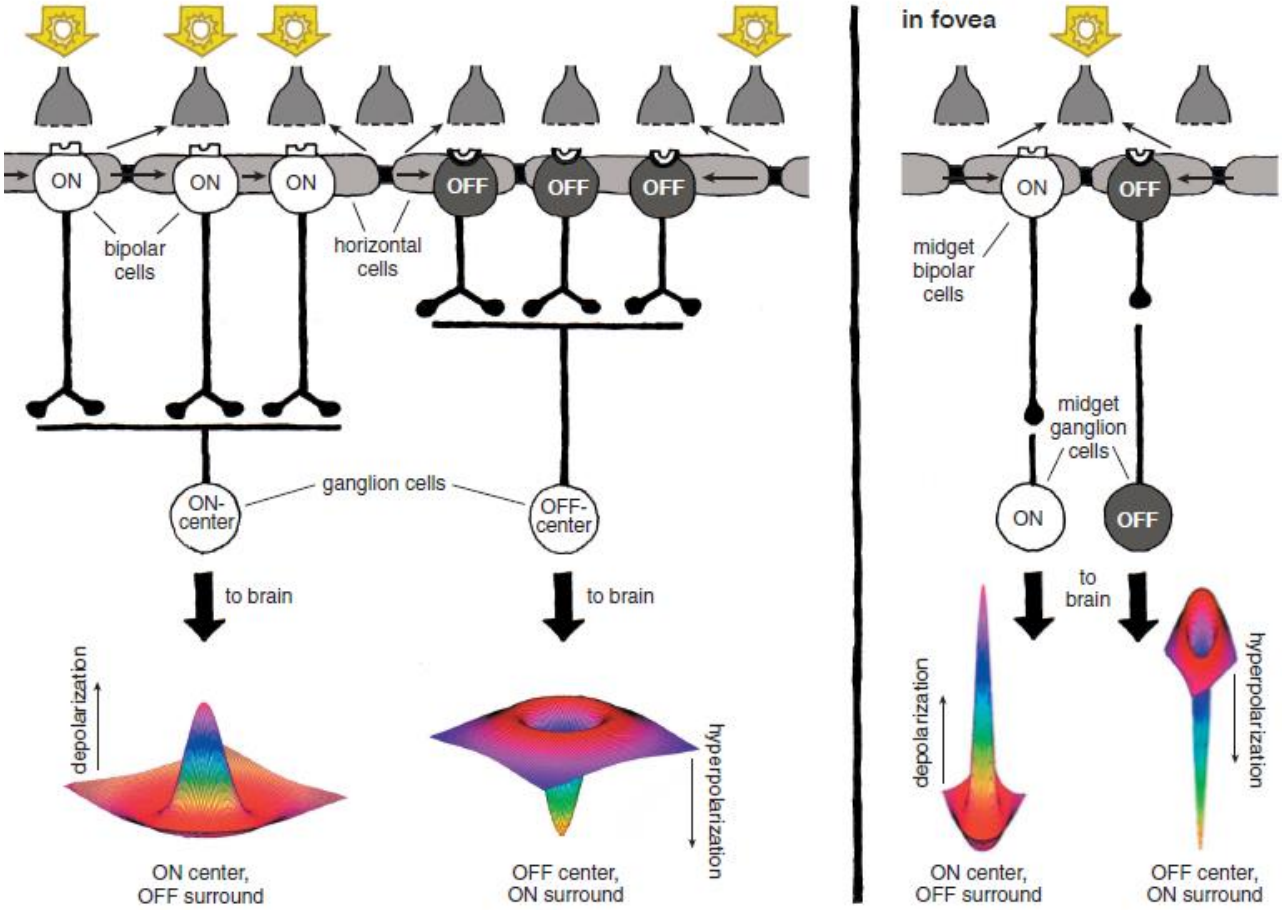

Figure 6. Processing of cone-derived signals along ON and OFF pathways. Human retinas have two types of ganglion cells: ON-center and OFF-center cells. ON center ganglion cells are activated when a spot of light falls in the center of their receptive fields, whereas OFF-center ganglion cells fire in response to light falling on their fields' periphery leaving their center dark. Horizontal cells convey antagonistic surround signals to bipolar cells and thence to ganglion cells. Ganglion cells have receptive fields with a Mexican-hat shape, reflecting their integration of opposing information about centers and surrounds. This kind of processing helps sharpen the boundaries of images. In the fovea (right), ganglion cells have much narrower receptive fields; in fact, each carries information from a single cone. A cone feeds information to two of these midget ganglion cells; at all times each foveal cone transmits either an ON or an OFF signal to the brain. This signal also carries a colour message regarding the type of cone (red or green) it comes from (Hauck, 2005).

1.2.3. Other retinal cells: Glia

The retina also contains glial cells: astro- and microglia. Three basic types of glial cell are found in the retina: Müller cells, microglia and astroglia (Ogden, 1978; Boycott & Hopkins, 1981). They primarily provide support for the neurons, but they can also act as part of the innate immune system, like the microglia which may phagocytose invasive molecules.

1.2.3.1. Müller cells

Considered the "oligodendrocytes of the retina", the Müller cells are the most numerous glial cells in the retina. Müller cell bodies are located in the inner nuclear layer (INL) and project irregularly thick and thin processes from the internal limiting membrane (ILM) to the bases of the rods and cones where they form a row of junctional complexes called the outer limiting membrane. Müller cells derive from the same progenitor cells as neurons during retinal development and they play a primordial role in the orientation, migration and movement of neurons (Rakic, 1981). Apart from critical architectural structural support, the functions of Müller cells are diverse: they provide metabolic support for retinal neurons, play a central role in the homeostatic regulation of the retina and maintain low synaptic levels of neurotransmitters protecting neurons from neurotransmitter excesses, mop up neural waste products (such as carbon dioxide and ammonium), phagocytose dead tissue, participate in the recycling of amino-acids, secrete neuroactive substances, such as GABA (γ-aminobutyric acid), taurine or dopamine, and synthesize retinoic acid from retinol (Newman *et al.*, 1984; Karwoski *et al.*, 1989; Edwards, 1994; Otori *et al.*, 1994; Derouiche & Rauen, 1995; Reichenbach & Robinson, 1995; Poitry-Yamate *et al.,* 1995; Poitry *et al.,* 2000). They contribute to the generation of the electroretinogram (ERG) b-wave (Miller & Dowling, 1970; Newman & Odette, 1984), the slow P3 component of the ERG (Karwoski & Proenza, 1977) and the scotopic threshold response (STR) (Frishman & Steinberg, 1989).

1.2.3.2. Astrocytes

Astrocytes are not intrinsic to the retina, but during development they migrate along the optic nerve and insert into the retina (Watanabe & Raff, 1988). These cells

are characterized by a flat cellular body and radial filaments. In the mature retina, astrocytes are found in the ganglion cell layer and optic fiber layer where also numerous blood vessels are situated (Bussow, 1980). Astrocytes play a role in the construction of the blood-retina barrier and can modulate the growth of endothelial cells (Jiang *et al.,* 1993) and it seems that they act to protect the synaptic surfaces of the neurons (Forrester *et al.,* 2005).

1.2.3.3. Microglial cells or microglia

Microglia defends against pathogenic agents by phagocytosis (Del Río-Hortega, 1949). During embryonic development, progenitor myeloid cells from bone marrow enter the CNS and specifically into the retina (Kreutzberg, 1996). Once there, they become microglial cells, in charge of the innate immune response in nervous tissue. In the healthy retina, these cells are quiescent in the inner layers, but they become active and migrate wherever there is degeneration, trauma, cell death, inflammation or any form of lesion (Hao *et al.,* 2002; Kolb, 2003).

1.3. RETINAL FUNCTION: PHOTOTRANSDUCTION

Light enters the mammalian eye through the pupil and after transversion of the vitreous body enters the retina. The photons pass all retinal layers and are finally absorbed at the outer segments of rod and cone photoreceptors where the electromagnetic wave is transformed into an electrochemical signal (phototransduction) (Wald, 1951; Molday, 1998; Fu & Yau 2007; Wensel 2008).

The phototransduction cascade (Figure 7) in rods is initiated by rhodopsin, a seven transmembrane-domains protein covalently linked to an 11-*cis* retinal chromophore. Photoexcitation converts 11-*cis* retinal to its all-*trans* isomer, creating Meta II rhodopsin, which catalyses the activation of the G-protein transducin. This in turn leads to the activation of phosphodiesterase (PDE), which hydrolyses cGMP to 5´-GMP. One rhodopsin molecule can activate multiple PDE molecules, amplifying the signal. The decrease in intracellular cGMP causes the cGMP-gated cation channels in the outer segment membrane to close (Kennan *et al.,* 2005). Without the balanced influx of Ca^{2+} the cell becomes hyperpolarized. This leads to inhibition of neurotransmitter glutamate release from the synaptic region of the photoreceptors and subsequently to

the generation of an electric signal in the downstream neuron of the activated photoreceptor (bipolar cell) (Massey, 1990) which is finally sent to the visual cortex. The resting phase is restored when Meta II rhodopsin is inactivated by rhodopsin kinase and binds to arrestin (S-antigen). Transducin and PDE are inactivated and disassociate due to the hydrolysis of the bound GTP by intrinsic GTPase activity of the transducin α subunit. The low level of intracellular Ca^{2+} caused by the closure of the cGMP-gated channels activates guanylate cyclase, which synthesizes cGMP. As intracellular levels of cGMP increase, the cGMP-gated Na^{+} and Ca^{2+} channels reopen and a depolarized dark state is reestablished. As Ca^{2+} levels raise again, guanylate cyclase activity is inhibited and cGMP synthesis returns to basal levels (Slaughter & Miller, 1981; Dowling, 1987; Pierce, 2001; Masland, 2001; Kolb, 2003; Forrester *et al.,* 2005).

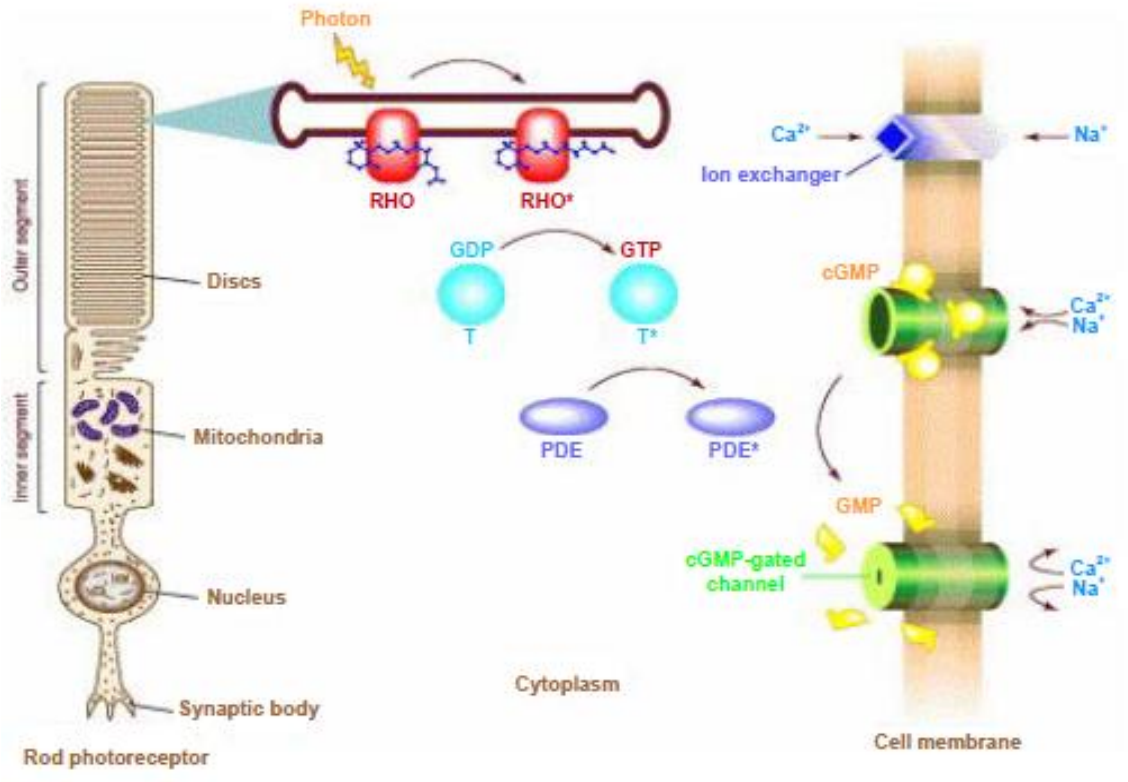

Figure 7. Phototransduction cascade. Light induced phototransduction takes place in the membrane discs of rod outer segments. The absorption of a photon induces the formation of activated rhodopsin, which then activates transducin. Activated transducin activates phosphodiesterase, which then catalyses the hydrolysis of cGMP to GMP. This leads to a closure of cGMP-gated cation channels in the outer membrane segment and to a hyperpolarisation of the photoreceptor cell (Farrar *et al.,* 2002).

1.4. ELECTRORETINOGRAPHY AND OPTICAL COHERENCE TOMOGRAPHY

1.4.1 Electroretinography

Electroretinography (ERG) is an important electrophysiological technique used to evaluate retinal function in humans and animals (Narfström & Nilsson, 1985; Ofri, 2002). It is used for the evaluation of retinal function prior to cataract surgery, as well as applied in the early diagnosis of inherited retinal degenerations, in the diagnosis of sudden acquired retinal degeneration and optic neuritis, and more recently, in the monitoring of therapeutic responses and in the evaluation of new drugs in toxicity studies (Ford *et al.,* 2003; Ropstad *et al.,* 2007; Messias *et al.,* 2008; Vaegan & Narfström, 2008; Marmor *et al.,* 2009; Jeong *et al.,* 2010).

The basic method of recording the electrical response known as the global or full-field ERG, is performed by stimulating the eye with a bright light source such as a flash produced by light emiting diodes (LEDs) or a strobe lamp. The flash light elicits a biphasic waveform recordable at the cornea similar to that illustrated below (Figure 8). The two components that are most often measured are the a- and b-waves. The a-wave is the first large negative component, followed by the b-wave which is corneal positive and usually larger in amplitude (http://webvision.med.utah.edu/).

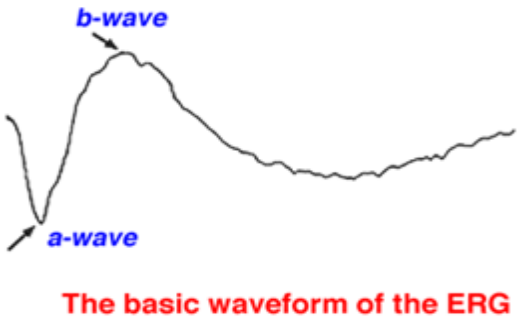

Figure 8. Biphasic waveform of a typical normal patient (http://webvision.med.utah.edu/).

The a-wave reflects the general physiological health of the photoreceptors in the outer retina (Penn & Hagins, 1969; Miller & Dowling, 1970). In contrast, the b-wave reflects the health of the inner layers of the retina, including the ON bipolar cells and the Müller cells (Miller & Dowling, 1970; Stockton & Slaughter, 1989; Xu & Karwoski,

1994; Robson & Frishman, 1995; Shiells & Falk, 1999; Jeong *et al.,* 2010; Jae *et al.,* 2013). Two other waveforms that are sometimes recorded in the clinic are the c-wave, originated in the pigment epithelium (Marmor & Hock, 1982) and the d-wave indicating activity of the OFF bipolar cells.

Two principal measures of the ERG waveform can be taken: 1) The amplitude (a) from the baseline to the negative trough of the a-wave, and the amplitude of the b-wave measured from the trough of the a-wave to the following peak of the b-wave; and 2) the time (t) from flash onset to the trough of the a-wave and the time (t) from flash onset to the peak of the b-wave (Figure 9). These times, reflecting peak latency, are referred to as "implicit times" in the jargon of electroretinography (http://webvision.med.utah.edu/).

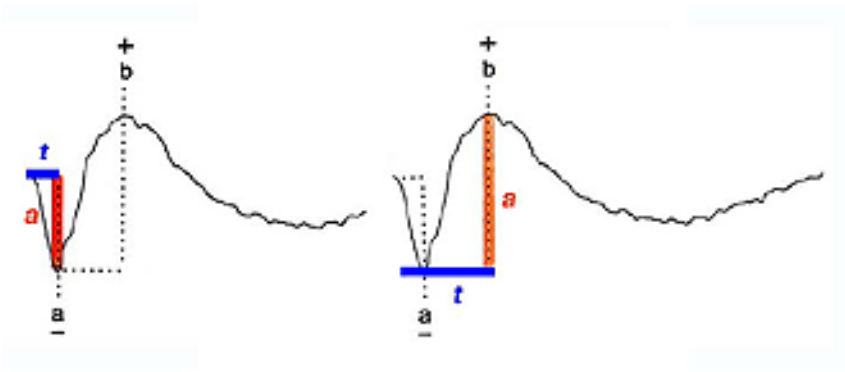

Figure 9. Amplitud and implicit time measurements of the ERG waveform (http://webvision.med.utah.edu/).

1.4.2 Optical Coherence Tomography

Optical coherence tomography (OCT) is a novel method of retinal imaging (Fujimoto, 2003; Fischer *et al.,* 2009). Recent studies with custom-made equipment support the applicability of OCT in rodents, the ability to detect retinal lesions, and the potential to follow disease processes over time (Kim *et al.,* 2008; Xu *et al.,* 2009).

Technically, OCT uses a weakly coherent infrared laser to analyze the reflectance properties of a sample (Fujimoto, 2003). Each resulting data set consists of a large number of topographically ordered depth profiles (A-Scans), which together represent a two-dimensional slice (similar to an ultrasound scan) across the sample. Several

consecutive slices together may be used to generate a three-dimensional data set (‘‘volume scan’’) (Fischer *et al.,* 2009).

The OCT provides a high-resolution depth profile based on reflectivity that correlates well with histomorphological sections (Figure 10) (Fischer *et al.,* 2009). However, the resulting image is different as conventional imaging and histology are based on absorption (light shade-little absorption, dark shade-strong absorption), whereas OCT is based on reflectivity (light shade-weak reflectivity, dark shade-strong reflectivity) (Fischer *et al.,* 2009). In particular, membranous surfaces are extremely well detected in OCT regardless of their physical extension. Consequently, membrane-rich but less optically dense layers like plexiform and nerve-fiber layers are represented in a darker shade of gray than optically more dense layers with less membrane content like the outer nuclear layer (Fischer *et al.,* 2009) (Figure 10).

The high resolution capability together with other properties such as ease of use, lack of ionizing radiation, patient comfort, and low cost have made OCT extremely popular for imaging retinal cell layers in the macular cube in both ophthalmology (Fujimoto *et al.,* 2003) and neurology (Jindahra *et al.,* 2010). For example, patients have quantitative abnormalities of different retinal layers in multiple sclerosis (MS) (Frohman *et al.,* 2008; Saidha *et al.,* 2011; 2012), type 1 diabetes (van Dijk *et al.,* 2009), Alzheimer’s disease (Kirbas *et al.,* 2013), Parkinson’s disease (Hajee *et al.,* 2009), and glaucoma (Guedes *et al.,* 2003).

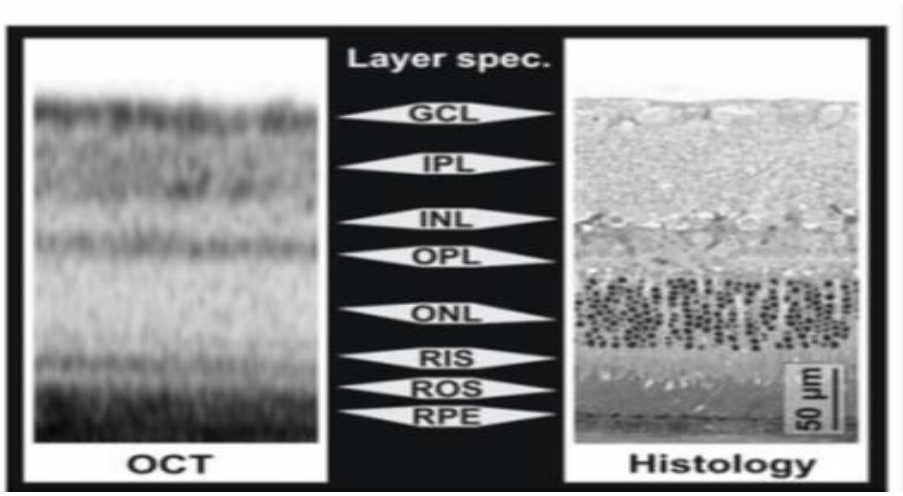

Figure 10. Representation of retinal layers in OCT and histology (Fischer *et al.,* 2009).

2. RETINITIS PIGMENTOSA

The disease named Retinitis pigmentosa (RP) is a genetically and phenotypically heterogeneous family of inherited blinding diseases that result in selective photoreceptor cell death. Inherited retinal degeneration is a broad term applied to dystrophies including retinitis pigmentosa, Ushers syndrome and others, which result in a progressive loss of vision.

RP is the most widespread of these disorders, affecting approximately 1.5 million individuals worldwide, and causing visual disability among younger people (Herse, 2005) with an incidence of 1 in 4000 (Bunker *et al.,* 1984).

It is believed to be the most prevalent cause of registered blindness in working populations in non-tropical countries (Doonan & Cotter, 2004).

Main symptoms of RP include progressive loss of visual functions with night-blindness or nyctalopia, which results from rod photoreceptor degeneration (typically, rod photoreceptor cells permit vision under dim light conditions). As rod cells die, cone photoreceptor viability (the source of high-resolution colour vision in daylight), is compromised and the disease progresses towards loss of peripheral fields, tunnel vision and finally blindness. Other characteristic features include pigmentary deposits in advanced RP and attenuation of the retinal blood vessels (Doonan & Cotter, 2004).

Mutations in 36 different genes have been found to cause RP, and mutations in many more cause widespread rod cell death in association with syndromes that have extraocular manifestations (Miranda *et al.,* 2010). Inheritance can follow an autosomal dominant (30-40% of cases), autosomal recessive (50-60%), Xlinked or digenic pattern (5-15%) (Hartong *et al.,* 2006).

The enormous genetic heterogeneity among the diseases that constitute RP is a problem for the development of treatments that deal with primary genetic defects.

Genes implicated in RP include, rhodopsin (RHO), the catalytic phosphodiesterase (PDE) subunits α (PDE6α) and β (PDE6β), the α subunit of rod cGMP-gated channel (CNGA1) and arrestin (SAG) (Danciger *et al.,* 1995; Dryja *et al.,* 1990a; 1990b; 1995, Huang *et al.,* 1995; McLaughlin *et al.,* 1993; 1995; Nakamachi *et al.,* 1998). Mutations in the rhodopsin gene are the most common and may affect protein folding, stability

or trafficking (Sung *et al.,* 1991). Structural components of photoreceptors, such as peripherin also harbour mutations causative of RP. Peripherin is located in rod and cone outer segment discs and appears to be critical for the formation and stabilization of photoreceptor outer segments (Goldberg *et al.,* 1998). Over 40 mutations have been identified within the peripherin gene of patients with retinal dystrophies (Keen & Inglehearn, 1996). Mutations in genes such as 11-*cis* retinal dehydrogenase (Yamamoto *et al.,* 1999) and cellular retinaldehyde binding protein (Maw *et al.,* 1997), required for recycling of the rhodopsin chromophore 11-*cis* retinaldehyde, also result in RP.

In particular, recessive defects in the gene encoding the β-subunit of cyclic GMP phosphodiesterase have been identified as causes not only of RP in humans (McLaughlin *et al.,* 1993; 1995) but also of retinal degeneration in mice widely used for eye research (rd1 mice) (Bowes *et al.,* 1990; Pittler *et al.,* 1991; Kaneko *et al.,* 2008).

Despite the diversity of retinal degeneration disorders, apoptosis of photoreceptors seems to be a feature common to all (Chang *et al.,* 1993; Dunaief *et al.,* 2002; Carella, 2003). The signalling pathways of apoptosis in photoreceptor cell death are still not fully understood (Sanvicens *et al.,* 2006). RP develops as a result of defects in genes responsible for upholding the structural and/or functional integrity of photoreceptors. In the most typical progression of RP, rods die first due to the mutation and this is followed by a mutation-independent cone cell death (Komeima *et al.,* 2006). It appears that cones depend upon rods for survival and that once rods die, the death of cones is inevitable, although the rate of cone death can vary greatly even among siblings with the same disease-causing mutation. In addition, the death of rods leads to a gradual change in retinal blood vessels that causes them to release a chemoattractant for RPE cells stimulating their transretinal migration. It is remarkable that the death of rods can have these remote effects long after they have departed (Komeima *et al.,* 2006).

Several studies have demonstrated that the eye is particularly sensitive to oxidative damage (Yu *et al.,* 2000, 2004; Shen *et al.,* 2005; Komeima *et al.,* 2006; Sanz *et al.,* 2007; Miranda *et al.,* 2010). Because of its high oxygen request and content of unsaturated lipids and its constant exposure to light, retina may be an elective site for

oxygen radical production and lipid peroxidation (Yamada *et al.,* 1999; 2001; Liang & Godley *et al*., 2003; Okoye *et al.,* 2003).

After the death of rods, cone photoreceptors begin to die and the mechanism remains unknown. Since rods is the most numerous cell type in the retina and also the most metabolically active and the biggest consumers of oxygen, as mentioned above, one would predict that oxygen levels would increase in the outer retina as rods die. Measurements with oxygen electrodes in models of RP have shown that prediction to be correct (Yu *et al.,* 2000; 2004). The increased levels of oxygen has been shown to result in progressive oxidative damage to cones in a transgenic pig model (Shen *et al.,* 2005) and a mouse model of RP, the rd1/rd1 mice (Komeima *et al.,* 2006).

3. OXIDATIVE STRESS

Oxidative stress was defined by Helmut Sies as: "a change in the prooxidant-antioxidant balance in favour of the former, potentially leading to biological damage" (Sies, 1991).

Free radicals are molecules with an unpaired electron in their outer orbit. The unpaired electron is highly reactive as it seeks to pair with another free electron.

The most common reported cellular free radicals are:

ROS (Reactive oxygen species)

- Superoxide Anion ($O_2^{-\cdot}$).
- Hidroxyl radical ($^{\cdot}OH$).

RNS (Reactive nitrogen species)

- Nitric oxide ($^{\cdot}NO$).
- Nitric dioxide ($NO_2^{\cdot}$).

RCS (Reactive chlorine species)

- Atomic Chlorine ($^{\cdot}Cl$).

Other molecules like hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), hyplochlorus acid (HOCL) are not free radicals but they are reported to generate free radicals through various chemical reactions (Packer *et al.,* 2000; Gilgun-Sherki *et al.,* 2001).

3.1 FREE RADICALS

Free radicals play a very important role in the origin of life and biological evolution, leaving beneficial effects on the organisms (McCord, 2000).

Oxygen free radicals are involved in many biochemical activities of cells such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity (Uttara *et al.,* 2009). Nitric oxide (·NO) is an important signaling molecule that essentially regulates the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Zheng & Storz , 2000).

Human body produce oxygen free radicals and other reactive oxygen species as by-products through numerous physiological and biochemical processes. Oxygen related free radicals (superoxide and hydroxyl radicals) and other reactive species (hydrogen peroxide, nitric oxide, peroxynitrile and hypochlorous acid), are produced in the body, primarily as a result of aerobic metabolism (Halliwell, 1994; Poulson *et al.,* 1998)

Humans are also constantly exposed to free radicals created by electromagnetic radiation from the man-made environment such as pollutants and cigarette smoke. Natural resources such as radon, cosmic radiation, as well as cellular metabolism (respiratory burst, enzyme reactions) also add free radicals to this microenvironment (Uttara *et al.,* 2009).

3.1.1 Physiological role of free radicals

It has been demonstrated the role of free radicals in the antimicrobial and antitumor defense, as well as messengers and inductors of genes (Satriano *et al.,* 1993; Zimmerman *et al.,* 2002; Al-Shabrawey *et al.,* 2008; Hardy, 2004). They may be involved in the inactivation or activation of certain enzymes, (Fillebeen & Pantopoulos, 2002; Minamiyama *et al.,* 2007), or contribute to the regulation of the extent of the inflammatory process (Bourbon *et al.,* 2004). In addition, it has been postulated that oxidant-antioxidant balance may be involved in the apoptotic process (Gupta *et al.,* 2007; Svensk, 2004). Nitric oxide is involved in regulating functions of the immune, cardiovascular and nervous systems (Mayer & Hemmens, 1997).

3.1.2 Pathophysiological role of free radicals

Production of free radicals have been related with hypertension (Touyz, 2004; Zalba *et al.,* 2001b; Droge, 2002), cardiovascular dysfunction (Ramachandran *et al.,* 2003a), inflammation (Telfer & Brock, 2004), diabetes (Muriach *et al.,* 2006; Miranda, 2004; Hermenegildo, 1993; Johnsen-Soriano *et al.,* 2007; Arnal *et al.,* 2010), acquired immunodeficiency syndrome (AIDS) (Jareño *et al.,* 2002), atherosclerosis (Madamanchi *et al.,* 2005; Mueller *et al.,* 2005), retinitis pigmentosa (Carmody *et al.,* 1999; Hackam et at., 2004), etc.

These radicals can directly or indirectly affect several cellular and physiological mechanisms, and may lead to alterations of vital molecules, including DNA (causing mutations), membrane lipids (causing their peroxidation) and protein (by altering enzyme activities) and ultimately can produce apoptosis and cell death (Carmody *et al.,* 1999; Liang *et al.,* 2003; Yamada *et al.,* 1999; 2001; Okoye *et al.,* 2003).

3.1.3 Free radicals can be produced from different sources

Some endogenous resources that can produce free radicals are:

- Electron transport chain in the mitochondria (Kas & Blattna, 1986);
- Excessive activity of NADPH phagocytosis (Gabig & Babior, 1979);
- Dislocation of transition metals (Fe^{2+}, Cu^{+}..) from their deposits (Halliwell & Gutteridge, 1986);
- Activation of the metabolism of arachidonic acid during the inflammatory process (Winyard *et al.,* 1994);
- Activation of the enzyme nitric oxide synthase (Beckman & Brent, 1990);
- Decreased capacity of protective antioxidants mechanisms (Giugliano *et al.,* 1996);
- Hyperactivity of xanthine oxidase (Chambers *et al.,* 1985).

And some exogenous resources of free radicals are**:**

- Electromagnetic or cosmic radiation (Fridovich, 1983);
- Increase of transition metals in metabolic diseases and inflammatory processes (Halliwell & Gutteridge, 1986);
- Drugs or toxic compounds (Trush *et al.,* 1982).

3.2 ANTIOXIDANT DEFENSES

An antioxidant is defined as any substance which, when present at low concentrations compared to the oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell & Gutteridge, 1986). The term "oxidizable substrate" includes almost any macromolecule that is found in living cells, such as proteins, lipids, carbohydrates and DNA.

Detoxification of oxygen free radicals is a prerequisite for aerobic life, so an important antioxidant defense system has been developed. Antioxidants defense systems co-evolved along with aerobic metabolism to counteract oxidative damage from oxygen free radicals (Yu, 1994). Antioxidants, such as glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A and tea polyphenols help to regulate the ROS thus generated. Antioxidant defense is further supported with antioxidant enzymes, e.g. superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase (Uday *et al.,* 1990; Poulson *et al.,* 1998; Yun-Zhong *et al.,* 2002).

Antioxidants are classified as exogenous (natural or synthetic) or endogenous compounds, both responsible for removal of free radicals, scavenging ROS or their precursors, inhibiting formation of ROS and binding metal ions needed for catalysis of ROS generation (Gilgun-Sherki *et al.,* 2001; Uttara *et al.,* 2009).

Natural antioxidant system is sorted in two major groups, enzymatic and non-enzymatic. Enzymatic antioxidants are comprised of limited number of proteins such as catalase, glutathione peroxidase as well as superoxide dismutase (SOD) along with some supporting enzymes. Non-enzymatic antioxidants include direct acting antioxidants, which are extremely important in defense against oxygen free radicals. Non-enzymatic antioxidants include ascorbic and lipoic acid, polyphenols and carotenoids, derived from dietary sources. The cell itself synthesizes a minority of these molecules. Indirectly acting antioxidants mostly include chelating agents that bind to redox metals to prevent free radical generation (Gilgun-Sherki *et al.,* 2001; Uttara *et al.,* 2009).

3.2.1 Metabolism of glutathione and other amino acids

3.2.1.1 Glutamic Acid

Glutamic acid or glutamate, its ionized form, is the major excitatory neurotransmitter in the brain and in the retina (Figure 11) (Michaelis, 1998; Thoreson & Witkovsky, 1999; Reis *et al*., 2009; Kalivas, 2009). But glutamate is also neurotoxic and therefore, glutamate concentrations have to be very closely regulated.

There are two classes of glutamate receptors, divided according to their primary signal transduction mechanism: ionotropic and metabotropic (Nakanishi, 1992; Forsythe & Barnes-Davies, 1997; Zhou *et al.,* 2011) (Figure 11). The ionotropic receptors work via ion channels. The metabotropic receptors are G-protein coupled receptors. There are three subclasses of ionotropic receptors: N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate type receptors. The NMDA receptors are the ones that are most associated with excitatory neurotoxicity and calcium entry into the cells. The calcium entry causes release of caspases from the mitochondria leading to apoptosis (Sucher *et al.,* 1997). NMDA receptors are made up of 3 different subunits, NR1, NR2A-D, and, in some cases, NR3A or B subunits (Chatterton *et al.,* 2002; Zhou *et al.,* 2011). The receptor is probably composed of a tetramer of these subunits. Alternative splicing further helps in adding pharmacologic differences to the action of the receptors (Chatterton *et al.,* 2002; Zhou *et al.,* 2011). There is a diversity of NMDA receptor types in different regions of the CNS (Monyer *et al.,* 1994; Sheng *et al.,* 1994; Laurie *et al.,* 1997).

At least eight metabotropic glutamate receptors (mGluR) are known (Nakanishi, 1994; Zhou *et al.,* 2011). These are subdivided into three subclasses (Conn & Pin, 1997; Schoepp *et al.,* 1999). Type I metabotropic receptors are associated with intracellular phosphatidyl inositol metabolism. Type II and III receptors are associated with an inhibitory cyclic adenosine monophosphate (cAMP) cascade as well as other postsynaptic cascades that lead to the release of Ca^{2+} from intracellular stores. There are some data that suggest that some of the type II mGluRs are neuroprotective (Miyamoto *et al.,* 1997; Lea *et al.,* 2005) (Figure 11).

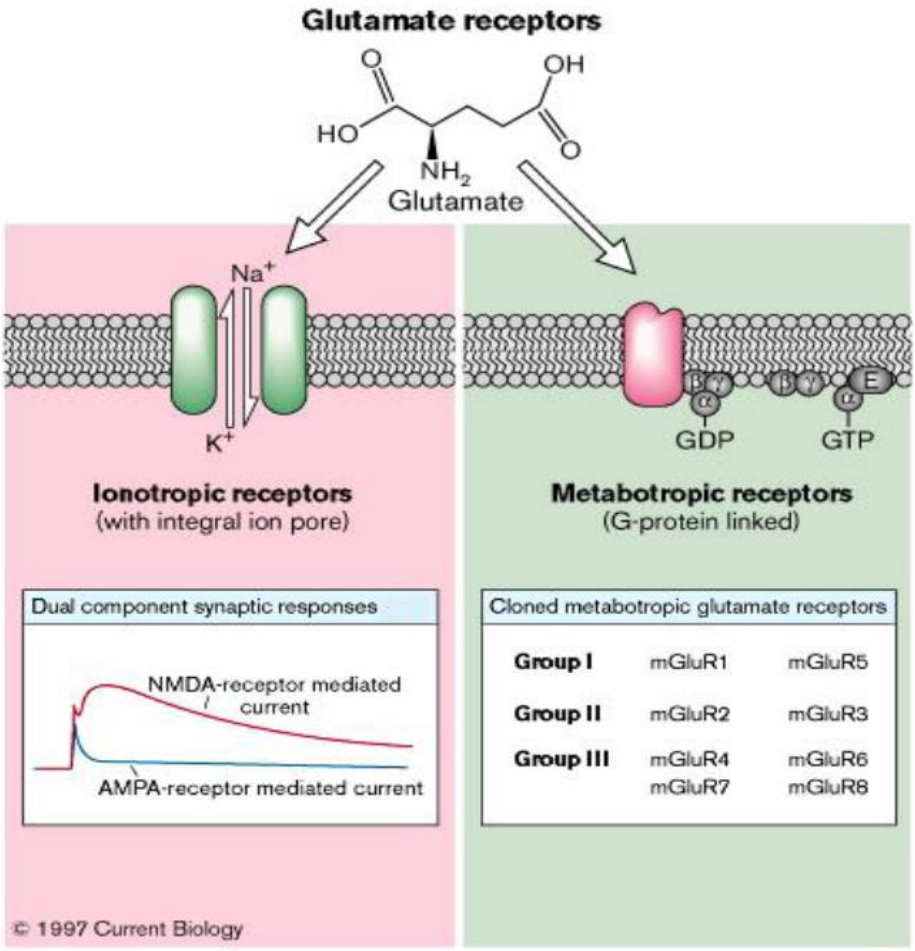

Figure 11. Glutamate activates two types of receptor: ionotropic receptors have an integral cation permeable pore, whereas metabotropic receptors activate G-protein-coupled second messenger cascades (Forsythe & Barnes-Davies, 1997).

Glutamate participates in many important physiological processes, such as developmental plasticity, long-term potentiation and development (Scheetz & Constantine-Paton, 1994; Bisti *et al.,* 1998).

The retina being an integral component of the CNS, shares many of its organizational features, including the preeminence of glutamate as its excitatory neurotransmitter. Excellent reviews of glutamate's actions in the vertebrate retina have been published (Massey, 1990; Wilson, 1994; Massey & Maguire, 1995; Thoreson & Witkovsky, 1998; Bringmann *et al.,* 2009) and other reviews dealing with retinal electrophysiology (Wu, 1994) or transmitter release (Matthews, 1996) have discussed retinal glutamatergic circuitry.

In the vertebrate retina, glutamate is released from photoreceptors and bipolar cell terminals and exerts its actions by activating postsynaptic ionotropic and/or metabotropic receptors that are expressed in most, if not all, retinal cells (Figure 12) (Thoreson & Witkovsky, 1999). Fine tuning of glutamate uptake and degradation in glial cells is essential to avoid neurotoxicity and to allow normal signal transmission between photoreceptor and bipolar cells (Derouiche & Rauen, 1995; Rauen *et al.,* 1998; Harada *et al.,* 1998; Delyfer *et al.,* 2005; Bringmann *et al.,* 2009).

Previous studies have clarified the mechanisms by which glutamate is accumulated in the extracellular space in retinal tissue (Lieth *et al.,* 2000; Li & Puro, 2002; Silva & Carvalho, 2013). In the retina, glutamate homeostasis is maintained by the glial L-glutamate/ L-aspartate transporter (GLAST) and by glutamine synthetase (GS), both being expressed in Müller glial cells (Linser *et al.,* 1984; Erecinska & Silver, 1990; Derouiche & Rauen, 1995; Rauen *et al.,* 1998; Harada *et al.,* 1998; Izumi *et al.,* 2002; Delyfer *et al.,* 2005; Bringmann *et al.,* 2009).

Glutamate toxicity has been demonstrated both in inner retinal cells and in photoreceptor terminals (Lucas & Newhouse, 1957; Olney, 1969; 1982; Yazulla & Kleinschmidt, 1980; Ingham & Morgan, 1983; Sattayasai & Ehrlich, 1987; Sattayasai *et al.,* 1989; Sahel *et al.,* 1991) and in ganglion cells (Vorwerk *et al.,* 1996; Sucher *et al.,* 1997).

Changes in glutamate metabolism have been observed in several different models of inherited retinal degeneration (Ulshafer *et al.,* 1990; Nakazawa *et al.,* 1993; Noro *et al.,* 1994; Fletcher & Kalloniatis, 1996; Kalloniatis & Tomisich, 1999; Fletcher *et al.,* 2000; Delyfer *et al.,* 2005). Ulshafer *et al.* (1990) were the first to demonstrate an increase in excitatory amino acid levels (glutamate and aspartate) around degenerating photoreceptors in the GUCY1* chick. In the Royal College of Surgeons (RCS) rat Müller's cells, Fletcher and Kalloniatis (1996; Kalloniatis & Tomisich, 1999; Fletcher *et al.,* 2000) observed an increase in aspartate and glutamine levels (both of which are either precursors or metabolites of glutamate), along with alterations in glutamate and GABA (y-aminobutyric acid) manufacturing pathways supposedly linked to abnormal Müller glial cell function. In the rds/rds mouse, an accumulation of

glutamate has also been observed in photoreceptor inner segments (Nakazawa *et al.,* 1993; Noro *et al.,* 1994).

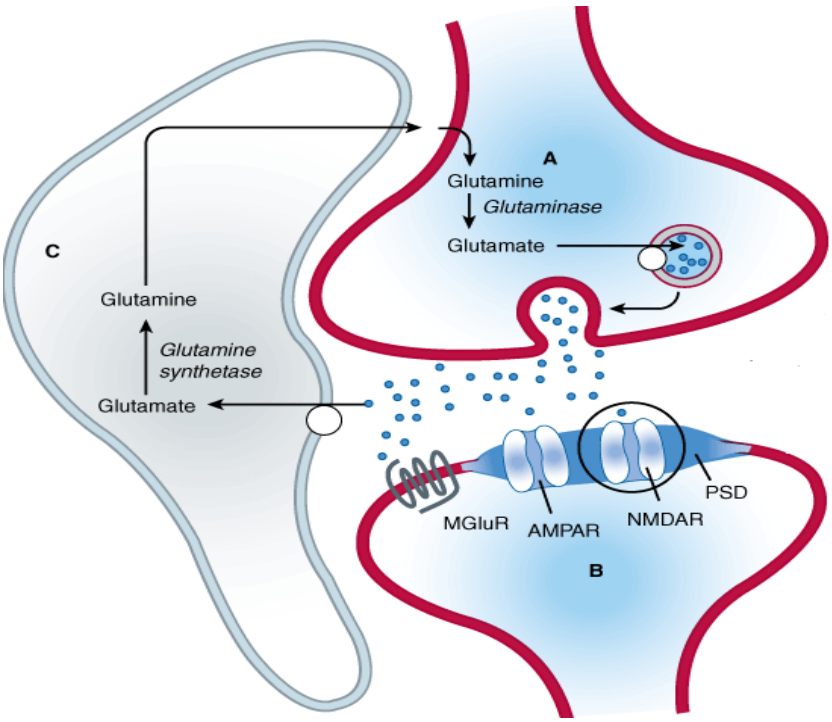

Figure 12. Schematic diagram of a glutamate synapse. Glutamine is imported into the glutamatergic neuron **(A)** and converted into glutamate by glutaminase. Upon release into the synapse, glutamate can interact with AMPA and NMDA ionotropic receptor channels and with metabotropic receptors (MGluR) on the postsynaptic cell **(B)**. Synaptic transmission is terminated by active transport of the glutamate into a neighboring glial cell **(C)** by a glutamate transporter (from Katzung *et al.,* 2010).

Finally, Delyfer and coworkers (Delyfer *et al.* 2005) studied glutamate metabolism and observed increases in glutamate and glutamine (a precursor of glutamate) levels and an increase in the expression of the L-glutamate/ L-aspartate transporter (GLAST) (similarly to the results obtained by Rauen in 1998 (Rauen *et al.,* 1998)) and in glutamine synthetase (Linser *et al.,* 1984; Erecinska & Silver, 1990; Delyfer *et al.,* 2005).

The same author demonstrated that injection of recombinant GDNF (glial cell line-derived neurotrophic factor) (with neuroprotective effect on photoreceptors) significantly slows down photoreceptor degeneration, but also partially preserves visual function in the rd1 mouse (Frasson *et al.,* 1999) and in several different models of photoreceptor degeneration (McGee-Sanftner *et al.,* 2001; Wu *et al.,* 2002;

Lawrence *et al.,* 2004). Although GDNF enhances the expression of GLAST (L-glutamate/ L-aspartate transporter) around the degenerating photoreceptor they failed to demonstrate whether GDNF promoted photoreceptor-survival via a decrease in free glutamate levels (Delyfer *et al.,* 2005).

The mechanisms underlying GDNF neuroprotection remain unknown (Delyfer *et al.,* 2005). In vitro, a direct survival-promoting effect on newborn rodent photoreceptors was observed (Jing *et al.,* 1996; Politi *et al.,* 2001). Now, several arguments support the hypothesis that GDNF exerts its trophic effect on photoreceptors in the rd1 mouse also indirectly through activation of intermediate Müller glial cells (Delyfer *et al.,* 2005) as other trophic factors (Zack, 2000; Harada *et al.,* 2000; Wahlin *et al.,* 2000; 2001).

Increased concentrations of extracellular glutamate have also been implicated in the pathophysiology of neuronal loss in ophthalmic disorders such as glaucoma, ischemia, diabetes, etc (Romano *et al.,* 1995; Ambati *et al.,* 1997; Brooks *et al.,* 1997; Sucher *et al.,* 1997; Lieth *et al.,* 1998; Dkhissi *et al.,* 1999; Kowluru & Kennedy, 2001; Martin *et al.,* 2002; Mattson, 2008; Bringmann *et al.,* 2009).

A dysfunctional electrogenic glutamate transport in Müller cells caused by oxidative stress has been also described in experimental diabetes (Li & Puro, 2002) and in Leber hereditary optic neuropathy (Beretta *et al.,* 2006). Human Müller cells from patients with various retinopathies such as retinal detachment, proliferative vitreoretinopathy, and glaucoma display an increase in the density of the glutamate transporter currents when compared to cells from healthy donors (Reichelt *et al.,* 1997). An increase in GLAST labeling has also been observed in experimental retinal detachment (Sakai *et al.,* 2001).

A downregulation of GLAST has been described in retinas of patients with glaucoma (Naskar *et al.,* 2000). Elevation of the intraocular pressure in experimental glaucoma causes a failure of GLAST activity resulting in a decreased accumulation of glutamate in Müller cells and a significant glutamate uptake by retinal ganglion cells; the failure of GLAST coincides with excitotoxic damage to the retina (Holcombe *et al.,* 2008). The increase in the intraocular pressure also causes inner retinal hypoxia (via compression of blood vessels) resulting in elevated formation of free radicals in the mitochondria

and lipid peroxidation that disrupts the glutamate transport in Müller cells (Bringmann *et al.,* 2009).

A malfunction of the glutamate transport into Müller cells will contribute to the increase in extracellular glutamate to excitotoxic levels (Delyfer *et al.,* 2005; Bringmann *et al.,* 2009). Transient retinal ischemia or diabetes do not significantly alter the expression of GLAST or the amplitude of GLAST-evoked membrane currents (Barnett *et al.,* 2001; Ward *et al.,* 2005; Pannicke *et al.,* 2005, 2006) but reduce the efficiency of the glutamate transport into Müller cells (Barnett *et al.,* 2001; Li & Puro, 2002); under these conditions, a high amount of glutamate is transported into photoreceptor, bipolar and ganglion cells (Barnett *et al.,* 2001).

3.2.1.2 Glutathione system

The glutathione system, includes glutathione (γ-glutamyl-Cysteinyl-glycine; GSH) and the enzymes related to its metabolism, which are also responsible for maintaining the redox state under physiological conditions (Meister & Anderson, 1983). Glutathione is a tripeptide (consisting of glutamic acid, Cysteine and glycine) of interesting biological properties due to two structural characteristics: the γ-glutamyl bond that protects it against peptidases, and the presence of a free thiol group which makes it a very reactive compound with all kinds of substances and even with itself (Meister & Anderson, 1983).

GSH (Figure 13) is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide (GSH/GSSG) is the major redox couple in animal cells (Dringen, 2000; Dickinson & Forman, 2002; Zeevalk *et al.,* 2008; Park *et al.,* 2009).

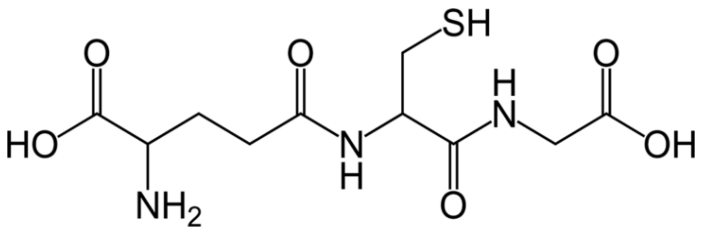

Figure 13. Chemical structure of glutathione (Filomeni *et al.,* 2005).

GSH is a small molecule found in almost every cell. It cannot enter most cells directly and therefore must be synthesized inside the cell, from its three constituent

amino acids. It is formed in a two-step enzymatic process including, first, the formation of γ-glutamylCysteine from glutamate and Cysteine (Cys), by the activity of the γ-glutamylCysteine synthetase; and second, the formation of GSH by the activity of GSH synthetase (GS) which uses γ-glutamylCysteine and glycine as substrates (Meister & Tate, 1976; Dringen *et al.,* 2000; Dikinson & Forman, 2002; Circu & Aw, 2011) (Figure 14). While its synthesis and metabolism occur intracellularly, its catabolism occurs extracellularly by a series of enzymatic and plasma membrane transport steps (Franco *et al.,* 2007).

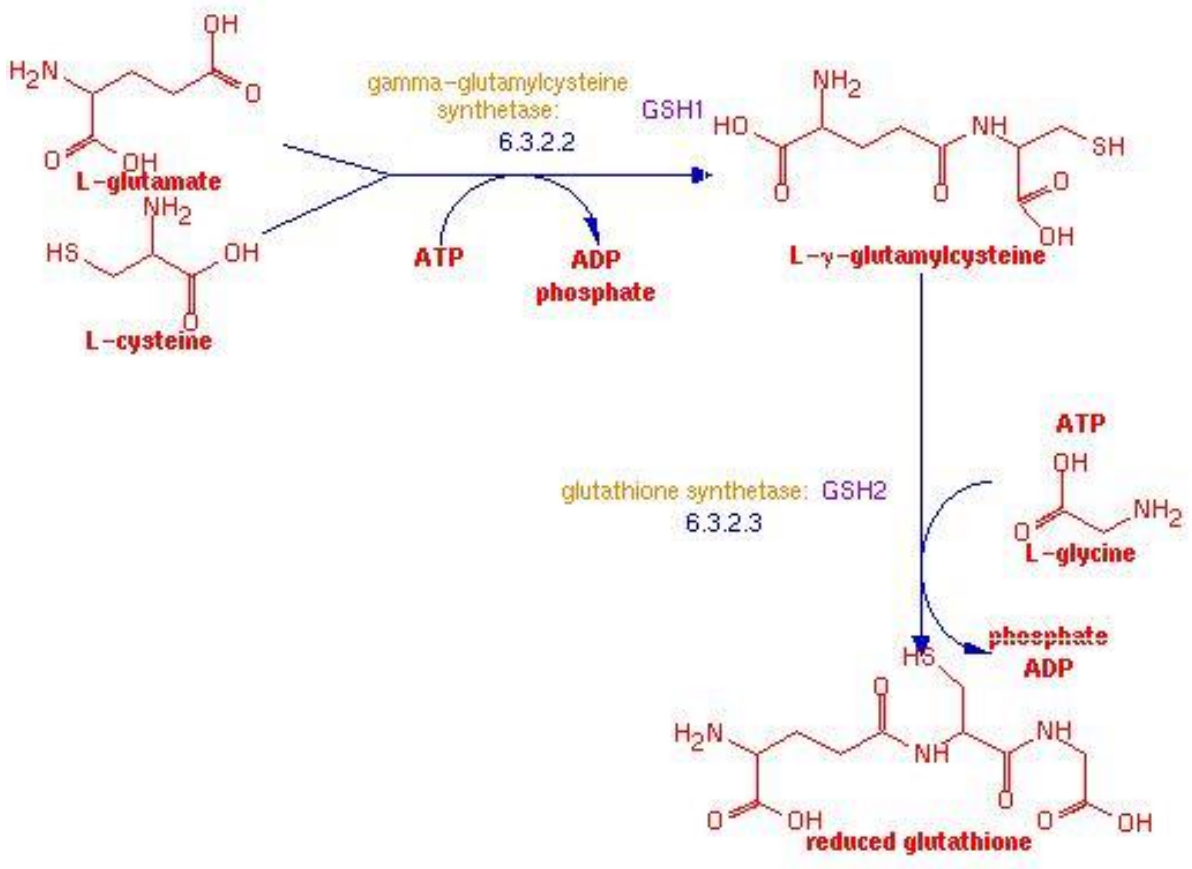

Figure 14. Schematic diagram of glutathione synthesis (Yeast Genome Pathway Analysis, 2006).

GSH is considered to be in equilibrium between the different torns that this tripeptide may occur (Kosower & Kosower, 1978): it can be found as reduced thiol (GSH), oxidized form (GSSG) and mixed disulfides GS-S-protein.

The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in nonenzymatic reactions and is the electron donor in the

reduction of peroxides catalyzed by glutathione peroxidase (GPx) (Figure 15) (Winterbourn & Metodiewa, 1994; Dringen, 2000; Dickinson & Forman, 2002). The product of the oxidation of GSH is glutathione disulfide (GSSG). GSH is regenerated from GSSG within cells in a reaction catalyzed by the flavoenzyme glutathione reductase (GR). This enzyme regenerates GSH by transferring reduction equivalents from NADPH to GSSG (Figure 15) (Filomeni *et al.,* 2005).

During the course of the reactions catalyzed by GPx and GR, GSH is recycled (Figure 15). In contrast, GSH is consumed during the generation of glutathione-S-conjugates by glutathione-S-transferases (Salinas & Wong, 1999) or by the release of glutathione from cells (Kaplowitz *et al.,* 1996). Both processes lower the level of total intracellular glutathione.

GSH, is the most important and effective endogenous antioxidant. It is considered as the body's first line of defense against oxidative stress. As an antioxidant, GSH has important functions, it is a transport and storage form of Cysteine, it is a reaction partner for the detoxification of xenobiotics, and it is a cofactor in isomerization reactions (Meister & Anderson, 1983; Dringen *et al.,* 2000). In addition, GSH maintains the thiol redox potential in cells keeping sulfhydryl groups of cytosolic proteins in the reduced form and it is also suggested that GSH plays a role in the regulation of apoptosis (van den Dobbelsteen *et al.,* 1996; Ghibelli *et al.,* 1998; Hall, 1999; Dringen, 2000; Jones, 2006).

More recent studies of the functions served by GSH in cells include modulation of protein function via thiolation which may control physiological and pathophysiological pathways including DNA synthesis and repair, protein synthesis, amino acid transport, modulation of glutamate receptors and neurohormonal signaling (Kamata & Hirata, 1999; Sen, 2000; Moran *et al.,* 2001; Jones, 2006; Zeevalk *et al.,* 2008).

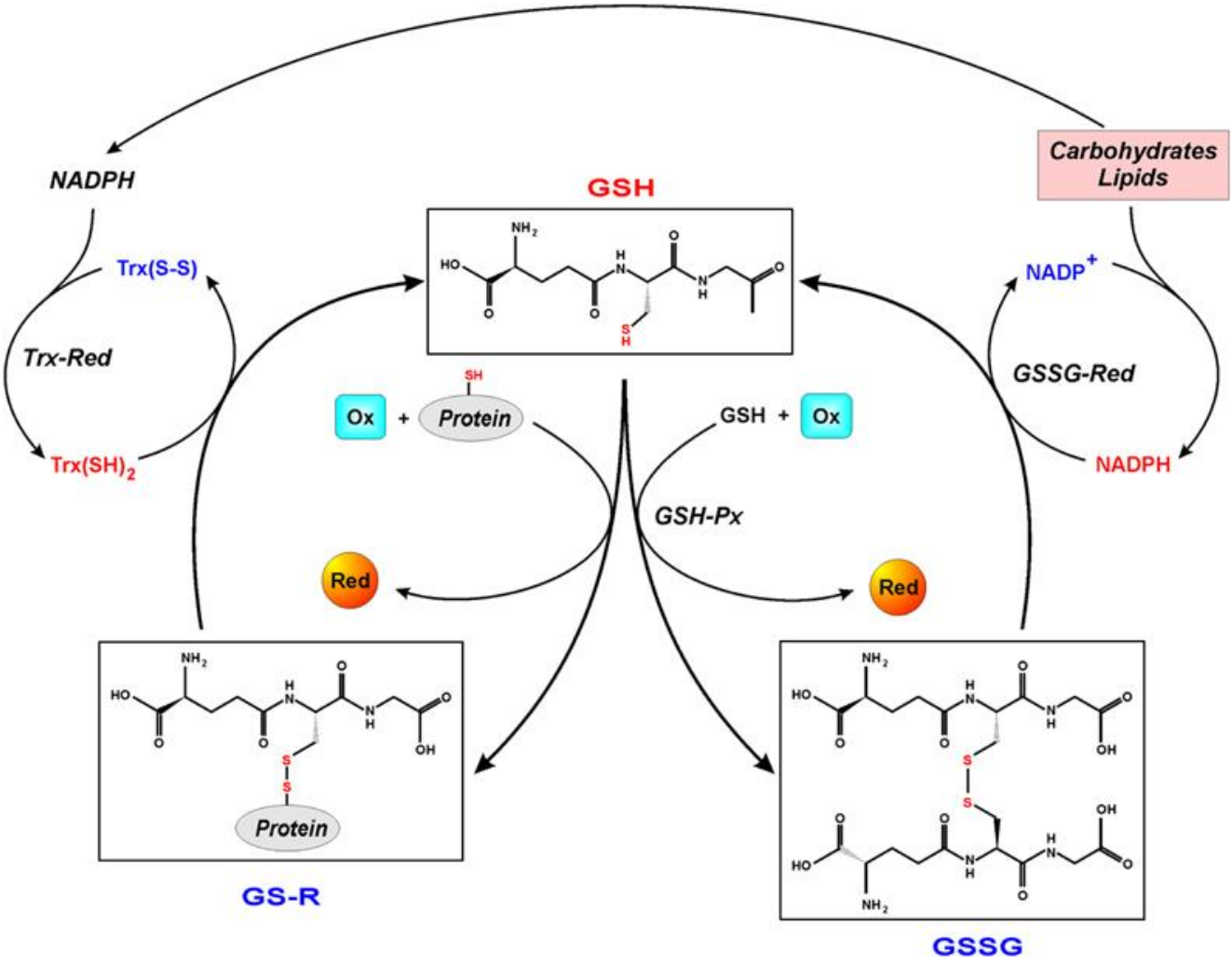

Figure 15. Function of GSH as an antioxidant. GSH reacts nonenzymatically with radicals and is the electron donor for the reduction of peroxides in the reaction catalyzed by GPx. GSH is regenerated from GSSG by GR which uses NADPH as cofactor (Filomeni *et al.,* 2005).

A number of neurodegenerative diseases and psychiatric disorders have been linked to abnormal GSH levels and S-glutathionylation of proteins, such as Parkinson's disease (Sian *et al.,* 1994; Chinta *et al.,* 2006), Alzheimer's disease (Zhu *et al.*, 2006; Di Domenico *et al.*, 2009), Huntington's disease (Klepac *et al.,* 2007), and schizophrenia (Steullet *et al.,* 2006; Raffa *et al.,* 2009; Radonjic *et al.,* 2010).

Modifications of the cellular redox state of the eye are believed to contribute to the pathogenesis of many diseases and it has been demonstrated that ROS are key signalling molecules in driving apoptosis both in in vitro and in vivo models of retinal diseases (Sanvicens *et al.,* 2004; Komeima *et al.,* 2008; Miranda *et al.,* 2010).

It is known that GSH is present in the retina (Hermann & Moses, 1945) and that this tissue also has enzymatic activity associated with GSH metabolism like glutathione peroxidase, glutathione disulfide reductase, and glutathione S-transferase (Reim et al., 1972; Castorina *et al.,* 1992; Puertas et al., 1993; Dickinson & Forman, 2002; Hayes *et al.,* 2005; Chinta *et al.,* 2007). In the mammalian retina, the GSH content has been studied by biochemical analysis of total retinal GSH (Kowluru *et al.,* 1994).

No GSH immunoreactivity has been reported in outer segments of rod and cone photoreceptors from rodent, primate and zebrafish retinas (Pow & Crook, 1995; Schuette & Werner, 1998; Marc & Cameron, 2001), but Müller cells and inner retinal neurons appear to contain substantial pools of this compound (Organisciak *et al.,* 1984; Pow & Crook, 1995; Huster *et al.,* 2000). It has been postulated that because of the lack of GSH in rod and cone segments, the most useful form to mitigate lipid peroxidation damage is phagocytosis and degradation of retinal photoreceptor outer segment material by the retinal pigment epithelium (RPE) (Winkler, 2008).

Oxidative damage has been reported to be present in cone photoreceptor degeneration (Delyfer *et al.,* 2005; Ristoff *et al.,* 2007; Miranda *et al.,* 2010).

Moreover, it has been suggested that glutathione alterations can also be observed in humans affected with photoreceptors dystrophy. Two sisters with severe glutathione synthetase deficiency, an autosomal recessive inborn error of metabolism resulting in very low intracellular levels of GSH showed progressive retinal dystrophy with hyperpigmentations and maculopathy. These findings agree with a rod/cone type of retinal dystrophy (Ristoff *et al.,* 2007).

3.2.1.3 Cysteine and Cystine

Cysteine (Figure 16) is the rate-limiting substrate for GSH synthesis (Meister & Anderson, 1983). The thiol/disulfide redox couple Cysteine (Cys) and its disulfide Cystine (CysS) are the most abundant low-molecular weight thiol/disulfide redox couple in plasma and are also present in tissues (Nkabyo *et al.,* 2005; Iyer *et al.,* 2009; 2009; Go & Jones, 2011).

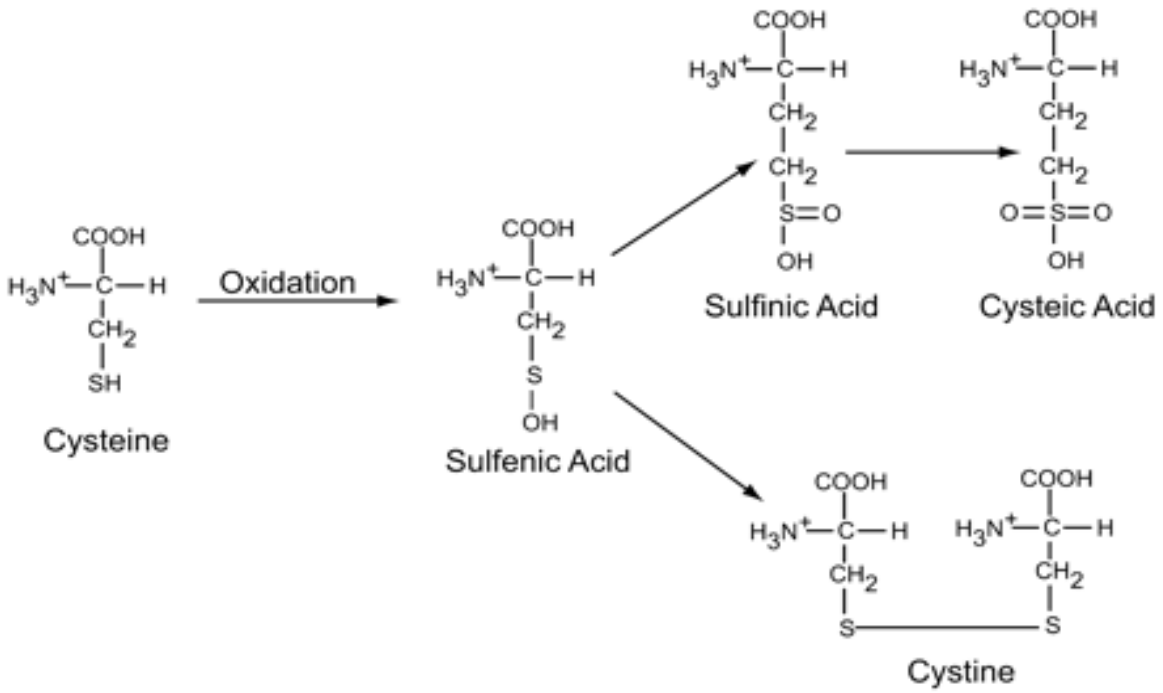

Figure 16. Chemical structure of Cysteine and Cystine (Griffiths *et al.,* 2002)

Cys and CysS, are transported into the cell via sodium dependent and independent transporters, respectively (Bannai, 1984; Bannai *et al.,* 1984).

Several in vitro studies demonstrate that reversible redox reactions of thiol/disulfide couples play roles in the regulation of important cellular processes such as proliferation, differentiation, and apoptosis and have been implicated in human disease (Georges-Labouesse *et al.,* 1996; Samiec *et al.,* 1998; Kirlin *et al.,* 1999; Jones *et al.,* 2002; Moriarty-Craige *et al.,* 2005).

However, cellular thiol/disulfide are not in redox equilibrium and respond differently to chemical toxicants and physiological stimuli (Jones, 2006). The reduction potentials for the redox couples GSH/GSSG and Cys/CysS in plasma are useful indicators of systemic oxidative stress (Moriarty *et al.,* 2003) and other medically relevant physiological states.

It has been demonstrated that modifications of extracellular Cys/CysS could directly regulate cell proliferation by acting as an oxidant-reductant redox switch (Jones *et al.,* 2000). A reducing extracellular redox state modulated by Cys/CysS has been demonstrated to increase cell proliferation through a growth factor-signaling pathway in colon carcinoma cells (Jonas *et al.,* 2002), whereas cells grown under oxidizing

conditions are more sensitive to oxidant-induced apoptosis (Jiang *et al.,* 2005). A study in lung fibroblasts showed that an oxidizing extracellular redox state modulated by Cys/CysS stimulated cell proliferation and extracellular matrix expression (Ramirez *et al.,* 2007).

These combined results suggest that extracellular Cys/CysS redox-dependent proliferation may be cell type specific (Chaiswing *et al.,* 2012).

3.3 RETINITIS PIGMENTOSA AND OXIDATIVE STRESS

Increasing evidence suggests that oxidative stress contributes to the pathogenesis of many retinal degenerative disorders, including age-related macular degeneration (AMD), glaucoma, diabetic retinopathy, and light damage (Berson *et al.,* 1993, 2004; Ganea & Harding, 2006; Bazan, 2006; Miranda *et al.,* 2010).

Like other tissues, the retina is subject to oxidative damage. However, the retina possesses several features which makes it especially vulnerable to oxidation. These are the very high oxygen consumption rate of the retina, the high content of polyunsaturated fatty acids in the photoreceptor outer segments, and the light exposure of the tissue (Anderson *et al.,* 1984; Handelman & Dratz, 1986; Dentchev *et al*., 2007).

Oxidative stress has also been implicated in the pathogenesis of RP. This is further supported by reports that show that oxidative damage is present in cone photoreceptor degeneration (Shen *et al.,* 2005; Cingolani *et al.,* 2006; Miranda *et al.,* 2010).

One hypothesis is that after rods die, oxygen utilization in the outer retina is reduced; but choroidal vessels, unlike retinal vessels, are incapable of autoregulation to decrease blood and oxygen level in the outer retina becomes markedly elevated (Usui *et al.,* 2009).

In this sense, Komeima *et al*. (2007) showed that antioxidants (α-tocopherol, ascorbic acid, Mn (III) tetrakis (4benzoic acid) porphyrin, and α-lipoic acid) decreased cone photoreceptor cell death in different mouse models of retinitis pigmentosa. Lu *et al*. (Lu *et al.,* 2009) demonstrated that Gpx4 provided strong protection of retinal

structure and function in an animal model of RP. Usui *et al*. (Usui *et al.,* 2009) showed that, in RP treatment, it may be needed an increase in multiple antioxidants as they found that overexpression of superoxide dismutase 1 (SOD1) in rd1 mice increased oxidative damage and accelerated cone cell death while increased expression of SOD2 and catalase at the same time in the mitochondria of photoreceptors of rd10+/+ mice reduced superoxide radicals and oxidative damage in the retina, provided significant preservation of cone function, and reduced cone cell death. Drack *et al*. evaluated the protective effect of tauroursodeoxycholic acid (TUDCA), the active component in bear bile, on photoreceptor degeneration in different models of retinal degeneration in mice and demonstrated that TUDCA appears to be protective in retinal degenerations with later onset and slower course, in rd10 mice (Drack *et al.,* 2012).

However, our group and others have also found significant protection when antioxidants are administered while rods are dying. We have used a different combination of antioxidants (zeaxanthin, lutein, α-lipoic acid and glutathione) that drastically reduced the number of rod photoreceptors displaying oxidatively damaged DNA, and delayed the degeneration process significantly in rd1 mice (Sanz *et al.,* 2007). A similar treatment (Miranda *et al.,* 2010) increased glutathione peroxidase (GPx) activity and GSH levels and decreased Cystine concentrations in rd1 retinas.

Others have also demonstrated an increased expression of genes involved in cell proliferation pathways and oxidative stress at post-natal day 14 (the peak of rod degeneration is between PN 11-14 days), at PN35 (considered as the terminal stage of rod degeneration) (Carter-Dawson *et al.,* 1978) and at PN50 (during cone degeneration) (Hackam *et al.,* 2004). Carmody *et al.* demonstrated an early and sustained increase in intracellular reactive oxygen species accompanied by a rapid depletion of intracellular glutathione in an in vitro model of photoreceptor apoptosis and that these early changes in the cellular redox state precede disruption of mitochondrial transmembrane potential, nuclear condensation, DNA nicking, and cell shrinkage, all of which are well-characterized events of apoptotic cell death (Kerr *et al.,* 1972; Carmody *et al.,* 1999).

3.4 THERAPEUTIC STRATEGIES IN RETINITIS PIGMENTOSA

Several strategies to preserve visual functions are being studied in different RP animal models. They comprise transplantation of stem cells, RPE or retina, delivery of a large number of neuroprotective cytokines, gene therapy including replacement of missing proteins or removal of harmful molecules, implantation of subretinal or epiretinal chips or cortically based electrodes (Eckmiller, 1997; Nishida *et al.,* 2000; Young *et al.,* 2000; Kurimoto *et al.,* 2001; Zrenner, 2001; Alteheld *et al.,* 2004; Lamba *et al.,* 2006; 2009; 2010; Osakada *et al.,* 2008; 2009; Hirami *et al.,* 2009; Meyer *et al.,* 2009; Zhou *et al.,* 2011).

At present, scientific research currently focuses on two alternative ways to find a cure for inherited retinal diseases such as RP: replacement and rescue. Replacement approaches include different techniques to substitute degenerating or lost photoreceptors. And on the other hand, the rescue strategy aims at preserving or prolonging the life-span of affected photoreceptor cells.

Neuroprotection and certain gene-therapies appear to be among the most promising approaches. Because apoptosis is the final pathway of cell removal, prevention of apoptosis is conceptually feasible for all types of mutations.

This is advantageous to gene therapy, where genetic screening for each patient needs to be performed before a therapy strategy can be developed. Either the inhibition of apoptosis as the downstream final event in retinal degeneration or prolongation of photoreceptor survival by neuroprotective molecules appears to be a reasonable approach. However, successful prevention of apoptosis will require, at least in part, the understanding of neuroprotective signalling and apoptotic death mechanisms in the retina.

3.4.1 Replacement

3.4.1.1 Transplantation

Numerous attempts have been made to transplant tissue into animal models of retinal degeneration. Two different types of tissue have been transplanted: RPE transplantation, which will help when this layer is affected (Li & Turner, 1988; Sheedlo

et al., 1989; Gouras & Lopez, 1989; Lavail *et al.,* 1992; Jiang & Hamasaki, 1994; Whiteley *et al.,* 1996), and retinal neuronal transplantation, which aims at replacing the degenerated neural tissue (Delyfer *et al.,* 2004).

Many research groups have attempted to transplant embryonic or adult dissociated retinal cells, layers of photoreceptors or even entire retinae in order to replace the function of the dead photoreceptors (Kurimoto *et al.,* 2001; Zhang *et al.,* 2003; Aramant & Seiler, 2004; McGill *et al.,* 2007; Lamba *et al.,* 2006; 2009; 2010; Osakada *et al.,* 2008; 2009; Zhou *et al.,* 2011). In most cases, however, even though the transplanted tissue survives, it does not conform to the architectural organization of the host retina (Hamel, 2006; Yao *et al.,* 2011; West *et al.,* 2012). This is due largely to the nature of the subretinal and epiretinal spaces, which are partially immune privileged (Jiang *et al.,* 1993; Lund *et al.,* 2003) and therefore unaffected by the regulatory functions of the immune system.

Despite the many challenges, there have been some cases demonstrating a certain degree of integration. Latest reports have revealed that post-mitotic rod precursor cells taken from the developing mouse retina at PN1 can integrate, differentiate into rod photoreceptors, form synaptic connections with the host retinal cells and improve overall visual function (MacLaren *et al.,* 2006; Lamba *et al.,* 2006; 2009; 2010; Osakada *et al.,* 2008; 2009; West *et al.,* 2009; 2012; Zhou *et al.,* 2011; Gonzalez-Cordero *et al.,* 2013).

3.4.2. Rescue

3.4.2.1 Gene therapy

Different gene-mediated therapy strategies have been developed for the treatment of inherited retinal degenerations. In mutations leading to loss of function, the principle of gene therapy is to correct the genetic defect by the introduction of a wild-type version of the mutated gene into the cells in which normal functioning of this gene is required (Delyfer *et al.,* 2004).

Lately, different gene-mediated therapy strategies have been developed for the treatment of inherited retinal degenerations, using either viral or non-viral vectors and has resulted in the slowing down of the photoreceptor degeneration process in the rd1

mouse (Bennett *et al.,* 1996; Jomary *et al.,* 1997; Kumar-Singh & Farber, 1998; Takahashi *et al.,* 1999), in the rds mouse (Ali *et al.,* 2000) and in the RCS rat (Vollrath *et al.,* 2001).

Much progress has been made over the past years, especially in some forms of inherited retinal degeneration, such as Leber's congenital amaurosis (LCA), which can be caused by a mutation in the RPE65 gene (Li *et al.,* 2009; Zheng *et al.,* 2012).

Since the genetic causes for RP are known in many cases, gene therapy would in principle be the first choice for treatment. However, due to the variety of genes and mutations, therapy would often need to be individually designed for each patient. Furthermore, RP causing mutations usually kill the cells harbouring the mutation. By the time a human subject experiences RP symptoms, the cells carrying the original mutation are lost and hence no longer available for gene therapy.

3.4.2.2. Light Protection

Clinical evidence and data from animal studies suggest that some pigmentary retinopathies are particularly susceptible to light damage (Wang *et al.,* 1997). Patients with RP are advised to wear dark glasses outdoors. The use of amber spectacles should block ultraviolet rays and visible wavelengths up to about 527 nm (Musarella & MacDonald, 2011).

Outdoors, it is ideal to use spectacles that block ultraviolet rays and light up to approximately 550nm to filter blue light (Musarella & MacDonald, 2011). However, this therapy has been substituted by others more effective.

3.4.2.3. Neurotrophic factors

One approach towards treatment is the application of neuroprotective substances, including neurotrophic factors, growth factors, cytokines or combinations of these, in order to prevent or delay photoreceptor cell loss (LaVail *et al.,* 1998; Caffé *et al.,* 2001b; Chaum, 2003; Rhee & Yang, 2003; Azadi *et al.,* 2007). The feasibility of such an approach is supported by evidence that, when the retina degenerates, there is an activation of endogenous neuroprotective pathways, inducing production of molecules known to promote cell survival (Wen *et al.,* 1995, 1998; Gao & Hollyfield, 1996; Akhtar

et al., 2004; Roth, 2004). However, the endogenous neuroprotective programs are apparently insufficient to rescue photoreceptors from cell death.

Several neuorotrophic factors have been shown to protect photoreceptors from degeneration, including ciliary neurotrophic factor (CNTF) (Musarella & MacDonald, 2011). In different animal models of retinal degeneration, CNTF was shown to delay photoreceptor degeneration (LaVail *et al.,* 2000; Liang *et al.,* 2001; Bok *et al.,* 2002; Frasson *et al.,* 2002). A thicker outer nuclear layer was observed in treated animals, reflecting preservation of the photoreceptors and anatomical rescue (Musarella & MacDonald, 2011). Electrophysiological recordings performed to evaluate retinal function demonstrated an improvement in the scotopic and photopic responses recorded from CNTF-treated eyes compared to untreated eyes (LaVail *et al.,* 2000; Bok *et al.,* 2002; Musarella & MacDonald, 2011). Glial cell line-derived neurotrophic factor (GDNF) has also been shown to have a neuroprotective effect on degenerating photoreceptors by slowing down the degeneration of rods while preserving visual function (Frasson *et al.,* 2002).

While neuroprotective factors may offer promising results in the treatment of RP in animal models, effective treatment strategies need to be developed for clinical delivery. Direct intravitreal or subretinal neurotrophic factor injections have been performed in animal models with therapeutic effect (Li *et al.,* 2010); however, an implantable device allows for long term delivery avoiding repeated injections with the risk of mechanical or infectious complications (Musarella & MacDonald, 2011).

3.4.2.4 Antioxidants

It has been reported in epidemiological studies that many antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities to greater or lesser extent (Mitscher *et al.,* 1996; Owen *et al.,* 2000; Sala *et al.,* 2002).

Several studies have shown that the use of antioxidants, in vitro and in vivo delayed the photoreceptor degeneration process significantly in different RP animal models (Komeima *et al.,* 2006; 2007; Sanz *et al.,* 2007; Miranda *et al.,* 2010; Berson *et al.,* 2012; Drack *et al.,* 2012).

Komeima *et al*. (2007) showed that antioxidants (α-tocopherol, ascorbic acid, Mn (III) tetrakis (4benzoic acid) porphyrin, and α-lipoic acid) decreased cone photoreceptor cell death in different mouse models of retinitis pigmentosa. Drack *et al*. evaluated the protective effect of tauroursodeoxycholic acid (TUDCA), the active component in bear bile, on photoreceptor degeneration in different models of retinal degeneration in mice and demonstrated TUDCA appears to be protective in retinal degenerations with later onset and slower course, like the rd10 mice (Drack *et al.,* 2012). Berson *et al*. (Berson *et al.,* 2012) demonstrate that w-3 rich diet should make it possible for many patients with typical retinitis pigmentosa to retain both visual acuity and central visual field for most of their lives.

Our group and others have also found significant protection when antioxidants are administered while rods are dying. We have used a different combination of antioxidants (zeaxanthin, lutein, α-lipoic acid and glutathione) that drastically reduced the number of rod photoreceptors displaying oxidatively damaged DNA, and delayed the degeneration process significantly in rd1 mice (Sanz *et al.,* 2007). A similar treatment (Miranda *et al.,* 2010) increased glutathione peroxidase (GPx) activity and glutathione (GSH) levels and decreased Cystine concentrations in rd1 retinas.

3.4.2.5 Neurosteroids

During the last decade, a significant amount of evidence has been accumulated with respect to the neuroprotective actions played by steroids such as dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), progesterone, testosterone, and estradiol (Schumacher *et al.,* 2000; Garcia-Segura *et al.,* 2001; Stein, 2001; Wise *et al.,* 2001; McEwen, 2002). It has been shown that steroids may prevent neuronal cell death triggered by different stimuli including excitotoxicity, and changes in steroidal environment may influence the neuronal fate in various neurological and mental disorders (Guarneri *et al.,* 2003). Progesterone and estrogen influence the outcome of ischemic and traumatic injury in female and male brain, and in different ways they promote the reduction in the consequences of the injury cascade (Garcia-Segura *et al.,* 2001; Stein, 2001; Wise *et al.,* 2001); estrogens prevent or delayed the onset of Alzheimer´s disease and cognitive deficit (Garcia-Segura *et al.,* 2001; Wise *et al.,* 2001) and local progesterone synthesis promotes

myelin formation during regenerating processes of injured peripheral nerves (Schumacher *et al.,* 2000).

Other studies demonstrate that 17β-estradiol protects against cell death after oxygen-glucose deprivation (OGD) in primary oligodendrocyte cultures and that protection by estrogen is dose-dependent (Takao *et al.,* 2004; Gerstner *et al.,* 2008). In both male and female astrocytes, physiological levels of 17β-estradiol added to the culture medium prevent cell death following OGD (Liu *et al.,* 2007; 2008). Other evidence indicates that both long-term and short-term pretreatments with 10 nM 17β-estradiol protect cerebral endothelial cells after OGD (Guo *et al.,* 2010).

In vitro, estrogen also protects neurons against insults induced by glutamate, and beta-amyloid peptide (Sribnick *et al.,* 2004; Yao *et al.,* 2007). A number of studies have suggested that estrogen may suppress microglial activation, an effect that could help mediate estrogen neuroprotection (Mor *et al.,* 1999).

Similar knowledge is now emerging in some forms of primary and secondary retinal degeneration: estrogen is thought to have a role in age- and gender-associated ocular disease and to prevent retinal ischemia reperfusion injury (Sator *et al.,* 1997; Ogueta *et al.,* 1999; Nonaka *et al.,* 2000; Wickham *et al.,* 2000; Munaut *et al.,* 2001); DHEA, DHEAS, progesterone and 17β-estradiol protect against retinal exocitotoxicity (Cascio *et al.,* 2000; 2002); administration of intraperitoneal (i.p.) injection of progesterone protected the inner nuclear and nerve fiber layers in a model of retinal ischemia in rats (Lu *et al.,* 2008) but neither i.p. administration (60 mg/kg) nor daily dosage (2.5 mg/day) with progesterone protected rat photoreceptors from cell death in models of light damage (O´Steen, 1977; Kaldi & Berta, 2004). However other studies have shown that administration of norgestrel protects mouse photoreceptor cells in both a light-induced and an inherited retinal degeneration model (Doonan *et al.,* 2011).

4. STRUCTURE AND FUNCTIONS OF PROGESTERONE

4.1 STEROIDS

According to most commonly used definition, neurosteroids cover both steroid hormones, which are synthesized *de novo* in neurons and glial cells, and their metabolites acting within the central nervous system (CNS) (Compagnone and Mellon, 2000).

Steroids are a group of structurally related compounds widely distributed in animals and plants. Examples of steroids include Vitamin D, cholesterol, the sex hormones, estradiol and testosterone, cholic acid, some carcinogenic hydrocarbons and certain sapogenins (Schroepfer, 1981).

Steroid hormones play a regulatory role in a variety of cellular processes such as reproduction, development, differentiation, apoptosis, and brain function (Tsai & O'Malley, 1994).

Evidence suggests a close relationship among steroid availability, neuronal susceptibility, and rate of occurrence of seizures, ischemic and traumatic injury, Alzheimer's disease, and also the likelihood of cognitive aging (Garcia-Segura *et al.,* 2001; Stein, 2001; Wise *et al.,* 2001; McEwen, 2002; Stein *et al.,* 2008) and finally some ophthalmic disease such as age-related macular degeneration, and retinal ischemic conditions (Ogueta *et al.,* 1999; Nonaka *et al.,* 2000; Wickham *et al.,* 2000; Munaut *et al.,* 2001).

4.2 PROGESTERONE SYNTHESIS

Neuroactive steroids or neurosteroids, are steroid hormones synthesized within the central nervous system from the precursor cholesterol (Figure 17) (Rupprecht *et al*., 1996).

One of these neurosteroids, progesterone, also known as P4 (pregn-4-ene-3, 20-dione) is a C-21 steroid hormone and the biosynthesis of this hormone is a multistep process (Garcia-Segura & Melcangi, 2006).

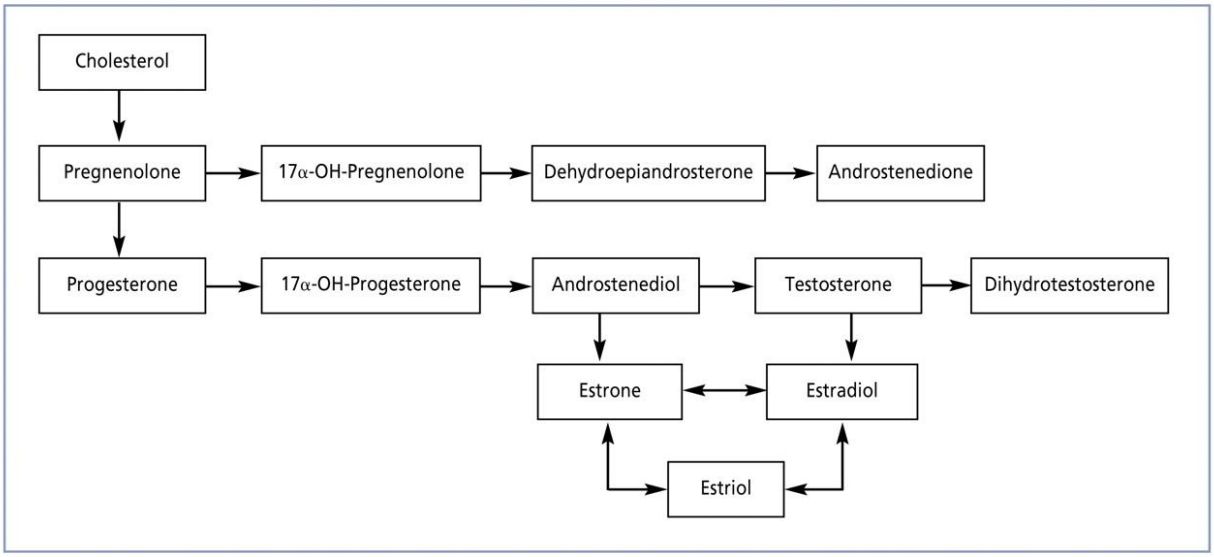

Figure 17. Scheme Progesterone Synthesis (Conaway, 2011).

In the CNS, the first step in the synthesis of all steroid hormones is the synthesis of pregnenolone (PREG) from cholesterol which is transformed into different steroid products through enzymatic reactions in a tissue-specific manner by a cytochrome P450 isozyme in myelinating glial cells (Figure 17) (Le Goascogne *et al.,* 1987; Baulieu, 1997; Feeser & Loira, 2011; Miller & Auchus, 2011; Li *et al.,* 2013). In this process, cholesterol mobilized from cytosolic lipid droplets or from lysosomes is transported to mitochondria and becomes localized to the inner mitochondrial membrane (Miller & Auchus, 2011; Miller, 2013). PREG is converted to progesterone in two reactions, both catalyzed by 3-beta-hydroxysteroid dehydrogenase/isomerase (3βHSD). Subsequent steps require several enzymes including, 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase/C17-20-lyase (17βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD). Specifically, pregnenolone is converted to 17-hydroxypregnenolone and dehydroepiandrosterone by CYP17 (17α-hydroxylase). Dehydroepiandrosterone is then converted to androstenedione by 3β-hydroxysteroid dehydrogenase. Androstenedione can have two fates, it can either be converted to estrone via CYP19 (aromatase/estrogen synthase) or it can be converted to testosterone via 17β-hydroxysteroid dehydrogenase. Alternatively, PREG may be converted to dehydroepiandrosterone or dehydroepiandrosterone sulfate (DHEA, DHEA-S) (Rupprecht *et al.,* 1996; Li *et al.,* 2013; Miller, 2013).

The retina is also capable of transforming cholesterol into pregnenolone; then in progestens, corticosteroids and sex stereoids. The retina is considered an authentic steroidogenic structure of the CNS in which steroids synthesis is integrated in its circuits for a conceivable role in the visual function (Guarneri *et al.,* 2003). Most cholesterol is synthetized in neuronal retina and transported to membranes of rod segments of photoreceptors where it is required for continuous renewal during life (Levin & Gordon, 2002). The renewal process also involves RPE where cholesterol is additionally provided from an extracellular source through LDL receptors and the apolipoprotein E (ApoE) synthetized locally (Ong *et al.,* 2001). ApoE is also formed in retinal Müller glial cells and/or internalized by ganglion cells from which it can be rapidly transported into the optic nerve and brain (Ong *et al.,* 2001). Recently high cholesterol in RPE, changes in ApoE expression by Müller glia of the human retina, and the apolipoprotein polymorphism have been suggested to increase the risk of AMD, the impairment of visual function during aging, and the progression of glaucoma (Anderson *et al.,* 2001; Ong *et al.,* 2001; Sen *et al.,* 2002).

Cholesterol is also used from retinal steroidogenesis in the adult rat. The transformation of cholesterol into pregnolone appears to occur at ganglion cells and in some cells of the inner nuclear layer, the amacrine cells, where the cytochrome p450 cholesterol side-chain cleavage (p450scc) is located (Guarneri *et al.,* 1994). In brain peripheral nerves, the cytochrome p450scc is mainly expressed in glial cells (Baulieu, 1997), the only exception is that of cerebellar Purkinje cells and hippocampal neurons which, like the retina, provide a neuronal-type pregnenolone synthesis (Compagnone & Mellon, 2000). This raises the possibility that diverse regulatory mechanisms could exist to control the enzyme expression and activity at different locations (Guarneri *et al.,* 2003).

4.3 METABOLISM OF PROGESTERONE

Progesterone is readily metabolized in the brain to 5α- dihydroprogesterone (DHP) by 5α-reductase, and then further reduced to 3α, 5α- tetrahyroprogesterone (ALLO) by the enzyme 3α-hydroxysteroid reductase (Celotti *et al.,* 1992; Mellon & Vaudry, 2001; Stoffel-Wagner, 2001; Belelli & Lambert, 2005).

4.4 FUNCTIONS OF PROGESTERONE IN THE CENTRAL NERVIOUS SYSTEM (CNS)

Baulieu *et al*. coined the term neurosteroid as a result of their discovery that steroids could be synthesized in the central nervous system (CNS) and affect neurotransmission by interaction with receptors (Baulieu *et al.,* 1996). Several biologic effects of neurosteroids have been observed, such as electrical stimulation of neurons (Arnold & Gorski, 1984), modulation of the function of y-aminobutyric acid (GABA)A receptor, (Mienville & Vicini, 1989) and growth and differentiation of glial cells in vitro (Bauliei, 1991).

Progesterone (P4) has multiple biological functions in the CNS, among which is its ability to afford neuroprotection; it is well documented to promote cell survival and proliferation and to have palliative and disease regression effects in non-neuronal tissue (Rider & Psychoyos, 1994; Makrigiannakis *et al.,* 2000; Svensson *et al.,* 2000; Olive & Pritts, 2001; Lange, 2008). Furthermore, following two decades of compelling preclinical research across several different injury and disease models, progesterone is now recognized as a powerful neurosteroid (Baulieu *et al.,* 2001). It has the ability to attenuate neuronal apoptosis associated with CNS damage such as Traumatic Brain Injury (TBI) (Shahrokhi *et al.,* 2010) and spinal cord injury (Gonzalez *et al.,* 2009) as well as to ameliorate the effects of stroke (Betz & Coester, 1990) and neurodegenerative conditions such as Alzheimer's disease (Frye & Walf, 2009).

Progesterone appears to act on multiple levels and pathways to interrupt or slow destructive processes such as up-regulating the inhibitory neurotransmitter GABA, decreasing lipid peroxidation and oxidative stress, limiting the release of inflammatory cytokines, and decreasing cellular apoptosis (Stein *et al.,* 2008).

Up-regulation of GABA:

Several research teams have reported that progesterone upregulates GABA, an inhibitory neurotransmitter in the central nervous system (Lambert *et al.,* 2003; Pierson *et al.,* 2005; Mani, 2006). GABA-mediated inhibition can decrease excessive injury-induced excitotoxicity caused by the release of glutamate or other excitatory neurotransmitters (Brann *et al.,* 2005).

Antioxidant Effects:

Progesterone does not have the characteristic chemical structure of an antioxidant, but high levels of the hormone appear to reduce free radical damage (Roof *et al.,* 1997; Pajovic *et al.,* 1999). Progesterone administration reduces lipid peroxidation in different types of in vitro free-radical-generating systems in a dose-dependent manner (Shimamura *et al.,* 1995; Sugino *et al.,* 1996). All these studies suggest that progesterone reduces lipid peroxidation and oxidative stress, most likely by decreasing the generation of free radicals and enhancing endogenous free radical scavenging systems (Stein *et al.,* 2008).

Reduction of Inflammatory Cytokines:

An anti-inflammatory role for progesterone has been reported. In particular, progesterone has been observed to suppress the expression of various pro-inflammatory cytokines following cerebral ischemia, minimizing the ischemia-induced increase in IL-1β (Gibson *et al.,* 2005), TNF-α (Aggarwal *et al.,* 2008), and TGF-β (Gibson *et al.,* 2005). Additionally, progesterone can decrease the expression of inducible nitric oxide synthase, iNOS or NOS, in vivo (Coughlan *et al.,* 2005; Gibson et al., 2005) and in vitro (Coughlan et al., 2005; Muller & Kerschbaum, 2006).

Progesterone and its active metabolites are also potent antagonists of inflammation in CNS after traumatic brain injury (Stein *et al.,* 2008). Two key mechanisms (McIntosh *et al.,* 1996) appear to play a role in a traumatic brain injury and stroke: antagonism of cytokine release and inhibition of immune cell activation and migration. Contusion injuries produce a marked inflammatory reaction, with gliosis seen in several brain areas, both proximal and distal to the injury. Progesterone acts at the earliest point in the cytokine cascade by modulating gene response elements (Grossman *et al.,* 2004) and preventing or slowing inflammatory reactions induced by cytokines (interleukin-1, interleukin-6, tumor necrosis factor, and others) (Chao *et al.,* 1994; Arvin *et al.,* 1996; Ehring*et al.,* 1998; Grossman *et al.,* 2004; Cutler *et al.,* 2006; Stein *et al.,* 2008).

Decreased Apoptosis

It is also known that progesterone appears to reduce neuronal apoptosis. Nuclear factor kB (NFkB) has been implicated in the initiation of neuron inflammation and

apoptosis after traumatic brain injury (Stein *et al.,* 2008). Progesterone reduces both the nuclear concentration of NFkB and expression of NFkB target genes. It has been shown that after progesterone treatment, mitochondrial RNA and protein for NFkB-regulated inflammatory factors such as interleukin-1β, C3, inducible nitric oxide synthase, and cyclooxygenase-2 are significantly reduced (He *et al.,* 2004; Djebaili *et al.,* 2004; 2005; Pettus *et al.,* 2005; Cutler *et al.,* 2006; Stein *et al.,* 2008).

4.5 PROGESTERONE IN THE RETINA

As with the brain, the retina is a target of steroids and a site of steroid production (Guarneri *et al.,* 1994).

The endocrine influence on visual processing was originally proposed in the late 1970s based on the evidence that susceptibility of retinal cells to light damage occurred concomitantly with sexual maturation in both male and female animals (O´Steen & Anderson, 1974). However, recent advances in research have proved the existence of hormone steroid receptors, steroid enzymes for the formation of ex novo steroids or neurosteroids, and their involvement in the physiology and pathology of visual function (Guarneri *et al.,* 2003; Cascio *et al.,* 2007).

In terms of retinal disease, evidence confirms that neurosteroidogenesis occurs in the retina, particularly de novo production of pregnenolone, the precursor for synthesis of progesterone and all other steroids (Guarneri *et al.,* 1994). Nuclear progesterone receptor mRNAs have been identified in the retina and choroid (Wickham *et al*., 2000) while membrane-associated progesterone receptor component 1 has been identified in photoreceptor and Müller glial cells of the retina (Swiatek-De Lange *et al.,* 2007).

Studies have shown that progesterone does indeed protect against apoptosis by binding to either or both of these receptor types (Friberg *et al.,* 2009; Peluso *et al.,* 2009).

There is a controversy about the beneficial effects of progesterone in retinal related disease. Several retinal studies have failed to show beneficial effects of progesterone, in a model of retinal ganglion cell injury (Nakazawa *et al.,* 2006) and in different studies

that tried to demonstrate its protective effects against light stress (O'Steen, 1977; Kaldi & Berta, 2004). In contrast Lu and colleagues found a beneficial effect of progesterone in a high intraocular pressure model (Lu *et al.,* 2008).

With regard to models of inherited retinal degeneration, literature strongly supports an antioxidant-based therapeutic approach in rd models (Komeima *et al.,* 2007; Usui *et al.,* 2009; Miranda *et al.,* 2010) and in several instances neurotrophic factors attenuate photoreceptor degeneration (Faktorovich *et al.,* 1990; Cayouette *et al.,* 1998; Bok *et al.,* 2002), however little is known about the effects of steroid sex hormones on disease progression.

On the other hand, Calcium (Ca^{2+}) is an essential second messenger that mediates multiple cellular responses in neurons to various stimuli including cell proliferation, neurotransmission, and cell death (Berridge *et al.,* 2000; 2003). Tight control of spatial and temporal Ca^{2+} signaling enables neurons to perform these different tasks appropriately. Progesterone is involved in multiple processes in neurons including neuroprotection (Roof *et al.,* 1994; Asbury *et al.,* 1998; Nilsen & Brinton, 2002; 2003; Kaur *et al.,* 2007) and there is increasing evidence from non-neuronal cell models suggesting that P4 is implicated in intracellular Ca^{2+} signaling (Wasserman *et al.,* 1980; Kirkman-Brown *et al.,* 2000). Furthermore, recent results in mouse models of retinal degeneration show that apoptosis and cell loss is delayed and endogenous survival pathways are activated in response to norgestrel (Doonan *et al.,* 2011).

5. ANIMAL MODELS

Rodents are commonly used as animal models to mimic the most prevalent neurodegenerative diseases of the retina. Since they are mainly nocturnal animals, most of the photoreceptor cells in rodent retina are rods. Cones are present, but in a low concentration (3-5%). The murine retina contains only two types of cones (S and M) which are distributed evenly throughout the ONL (in comparison to the human retina where density varies, e.g. in the cone-rich fovea). A large number of rodent retinal degeneration models are available for studies on inherited retinal degenerations (Chang *et al.,* 2002; Dalke & Graw, 2005). These include: rhodopsin mutants (Tam & Moritz, 2007; Trumpler *et al.,* 2008), light-induced damage models

(Reme *et al.,* 1998; Hao *et al.,* 2002; Wenzel *et al.* ,2005), and Royal College of Surgeons (RCS) rats, with a mutation in the Mertk gene (D'Cruz *et al.,* 2000). However the animal model best characterized to date, is the rd1 mouse.

5.1. rd1 MOUSE

The rd1 mouse is also known as retinal degeneration mouse or simply "rd", and was first discovered by Keeler in the mid-twenties (Keeler, 1924).

The rd1 mouse model is characterized by a loss-of-function mutation in the gene encoding for the β-subunit of rod photoreceptor cGMP phosphodiesterase 6 (PDE6) (Bowes et al., 1990; Pittler& Baehr, 1991) which leads to an accumulation of cGMP in rod photoreceptors (Farber & Lolley, 1974). The CNG channels then remain open, allowing an overload of Ca^{2+} into the cytoplasm. The uncontrolled Ca^{2+} influx is believed to be the starting point of an elaborate pathway that involves numerous signalling mechanisms, culminating with widespread cell death (Chang *et al.,* 1993; Fox *et al.,* 1999; Sancho-Pelluz *et al.,* 2008).

The degenerative process in this model starts very early, even before the retina becomes mature, around post-natal (PN) day 10 (Figure 18) (Portera-Cailliau *et al.,* 1994; Otani *et al.,* 2004). The peak of rod photoreceptor cell death occurs between PN 11-14 (Punzo & Cepko, 2007; Paquet-Durand *et al.,* 2007). By PN30 the mutation-dependent degeneration is almost completed, with one or two rows of photoreceptors remaining, mainly cones, which will eventually die within the 6 next months (Carter-Dawson *et al.,* 1978; Farber *et al.,* 1994; LaVail *et al.,* 1997; Pierce, 2001).

Mutations in the PDE6-β and PDE6-α gene are present in about 10% of RP human patients (Bayes *et al.,* 1995; Dryja *et al.,* 1999), and, for this reason the rd1/rd1 mouse serves as an adequate model for human inherited retinal degenerative (McLaughlin *et al.,* 1995).

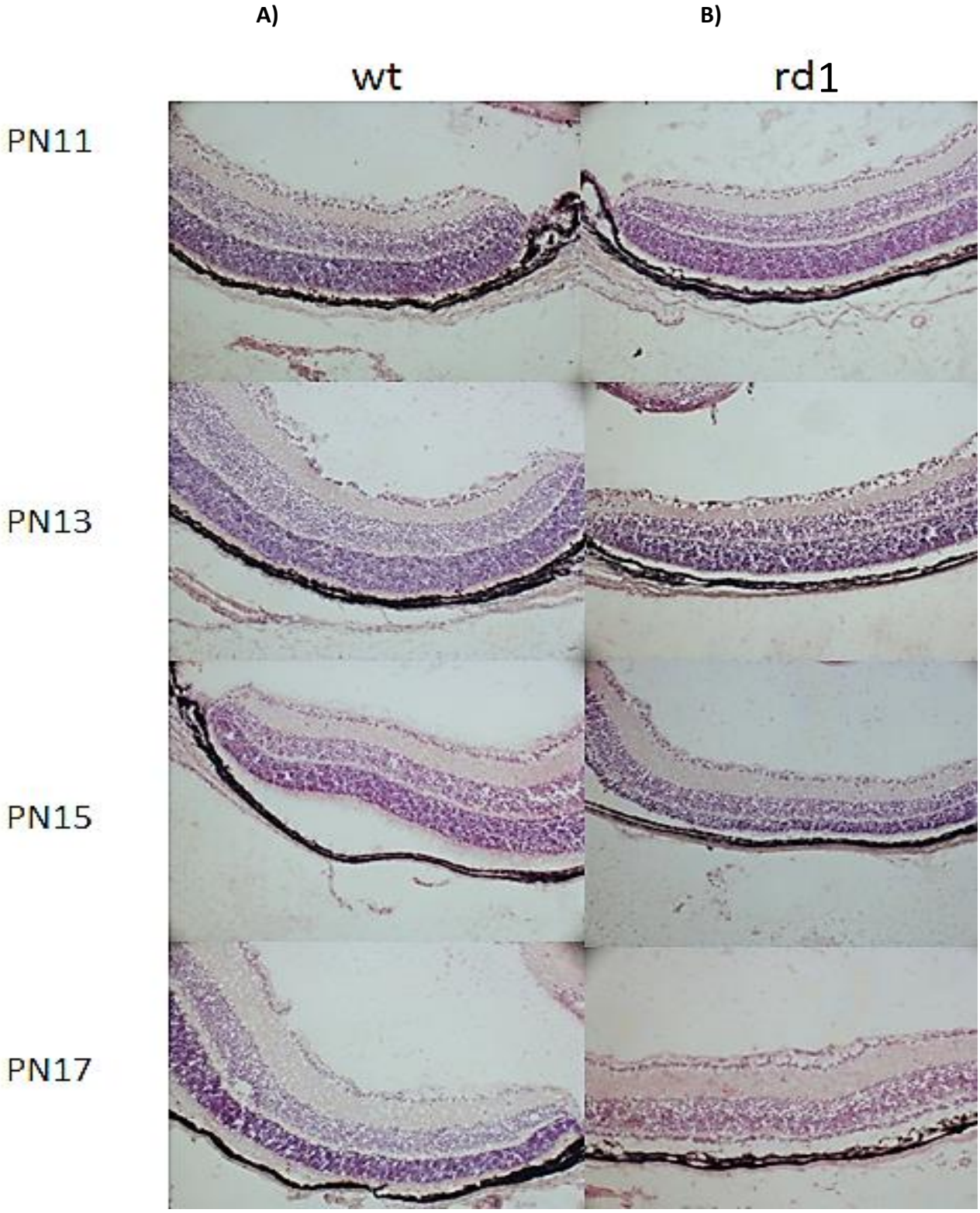

Figure 18. ONL reduction in rd1 retina compared to wild type (wt or control)**.** When compared, wild type (left picture) and rd1 (right picture) retinae at PN11 seemed to be equally developed and the ONL had the same thickness. Four days later (PN15), the wild type retina remained the same as before, whereas the rd1 ONL had been radically reduced. At PN17, wild type ONL did not change, but in the rd1 there were only one to two rows of photoreceptors left. (Scale bar = 50 μm).

-HYPOTHESIS

Hereditary retinal degenerations (Retinitis Pigmentosa and related diseases) are characterized by a progressive loss of photoreceptors and result in severe visual impairment and blindness.

As inherited degenerative diseases of the retina are due to genetic defects, gene therapy appears as the most rational approach for curative treatment. However, gene therapy will only work for patient groups with known mutations, hence excluding many from this kind of treatment. Furthermore, a large proportion of patients with RP seek clinical help when photoreceptor degeneration has progressed and night blindness is already prominent. At this stage the rod photoreceptors have largely died, which implicates that many of the cells that harbour the original genetic defect are no longer available for gene therapy.

In the retina of rd1 mice, a model of retinitis pigmentosa, it has been shown changes in glutamate and glutathione concentrations; with this in mind, we propose that regulation of GSH and glutamate concentrations (with oral progesterone treatment), as a mutation-independent therapeutic regime, is not only a means of extending the window of opportunity for other therapies or cures, but a feasible approach on its own towards maintaining survival- and function-upholding of the neuroretina.

-OBJECTIVES

1. To characterize the time course of changes in glutamate and thiol metabolism GSH, GSSG and Cys in the retina of rd1 and wt mouse.

2. To determine the possible therapeutic effect of oral administration of progesterone *in vivo* in an experimental model of RP: the rd1 mouse.

2.1 To characterize if oral progesterone treatment is able to restore the alterations in glutamate concentration and thiol metabolism observed in the retina of rd1 mice.

2.2 To determinate if oral progesterone treatment is able to protect photoreceptors in the retina of rd1 mice from death.

2.3 To study if oral progesterone treatment is able to revert the characteristic gliosis observed in the retina of rd1 mice.

2.4 To determine if oral progesterone treatment is able to improve retinal functionality in rd1 mice.

-MATERIALS AND METHODS

1. CHEMICAL PRODUCTS AND SOLUTIONS

-Alexa 488 goat anti-mouse (Invitrogen, Oregon, USA).

-Alexa 488 goat anti-rabbit (Invitrogen, Oregon, USA).

-Cysteine (Sigma Aldrich, Madrid, Spain).

-Diamount (mounting xilene free) (Diapath, Martinengo, Italy).

-2,4-Dinitrofluoro-bencene (DNFB) (Sigma Aldrich, Madrid, Spain).

-Eosine (Química Clínica Aplicada, Tarragona, Spain).

-Ethanol 96% (J.T. Baker, Deventer, Holland).

-Ethanol Absolute (J.T. Baker, Deventer, Holland).

-Folin-Ciocalteu´s phenol (Sigma Aldrich, Madrid, Spain).

-Glacial acetic acid (J.T. Baker, Deventer, Holland).

-Glial fibrilary Acidic protein (Dako Cytomation, Denmark).

-Glutamic acid (Sigma Aldrich, Madrid, Spain).

-Reduced Glutathione (Sigma Aldrich, Madrid, Spain).

-Oxidized Glutathione (Sigma Aldrich, Madrid, Spain).

-Hematoxiline (Química Clínica Aplicada, Tarragona, Spain).

-Iodoacetic acid (Sigma Aldrich, Madrid, Spain).

-m-cresol (Acros, New Jersey, USA).

-Methanol (MeOH) (J.T. Baker, Deventer, Holland).

-Monoclonal Anti-Glutamic Acid Decarboxylase 65 (Sigma Aldrich, Madrid, Spain).

-Normal Goat Serum (ATOM, Vector laboratories, Burlingame, USA).

-Perchloric Acid (Panreac, Barcelona, Spain).

-Paraformaldehide (J.T. Baker, Deventer, Holland).

-Phosphate buffer (Sigma Aldrich, Madrid, Spain).

-Phosphate buffered saline (Sigma Aldrich, Madrid, Spain).

-Potassium bicarbonate (Sigma Aldrich, Madrid, Spain).

-Potassium hydroxide (Sigma Aldrich, Madrid, Spain).

-Potassium sodium tartrate tetrahydrate (Sigma Aldrich, Madrid, Spain).

-Protein Standar bovine serum albumin (Sigma Aldrich, Madrid, Spain).

-Rabbit polyclonal antibody to Glutathione (Abcam, Cambridge, UK).

-Sacharose (Panreac, Barcelona, Spain).

-Sodium acetate trihydrate (Sigma Aldrich, Madrid, Spain).

-Sodium bicarbonate (Sigma Aldrich, Madrid, Spain).

-Sodium hydroxide (Sigma Aldrich, Madrid, Spain).

-Triton X-100 (Sigma Aldrich, Madrid, Spain).

-Tris (tris (hydroxymethyl aminomethane) (Sigma Aldrich, Madrid, Spain).

-Tween 20 (Sigma Aldrich, Madrid, Spain).

-In situ Cell Death Detection Kit (Roche, Mannheim, Germany.)

-Vectashield with Dapi (ATOM, Vector laboratories, Burlingame, USA).

-Water (J.T. Baker, Deventer, Holland).

-Xilol (Scharlab, Barcelona, Spain).

2. RESEARCH DESIGN AND METHODS

2.1 ANIMALS

Rd1 and C3H (wild type, wt) mice have been used in this work, donation from Dr. Theo Van Veen, Lund University (Sweden).

2.2 ANIMAL CARE

Mice housing was held in cages under controlled conditions of temperature (20° C) and humidity (60%) under standard white cycling lighting (8:00 to 20:00 and 20:00 to 8:00). During housing, the animals had free access to water and a standard diet by Harlan Ibérica SL (Barcelona, Spain).

Animal care and protocols were in accordance and approved by the Animal Ethics committee of the institution and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research as well as Spanish law regulating animal experiments.

All efforts were made to reduce discomfort and the number of animals used.

At post-natal day (PN) 11, previous observations have detected dramatic and characteristic changes when comparing wt and rd1 retinae (Hauck *et al*., 2006; Paquet-Durand *et al.*, 2006). Therefore, most comparisons were conducted at this age. Day of birth was considered as PN0. Pups were sacrificed by decapitation prior to age PN9 or decapitated after asphyxiation in a CO_2 chamber for mice older than PN9.

2.3 COLLECTION OF SAMPLES

The processing of the samples varies according to the kind of the determinations to which they are to be used:

2.3.1 Characterization of Glutamate and thiol metabolism in rd1 mice retina

For the analysis of biochemical parameters, following decapitation, the eyes were quickly enucleated and the retina was dissected and homogenized at 13.000 rpm for 1 minute with an ULTRA TURRAX T25 (Labortech, IKA, Staufen, Germany) in prechilled 0.2 M phosphate buffer (PB), pH 7.0. At this point, this homogenate was centrifuged at

6000 rpm during 2 minutes at 4° C. Immediately after, 180 µL of homogenate were acidified with 20 µl of 20% of perclhoric acid (PCA) and supernatant was collected and store at −20° C until used.

2.3.2 Evaluation of progesterone effect in rd1 mice retina

Rd1 and wild type mice were used to evaluate the possible progesterone protective action. Mice were divided in 3 groups:

- Group 1, rd1 mice (n = 6) received an oral administration of 100 mg/kg body/weight of progesterone on alternate days starting at PN7. Animals were sacrificed at different post-natal days (PN11, PN13, PN15 and PN17). Day of birth was considered as PN0.
- Group 2, rd1 mice (n = 6) were treated with vehicle (olive oil) on alternate days starting at PN7. Animals were sacrificed at different post-natal days (PN11, PN13, PN15 and PN17). Day of birth was considered as PN0.
- Group 3, wt mice (n = 6) were treated with vehicle (olive oil) on alternate days starting at PN7. Animals were sacrificed at different post-natal days (PN11, PN13, PN15 and PN17). Day of birth was considered as PN0.

Administration of progesterone in wild type mice group has not showed differences compared to wt treated with olive oil (group 3).

For biochemical determinations, retinas were also quickly enucleated and the retina was dissected and homogenized at 13.000 rpm for 1 minute by ULTRA TURRAX T25 (Labortech, IKA, Staufen, Germany) in prechilled 0.2 M phosphate buffer, pH 7.0. The homogenate was centrifuged at 6000 rpm during 2 minutes at 4° C. 180 µl of this homogenate were acidified with 20 µl of 20% of PCA and supernatant was collected and store at −20° C as above.

For immunohistochemistry analysis, the retinas were fixed, cryoprotected, and cryosectioned. The retinas were fixed for 2 hours by immersion in 4% paraformaldehyde (PFA), washed 3 times in phosphate buffered saline (PBS) 0.1 M, pH

7.4, and cryoprotected using PBS containing increasing concentrations of sucrose (10, 20 and 30%).

10 µm cross sections of the retinas were then cut on a cryostat (Leica CM 1850 UV, Ag protect, Barcelona, Spain) collected on slides and stored at −4° C until used.

2.4 EXPERIMENTAL DESIGN

2.4.1 Time course Glutamate and thiol metabolism in rd1 mice

Retinas from rd1 and wild type mice (wt) at different post natal days (PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28) have been used to determinate Glutamate, GSH, GSSG Cys and Cyss, concentrations by high-performance liquid chromatography (HPLC) (Gilson, detector UV, ViS 156 Middleton, USA).

2.4.1.1. Biochemical determinations:

-Determination of proteins by Lowry's method:

This type of analysis is performed according to the procedure described by Lowry *et al.* (Lowry *et al.,* 1951) with the modifications routinely used in our laboratory (Peterson, 1977).

- *Solutions:*

Solution 1: Na_2CO_3 4% + NaOH 0.8% (in 1 l distilled water).

Solution 2: ●Solution 2a: $CuSO_4$ 1.25 mM (in 1 l of distilled water).

●Solution 2b: $C_4H_4KNaO_6$ 1.42 mM (potassium sodium tartrate tetrahydrate) (dissolved in 1 l of water).

Solution 3: Folin-Reagent 1 N 1.5 ml + 3 ml distilled water (Sigma Aldrich, Madrid, Spain).

Solution 4: Standar solution of 1 mg/ml of bovine serum albumin (BSA) (Sigma Aldrich, Madrid, Spain).

(SOLUTION 1+2 = solution1: solution 2a: solution 2b (2:1:1)).

- *Procedure:*

Test tubes are prepared with linear growing amounts of solution 4 (0, 10, 25, 50, 75, 100 µl) to obtain a calibration curve. Each test tube is filled with 1 ml solution 1+2 and distilled water. Then BSA is added to each tube according to the linear growing value of concentration indicated before. Finally each tube is vortexed. It is necessary to wait 10 minutes before continuing.

At the same time test tubes are also prepared with homogenate samples, they contain 1 ml of solution 1+2, 175 µl of distilled water and 25 µl of homogenate. The resulting solutions are vortexed. Both, test tubes for calibration curve and for homogenate samples, are prepared in duplicate.

Finally 100 ml of solution 3 (Folin reagent) is added to each tube. Tubes are vortexed. 25-30 minutes are necessary to allow complete reaction. After this time, the absorbance of each test tube is measured in a spectrophotometer at a wavelength of 696 nm.

The absorbance at 696 nm is directly proportional to the concentration of protein.

The absorbance values of the standard solution tubes are used to obtain a calibration curve:

Y = Absorbance $Y = ax + b$;

It is possible to extrapolate the concentration of proteins in the homogenate sample from the calibration curve values:

$X = ((Y-b)/ a) / Vm$ Vm = volume homogenate

-Determination of Glutamate, Glutathione, Glutathione disulfide and Cysteine concentrations

Concentrations of Glutamate, Glutathione (GSH), Glutathione disulfide (GSSG) and Cysteine (Cys) in the retina are determined by the procedure described by Reed (Reed *et al.*, 1980). The method is based on the iodoacetic acid reaction with thiol groups to form carboxymethyl derivatives followed by chromophore derivatization of amino groups with Sanger's reagent (2,4-dinitrofluoro-bencene). These N-DNP derivatives are

readily separated by HPLC, which allows the quantification of nanomolar levels of Glutamate, GSH, GSSH and Cys.

- *Equipment:*

We worked with a Gilson HPLC consisting of the following components:

-Pumps for Chromatography: Gilson 322 Pumps.

-Detector: Gilson 156UV/VIS.

-UV and visible lamp.

-Gilson 864 Degasser.

-Gilson 234 Auto-injector loop of 100 Pl.

-Gilson serial Input/ Output Chanel (GSIOC).

-Computer 486/50 Hz

-Software: Unipoint ^{TM} System.

For the chromatographic separation we used a Kromasil Amino 5 µm, 250 x 4.6 mm column (Análisis Vínicos, Barcelona, Spain).

The initial conditions of the flow are 1.0 ml/min, 80% mobile phase A and 20% mobile phase B.

These initial conditions are maintained for 10 minutes, and then are followed by a linear gradient to 95% mobile phase B for 40 minutes, finally rebalanced with the initial conditions for 10 minutes before starting with the next sample analysis. The wavelength used is 365 nm.

The range of the UV detector use is 0,005 and the response time is 5 seconds. The injection of each sample is performed automatically with an auto-injector for up to 45 samples.

The mobile phase A is prepared with 80% methanol (800 ml HPLC grade water + 3.2 l of methanol) and mobile phase B is prepared with 5 M sodium acetate 80% methanol (800 ml of sodium acetate + 3.2 l methanol). Subsequently both phases are filtered through a filter membrane (Schleicher und Schuell, Barcelona, Spain) 0.45 micron pore and 47 mm in diameter.

- *Solutions:*

-Calibration stock solution: It is prepared daily and stored in a refrigerator at 4° C. These stocks are concentrations of 10 mM of Glutamate, GSH, GSSG and Cys in ultra-pure water.

-Solution 1**:** It is prepared with iodoacetic acid, IAA (100 mM) and 0.2 mM m-cresol.

-Solution 2: It is prepared with KOH 10 M and $KHCO_3$ 3 M.

-Solution 3: It is prepared with DNFB (1-fluoro-2, 4-dinitrobenzene) 1.5% v/v in pure ethanol.

This solution is made fresh daily and stored at 4° C protected from light.

- *Procedure:*

1. 90 µl of the sample and 10 µl of Solution 1 are pipetted in 2 ml Eppendorf tubes.
2. Each solution is adjusted to a pH of 8.5 to 9 by adding 30 µl of Solution 2.
3. Tubes are incubated for 30 min in darkness, at room temperature.
4. 100 µl of Solution 3 is added. N-DNP derivatives are then formed, but require a minimum of four hours in darkness at 4° C for their formation.

After this time tubes are centrifuged at 13.000 rpm for 10 min and the supernatant is used for analysis in HPLC equipment. The minimum sample of amount injected is 115 µl.

In each analysis a white and a standard calibration curve (0, 20, 40, 50, 60, 80 and 100 mM) is prepared.

The peak area obtained is directly proportional to the concentrations of each thiol derivates in the sample. Concentrations are calculated from the standard curve.

-Optical Coherence Tomography:

Optical coherence tomography (OCT) is a fundamentally new type of optical imaging modality. It performs high-resolution, cross-sectional tomographic imaging of the internal microstructure in materials and biological systems by measuring backscattered or backreflected light. OCT images are two-dimensional data sets which

represent the optical backscattering in a cross-sectional plane through the tissue. Image resolutions of 1 to 15 µm can be achieved one to two orders of magnitude higher than conventional ultrasound. Imaging can be performed *in situ* and in real time.

Spectral Domain OCT:

Spectral domain OCT, also known as fourier-domain OCT, is a significant improvement on time domain OCT. Spectral domain OCT eliminates the moving reference mirror found in time domain, enabling scan rates up to 100 times faster. With spectral domain OCT, all of the wavelengths of returning light are analyzed simultaneously, resulting in faster collection of more data. Faster scan rates are desirable to help limit motion artifact and to obtain more information in order to locate the position of the scan in the eye.

This method was used according to manufacturer instructions (Heidelberg Engineering) that selected a long wavelength superluminescent diode (SLD) with a peak wavelength of 870 nm to enable the best penetration of infrared light through cloudy media.

Animals were anesthetized with intraperitoneal injection of ketamine and azepromazine (100 mg/kg body weight and 2.5 mg/kg body weight, respectively). Pupils were dilated with 1% tropicamide (Alcon Labs, Barcelona, Spain) followed by the administration of topical artificial tear gel (Hyabak, Thea Labs, Barcelona, Spain). Mice were then placed on a pad heated to 37-39° C.

Mouse eyes were subjected to SD-OCT with a commercially available Spectralis™ HRA+OCT device from Heidelberg Engineering featuring a broadband superluminescent diode at λ= 880 nm as a low coherent light source. Each two-dimensional B-scan recorded at 30° field of view consists of 1536 A-Scans, which are acquired at a speed of 40.000 scans per second. Optical depth resolution is ca. 7 µm, with digital resolution reaching 3.5 µm (Wolf-Schnurrbusch *et al.*, 2008).

We mounted a commercially available 78-D double aspheric fundus lens (Volk Optical, Inc., Mentor, OH) directly in front of the camera unit to adapt the equipment to the special characteristics of the mouse eye. Imaging was performed using the

proprietary software package Eye Explorer, version 3.2.1.0 from Heidelberg Engineering. Length of the reference pathway was adjusted manually according to manufacturer's instructions using the "OCT debug window" to adjust for the optical length of the scanning pathway. The combination of scanning laser retinal imaging and SD-OCT allows for real-time tracking of eye movements and real-time averaging of OCT scans, reducing speckle noise in the OCT images considerably (Wolf-Schnurrbusch *et al*., 2008).

To quantify central retinal thickness based on high-resolution volume scans, we used the proprietary software Eye Explorer. Briefly, each volume scan consisted of at least 70 B-scans recorded at 30° field of view centered on the optic disc, which were used to calculate an interpolate retinal thickness map across the scanned retinal area. Central retinal thickness was quantified using the circular OCT grid subfield at 3 mm diameter with the center located on the optic disc.

2.4.1.2 Hematoxylin and Eosin Staining:

-Hematoxylin and Eosin Staining:

Hematoxylin and eosin (H-E) is a routine staining procedure of tissue sections. This staining method uses two separate dyes, one staining the nucleus and the other staining the cytoplasm and connective tissue. Hematoxylin is a dark purplish dye that stains the chromatin (nuclear material) within the nucleus. Eosin is an orangish-pink to red dye that stains the cytoplasmic material including connective tissue and collagen.

- *Solutions:*

-Hematoxylin (Química Clínica Aplicada, Tarragona, Spain).

-Eosine (Química Clínica Aplicada, Tarragona, Spain).

-Ethanol (Et-OH) 96% and Et-OH 100% (J.T. Baker, Deventer, Holland).

-Xilol (Scharlab, Barcelona, Spain).

- *Procedure:*

1. Stain retinal sections in hematoxylin (Química Clínica Aplicada, Tarragona, Spain) for 3 minutes.

2. Rinse in running tap water for 5 minutes.

3. Differentiate with eosine (Química Clínica Aplicada, Tarragona, Spain) for 1-2 minutes.

4. Rinse in running tap water.

5. Wash retinal sections in 70% ethanol (Et-OH) (diluted from Et-OH 96% (J.T. Baker, Deventer, Holland)) for 1 minute.

6. Wash retinal sections in 96% Et-OH (J.T. Baker, Deventer, Holland) for 2 minutes.

7. Wash retinal sections in 100% Et-OH (J.T. Baker, Deventer, Holland) for 1 minute.

8. Wash retinal sections in 100% Et-OH (J.T. Baker, Deventer, Holland) for 1 minute.

9. Wash retinal sections in Xilol (Scharlab, Barcelona, Spain) for 5 minutes.

10. Wash retinal sections in Xilol (Scharlab, Barcelona, Spain) for 5 minutes.

11. Dehydrate, clear and mount with Diamount (DPX) (mounting xilene free) (Diapath, Martinengo, Italy).

- *Microscopy and cell counting*

To analyze the thickness of the retina, images were captured using Nikon DS-Fi1 camera attached to Leica DM2000 microscope, Leica application Suite version 2.7.0 R1 software (Leica Microsystem); the study of photoreceptor cell death requires observation in the same exact region of the retina over time; for that reason we have divided the retina in three sections: far periphery, middle periphery and central area. Adobe Photoshop CS5 was used for primary image processing.

The number of rows in the outer nuclear layer (ONL) was counted manually in the three areas described above. All data given represent the means and standard error of the means from three retinal sections each, for at least 6 different animals.

c) Immunohistochemical detections:

-TUNEL assay:

TUNEL method (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) identifies apoptotic cells in situ. It shows the free 3'-OH that have been exposed during the process of fragmentation of double-stranded DNA by activation of endogenous endonuclease, during the process of apoptosis. The enzyme terminal transferase (TdT) incorporates label nucleotides (biotindUTP) to these ends. The site that is marked with biotin is revealed with fluorescein conjugated avidin (avidin-FITC).

Although initially thought to detect only apoptosis (Gavrieli *et al*., 1992), the TUNEL assay was later on shown to label cells undergoing diverse types of cell death, including necrosis (Grasl-Kraupp *et al*., 1995).

To detect TUNEL positive cells in retinal sections, the In situ Cell Detection Kit (Roche, Mannheim, Germany) was used according to manufacturer instructions.

- *Solutions:*

  - PBS buffer pH 7.1.

  - Normal Goat Serum.

  - BSA, Protein Standard (Sigma, Madrid, Spain).

  - In situ Cell Detection Kit (Roche, Mannheim, Germany).

  - Vectashield with DAPI (Vector).

- *Procedure:*

Retinal sections were dried for 15-30 minutes at room temperature (RT) and washed 3 times during 5 minutes in PBS. Subsequently retinas were blocked with 20% Normal Goat Serum, diluted in PBS + BSA 1% pH 7.1 for 45 min at 37° C.

Then, sections were washed 3 times in PBS at RT for 5 min and incubated with TUNEL solution. TUNEL solution is obtained by dilution of the contents of vial 1 (blue): enzyme solution (terminal deoxynucleotidyl transferase from calf thymus, recombinant in E.coli, in storage buffer) and vial 2 (violet): label solution (nucleotide

mixture in reaction buffer) with PBS and subsequent mixing. The incubation is done at 37° C in dark for 1 hour. Then the last washing step was done and slides were mounted with Vectashield® with DAPI (Vector Laboratories), and observed under the microscope.

- *Microscopy and cell counting*

Light and fluorescence microscopy was performed on a Nikon DS-Fi1 camera attached to Leica DM2000 microscope, Leica application Suite version 2.7.0 R1 software (Leica Microsystem); representative pictures were taken from far periphery, middle periphery and central areas of the retina. Adobe Photoshop CS5 was used for primary image processing.

Positive TUNEL cells in retinal sections were counted manually in the three areas described above (far periphery, middle periphery and central area) at 20 × magnification and this result was divided by the value of the outer nuclear layer (ONL) area. All data given represent the means and standard error from three retinal sections each, for at least 6 different animals.

-Immunohistochemistry:

Immunohistochemistry is a method of detection for proteins or peptides in tissue sections by the principle of antigen-antibody specificity. For that, a specialized antigen recognizing antibody is added to the tissue. After that, a secondary antibody that binds specifically to the primary is included. Instead the secondary antibody may also be linked to a fluorophore which emits fluorescence when exposed to a particular wavelength. In this case, the technique is also known as immunofluorescence.

- *Procedure:*

Frozen retinal sections were dried at room temperature (RT) or 37° C. Subsequently, the tissue was rehydrated in PBS and pre-incubated for 1 hour at RT in blocking solution: 20% of normal goat serum in PBS-BSA 1% and Triton 0,3%.

Immunohistochemistry was performed overnight at 4° C, using primary antibodies (Table 2) (GSH, 1:100 (Sigma Aldrich, Madrid, Spain); Gad-65, 1:200 (Sigma Aldrich, Madrid, Spain); GFAP, 1:500 (Dako cytomation, Denmark)) diluted in PBS-BSA-Triton

0,3%. Then, sections were washed next day and incubated for 60 min in dark at RT with secondary antibodies (Alexa 488 goat anti-rabbit, 1:200 and Alexa 488 goat anti-mouse, respectively) diluted in buffer. Tissue was rinsed again and mounting medium was added (Vectashield with DAPI) to avoid bleaching.

Antibody	Dilution	Name	Company
GSH	1:100	Glutathione	Sigma Aldrich, Madrid, Spain
GAD-65	1:200	Anti-Glutamic Acid Decarboxylase 65	Sigma Aldrich, Madrid, Spain
GFAP	1:500	Glial fibrilary acidic protein	Dako Cytomation, Denmark
Alexa Fluor® 488	1:200	Goat anti-rabbit IgG (H+L)	Invitrogen, Oregon USA
Alexa Fluor® 488	1:200	Goat anti-rabbit IgG (H+L)	Invitrogen, Oregon USA

Table 2. Antibodies used.

Negative controls were performed by omitting the primary antibody and incubating tissue sections with secondary antibodies alone.

- *Microscopy*

Light and fluorescence microscopy was performed on a Nikon DS-Fi1 camera attached to Leica DM2000 microscope, Leica application Suite version 2.7.0 R1 software (Leica Microsystem); representative pictures were taken from far periphery, middle periphery and central areas of the retina. Adobe Photoshop CS5 was used for primary image processing. Measurement of the area immunolabeled of the ONL was determined with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

d) Electrophysiological recording tecniches:

-Electroretinograms:

ERGs were performed under dim red illumination (Advanced Headlight LED x6, Energizer®, Barcelona, Spain).

Animals were anesthetized with intraperitoneal injection of ketamine and azepromazine (100 mg/ kg body weight and 2,5 mg/ kg body weight, respectively) after dark-adapted overnight. Pupils were dilated with 0,1% tropicamide (Alcon Labs, Barcelona, Spain) followed by the administration of topical artificial tear gel (Hyabak, Thea Labs, Barcelona, Spain). Mice were then placed on a pad heated to 37-39° C.

The ERG was carried out only in one eye and always during the morning. The corneal electrode was a gold wire loop (Roland Consult, Brandenburg, Germany); the reference needle electrode was placed on the forehead and a ground electrode was put subcutaneous near the tail.

Recordings were taken in a darkened room under dim red illumination to ensure a dark-adapted state and with electrical impedance balanced. Responses were analyzed using a RetiScan RetiPort electrophysiology unit (Roland Consult, Brandenburg, Germany). Each ERG session consisted of one scotopic response. Stimuli consisted of white LED-flashes with an intensity 3 cd.s/m^2. The electrodes were connected to a preamplifier, and signals were amplified with a band pass filter between 1 and 300 Hz. Responses were amplified, averaged and stored with the system interface and the amplitude (µV) and implicit times (ms) of the different ERG components were analysed.

The amplitude of the *a*-wave was calculated from the baseline to the first negative deflection, and the amplitude of the *b*-wave was measured from the trough of the *a*-wave to the peak of the *b*-wave. Implicit times of *a*- and *b*-waves were calculated from the onset of light stimulus to the peak of each wave respectively.

3. STATISTICAL ANALYSIS

Statistical analysis of the data from this study was carried out using SPSS version 15. The results are presented as mean values ± error standard from at least six animals in each group.

To determine statistical differences in glutamate, GSH, GSSG and Cys retinal concentrations at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28, we applied a two-way analysis of variance (ANOVA) with primary treatment (age) as the first factor and secondary treatment (strain) as the second factor. When the ANOVA indicated a significant difference, a post hoc Dunnett's T3 test was applied to demonstrate the differences. In order to simplify the results, only the statistical differences between the rd1 and wild type retinas at each age are noted in each figure.

To determine statistical differences in all the other parameters evaluated in this work, one way ANOVA with LSD or Dunnett's post hoc analysis was applied.

A 95% confidence interval was used to assess significance.

-RESULTS

1. CHARACTERIZATION OF GLUTAMATE AND THIOL METABOLISM IN RD1 MICE

1.1. Glutamate

Figure 19 shows that, glutamate concentrations were significantly decreased in rd1 retinas when compared to wt (wild type or control) retinas at PN3 day (PN3 ($F(1) = 7.494$, $p < 0.05$ vs rd1)).

At the onset of degeneration (PN7), glutamate levels started to be slightly higher in rd1 retinas wich was statistically significant at PN7 (PN7 ($F(1) = 18.138$; $p < 0.05$ vs wt) and PN11 (PN11 ($F(1) = 48.470$; $p < 0.05$ vs wt), at PN15 (PN15 ($F(1) = 31.048$; $p < 0.05$ vs wt), and PN17 (PN17 ($F(1) = 10.629$; $p < 0.05$ vs wt).

Glutamate concentrations in wt and rd1 retinas were comparable at PN day 21 and day 28.

There was a statistically significant interaction between strain and age on glutamate concentration ($F(7) = 11.752$; $p < 0.05$).

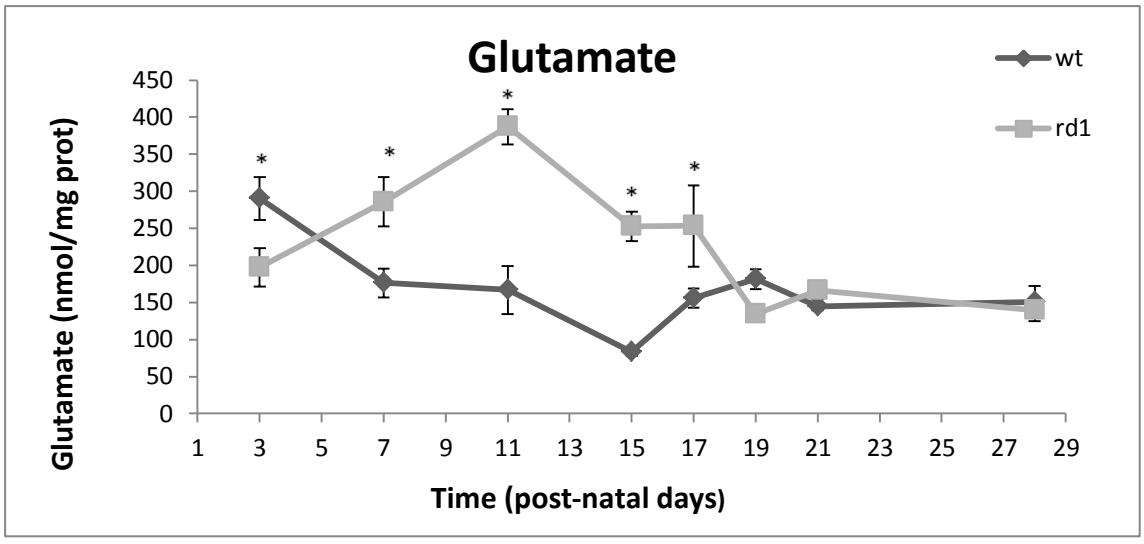

Figure 19. Retinal glutamate concentrations between the two groups of study, rd1 and wild type (wt) mice at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 as determined by HPLC. The markers represent the mean (at least n = 8 for each age and strain) of glutamate concentrations. The error bars represent the standard error of the mean (* $p < 0.05$ vs rd1 and wt, respectively).

1.2 Glutathione (GSH)

Figure 20 shows GSH concentrations in rd1 and wt mice retinas at different post-natal (PN) days (PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28).

A decrease in GSH concentration with increasing age in control mice was observed; however, this pattern was not observed in the rd1 retinas.

No significant changes were observed in GSH retinal concentrations between rd1 and wt mice when the degenerative process became detectable (PN3-PN11) and rods die (PN11) (Figure 20).

At PN15 the concentration of GSH in retina from rd1 mice was statistically lower compared to retinas concentrations in wt (wild type or control) mice ($F(1) = 4.575$; $p < 0.05$ vs rd1).

Immediately after PN15, GSH concentration started to increase in rd1 retinas when compared to wild type retinas ($F(1) = 10.821$; $p < 0.05$ vs wt) and PN19 ($F(1) = 13.939$; $p < 0.05$ vs wt) (Figure 20).

At PN28, GSH concentrations from wt and rd1 mice retinas were again similar (Figure 20).

There was a statistically significant interaction between strain and age on GSH concentration ($F(7) = 4.108$; $p < 0.05$).

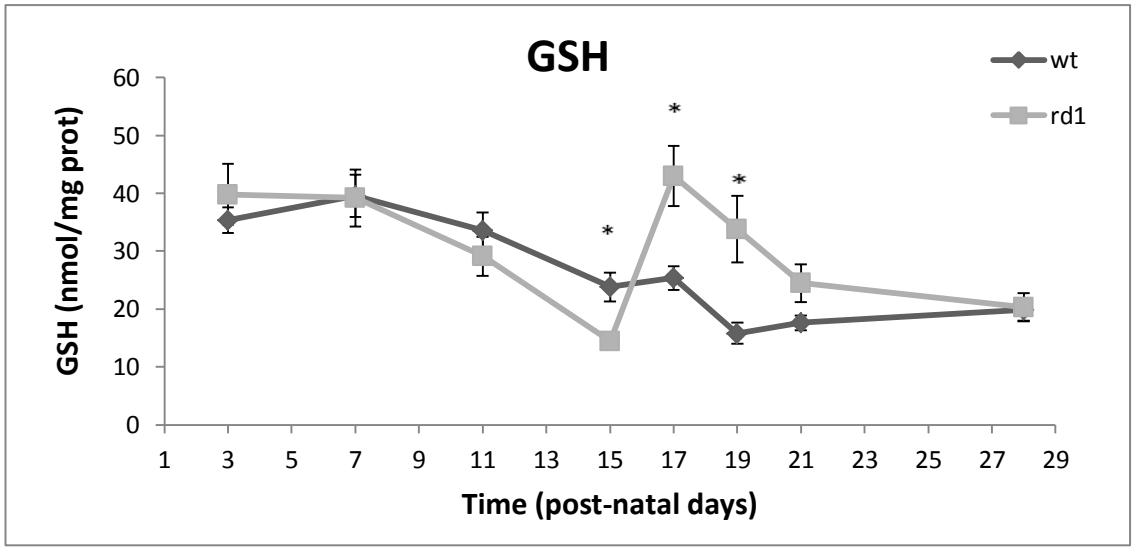

Figure 20. Retinal glutathione (GSH) concentrations in the two groups of study, rd1 and wild type (wt) mice at post-natal (PN) PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 as determined by HPLC. The markers represent the mean (at least n = 8 for each age and strain) of GSH concentrations. The error bars represent the standard error of the mean (* $p < 0.05$ vs rd1 and wt, respectively).

1.3 Glutathione Disulfide (GSSG)

Figure 21 represents GSSG concentrations in the retina of the two groups of mice studied, wt and rd1 at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28.

At the beginning of the experiment (PN3-PN9) and when the degenerative process became detectable (PN11-PN15), there were no differences in GSSG concentrations between wt and rd1 mice retinas (Figure 21).

At PN17, GSSG concentrations, started to be slightly higher in rd1 mice retinas when compared with wt mice retinas, finding statistically significant differences between strains at PN21 ($F(1) = 8.948$; $p < 0.05$ vs wt) and PN28 ($F(1) = 69.885$; $p < 0.05$ vs wt) (Figure 21).

These results were in accordance with the decrease found in GSH concentrations in the retina of rd1 mice at PN19, PN21 and PN28 (Figure 21).

There was a statistically significant interaction between strain and age on GSSG concentration ($F(7) = 9.272$; $p < 0.05$).

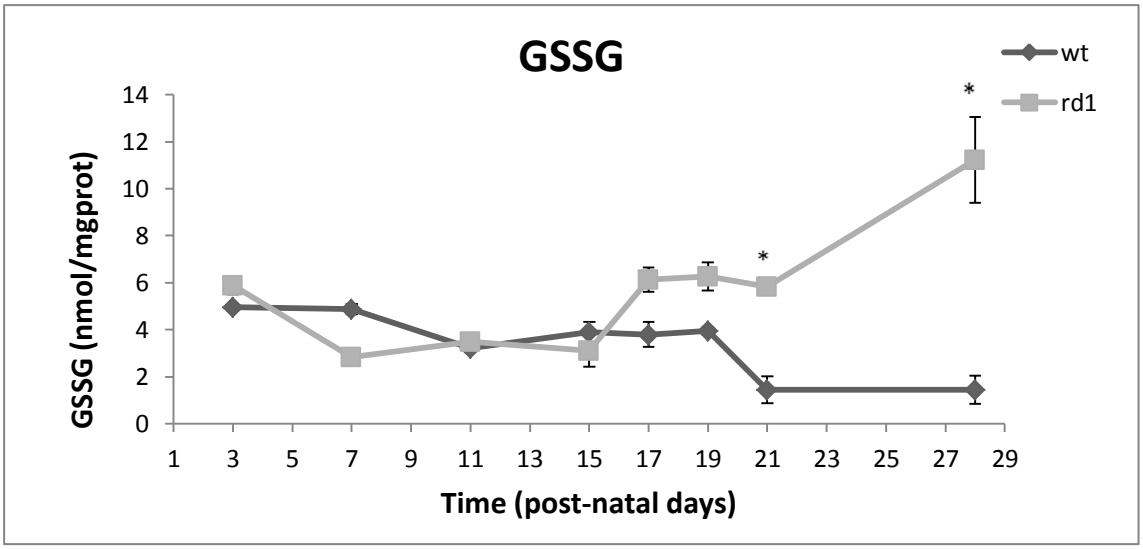

Figure 21. Retinal glutathione disulfide (GSSG) concentrations in the two groups of study, rd1 and wild type (wt) mice at post-natal (PN) days PN3, PN7, PN11, PN13, PN15, PN17, PN19, PN21 and PN28 as determined by HPLC. The markers represent the mean (at least n = 8 for each age and strain) of GSSG concentrations. The error bars represent the standard error of the mean (* $p < 0.05$ vs wt).

1.4 Cysteine (Cys)

Cys concentrations were measured in rd1 and wt mice retinas at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 days by HPLC, but no significant changes were observe between the two genotypes (wt and rd1) and age (Figure 22), though a tendency to decrease with age can be observed ($F(7) = 1.812$; $p = 0.087$) in both groups of study (Figure 22).

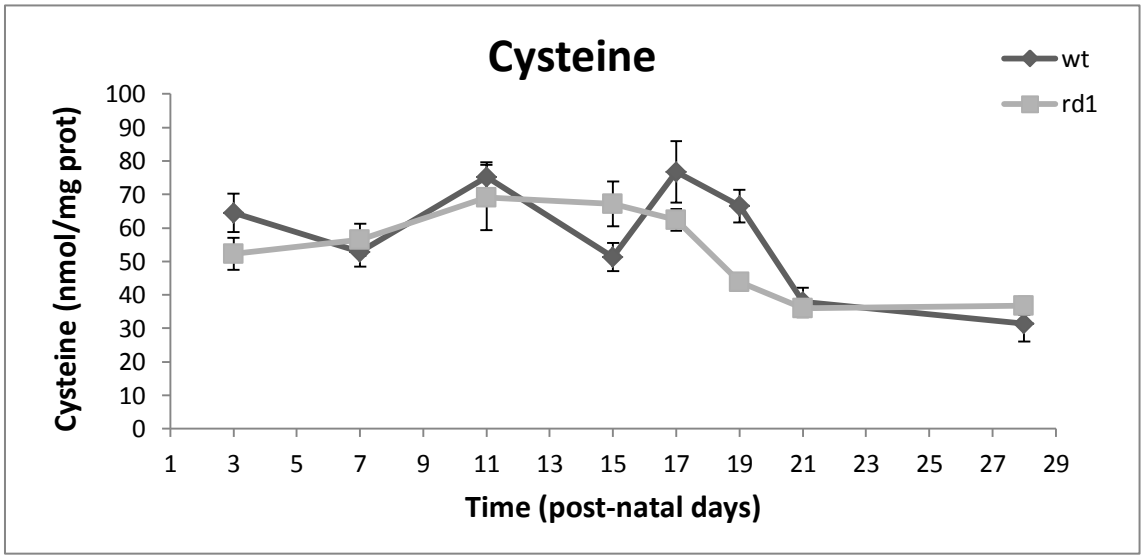

Figure 22. Retinal Cysteine (Cys) concentrations in the two groups of study, rd1 and wild type mice (wt) at post-natal (PN) days PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 days as determined by HPLC. The markers represent the mean (at least n = 8 for each age and strain) of Cys concentrations. The error bars represent the standard error of the mean.

2. EVALUATION OF PROGESTERONE EFFECT IN RD1 MICE RETINA

2.1 Progesterone reduces oxidative stress

2.1.1 Determination of glutamate and thiol metabolism in wt, rd1 and rd1 treated mice

- *Glutamate*

Differences in retinal glutamate concentrations in the three groups of mice studied in this work (wt, rd1 and treated with progesterone rd1 mice) at PN11, PN13, PN15 and PN17 are shown in Figure 23.

At PN11 retinal glutamate concentrations were significantly increased in untreated rd1 mice when compared to wild type mice (* $p< 0.05$ vs wt). Treatment with progesterone restored retinal glutamate concentrations to values similar to the concentrations observed in retinas from wild type mice (# $p< 0.05$ vs treated rd1).

At PN13 there was no difference in retinal glutamate concentrations in untreated or treated with progesterone rd1 mice compared to glutamate concentrations in the retina from wt mice. However retinal glutamate concentrations were decreased in treated mice compared to untreated mice.

After the peak of cell death in this animal model (PN15 and PN17), retinal glutamate concentrations were significantly increased in rd1 mice when compared to wild type mice (* $p< 0.05$ vs wt).

Treatment with progesterone significantly reduced retinal glutamate concentrations at PN15 (* $p< 0.05$ vs wt; # $p< 0.05$ vs treated rd1) and PN17 (* $p< 0.05$ vs wt; # $p< 0.05$ vs treated rd1) suggesting progesterone may induce some protection against glutamate toxicity (Figure 23).

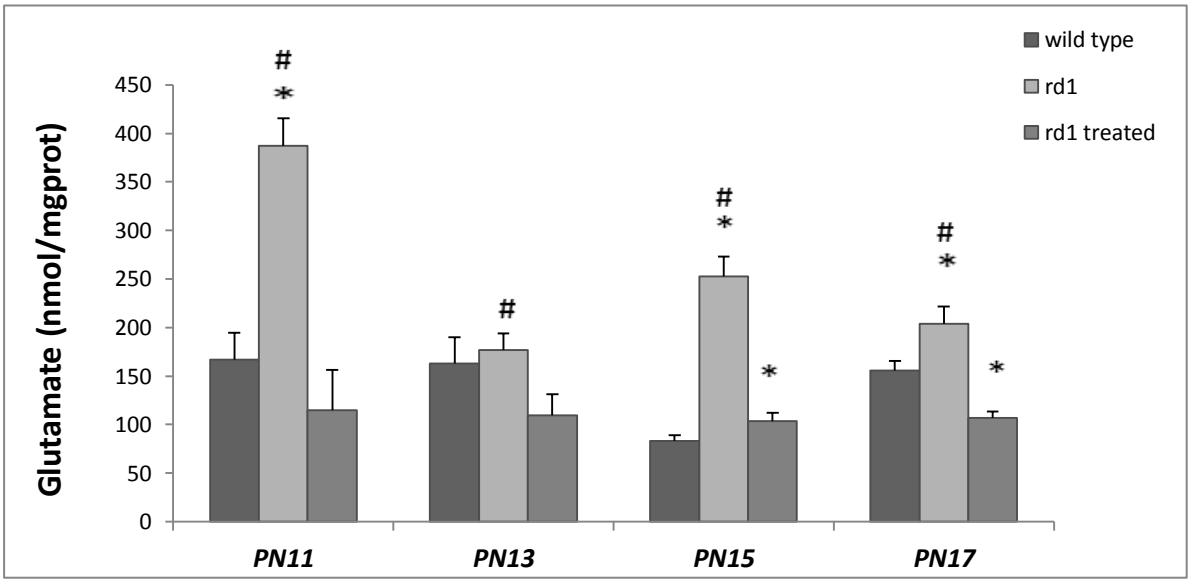

Figure 23. Glutamate concentrations (nmol/mgprot) in retinas from wild type (wt), rd1 and rd1 treated mice. Glutamate was measured at different post-natal days (PN11, PN13, PN15 and PN17) by HPLC. The bars represent the mean concentration (at least n = 6 animals for each age and strain); the error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

- *GSH*

GSH concentrations in the retina from wt, untreated and progesterone-treated rd1 mice are shown in Figure 24.

There were no differences in retinal GSH concentrations in the three groups of mice studied at PN11 and PN13, the peak of photoreceptor cell death in this animal model.

At PN15, just after the peak of degeneration, we could observe a significant decrease in GSH concentrations in the retina from untreated rd1 mice when compared with the concentrations observed in the retina from wt mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1) (Figure 24).

At PN17, GSH concentrations in retinas from untreated and progesterone-treated rd1 mice were significantly higher than those concentrations observed in retinas from wt mice (* p< 0.05 vs wt) (Figure 24).

Interestingly, treatment with progesterone in rd1 mice significantly increase GSH retinal concentrations at PN15 and PN17.

These results may suggest that at least some of the protection effect against photoreceptor degeneration elicited by progesterone may be due, in part, to the activation of the GSH system.

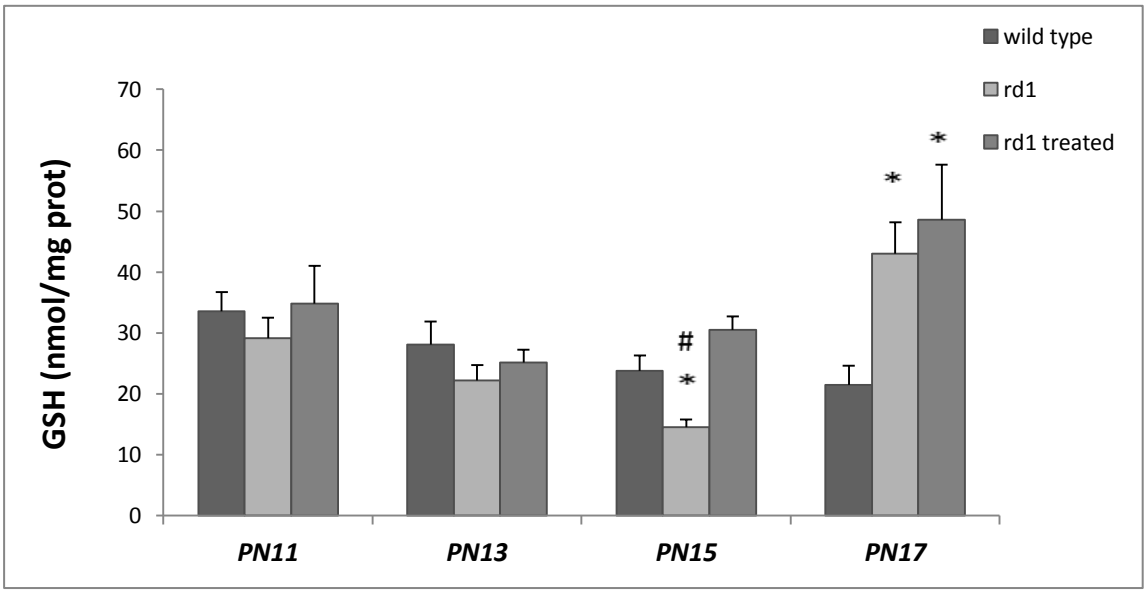

Figure 24. GSH concentrations (nmol/mg prot) in retinas from wild type, rd1 and treated rd1 mice. GSH was measured at different post-natal (PN) days (PN11, PN13, PN15 and PN17) by HPLC. The bars represent the mean concentration (at least n = 6 animals for each age and strain). The error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

- *GSSG*

GSSG concentrations in the retina from wt, untreated rd1 and progesterone-treated rd1 mice are shown in Figure 25.

At PN11 and PN13, GSSG concentrations in retinas from wt and untreated rd1 mice were similar. At PN15 and PN17, GSSG concentrations in retinas from untreated rd1 were higher than in retinas from wt mice (* p< 0.05 vs wt) (# p< 0.05 vs treated rd1).

GSSG concentrations in retinas from rd1 mice treated with progesterone were significantly higher compared to GSSG concentrations of retinas from wt and untreated rd1 mice at PN11, PN13, PN15 and PN17.

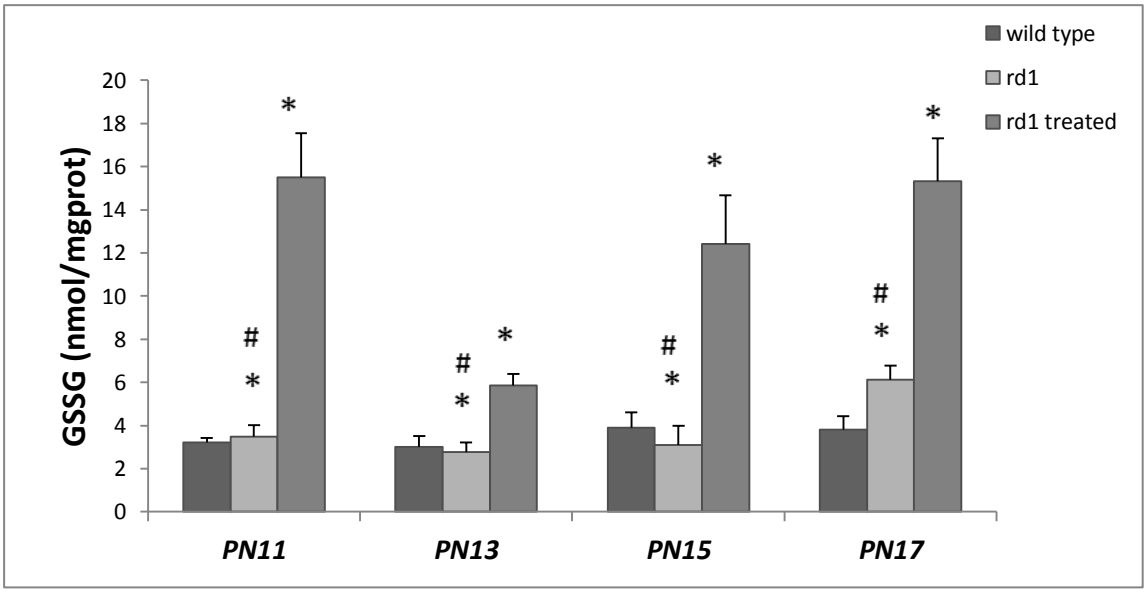

Figure 25. Glutathione disulfide (GSSG) concentrations (nmol/mg prot) in retinas from wt, rd1 and treated rd1 mice at different post-natal (PN) days. GSSG was measured at different PN days (PN11, PN13, PN15 and PN17) by HPLC. The bars represent the mean concentration (at least n = 6 animals for each age and strain). The error bars represent the standard error of the mean (* $p < 0.05$ vs wt; # $p < 0.05$ vs treated rd1).

- *Cys*

Figure 26 shows retinal Cysteine (Cys) concentrations from wt, untreated and progesterone-treated rd1 mice at different PN days (PN11, PN13, PN15 and PN17).

At PN11, PN15 and PN17 there was no difference in Cys concentrations in retinas from wt compared to those found in the retina from untreated and progesterone-treated rd1 mice.

However, Cys concentrations at PN13 were significantly decreased in retinas from progesterone-treated rd1 mice compared to those found in the retina from untreated rd1 mice (# $p < 0.05$ vs treated rd1).

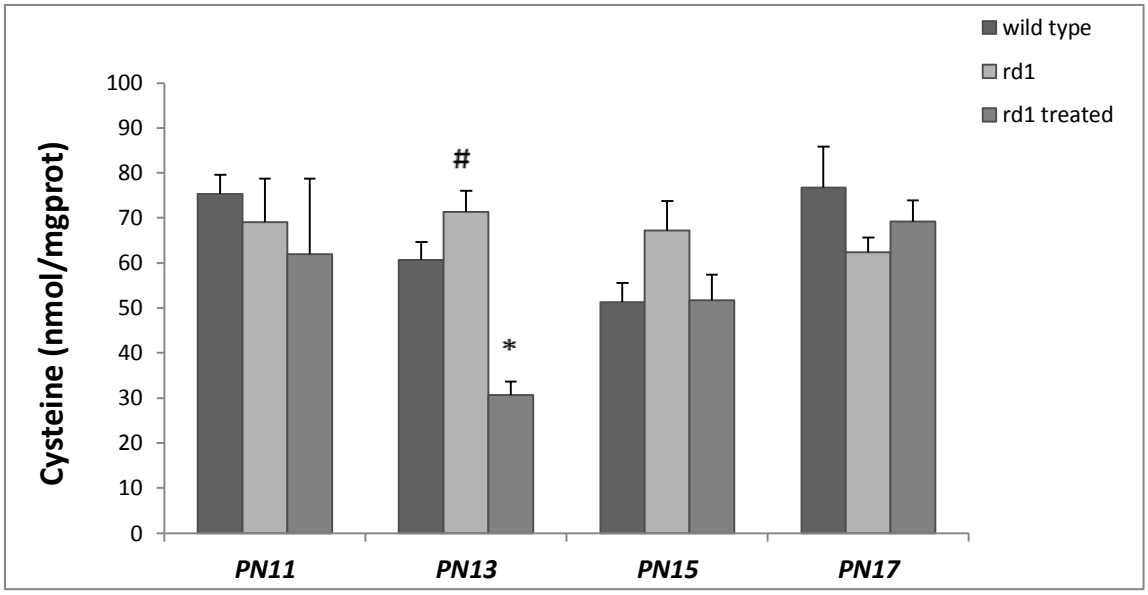

Figure 26. Cysteine (Cys) concentrations (nmol/mgprot) in retinas from wild type, rd1 and treated rd1 mice. Cysteine was measured at different post-natal days (PN11, PN13, PN15 and PN17) by HPLC. The bars represent the mean concentration (at least n = 6 animals for each age and strain). The error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

2.1.2 GSH Immunohistochemistry

Figure 27 shows GSH immunostaining in the retina from wt, untreated and progesterone-treated rd1 mice at PN11, PN13, PN15 and PN17.

At PN11 retinas from wt, untreated and progesterone-treated rd1 mice exhibit a similar GSH pattern of labeling: prominent immunoreactivity was found mainly in inner nuclear layer (INL) and in outer plexiform layer (OPL) and an intense staining was also found in cells located within the ganglion cell layer (GCL) and in processes from Müller cells.

With age, the pattern of GSH staining in retinas from wt mice changed and the intense of staining gradually decreased in the INL and GCL. Interestingly, at PN17, GSH inmunolabeling was almost restricted to the OPL and Müller cells in control retinas.

This same pattern of GSH inmunostainig was observed in retinas from treated with progesterone rd1 mice. However, retinal GSH distribution exhibit a different pattern of inmunostainning in the rd1 mice at PN17, and GSH was still observed in the GCL and INL, in addition to the staining observed in the OPL (Figure 27).

Table 3. Measurement of the area occupied by GSH immunolabeling in the ONL of the retina from wild type, untreated and treated by progesterone rd1 mice at PN11 and PN17 (areas were calculated with Image J software).

Post-natal days	*wild type*	*rd1*	*rd1 treated*
PN11	18%	13%	13%
PN17	4%	14%	3%

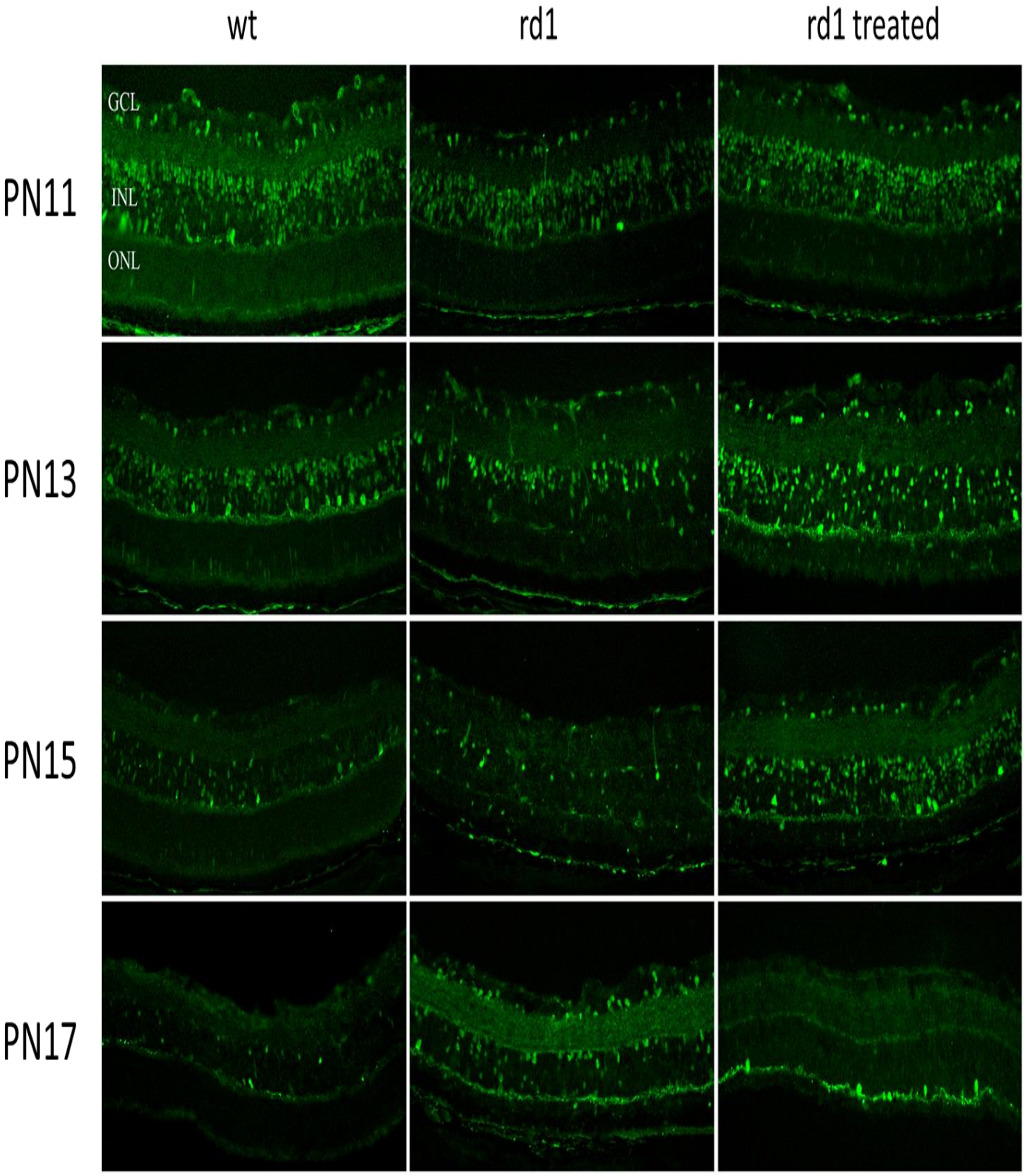

Figure 27. Mice mid-peripheral retina immunolabelled for GSH from the three groups studied, wild type, rd1 and progesterone-treated rd1 mice at different post-natal (PN) days (PN11, PN13, PN15 and PN17). (Scale bar = 50).

2.1.3 GAD-65 Immunohistochemistry

Figure 28 shows GAD-65 immunostaining in the retina from wt, untreated and progesterone-treated rd1 mice at PN11, PN13, PN15 and PN17.

At PN11 and PN13, in retinas from wt, untreated and progesterone-treated rd1 mice, GAD65-immunoreactive profile was observed in the OPL, INL, inner plexiform layer (IPL), GCLs and in retinal pigment epithelium (RPE) (Figure 28).

With age (PN15 and PN17), the pattern of GAD-65 staining in retinas from wt, rd1 and progesterone-treated rd1 mice seemed to change and the intensity of staining increased in the IPL.

However, no differences were observed in the retina from the different groups studied (wt, untreated and progesterone-treated rd1 mice).

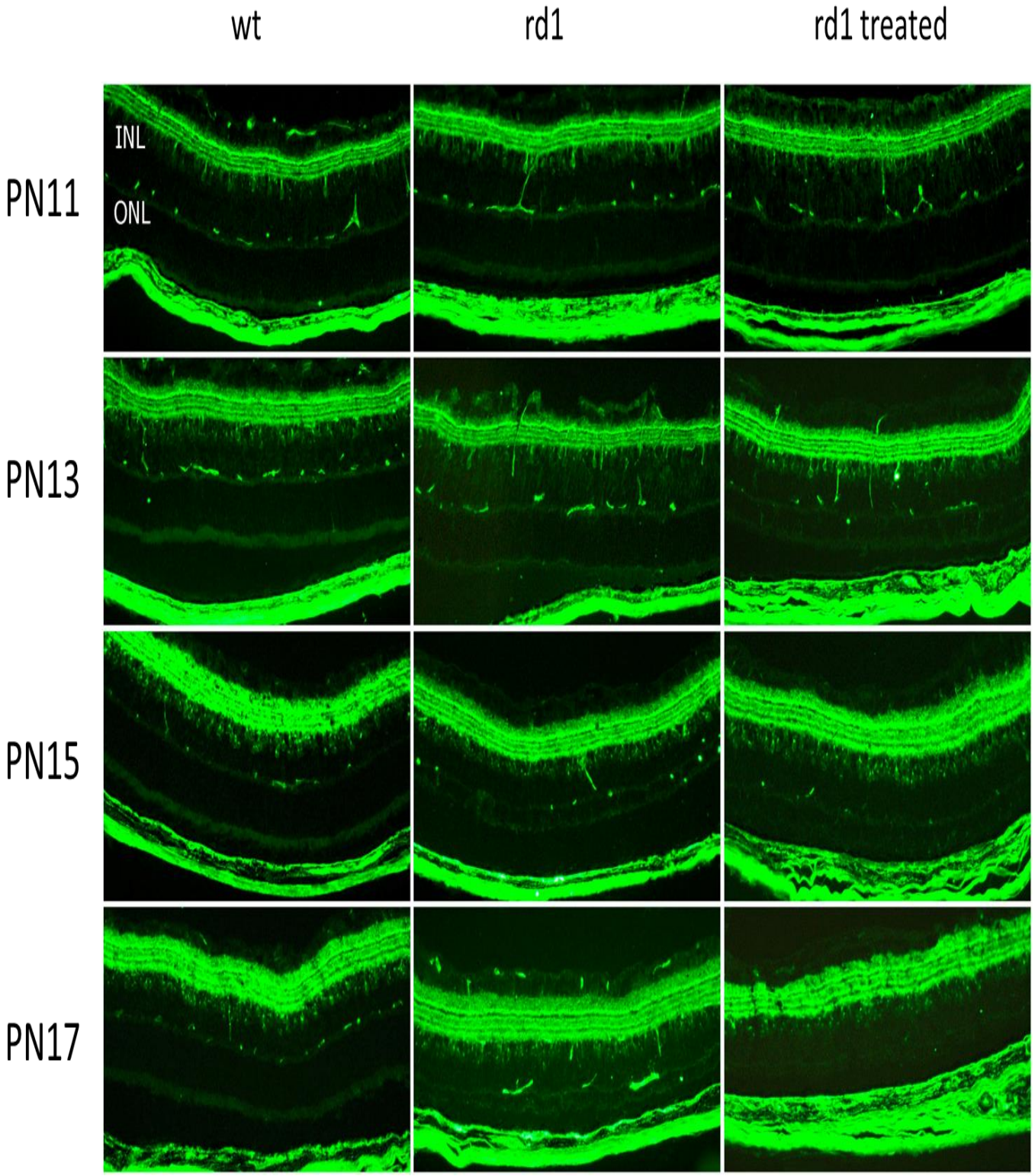

Figure 28. Mice retina immunolabeled for GAD-65 at different post-natal (PN) days (PN11, PN13, PN15 and PN17) from retinas of the three groups studied (wild type, untreated and progesterone-treated rd1 mice). (Scale bar = 50).

2.2 Progesterone delays rod and cone cell death in rd1 mice

2.2.1. Optical coherence tomography

Figure 29 shows changes in retinal thickness during the degeneration process in rd1 mice, showing that the thickness of the retina is decreased in the rd1 mice model over time.

Optical coherence tomography (OCT) of representative animals (Figure 30) showed a total retinal thickness of 0.177 mm on average in rd1, and progesterone-treated rd1 mice at PN17. At this time, the retinal thickness as measured on OCT decreased in both groups, with the progesterone-treated eyes ultimately showing a trend toward less thinning of the retinas compared with the rd1 untreated group.

Although OCT could resolve all the major retinal layers in mice and it has the capability to image the morphology of the rodent retina non-invasively and thereby to monitor progressive changes caused by degeneration in rd1 mice, it cannot reach the resolution of standard histological methods.

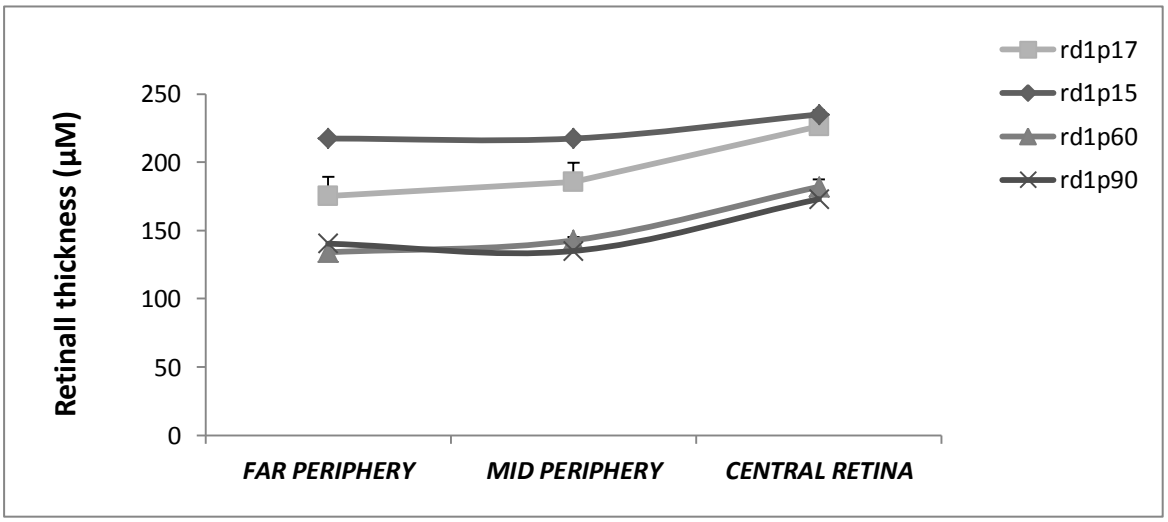

Figure 29. Changes of rd1 mouse retinal thickness during degeneration at different postnatal (PN) days (PN15, PN17, PN60 and PN90). The figure represents the thicknesses of the whole retina divided in three areas: far peripheral, mid peripheral and central retina. The markers represent the mean (at least n = 8 animals for each age and strain). The error bars represent the standard error of the mean.

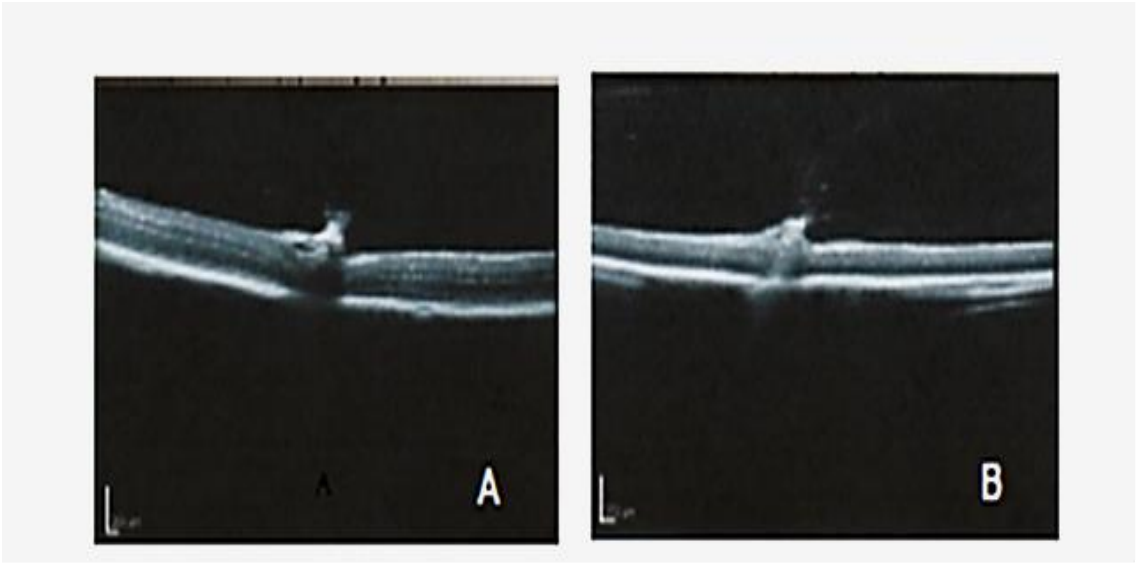

Figure 30. Optical coherence tomography (OCT) of representative animals of progesterone-treated rd1 mice compare to untreated rd1 groups at post-natal (PN) day PN17. (A) progesterone-treated rd1 and (B) untreated rd1 mice.

2.2.2 Histological study

Figure 31 shows hematoxilin-eosin stainings of the retina from the three groups studied (wt, rd1 and progesterone-treated rd1 mice) at PN11, PN13, PN15, and PN17 days. Ocular sections through the far peripheral, mid peripheral and central retina were used (n = 6).

At PN11, no differences between the number of rows in the ONL in the retina from wt, untreated rd1 and progesterone-treated rd1 mice were observed. At PN13, PN15 and PN17 it appears to be more rows in the ONL of the retina from progesterone-treated rd1 compared to untreated rd1 mice.

In the rd1 mice, the photoreceptor cell death occurs in a characteristic topographical pattern starting in the posterior retina and spreading anteriorly and further posteriorly towards the optic nerve. For that reason, the results are expressed in graphics where the different regions (far peripheral, mid peripheral and central retina) at the different PN days (PN11, PN13, PN15, and PN17) were shown (Figure 32, Figure 33 and Figure 34). Regions shown in Figure 31 are far peripheral retina.

At the far peripheral retina, differences in the number of rows in the ONL of the retinas from the different groups studied (wt, untreated and progesterone-treated rd1

mice) were not initially appreciated (PN11) (Figure 31 and Figure 32). However, at PN13, PN15 and PN17, differences were notable (Figure 32), showing a statistically significant increase in the number of rows in the ONL of the retina from the oral administration of progesterone rd1 mice compared to a number of rows in the ONL of the retina from untreated rd1 mice (* $p< 0.05$ vs wt; # $p< 0.05$ vs treated rd1).

At mid peripheral retina, the results were similar to those found in the far peripheral retina. At PN11, differences between groups (wt, rd1 and treated rd1 mice) were not appreciated, but at PN13, PN15 and PN17 (Figure 31 and Figure 33) the number of rows in the ONL of the retinas from progesterone-treated rd1 mice had significantly increased compared to those in rd1 untreated mice (* $p< 0.05$ vs wt; # $p< 0.05$ vs treated rd1 mice).

When we observed the area of the retina close to the optic nerve (central retina), there were no changes in the number of rows in the ONL of the retinas from untreated rd1 compare to progesterone-treated rd1 mice at PN11 and PN13 (Figure 34). At PN15 and PN17, the number of rows in the ONL of the retinas from progesterone-treated rd1 mice had increased compared to those found in the retina from rd1 untreated mice, and was statically significant (Figure 34) (* $p< 0.05$ vs wt; # $p< 0.05$ vs treated rd1 mice).

From these results, it can be suggested that treatment with progesterone significantly preserves photoreceptor cell number in the retina from rd1 mouse model compared to untreated rd1 mice.

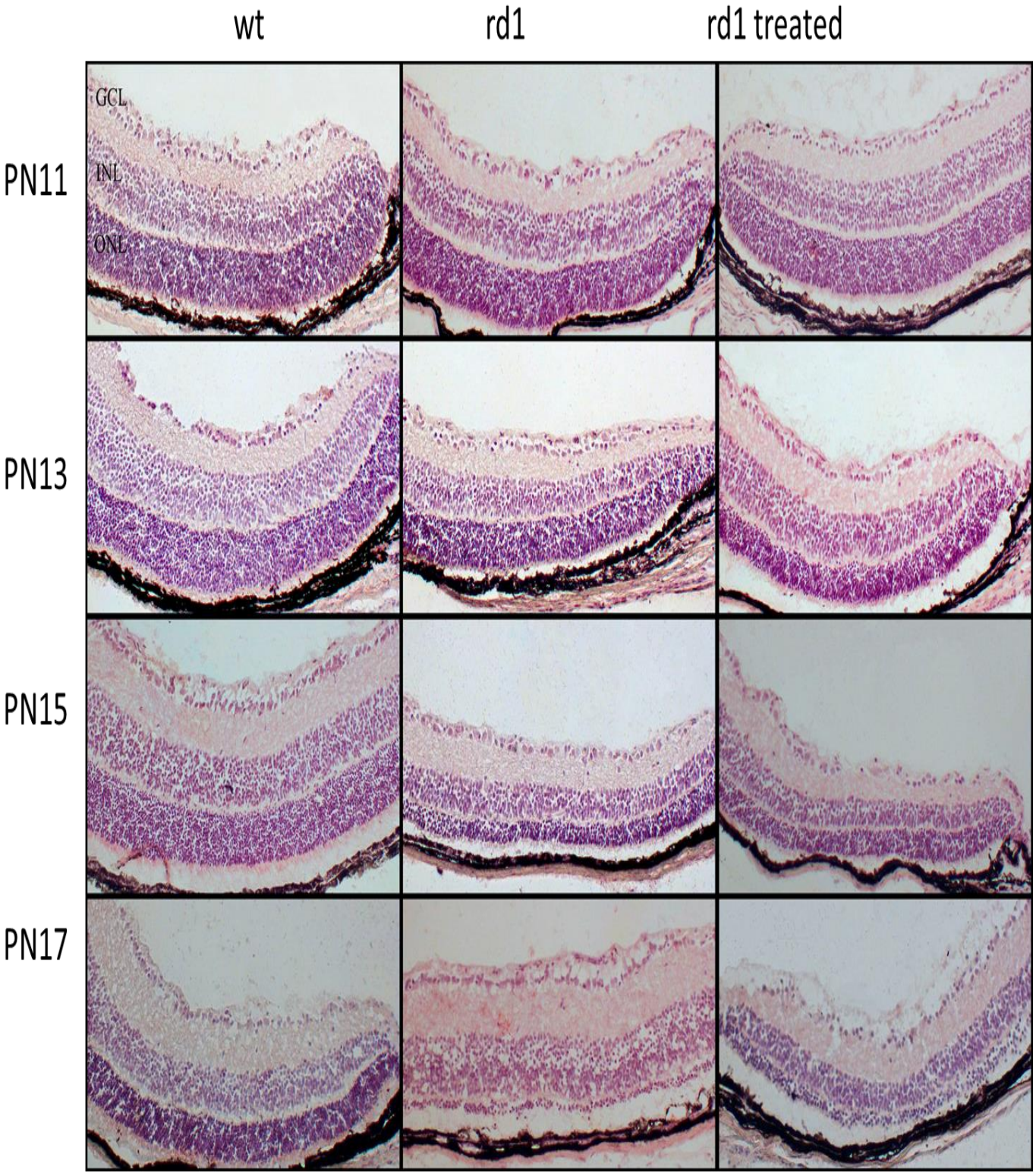

Figure 31. Hematoxilin-eosin staining of retinal sections shows retinas from control, rd1 and progesterone-treated rd1 mice, at post-natal (PN) days PN11, PN13, PN15 and PN17 in far peripheral retina. Progesterone treatment delays rod and cone cell death in rd1 mice. (Scale bar = 50).

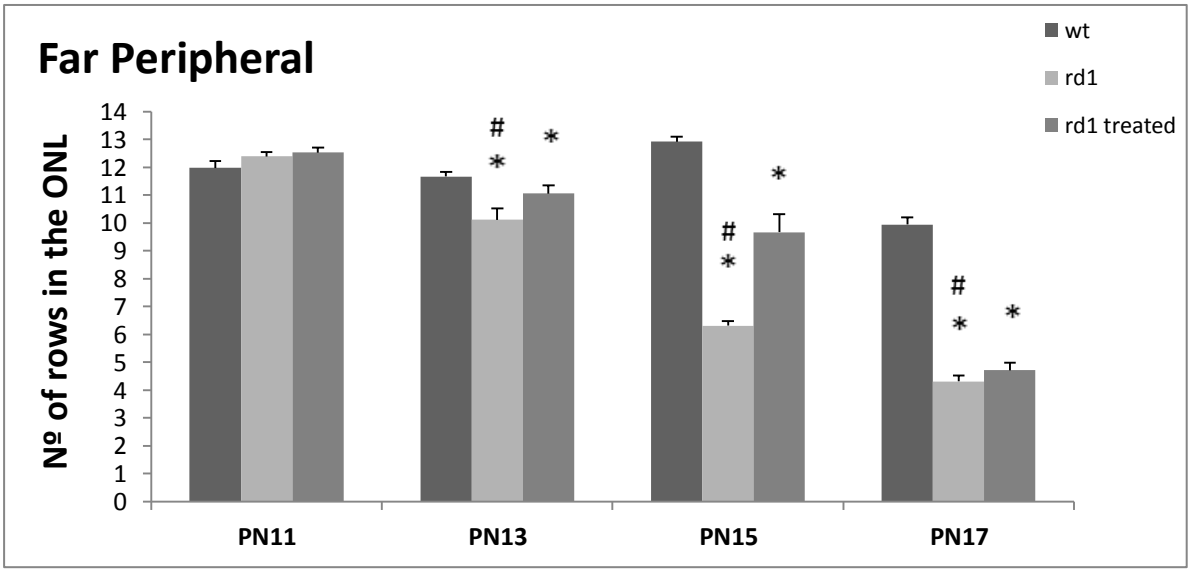

Figure 32. Graphical representation of the number of rows in the far peripheral retina of the outer nuclear layer (ONL) in the three groups studied (wild type (wt), rd1 and treated rd1 mice). The bars represent the mean (at least n = 8 animals for each age and strain) of the number of rows. The error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

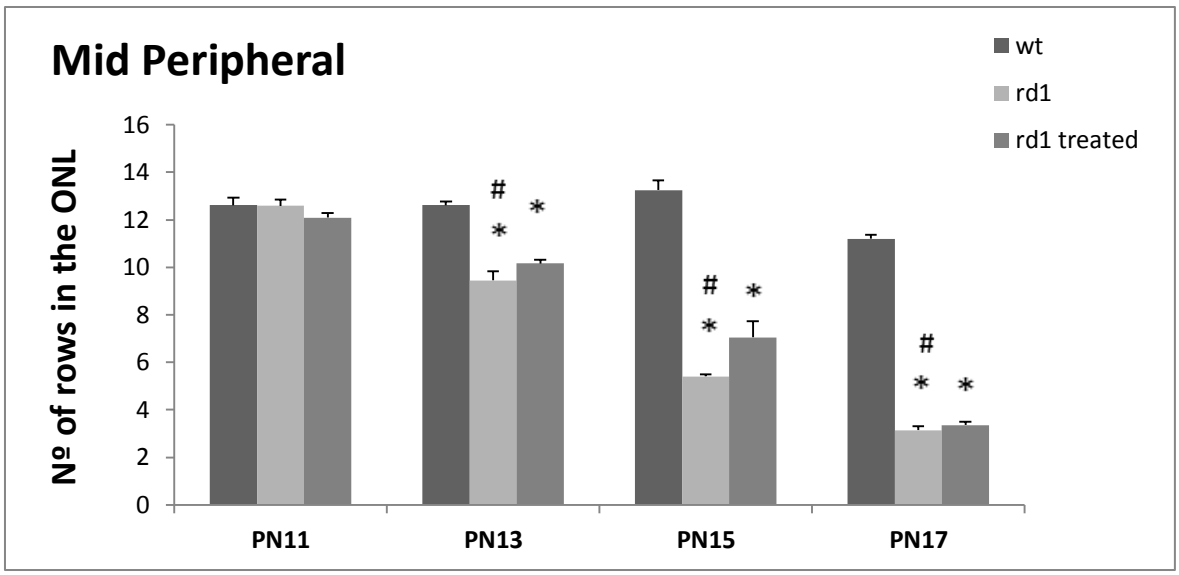

Figure 33. Graphical representation of the number of rows in the mid peripheral retina of the outer nuclear layer (ONL) in the three groups studied (wild type (wt), rd1 and treated rd1 mice). The bars represent the mean (at least n = 8 animals for each age and strain) of the number of rows. The error bars represents the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

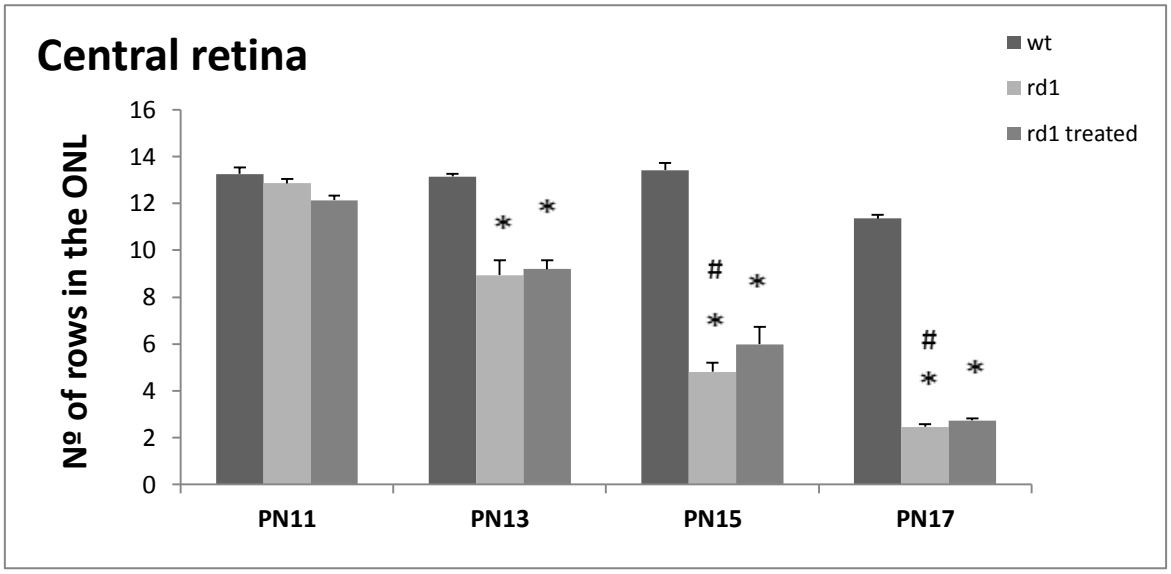

Figure 34. Graphical representation of the number of rows in the area of the retina close to the optic nerve in the outer nuclear layer (ONL) in the three groups studied (wild type (wt), rd1 and treated rd1 mice). The bars represent the mean (at least n = 8 animals for each age and strain) of the number of rows. The error bars represent the standard error of the mean (* $p < 0.05$ vs wt; # $p < 0.05$ vs treated rd1).

2.3. Progesterone treatment reduces apoptosis in the rd1 mice model

The distribution of TUNEL-positive cells is not uniform throughout the degenerating rd1 retina. The photoreceptor cell death occurs in a characteristic topographical pattern starting in the posterior retina and spreading anteriorly and further posteriorly towards the optic nerve. As mentioned above, for that reason the results are expressed in figures separated by different regions (far peripheral, mid peripheral and central retina) at the different PN days (PN11, PN13, PN15, and PN17) (Figure 35, Figure 36, Figure 37 and Figure 38). Regions shown in Figure 35 are far peripheral regions.

At the far peripheral retina, the number of TUNEL-positive cells in the ONL of the retinas from the groups studied (wt, untreated and progesterone-treated rd1 mice) were notable at PN11, PN13, PN15 and PN17. We found a significant decrease in the number of TUNEL-positive cells in the ONL of the retina from treated rd1 mice compared to untreated rd1 mice (* $p < 0.05$ vs wt; # $p < 0.05$ vs treated rd1) (Figure 35 and Figure 36).

At the mid peripheral retina, the results were similar to those found in the far peripheral retina. At PN11, PN13, PN15 and PN17 (Figure 37), the number of TUNEL-positive cells in the ONL of the retinas from progesterone-treated rd1 mice had significantly decreased compared to those in rd1 untreated mice (* $p < 0.05$ vs wt; # $p < 0.05$ vs treated rd1).

At PN11, PN13, PN15 and PN17, the number of TUNEL-positive cells in the ONL of the retinas from rd1 mice treated with progesterone had significantly decreased compared to those found in the retina from rd1 untreated mice (* $p < 0.05$ vs wt; # $p < 0.05$ vs treated rd1) (Figure 37).

The total number of TUNEL-positive cells in the ONL of the retina in the different regions (far peripheral, mid peripheral and central retina) was counted to give an accurate representation of the protection afforded by progesterone (Table 4). These results suggest that progesterone may delay cell death in the rd1 mice model, decreasing the number of TUNEL-positive cells in the ONL.

These studies were completed on both male and female populations, with similar protection achieved for both sexes, which indicated that the effects we observed were not gender specific (data not shown).

While it is evident, thus far, that progesterone has neuroprotective properties for photoreceptors, it is important to elucidate whether this neuroprotection translates into improved function.

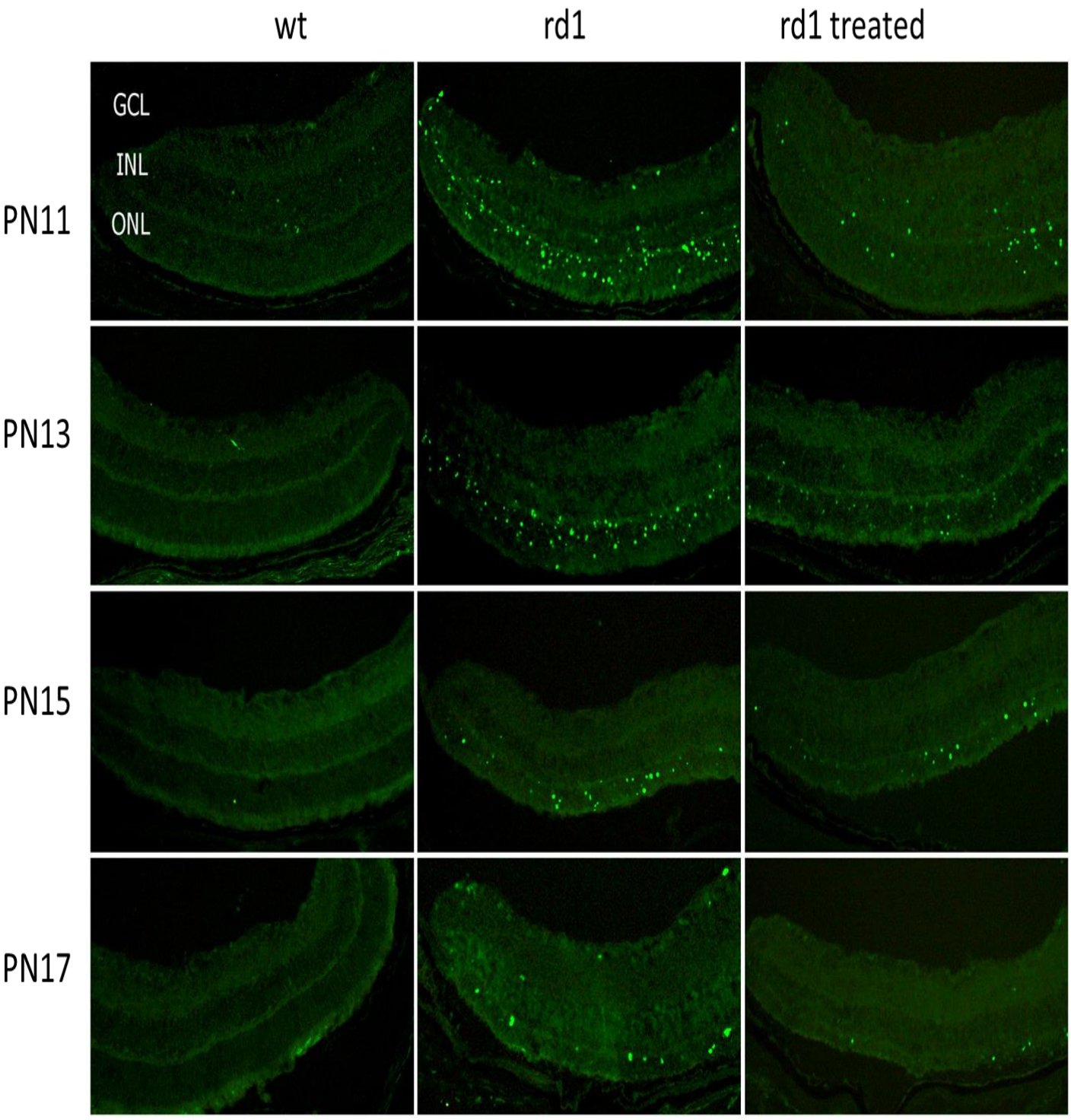

Figure 35. TUNEL staining in retinas from wild type, untreated and progesterone-treated rd1 mice at different post-natal (PN) days (PN11, PN13, PN15 and PN17). The number of positive cells were reduced in the retinas of progesterone-treated rd1 mice compared to those found in the retinas from untreated rd1 mice. (Scale bar = 50).

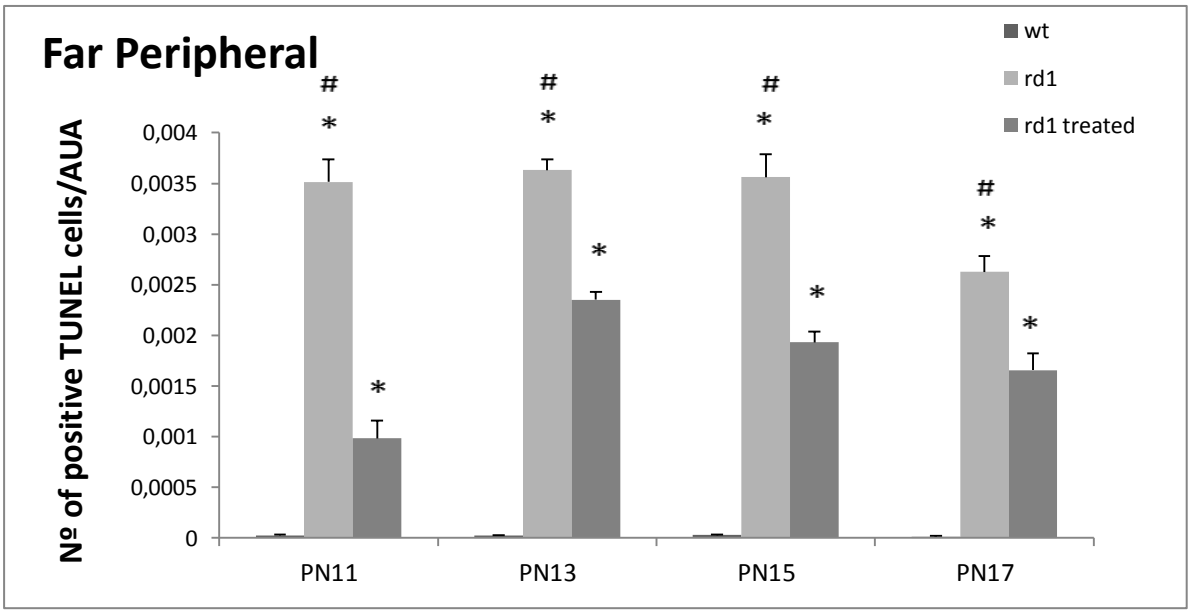

Figure 36. Graphical representation of the number of TUNEL-positive cells at post-natal (PN) days PN11, PN13, PN15 and PN17 (postive cells/AUA) in the retina from the different groups studied, wild type (wt), rd1 and progesterone-treated rd1 mice) (six retinas for each age and group) in the far peripheral region of the ONL. The bars are means of the number of TUNEL-positive cells. The error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

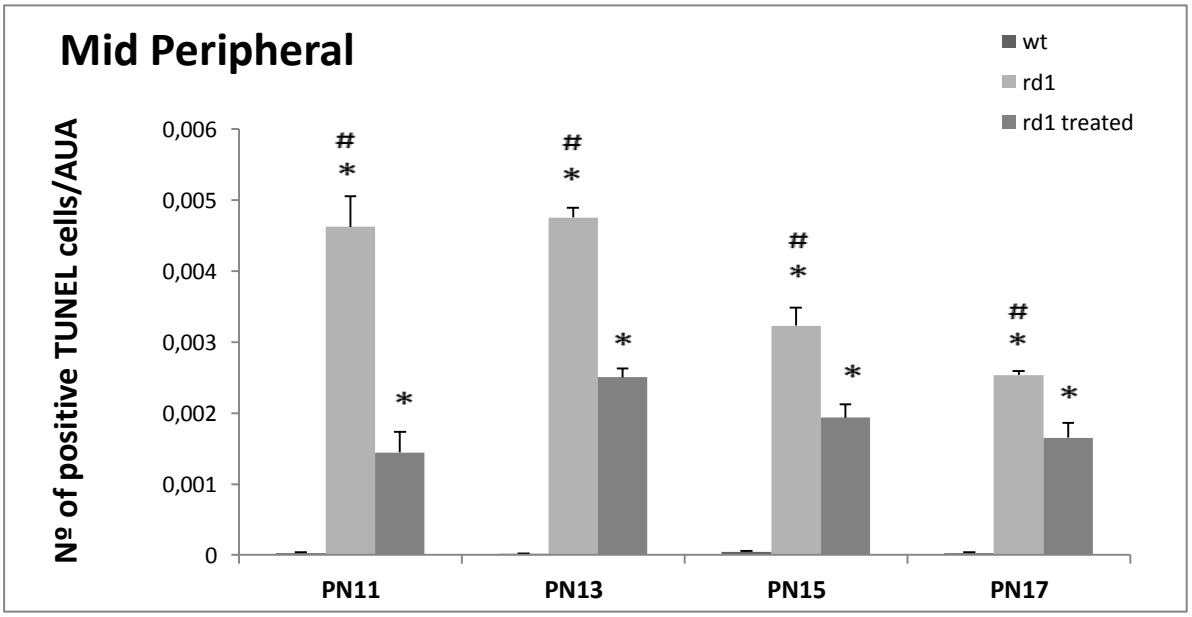

Figure 37. Graphical representation of the number of TUNEL-positive cells at post-natal (PN) days PN11, PN13, PN15 and PN17 (postive cells/AUA) in the retina from the different groups studied (wild type (wt), rd1 and progesterone-treated rd1 mice (six retinas for each age and group) in the mid peripheral retina of the ONL. The bars are means of the number of positive cells. The error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

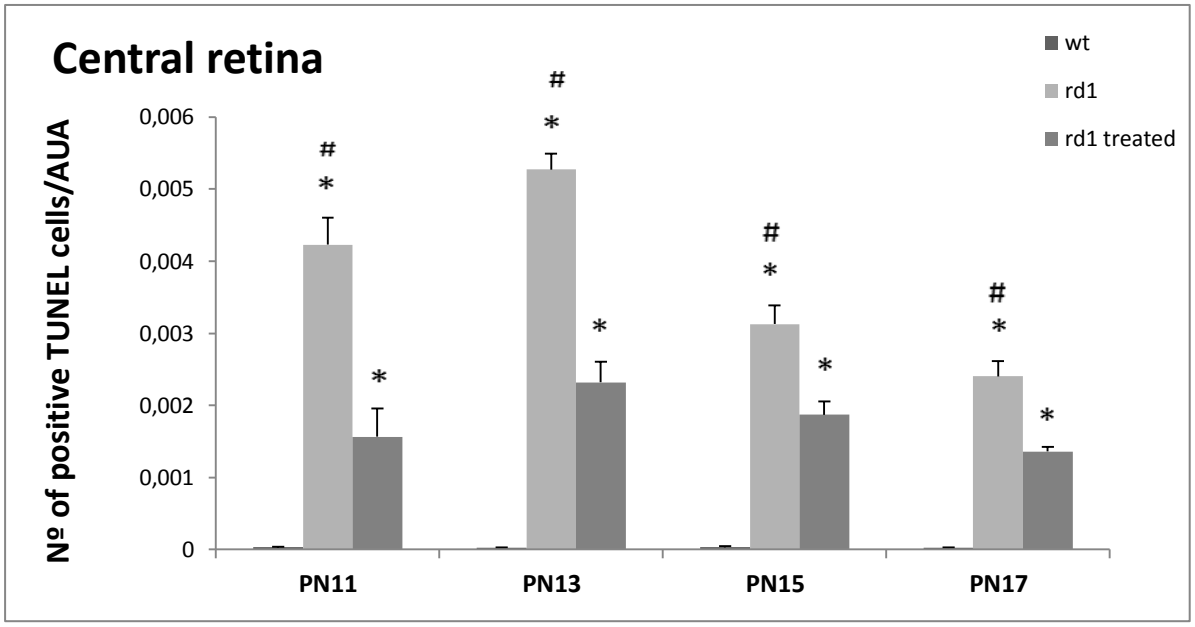

Figure 38. Graphical representation of the number of TUNEL-positive cells at post-natal (PN) days PN11, PN13, PN15 and PN17 (postive cells/AUA) in the retina from the different groups studied (wild type (wt), rd1 and progesterone-treated rd1 mice) (six retinas for each age and group) in the central retina of the ONL. The bars are means of the number of positive cells. The error bars represent the standard error of the mean (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

Table 4. Percentage of the cells in the outer nuclear layer (ONL) that are TUNEL-positive in the retina from the different groups studied (wild type (wt), untreated and progesterone-treated rd1 mice) in far peripheral, mid peripheral and central retina at post-natal (PN) days PN11, PN13, PN15 and PN17.

PN11	*Far peripheral*	*Mid peripheral*	*Central retina*
wt	4%	6%	6%
rd1	59%	76%	70%
treated rd1	17%	24%	27%

PN13	*Far peripheral*	*Mid peripheral*	*Central retina*
wt	4%	3%	6%
rd1	64%	68%	67%
treated rd1	33%	32%	30%

PN15	*Far peripheral*	*Mid peripheral*	*Central retina*
wt	4%	9%	6%
rd1	34%	27%	26%
treated rd1	17%	24%	27%

PN17	*Far peripheral*	*Mid peripheral*	*Central retina*
wt	2%	5%	4%
rd1	17%	17%	16%
treated rd1	9%	10%	10%

2.4. Progesterone reduces gliosis in rd1 mice model

2.4.1 Immunohistochemistry GFAP

Figure 39, shows glial fibrillary acidic protein (GFAP) immunoreactivity in the retina from the three different groups studied (wt, untreated and progesterone-treated rd1 mice) at PN11, PN13, PN15 and PN17.

The study shows an increase in GFAP expression in the retina of the untreated rd1 mice at PN11, PN13, PN15 and PN17 compared to wt mice, while GFAP expression in progesterone-treated rd1 mice presents a slight decrease over time compared to untreated rd1 mice (Figure 39).

Rd1 mice treated orally with progesterone starting at PN7 to PN11, PN13, PN15 and PN17, showed gliosis in the retina. The pattern followed is closer to that of the rd1 group than to wt mice; however, gliosis seemed to be decreased, suggesting that progesterone decreases gliosis in RP.

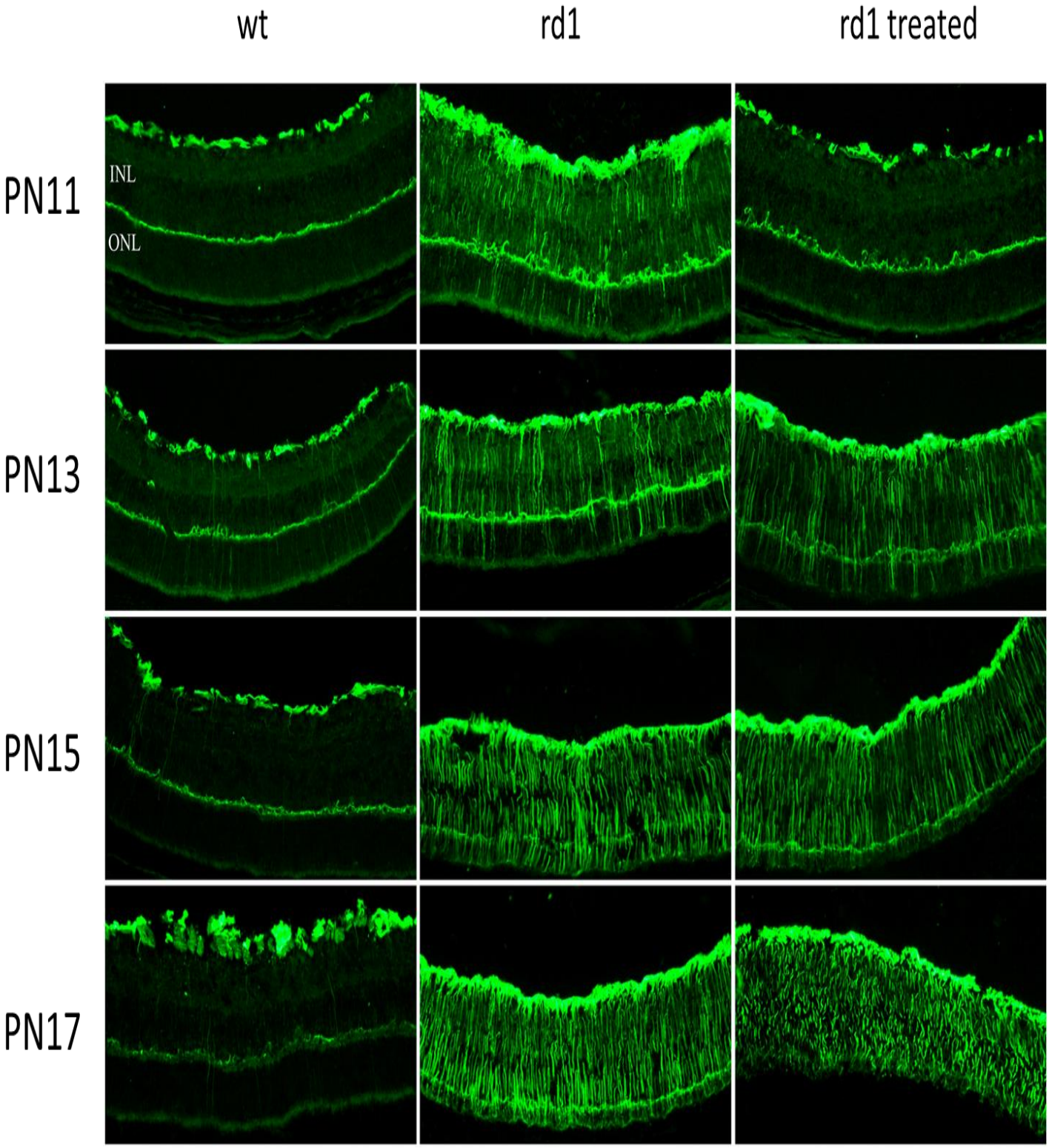

Figure 39. Photographs representative of immunofluorescent labelling for glial fibrillary acidic protein (GFAP) in selected areas of the retina from wild type, rd1 and progesterone-treated rd1 mice, at different post-natal (PN) days (PN11, PN13, PN15 and PN17). Gliosis is reduced in the retina of progesterone-treated rd1 mice compared to untreated rd1 mice. (Scale bar = 50).

2.5 Progesterone preserves photoreceptor function in rd1 mice

Electroretinography (ERG) analysis was carried out to compare retinal function in the different groups studied (wt, untreated rd1 and progesterone-treated rd1 mice) at PN13, PN15 and PN17. Alternate-day dosing with 100 mg/kg progesterone from PN7 to PN13, PN15 and PN17, respectively, improved the scotopic b-wave amplitude and a-wave amplitude compared to untreated rd1 mice (Figure 40, Figure 41 and Figure 43).

Figure 41 and Figure 42 show values of ERG scotopic a-wave amplitude, and values of ERG a-wave latency time at PN13, PN15 and PN17, respectively. At PN17, statistical changes of ERG a-wave values were observed in untreated rd1 mice and progesterone-treated rd1 mice compared to wt mice (*$p \leq 0.05$ vs wt), although we observed a tendency in the a-wave amplitude to increase after progesterone treatment.

Figure 43 and Figure 44 show values of ERG scotopic b-wave amplitude and values of ERG b-wave latency time at PN13, PN15 and PN17, respectively. B-wave amplitude was significantly decreased at PN15 and PN17 in untreated mice compared to wt mice (*$p \leq 0.05$ vs wt). At PN15 and PN17, treatment with progesterone restored retinal values of ERG b-wave amplitude to values similar to the ERG b-wave amplitude observed in retinas from wt mice (Figure 43). B-wave latency time was significantly decreased in the retina of treated rd1 mice compared to rd1 untreated mice at PN15 (# $p< 0.05$ vs rd1 treated) (Figure 44). At PN17, statistical changes of ERG b-wave latency time values were observed in untreated rd1 mice and progesterone-treated rd1 mice compared to wt mice (*$p \leq 0.05$ vs wt).

Taken together, these results suggest that treatment with progesterone may provide a certain improvement in photoreceptor function in the rd1 mouse model.

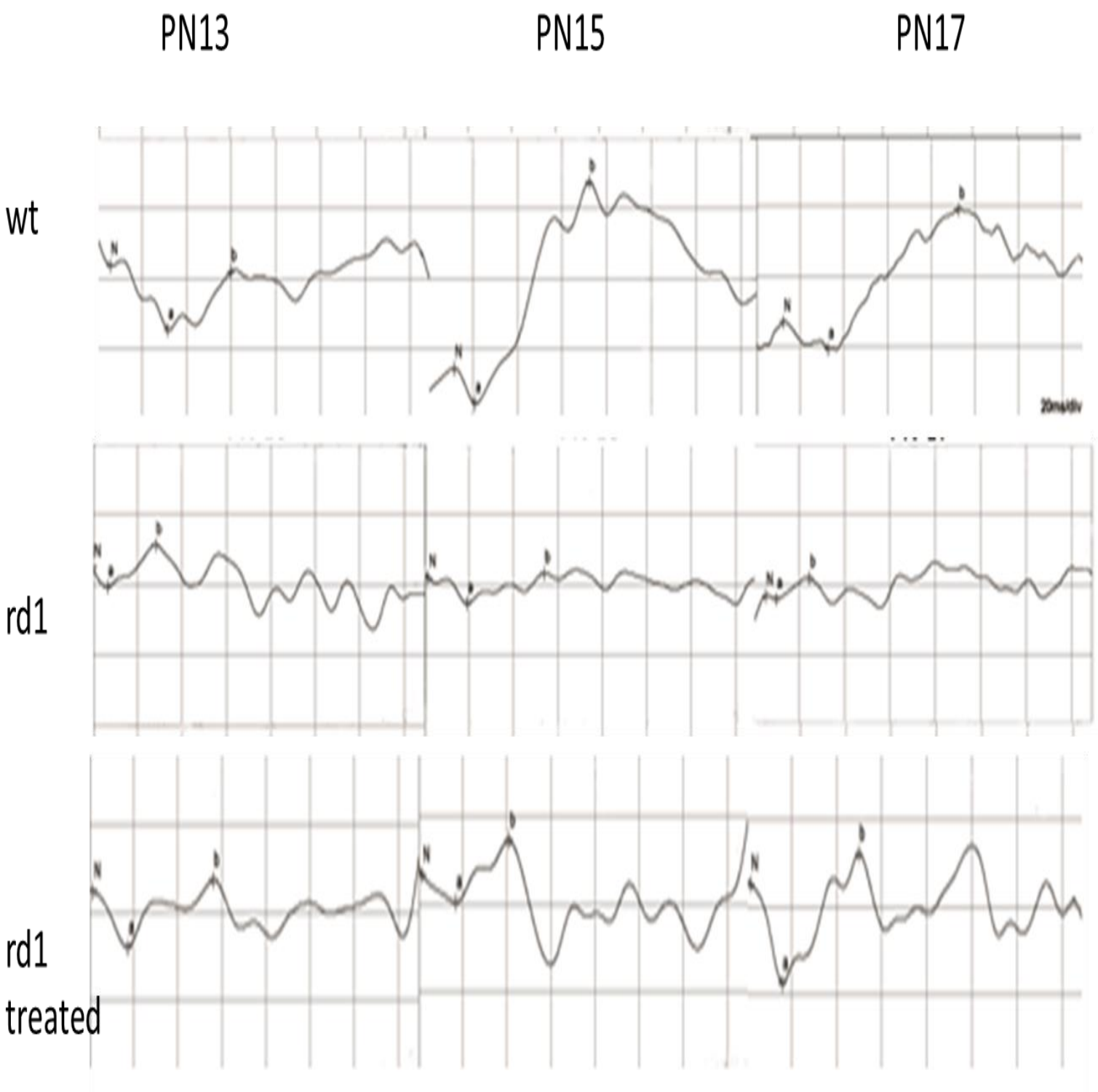

Figure 40. Progesterone significantly improved the a- and b-wave amplitude in progesterone-treated rd1 mice compared to untreated mice, indicating improved retinal function. Scotopic electroretinographies (ERGs) were done at PN13, PN15 and PN17, as described in the Materials and Methods sections. Representative wave forms are shown for the wild type (wt), rd1 and treated rd1 mice.

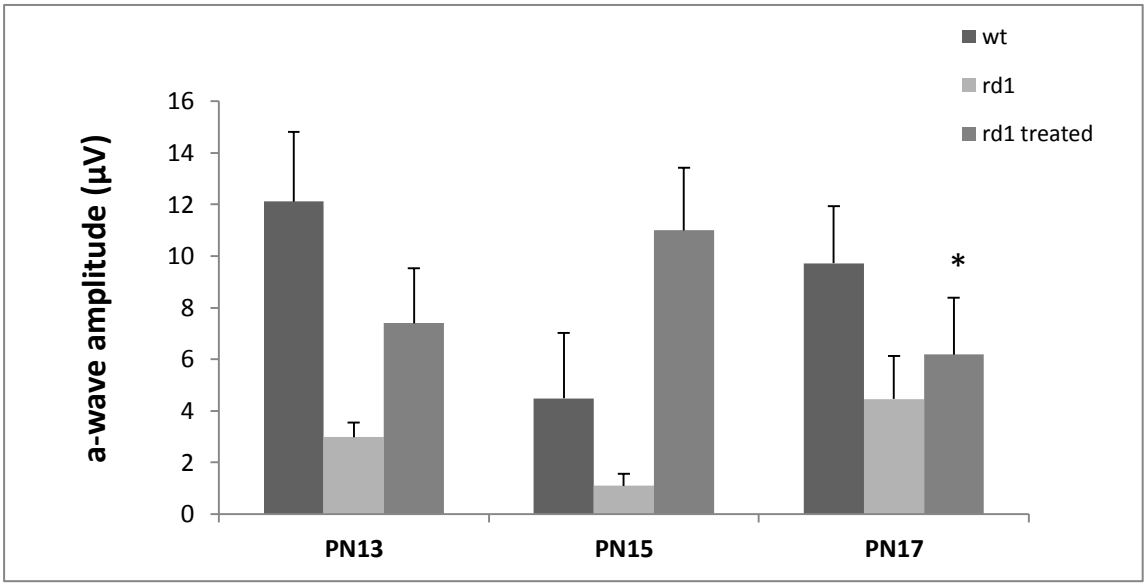

Figure 41. Graphical values of electroretinography (ERG) a-wave amplitude from the different groups studied (wild type (wt), rd1 and treated rd1 mice) at different postnatal (PN) days (PN13, PN15 and PN17). Significant differences were found at PN17. The bars are means of ERG a-wave amplitud. The error bars represent the standard error of the mean (* $p < 0.05$ vs wt).

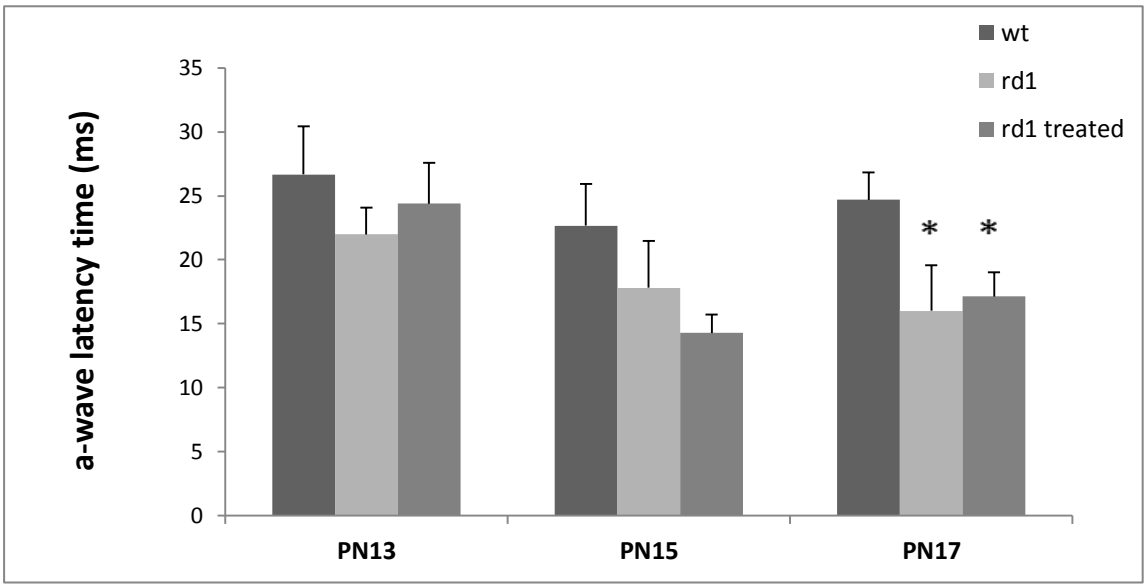

Figure 42. Graphical values of electroretinography (ERG) a-wave latency time from the different groups studied (wild type (wt), rd1 and treated rd1 mice) at different postnatal (PN) days (PN13, PN15 and PN17). Significant differences were found at PN17. The bars are means of ERG a-wave latency. The error bars represent the standard error of the mean (* $p < 0.05$ vs wt).

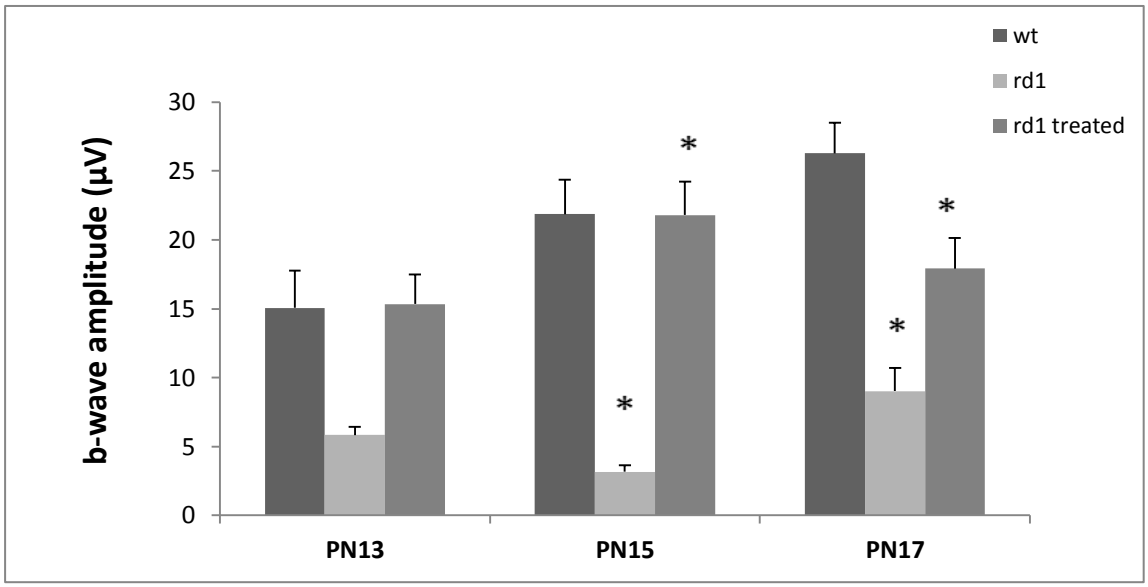

Figure 43. Graphical values of electroretinography (ERG) b-wave amplitude from the different groups studied (wild type (wt), rd1 and treated rd1 mice) at different postnatal (PN) days (PN13, PN15 and PN17). Significant differences were found at PN15 and PN17. The bars are means of ERG b-wave amplitude. The error bars represent the standard error of the mean (* $p < 0.05$ vs wt and treated rd1).

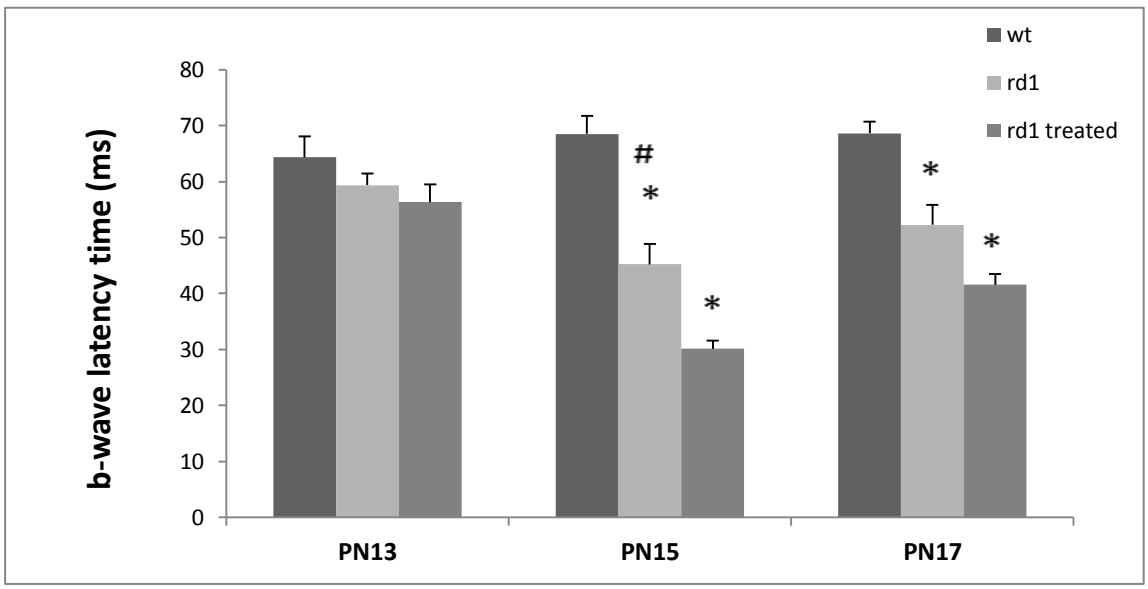

Figure 44. Graphical values of electroretinography (ERG) b-wave latency time from the different groups studied (wild type (wt), rd1 and treated rd1 mice) at different postnatal (PN) days (PN13, PN15 and PN17). Significant differences were found at PN15 and PN17. The bars are means of ERG b-wave latency. The error bars represent the standard error of the mean (* $p < 0.05$ vs wt ; # $p < 0.05$ vs treated rd1).

-DISCUSSION

Retinitis pigmentosa (RP) refers to a group of diseases in which a mutation results in death of rod photoreceptors followed by gradual death of cones. Mutations in different genes have been found to cause RP, and mutations in many more cause widespread rod cell death in association with syndromes that have extraocular manifestations. The enormous genetic heterogeneity among the diseases that constitute RP is a problem for the development of treatments that deal with primary genetic defects (Miranda *et al.,* 2010).

Despite the diversity of retinal degeneration disorders, apoptosis of photoreceptors seems to be a common feature to all (Chang *et al.,* 1993; Dunaief *et al.,* 2002; Carella *et al.,* 2003; Miranda *et al.,* 2010). However, the underlying cell death mechanisms remain unknown and at present there is no treatment available (Kennan *et al.,* 2005; Komeima *et al.,* 2006; 2007; Paquet-Durand *et al.,* 2009; Miranda *et al.,* 2010; Cepko, 2012).

Oxidative stress has been implicated in the pathogenesis of RP (Shen *et al.,* 2005; Komeima *et al.,* 2006; Miranda *et al.,* 2007*;* 2010). One hypothesis is that after rods die, oxygen utilization in the outer retina is reduced; but choroidal vessels, unlike retinal vessels, are incapable of autoregulation to decrease blood and oxygen levels in the outer retina and they become markedly elevated (Yu *et al.,* 2000; 2004; Usui *et al.,* 2009). Because of its high oxygen request and content of unsaturated lipids and its constant exposure to light, retina may be an elective site for oxygen radical production and lipid peroxidation (Yamada *et al.,* 1999; 2001; Liang & Godley, 2003; Okoye *et al.,* 2003).

In the rd1 model, as well as in human forms of RP, cone photoreceptors die even though they do not express the mutated protein. Cone death is thus a secondary event occurring after extensive rod cell degeneration; the mechanism for this phenomenon is not known. Since cone photoreceptor cells are more crucial for human vision than rods, one may thus argue that rescuing the cones should be the primary goal for any RP therapy. Of course, saving the rods could also promote cone survival (Sanz *et al.,* 2007; Miranda *et al.,* 2010).

Furthermore, Leveillard & Sahel reported that rods secrete factors that promote cone survival (Leveillard & Sahel, 2010; Lambard *et al.,* 2010); rods secrete a rod-

derived cone viability factor (RdCVF), which is necessary for sustaining cones (Leveillard *et al.,* 2004; Chalmel *et al.,* 2007). Interestingly the authors identified RdCVF as a truncated form of thioredoxin (Trx) –like a protein. Trx is an oxidation/reduction regulatory protein. The similarities between RdCVF and Trx could indicate that photoreceptors in general are very sensitive to oxidative stress (Sanz *et al.,* 2007) and agree very well with our demonstration here that a treatment with progesterone has a beneficial effect on photoreceptor survival.

1. CHARACTERIZATION OF TEMPORAL GLUTAMATE AND THIOL METABOLISM IN rd1 MICE

The cellular response to stress often involves changes in thiol content, which is first consumed in reactions that protect the cell by removing the deleterious compound and is then replaced through either enzymatic reduction of a disulfide, when that is possible, or by de novo synthesis (Dickinson & Forman, 2002).

Considerable evidence is available showing that thiols have important roles in the regulation of fundamental cellular processes, such as cell proliferation, differentiation, and apoptosis (Samiec *et al.,* 1998; Hall, 1999; Kirlin *et al.,* 1999; Nkabyo *et al.,* 2002; Miranda *et al.,* 2010).

Although previous studies have investigated some aspects of GSH metabolism in RP (Sanz *et al.,* 2007; Lu *et al.,* 2009; Miranda *et al.,* 2010), relatively little is known about possible changes in Cys and glutamate concentrations in RP, as well as changes in disulfides, such as GSSG.

1.1 Glutamate

L-glutamate is the primary excitatory neurotransmitter in the vertebrate central nervous system (CNS) (Michaelis, 1998; Thoreson & Witkovsky, 1999; Reis *et al.,* 2009; Kalivas, 2009). Glutamate participates in many important physiological processes, such as developmental plasticity, long-term potentiation and in development (Scheetz & Constantine-Paton, 1994; Bisti *et al.,* 1998). In the vertebrate retina, glutamate is released from photoreceptors and bipolar cell terminals and exerts its actions by activating postsynaptic ionotropic and/or metabotropic receptors that are expressed in most, if not all, retinal cells (Thoreson & Witkovsky, 1999). The fine tuning of glutamate uptake and degradation in glial cells is essential to avoid neurotoxicity and to allow normal signal transmission between photoreceptors and bipolar cells (Derouiche & Rauen, 1995; Rauen *et al.,* 1998; Harada *et al.,* 1998; Delyfer *et al.,* 2005; Bringmann *et al.,* 2009).

The increase of glutamate concentrations in the retina have been implicated in several diseases such as glaucoma, diabetes mellitus, and retinal detachments (Romano *et al.,* 1995; Ambati *et al.,* 1997; Brooks *et al.,* 1997; Sucher *et al.,* 1997; Lieth

et al., 1998; Dkhissi *et al.,* 1999; Kowluru & Kennedy, 2001; Martin *et al.,* 2002; 2005; Pulido *et al.,* 2007; Mattson, 2008).

Moreover, alterations in glutamate metabolism have been demonstrated in different models of inherited retinal degeneration (Ulshafer *et al.,* 1990; Nakazawa *et al.,* 1993; Noro *et al.,* 1994; Fletcher & Kalloniatis, 1996; Kalloniatis & Tomisich, 1999; Fletcher *et al.,* 2000). In the GUCY1* chick, an increase in glutamate and aspartate was found in the photoreceptor layer of blind chicks (Ulshafer *et al.,* 1990). In the RCS rat an increase in aspartate and glutamine levels was observed (Fletcher & Kalloniatis, 1996; Kalloniatis & Tomisich, 1999; Fletcher *et al.,* 2000). In the rds/rds mouse an accumulation of glutamate has also been observed in photoreceptors (Nakazawa *et al.,* 1993; Noro *et al.,* 1994).

However, other studies, for example, Orr and colleagues, did not observe any differences in glutamate concentrations between dystrophic and wt retinas (Orr *et al.,* 1976).

Our results (see Figure 19) indicate an increase in glutamate concentrations in the retina from rd1 mice compared to those concentrations found in the retinas from wt mice at PN7, PN11, PN15 and PN17. After PN17, glutamate concentrations decrease until PN19, where glutamate concentrations in rd1 and wt mice were similar at PN21 and PN28. These results are in agreement with the results obtained by Delyfer and coworkers, who found an increased in glutamate concentrations in the retinas from rd1 mice compared to retinas from wt mice (Delyfer et al., 2005). However, our results do not coincide in time with the results observed by Delyfer *et al*. as we observed a normalization of glutamate concentrations after PN19, and their study showed a continuous increase in this parameter until PN35. The differences might be explained by the different analytical techniques used.

In the CNS, neurotransmitter activity is crucial to regulate synaptic connectivity (Hollmann & Heinemann, 1994; Gan, 2003). Perturbation in glutamate levels during a critical period of development may therefore account for abnormal synaptogenesis in the rd1 mouse (Blanks *et al.,* 1974; Delyfer *et al.,* 2005). The possibility that glutamate excess may retrogradely affect photoreceptors is further supported by the severe

disruption of photoreceptor outer segments and terminals following intravitreal injections of kainate in the chicken eye (Barrington *et al.,* 1989; Sattayasai *et al.,* 1989).

In photoreceptors, glutamate has been reported to elicit a current generated by a transporter coupled to chloride channels (Picaud *et al.,* 1995a; 1995b) and to activate some metabotropic receptors, and thereby modulating intracellular calcium concentration (Koulen *et al.,* 1999; Marc *et al.,* 2003; Delyfer *et al.,* 2005). It is also known that excessive activation of ionotropic glutamate receptors leads to neuronal cell death (Olney *et al.,* 1974).

1.2 Thiol metabolism

GSH is the most abundant low molecular weight thiol of bacteria, plant and animal cells (Meister, 1988; Aoyama *et al.,* 2008; Forman *et al.,* 2009). GSSG is reduced to GSH by NADPH through the GSH reductase reaction (Dickinson & Forman, 2002; Townsend *et al.,* 2003). Cys is a precursor of GSH, and Cys or CysS can independently affect cell proliferation by altering the available Cys-CysS pool (Nkabyo *et al.,* 2005).

Several researchers postulate that within cells there are three redox systems (Cys/ CysS; GSH/GSSG and Trx-SH/Trx-sS) that are independently controlled and compartmentalized (Filomeni *et al.,* 2002; Circu & Aw, 2008). Cys, CysS, GSH and GSSG comprise the major low-molecular weight thiol/disulfide redox control systems in mammals (Iyer *et al.,* 2009).

The reduction potentials for the redox couples GSH/GSSG and Cys/CysS in plasma are useful indicators of systemic oxidative stress (Navarro *et al.,* 1997 Jonas, 2000; 2002) and other medically relevant physiological states such as Alzheimer's and Parkinson's diseases, diabetes, Cystic fibrosis, and HIV infection (Buhl *et al.,* 1989; Roum *et al.,* 1993; Bains & Shaw, 1997; Samiec *et al.,* 1998; Jiang *et al.,* 2005; Sanz *et al.,* 2010).

Considerable evidence is available that shows that the maintenance of cellular GSH pools protect against oxidant-induced apoptosis (Hall, 1999; Miranda *et al.,* 2010).

Reduced GSH concentration and/or a disproportionate ratio of GSH:GSSG has been associated with a number of neuropathological processes, such as Parkinson´s disease,

Alzheimer´s diseases, RP, diabetes, etc (Perry *et al.,* 1982; Smith *et al.,* 1991; Sotic *et al.,* 1992; Pow *et al.,* 1995; Jiang *et al.,* 2005; Miranda *et al.,* 2010).

Moreover, GSH depletion has been reported to induce apoptosis in certain cell systems (Fernandes & Cotter, 1994; Jenner & Olanow, 1996; Miranda *et al.,* 2010), and an increase in GSSG has been shown to induce directly apoptosis (Franco & Cidlowski, 2009) because caspases, the main players in the execution phase of apoptosis, can suffer S-glutathiolation (Circu & Aw, 2008).

GSH is synthesized from the amino acids Cys, glutamate and glycine. De *novo* synthesis of GSH occurs exclusively in the cytosolic compartment and is initiated by the synthesis of γ-glutamylcysteine from glutamate and Cys by the γ-glutamylcysteine synthetase; glycine is subsequently added by the activity of GSH synthetase (Franco & Cidlowski, 2009).

In our study, GSH concentrations showed a tendency to decrease in the retina from rd1 mice compared to those found in the retina from control mice until PN15 (Figure 20). It is at this point (after the onset of degeneration), when GSH concentrations in rd1 mice start to increase, finding significant differences at PN15, PN17 and PN19 compared to those concentrations found in wt mice. With respect to GSSG concentrations, the increase in rd1 mice just occurs after the onset of degeneration compared to wt mice, with significant differences occurring at PN21 and PN28 (Figure 21).

It could be concluded that the early increase in retinal glutamate amounts in the rd1 retina is a direct consequence of the mutation; that this increase may damage photoreceptors facilitating their death by apoptosis; and that the increase in GSH concentration is also a consequence of the excess of glutamate. Our results show that the increase in retinal GSH concentration in rd1 mice occurs at the same time that the decrease in glutamate (PN17), but occurs too late to minimize the disturbances that glutamate may have caused.

However, whether these changes are due to alterations in its synthesis or the degradation to better explain the biological significance of GSH changes should be more deeply analyzed.

2. EVALUATION OF PROGESTERONE ACTIVITY IN rd1 MICE RETINA

Progesterone is a neurosteroid that has multiple biological functions in the CNS among which is its ability to afford neuroprotection; it is also well documented that it promotes cell survival and proliferation and has palliative and disease regression effects in non-neuronal tissue (Rider & Psychoyos, 1994; Makrigiannakis *et al.,* 2000; Svensson *et al.,* 2000; Olive & Pritts, 2001; Lange, 2008).

There is evidence documenting that the retina is not only another CNS structure targeted by steroids, but also a probable site of neurosteroid production (Lanthier & Patwardhan, 1988; Guarneri et al., 1994; Compagnone et al., 1995; Guarneri et al., 1998; Cascio et al., 2000; Compagnone & Mellon, 2001; Sakamoto et al., 2001; Guarneri et al., 2003; Cascio et al. 2007).

Estrogen and progesterone have significant neuroprotective properties against various neurodegenerative disorders (Tang *et al.,* 1996; Slooter *et al.,* 1999; Waring *et al.,* 1999; Behl, 2002; Zlotnik *et al.,* 2011). Estrogen is reported to influence the architecture and function of the eye (Salyer *et al.,* 2001), the incidence and severity of many ocular diseases (Smith *et al.,* 1997; Snow & Seddon, 2000), and to protect from retinal degenerations (Nonaka *et al*., 2000; Marin-Castaño *et al.,* 2003; Bucolo & Drago, 2004; Yu *et al.,* 2004).

Progesterone treatment has shown neuroprotective beneficial effects in different studies (Stein *et al.,* 2008) conducted by different research groups in rats (Pascual *et al.,* 2002; Leonelli *et al.,* 2007), mice (Gibson & Murphy, 2004; Schumacher *et al.,* 2004), cats (Cervantes *et al.,* 2002) and rabbits (Chavez-Delgado *et al.,* 2003). Schumacher *et al.* reported that progesterone treatment of symptomatic Wobbler mice with motoneuron degeneration reduced neuropathology and up-regulated myelination in Schwann cells and oligodendrocytes in the CNS (Schumacher *et al.,* 2004). Ghoumari *et al*. demonstrated that progesterone treatment increases myelin production, especially in injured animals (Ghoumari *et al.,* 2003).

Similarly, Pascual *et al*. reported that progesterone treatment can reverse the effects of hypoxic injury to the solitary tract nucleus of rats (Pascual *et al.,* 2002). And most recently, Leonelli and coworkers have shown that long-term treatment with progesterone can reduce experimentally induced diabetic neuropathy in rats, a finding

consistent with the hormone's reported effects on myelin production (Leonelli *et al.,* 2007).

However, some studies have failed to show positive effects for progesterone in rats (Azcoitia *et al.,* 1998; Murphy *et al.,* 2000; Toung *et al.,* 2004; Gilmer *et al.,* 2008).

In models of inherited retinal degenerations (rd1), little is known about the effects of steroid sex hormones on disease progression. The literature strongly supports an antioxidant-based therapeutic approach in rd models (Komeima *et al.,* 2007; Usui *et al.,* 2009; Miranda *et al.,* 2010) and in several instances neurotrophic factors attenuate photoreceptor degeneration (Faktorovich *et al.,* 1990; Cayouette *et al.,* 1998; Bok *et al.,* 2002; Doonan *et al.,* 2011).

The present study describes progesterone as possible treatment in retinal degenerative diseases, such as RP.

2.1 Progesterone reduces oxidative stress

Sex steroid hormones regulate the activities of a number of enzymes of the antioxidant defence system (Huh *et al.,* 1994; Pajović *et al.,* 1999; 2002; Azevedo *et al.,* 2001; Pajovic *et al.,* 2003; Ozacmak & Sayan, 2009). Different studies have demonstrated that progesterone can reduce the level of malondialdehyde, a marker of oxidative stress, and prevent cerebral ischemia as well as traumatic brain injury (Roof & Hall, 2000; Morali *et al.,* 2005; Ozacmak & Sayan, 2009). Similarly, increased expression of the antioxidant enzymes superoxide dismutase, GSH peroxidase, and catalase has been reported following progesterone administration after bilateral common carotid artery occlusion (Aggarwal *et al.,* 2008).

It has been demonstrated, in *in vivo* and *in vitro* studies, that progesterone and estrogens are neuroprotective in models of acute neuronal stress and neurodegeneration (Amantea *et al.,* 2005). Estrogens suppress lipid peroxidation induced by amyloid β-peptide and ferrous sulfate (Kii *et al.,* 2005) and attenuate cell death caused by oxidative stress in a hippocampal cell line (Vedder *et al.,* 1999). The mechanism for the anti-oxidative action of estrogens is proposed to reduce the elevation of intracellular Ca^{2+} concentration, which is a major element in the

development of ischemic damage by reactive oxygen species (Behl *et al.,* 1995; Ozacmak & Sayan, 2009).

We have investigated whether progesterone was able to reverse the observed alterations in glutamate and thiol retinal metabolism in the rd1 mice.

2.1.1 Determination of glutamate and thiol metabolism in wt, rd1 and treated rd1 mice

- *Glutamate*

Progesterone (P4) is protective against excitotoxicity in dissociated cultured neurons (Goodman *et al.,* 1996; Ogata *et al.,* 1993; Nilsen & Brinton, 2002; 2003; Atif *et al.,* 2009; Mannella *et al.,* 2009; Luoma *et al.,* 2011; 2012), and has been shown to protect organotypic explants of the cerebral cortex against glutamate-induced cytotoxicity (Jodhka *et al.,* 2009; Liu *et al.,* 2010). Studies from Kaur *et al*. have demonstrated that protection from glutamate toxicity is associated with increased levels of brain-derived neurotrophic factor (BDNF) transcript and protein in cortical neurons using an organotypic slice model (Kaur *et al.,* 2007).

Progesterone protects hippocampal neurons from FeSO4-, amyloid β- as well as glutamate-induced cell death (Goodman *et al.,* 1996; Nilsen & Brinton, 2002; 2003; Kaur *et al.,* 2007; Hwang *et al.,* 2009). Furthermore, progesterone is effective in reducing secondary neuronal loss and the associated cognitive impairment following cortical contusion injury (Roof *et al.,* 1994; Asbury *et al.,* 1998; Hwang *et al.,* 2009).

Our results are in agreement with all these previous observations as we have found that treatment with progesterone significantly reduced retinal glutamate concentrations in the retina from rd1 mice at PN11, PN13, PN15 and PN17 (Figure 23), suggesting some protection against glutamate toxicity.

Excitotoxic death of neurons is a complex process that has been shown to occur through a variety of mechanisms that are not yet fully understood. However, it is thought that release of this neurotransmitter into the extracellular space, leads to the activation of both ionotropic and metabotropic glutamate receptor-mediated signalling pathways. Subsequent intracellular calcium elevations and calcium-dependent signalling pathways induced by this sharp increase in extracellular

glutamate is a major cause of injury-induced neuronal death (Choi, 1985; Tymianski, & Tator, 1996; Bender *et al.,* 2009; Xu *et al.,* 2009; Luoma *et al.,* 2012). Since excitotoxicity often leads to calcium-dependent death and progesterone is neuroprotective, it has been suggested that progesterone may prevents excitotoxic neuronal death by preventing the resulting calcium overload (Szydlowska & Tymianski, 2010; Luoma et al., 2012).

- *Thiol metabolism*

Different studies have shown the relationship between GSH and progesterone. Kangf & Uthus found a depletion of GSH and depression of estrogen and progesterone in female rats plasma after intraperitoneal injection of BSO (Kangf & Uthus, 1996). Ozacmak & Sayan, have shown that combination of 17α estradiol- and progesterone, restored GSH levels in hippocampus in cerebral ischemia in rats (Ozacmak & Sayan, 2009). Faddah *et al.* observed that intramuscular injection of Depo-medroxy progesterone acetate (DMPA) significantly decreased GSH concentrations in humans (Faddah et al., 2005). Similar results were observed by Yu in 1994 (Yu, 1994)

In this sense, in our study, we have found a significant increase in GSH (Figure 25), suggesting some protection against degeneration, may be by activation of GSH synthesis.

2.1.2 GSH Immunohistochemistry

It has been demonstrated that GSH is present in the retina (Hermann & Moses, 1945; Kowluru *et al.,* 1994). In the mammalian retina, it has also been reported that there is no GSH immunoreactivity in outer segments of rod and cone photoreceptors from rodent, rabbit, primate and zebrafish retinas (Pow & Crook, 1995; Schuette & Werner, 1998; Marc & Cameron, 2001), but Müller cells and inner retinal neurons appear to contain substantial pools of this compound (Organisciak *et al.,* 1984; Pow & Crook, 1995; Reichelt *et al.,* 1997b; Schuette & Werner, 1998; Huster *et al.,* 2000).

Interestingly, the staining of GSH and GSSG in the retinal section of different age wt and rd1 mice showed a change in its localization at different ages (Figure 27). At PN11, retinas from wt and rd1 mice exhibit a similar GSH labelling pattern: prominent immunoreactivity was found mainly in INL and OPL; an intense staining was also found

in cells located within the GCL and in some Müller cell processes. With age, the pattern of GSH and GSSG staining in retinas from wt mice changed and the intense of staining gradually decreased in the INL and GCL. Interestingly, at PN17, GSH immunolabelling was almost restricted to the OPL and Müller cells in wt retinas. However, retinal GSH distribution exhibits a different pattern of immunostaining in rd1 mice at PN17, and GSH and GSSG was still observed in the GCL and the INL in addition to the staining observed in the OPL.

Our main objective was to evaluate not only quantitative changes in GSH concentrations in the rd1 retina when compared with wt retinas, but also to study these changes by immunohistochemistry. In this thesis, we could observe that at PN15 (immediately after the peak of cell death) GSH concentration was decreased in rd1 retinas when compared to control retinas and then increased at PN17 and PN19; whereas GSH retinal concentrations were again similar at PN21 and PN28 (Figure 20). GSSG levels in rd1 retinas were similar from PN3 to PN19, and then significantly increased at PN21 and PN28, which is considered to be the terminal stage of rod degeneration (Figure 21).

It is well known that under normal conditions, retinal GSH is almost exclusively confined to Müller cells, astrocytes, and horizontal cells, and that under conditions associated with oxidative stress, GSH is rapidly released from Müller cells and provided to neurons (Pow & Crook, 1995; Schütte & Werner, 1998; Bringmann et al., 2009). Progesterone-treated rd1 mice exhibited the same pattern of GSH inmunostainig observed in the retinas from wt mice (Figure 27).

2.1.3 GAD-65 Immunohistochemistry

Excellent reviews of glutamate's actions in the vertebrate retina have been published (Massey, 1990; Wilson, 1994; Massey & Maguire, 1995; Thoreson & Witkovsky, 1998; Bringmann *et al.,* 2009). Glutamate toxicity has been demonstrated both on inner retinal cells and on photoreceptor terminals (Lucas & Newhouse, 1957; Olney, 1969; 1982; Yazulla & Kleinschmidt, 1980; Ingham & Morgan, 1983; Sattayasai & Ehrlich, 1987; Sattayasai *et al.,* 1989; Sahel *et al.,* 1991) and in ganglion cells (Vorwerk *et al.,* 1996; Sucher *et al.,* 1997).

Gamma-aminobutyric acid (GABA) is synthesized by two isoenzymes of glutamic acid decarboxylase, GAD65 and GAD67 (Ricci *et al.,* 2005), which differ in their contributions to the synaptic and non-synaptic pools of GABA (Soghomonian & Martin, 1998; Sheikh *et al.,* 1999; Souza *et al.,* 2009). While GAD65 is highly enriched in the nerve terminals and is believed to regulate vesicular GABA synthesis, GAD67 is found throughout the GABAergic neurons and may be involved in the synthesis of cytosolic or "metabolic" GABA (Sheikh *et al.,* 1999). Most studies describe GABA- and GAD-immunoreactive in retinal horizontal cells in some mammals: rabbit, guinea pig, opossum, cat and monkey (Mosinger et al., 1986; Osborne *et al.,* 1986; Agardh *et al.,* 1987; Mosinger & Yazulla, 1987; Chun & Wässle, 1989; Pourcho & Owczarzak, 1989; Wässle & Chun, 1989; Grünert & Wässle, 1990; Vardi *et al.,* 1994; Johnson & Vardi, 1998; Marc *et al.,* 1998; Calaza *et al.,* 2006; Guo *et al.,* 2010). In contrast, there have been failures to consistently demonstrate either GABA or GAD-immunoreactivity in horizontal cells of both adult mouse and rat retinas. In these species, GABA and GAD-immunoreactivities are transiently expressed by horizontal cells during the first several weeks of postnatal retinal development, and immunoreactivity is consistently reported to be absent in adult retinas (Vaughn *et al.,* 1981; Lin *et al.,* 1983; Schnitzer & Rusoff, 1984; Versaux-Botteri *et al.,* 1989; Fletcher & Kalloniatis, 1997; Dkhissi *et al.,* 2001). In our results, GAD65-immunoreactive profile was observed in the OPL, INL, IPL and GCLs, and with age (PN15 and PN17), the pattern of GAD-65 staining seems to change; the intensity of staining increased in the IPL in the retina from wt, rd1 and progesterone-treated rd1 mice (Figure 28).

2.2 Progesterone delays rod and cone cell death in rd1 mice

With regard to hormone therapy, some studies have demonstrated some protection of different hormones in many ocular diseases. Rex *et al.* found that the glycoprotein hormone erythropoietin afforded substantial protection from light damage but had no beneficial effects in the rd10 model (Rex *et al.,* 2004). Corrochano *et al.* observed a marked preservation of photoreceptor cell number and function in the rd10 mouse retina in response to pro-insulin (Corrochano *et al.,* 2008), indicating the potential for endocrine-induced neuroprotection. Doonan et al. have shown that

norgestrel significantly preserves the photoreceptor cell number and morphology in the same mouse model: the rd10 mouse (Doonan *et al.,* 2011).

Consistent with this, and knowing that in the rd1 mouse the photoreceptor cell death occurs in a characteristic topographical pattern starting in the posterior retina and spreading anteriorly and further posteriorly toward the optic nerve, herein we have observed significant differences in the number of rows in the ONL at PN13, PN15 and PN17 in the three areas of the retina studied: far peripheral, mid peripheral and central retina in the progesterone-treated rd1 mice when compared with the retinas from wt mice and untreated rd1 mice. We have shown that oral administration of 100 mg/kg body/weight of progesterone every 2 days, starting at PN7 significantly preserves the photoreceptor cell number in the rd1 mouse model (Figure 31, Figure 32, Figure 33 and Figure 34).

We have observed that in the peripheral retina, progesterone protection was more effective than in the central retina at PN15 and PN17, possibly because this area degenerates more slowly in this model.

2.3 Progesterone treatment reduces apoptosis in the rd1 model

Apoptosis, or programmed cell death, is an important form of cell death involving the activation of an endogenous cell suicide programme by either intrinsic or extrinsic stimuli (Carmody *et al.,* 1999; Drack *et al.,* 2012). Both embryogenesis and tumorigenesis depend, in part, on a skewing of the balance between apoptosis and maintenance. Apoptosis can be a biological defence to rid the body of unwanted or unneeded cells, and in normal retinal development, considerable apoptotic pruning occurs after the first post-natal week; however, disorders of apoptosis can also cause disease (Drack *et al.,* 2012).

Despite the diversity of retinal degeneration disorders, apoptosis of photoreceptors seems to be a common feature to all (Chang *et al.,* 1993; Dunaief *et al.,* 2002; Carella, 2003). RP develops as a result of defects in genes responsible for upholding the structural and/or functional integrity of photoreceptors.

Steroid hormones play a regulatory role in a variety of cellular processes such as reproduction, development, differentiation, apoptosis and brain function (Tsai & O'Malley, 1994). Progesterone has multiple biological functions in the CNS, among which is its ability to afford neuroprotection; it is well documented to promote cell survival and proliferation and to have palliative and disease regression effects in non-neuronal tissue (Rider & Psychoyos, 1994; Makrigiannakis *et al.,* 2000; Svensson *et al.,* 2000; Olive & Pritts, 2001; Lange, 2008). Progesterone has the ability to attenuate neuronal apoptosis associated with CNS damage (Gonzalez *et al.,* 2009; Shahrokhi *et al.,* 2010), to ameliorate the effects of stroke (Betz & Coester, 1990) and neurodegenerative conditions (Frye & Walf, 2009; Doonan *et al.,* 2011).

In this sense we have found a very substantial reduction in retinal cell death in progesterone-treated rd1 mice compared to untreated rd1 mice in the different retinal areas studied (far peripheral, mid peripheral and central retina) at PN11, PN13, P15 and PN17 (Figure 35, Figure 36, Figure 37, Figure 39 and Table 4).

Work by Doonan and colleagues (Doonan *et al.,* 2011), demonstrated that intraperitoneal injection of 100 mg/kg norgestrel on alternate days (a synthetic progestin) induced preservation of photoreceptors in rd10 mice on alternate days from PN18 to PN35 (Doonan *et al.,* 2011). However, in contrast, several retinal studies have failed to show beneficial effects of progesterone. Nakazawa *et al.* found that it did not afford protection in a model of retinal ganglion cell injury (Nakazawa *et al.,* 2006); O'Steen and colleagues, failed to demonstrate the protective effects of progesterone against light stress (O'Steen, 1977) and Kaldi and Berta, did not find improvement in retinal function or photoreceptor cell loss with the administration of progesterone for 4 days prior to a 24-h light induction (Kaldi & Berta, 2004).

However, our observations suggest that oral treatment with 100 mg/kg of progesterone substantially preserves the photoreceptor in rd1 mouse model.

2.4 Progesterone reduces gliosis in the rd1 mice model

Gliosis is the response of glial cells within neuronal tissue, including the retina, to any insult. It is characterized by the activation of glial cells, but the final outcome may

be beneficial or detrimental to the surrounding tissue depending on the time scale involved (Bringmann *et al.,* 2006; 2009).

The recruitment of glia to damaged regions occurs in almost every pathological condition in the CNS (Kim & de Vellis, 2005; Wirenfeldt *et al.,* 2011; Rutar *et al.,* 2012) and is apparent in a range of prominent human retinal pathologies, including age-related macular degeneration (AMD) (Penfold *et al.,* 1986; Wong *et al.,* 2001; Gupta *et al.,* 2003; Ezzat *et al.,* 2008; Cherepanoff *et al.,* 2009), RP (Gupta *et al.,* 2003), late-onset retinal degeneration (Gupta *et al.,* 2003), retinal detachment (Lewis *et al.,* 2005), glaucoma (Vrabec, 1975; Neufeld, 1999; Yuan & Neufeld, 2001), and diabetic retinopathy (Vrabec, 1975; Zeng *et al.,* 2008), as well as in many experimental models of retinal degeneration (Langmann, 2007; Rutar *et al.,* 2012).

Müller cells are the predominant type of glial cells in the vertebrate retina and its cell bodies are localized in the middle of the INL. In contrast to glial cells in other regions of the CNS, Müller cells account for only 2-5% of the total retinal cell population (Young, 1985; Jeon *et al.,* 1998).

Müller cells respond to insults to the retina through a process referred to as gliosis (Bringmann & Reichenbach, 2001; Bringmann *et al.,* 2006). The function of gliosis is poorly understood (Roesch *et al.,* 2012). Even fundamental aspects, such as whether gliosis is protective or destructive, are not understood (Bringmann *et al.,* 2006; Nakazawa *et al.,* 2007). The most well-known aspect of the glial response is that Müller cells upregulate the intermediate filament protein, GFAP, a protein considered to be a marker for reactive gliosis, not only in the retina but also across the entire CNS (Bringmann & Reichenbach, 2001; Pekny & Nilsson, 2005). In the retina, Müller cells maintain the microenvironment and support photoreceptor cell function. Usually, astrocytes and microglia in the healthy retina are confined to the innermost retinal layers (nerve fiber layer, GCL, IPL) and they alter their characteristics to become reactive glial cells and express GFAP in their cell bodies when pathologic events occur (Peterson *et al.,* 2000; Lewis & Fisher, 2003; Kurihara *et al.,* 2006; Sasaki *et al.,* 2009).

In our study, we have observed an increase in GFAP expression in the retina of the untreated rd1 mice at PN11, PN13, PN15 and PN17 compared to GFAP expression

found in the retina from wt mice (Figure 39). The induction of GFAP in progesterone-treated rd1 mice was partially or slightly inhibited.

2.5 Progesterone improves function in a model of retinitis pigmentosa: rd1

ERG has been used as a reliable diagnostic tool for the evaluation of retinal function in RP and other ocular diseases (Gouras & Carr, 1964; Krill, 1972; Narfström *et al.,* 2012; Jae *et al.,* 2013). Essentially, when a surface electrode is placed on the front of the eyeball and a light is shone through the pupil, distinct electrical potentials are recorded that reflect the electrical activity in the retina in response to photic stimulation (Armington & Bloom, 1974). The ERG is largely composed of a negative-going a-wave generated by rod photocurrents (Penn & Hagins, 1969), and a positive-going b-wave arising from bipolar cell or Müller cell activity (Miller & Dowling, 1970; Stockton & Slaughter, 1989; Xu & Karwoski, 1994; Robson & Frishman, 1995; Shiells & Falk, 1999; Jeong *et al.,* 2010; Jae *et al.,* 2013).

ERGs at PN13, PN15 and PN17 were recordable, showing that the rescued rod photoreceptors were able to function producing visual responses in progesterone-treated rd1 mice (Figure 40, Figure 41 and Figure 43). However, the electrical response of the rescued photoreceptors from treated rd1 mice was lower than that of photoreceptors of wt mice (Figure 40).

Therefore, the oral administration of progesterone in the early phases of RP under the conditions used in the present study had a direct and positive effect on photoreceptors, rescuing them structurally and functionally in rd1 mice.

While this treatment does not seem to entirely inhibit the pathological course of the mutation, it clearly slows down its progression. Therefore, we believe that the rd1 degenerative processes causes oxidative stress, which exacerbates the situation. Progesterone treatment would then help by alleviating the composite effects of oxidative stress, thereby reducing the rate of cell death. Photoreceptor degeneration in the rd1 retina is very rapid, and is essentially complete in about 5 days. Taking this into account, every day that the rod cell death is delayed would suggests at least a 20% reduction due to progesterone treatment compared with the normal degeneration period. By extrapolation to the human situation, in which the degeneration can take

many years, this could mean that loss of vision by such treatment could be delayed by several years, extending quality of life and giving time for other and more effective treatments to be developed.

In summary, our results demonstrate that oral administration of 100 mg/kg body/weight of progesterone appears to act on multiple levels to interrupt or delay destructive processes.

-CONCLUSIONS

1. There is an alteration in glutamate, GSH and GSSG concentrations in the retina of rd1 mice at different post-natal days (PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28).

1.1 Glutamate concentration is increased in the retina from rd1 mice compared to the glutamate concentration found in the retina from wt mice at PN7, PN11, PN15 and PN17. However, glutamate concentrations in rd1 mice starts to decrease at PN17, which is comparable with glutamate concentrations in the retina from wt mice at PN21 and PN28.

1.2 GSH concentration is decreased in the retina of rd1 mice compared to the concentration found in the retina from wt mice at PN11 and PN15. Immediately after the peak of cell death, GSH concentration in rd1 mice is increased at PN17 compared to GSH concentration from wt mice. However, GSH concentrations in rd1 mice start to decrease at PN17, which is comparable with GSH concentrations in the retina from wt mice at PN28.

1.3 GSSG concentration is increased in the retina of rd1 mice compared to that concentration found in the retina from wt mice at PN17, PN19, PN21 and PN28.

1.4 There were no changes in Cys concentrations in the retina from rd1 mice compared to those concentrations found in the retina from wt mice.

2. Progesterone treatment delays cell death in an experimental model of RP, the rd1 mouse.

2.1 Progesterone treatment is able to restore the alterations in glutamate concentrations and thiol metabolism observed in the retina of rd1 mice.

2.2 Progesterone oral treatment is able to transiently protect photoreceptors from death in the retina of rd1 mice.

2.3 Progesterone treatment is able to prevent the characteristic gliosis observed in the retina of the rd1 mouse model.

2.4 Oral progesterone treatment is able to improve retinal functionality in rd1 mice.

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-ANNEX

Table 5. Glutamate concentrations at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 in rd1 and wild type mice retina (* p< 0.05 vs rd1 and wt).

Glutamate (nmol/mgprot)

(PN) day	*wt*	*rd1*
PN3	290,460 ± 28,919*	197,693 ± 25,906
PN7	176,373 ± 19,551	285,858 ± 33,362*
PN11	166,9815 ± 32,318	387,112 ± 23,784*
PN15	83,351 ± 5,016	252,620 ± 19,868*
PN17	155,809 ± 13,311	253,461 ± 54,843*
PN19	181,396 ± 13,607	133,894 ± 6,780
PN21	144,338 ± 5,117	165,894 ± 12,064
PN28	150,990 ± 21,140	139,866 ± 15,295

Table 5.1. Glutamate concentrations at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 in the retina from wild type and rd1 mice. Pairwise comparisons.

Strain	*Age*	*Age*	*P*
wt	3,00	7,00	**,001***
		11,00	**,001***
		15,00	**,000***
		17,00	**,000***
		19,00	**,006***
		21,00	**,001***
		28,00	**,000***
	7,00	3,00	**,001***
		11,00	1,000
		15,00	**,022***
		17,00	1,000
		19,00	1,000
		21,00	1,000
		28,00	1,000
	11,00	3,00	**,001***
		7,00	1,000
		15,00	,018
		17,00	1,000
		19,00	1,000
		21,00	1,000
		28,00	1,000
	15,00	3,00	**,000***
		7,00	**,022***
		11,00	,018
		17,00	,340

		19,00	**,028***
		21,00	1,000
		28,00	,840
	17,00	3,00	**,000***
		7,00	1,000
		11,00	1,000
		15,00	,340
		19,00	1,000
		21,00	1,000
		28,00	1,000
	19,00	3,00	**,006***
		7,00	1,000
		11,00	1,000
		15,00	**,028***
		17,00	1,000
		21,00	1,000
		28,00	1,000
	21,00	3,00	**,001***
		7,00	1,000
		11,00	1,000
		15,00	1,000
		17,00	1,000
		19,00	1,000
		28,00	1,000
	28,00	3,00	**,000***
		7,00	1,000
		11,00	1,000
		15,00	,840
		17,00	1,000
		19,00	1,000
		21,00	1,000
rd1	3,00	7,00	,235
		11,00	**,000***
		15,00	1,000
		17,00	1,000
		19,00	1,000
		21,00	1,000
		28,00	1,000
	7,00	3,00	,235
		11,00	**,028***
		15,00	1,000
		17,00	1,000
		19,00	**,000***
		21,00	**,001***
		28,00	**,000***
	11,00	3,00	**,000***
		7,00	**,028***

		15,00	**,001***
		17,00	**,002***
		19,00	**,000***
		21,00	**,000***
		28,00	**,000***
	15,00	3,00	1,000
		7,00	1,000
		11,00	**,001***
		17,00	1,000
		19,00	**,006***
		21,00	,114
		28,00	**,013***
	17,00	3,00	1,000
		7,00	1,000
		11,00	**,002***
		15,00	1,000
		19,00	**,009***
		21,00	,151
		28,00	**,018***
	19,00	3,00	1,000
		7,00	**,000***
		11,00	**,000***
		15,00	**,006***
		17,00	**,009***
		21,00	1,000
		28,00	1,000
	21,00	3,00	1,000
		7,00	**,001***
		11,00	**,000***
		15,00	,114
		17,00	,151
		19,00	1,000
		28,00	1,000
	28,00	3,00	1,000
		7,00	**,000***
		11,00	**,000***
		15,00	**,013***
		17,00	**,018***
		19,00	1,000
		21,00	1,000

Table 6. Glutathione (GSH) concentrations in the retina from rd1 and wild type mice at different post-natal days (PN3, PN7, PN11, PN13, PN15, PN17, PN19, PN21 and PN28) (* p< 0.05 vs rd1 and wt).

Glutathione (nmol/mgprot)

(PN) day	*wt*	*rd1*
PN3	35,309 ± 2,223	39,820 ± 5,254
PN7	39,529 ± 3,621	39,168 ± 4,910
PN11	33,545 ± 3,152	29,106 ± 3,343
PN15	23,802 ± 2,522*	14,456 ± 1,257
PN17	25,359 ± 2,050	42,980 ± 5,153*
PN19	15,817 ± 1,828	33,773 ± 5,771*
PN21	17,616 ± 1,276	24,471 ± 3,286
PN28	19,869 ± 2,022	20,349 ± 2,338

Table 6.1. GSH concentrations at PN3, PN7, PN11, PN13, PN15, PN17, PN19, PN21 and PN28 in the retina from rd1 and wild type mice. Pairwise comparisons.

Strain	*Age*	*Age*	*P*
wt	3,00	7,00	1,000
		11,00	1,000
		15,00	,795
		17,00	1,000
		19,00	**,014***
		21,00	**,026***
		28,00	,353
	7,00	3,00	1,000
		11,00	1,000
		15,00	**,003***
		17,00	,084
		19,00	**,000***
		21,00	**,000***
		28,00	**,004***
	11,00	3,00	1,000
		7,00	1,000
		15,00	1,000
		17,00	1,000
		19,00	**,020***
		21,00	**,037***
		28,00	,553
	15,00	3,00	,795

			7,00	,003*
			11,00	1,000
			17,00	1,000
			19,00	1,000
			21,00	1,000
			28,00	1,000
		17,00	3,00	1,000
			7,00	,084
			11,00	1,000
			15,00	1,000
			19,00	1,000
			21,00	1,000
			28,00	1,000
		19,00	3,00	**,014***
			7,00	**,000***
			11,00	**,020***
			15,00	1,000
			17,00	1,000
			21,00	1,000
			28,00	1,000
		21,00	3,00	**,026***
			7,00	**,000***
			11,00	**,037***
			15,00	1,000
			17,00	1,000
			19,00	1,000
			28,00	1,000
		28,00	3,00	,353
			7,00	**,004***
			11,00	,553
			15,00	1,000
			17,00	1,000
			19,00	1,000
			21,00	1,000
	rd1	3,00	7,00	1,000
			11,00	,828
			15,00	**,000***
			17,00	1,000
			19,00	1,000
			21,00	,093
			28,00	**,008***
		7,00	3,00	1,000
			11,00	1,000
			15,00	**,000***
			17,00	1,000
			19,00	1,000
			21,00	,176

		28,00	**,017***
	11,00	3,00	,828
		7,00	1,000
		15,00	**,031***
		17,00	,109
		19,00	1,000
		21,00	1,000
		28,00	1,000
	15,00	3,00	**,000***
		7,00	**,000***
		11,00	**,031***
		17,00	**,000***
		19,00	**,002***
		21,00	,987
		28,00	1,000
	17,00	3,00	1,000
		7,00	1,000
		11,00	,109
		15,00	**,000***
		19,00	1,000
		21,00	**,008***
		28,00	**,000***
	19,00	3,00	1,000
		7,00	1,000
		11,00	1,000
		15,00	**,002***
		17,00	1,000
		21,00	1,000
		28,00	,191
	21,00	3,00	,093
		7,00	,176
		11,00	1,000
		15,00	,987
		17,00	**,008***
		19,00	1,000
		28,00	1,000
	28,00	3,00	**,008***
		7,00	**,017***
		11,00	1,000
		15,00	1,000
		17,00	**,000***
		19,00	,191
		21,00	1,000

Table 7. Glutathione disulfide (GSSG) concentrations at PN3, PN7, PN11, PN13, PN15, PN17, PN19, PN21 and PN28 in the retina from wild type and rd1 mice (* p< 0.05 vs wt).

Glutathione disulfide (nmol/mgprot)

(PN) day	*wt*	*rd1*
PN3	4,956 ± 0,132	5,887 ± 0,377
PN7	4,886 ± 0,216	2,841 ± 0,271
PN11	3,216 ± 0,154	3,490 ± 0,330
PN15	3,902 ± 0,441	3,110 ± 0,670
PN17	3,802 ± 0,535	6,131 ± 0,509
PN19	3,956 ± 0,1593	6,270 ± 0,606
PN21	2,262 ± 0,437	5,840 ± 0,285*
PN28	1,442 ± 0,569	11,217 ± 1,828*

Table 7.1. GSSG concentrations in the retina from rd1 and wild type mice at different post-natal days (PN3, PN7, PN11, PN13, PN15, PN17, PN19, PN21 and PN28). Pairwise comparisons.

Strain	*Age*	*Age*	*P*
wt	3,00	7,00	1,000
		11,00	1,000
		15,00	1,000
		17,00	1,000
		19,00	1,000
		21,00	,830
		28,00	,135
	7,00	3,00	1,000
		11,00	1,000
		15,00	1,000
		17,00	1,000
		19,00	1,000
		21,00	,963
		28,00	,159
	11,00	3,00	1,000
		7,00	1,000
		15,00	1,000
		17,00	1,000
		19,00	1,000
		21,00	1,000
		28,00	1,000
	15,00	3,00	1,000

			7,00	1,000
			11,00	1,000
			17,00	1,000
			19,00	1,000
			21,00	1,000
			28,00	1,000
		17,00	3,00	1,000
			7,00	1,000
			11,00	1,000
			15,00	1,000
			19,00	1,000
			21,00	1,000
			28,00	1,000
		19,00	3,00	1,000
			7,00	1,000
			11,00	1,000
			15,00	1,000
			17,00	1,000
			21,00	1,000
			28,00	1,000
		21,00	3,00	,830
			7,00	,963
			11,00	1,000
			15,00	1,000
			17,00	1,000
			19,00	1,000
			28,00	1,000
		28,00	3,00	,135
			7,00	,159
			11,00	1,000
			15,00	1,000
			17,00	1,000
			19,00	1,000
			21,00	1,000
rd1		3,00	7,00	,393
			11,00	1,000
			15,00	,448
			17,00	1,000
			19,00	1,000
			21,00	1,000
			28,00	1,000
		7,00	3,00	,393
			11,00	1,000
			15,00	1,000
			17,00	,228
			19,00	,165
			21,00	,549

		28,00	**,000***
	11,00	3,00	1,000
		7,00	1,000
		15,00	1,000
		17,00	,733
		19,00	,544
		21,00	1,000
		28,00	**,000***
	15,00	3,00	,448
		7,00	1,000
		11,00	1,000
		17,00	,251
		19,00	,178
		21,00	,644
		28,00	**,000***
	17,00	3,00	1,000
		7,00	,228
		11,00	,733
		15,00	,251
		19,00	1,000
		21,00	1,000
		28,00	**,001***
	19,00	3,00	1,000
		7,00	,165
		11,00	,544
		15,00	,178
		17,00	1,000
		21,00	1,000
		28,00	**,002***
	21,00	3,00	1,000
		7,00	,549
		11,00	1,000
		15,00	,644
		17,00	1,000
		19,00	1,000
		28,00	**,001***
		3,00	**,000***
	28,00	7,00	**,000***
		11,00	**,000***
		15,00	**,000***
		17,00	**,000***
		19,00	**,002***
		21,00	**,001***

Table 8. Cysteine (Cys) concentrations at PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28 in the retina from rd1 and wild type mice.

Cysteine (nmol/mgprot)

(PN) day	*wt*	*rd1*
PN3	64,511 ± 5,741	52,280 ± 4,720
PN7	52,807 ± 4,372	56,431 ± 4,780
PN11	75,270 ± 4,325	69,074 ± 9,694
PN15	51,321 ± 4,273	67,164 ± 6,651
PN17	76,711 ± 9,193	62,369 ± 3,214
PN19	66,557 ± 4,852	43,779 ± 2,595
PN21	37,998 ± 4,077	36,029 ± 2,638
PN28	31,330 ± 5,287	36,749 ± 2,745

Table 8.1. Cysteine concentrations in the retina from wild type and rd1 mice at different post-natal days (PN3, PN7, PN11, PN15, PN17, PN19, PN21 and PN28). Pairwise comparisons.

Strain	*Age*	*Age*	*P*
wt	3,00	7,00	,991
		11,00	,600
		15,00	1,000
		17,00	,544
		19,00	,998
		21,00	**,015***
		28,00	**,011***
	7,00	3,00	,991
		11,00	,108
		15,00	,990
		17,00	,087
		19,00	1,000
		21,00	,116
		28,00	,081
	11,00	3,00	,600
		7,00	,108
		15,00	,633
		17,00	1,000
		19,00	,181
		21,00	**,000***
		28,00	**,000***
	15,00	3,00	1,000
		7,00	,990

		11,00	,633
		17,00	,578
		19,00	,998
		21,00	**,016***
		28,00	**,012***
	17,00	3,00	,544
		7,00	,087
		11,00	1,000
		15,00	,578
		19,00	,150
		21,00	**,000***
		28,00	**,000***
	19,00	3,00	,998
		7,00	1,000
		11,00	,181
		15,00	,998
		17,00	,150
		21,00	,086
		28,00	,060
	21,00	3,00	**,015***
		7,00	,177
		11,00	**,000***
		15,00	**,016***
		17,00	**,000***
		19,00	,086
		28,00	1,000
	28,00	3,00	**,011***
		7,00	,081
		11,00	**,000***
		15,00	**,012***
		17,00	**,000***
		19,00	,060
		21,00	1,000

Table 9. Means ± error standard of number of rows at PN11, PN13, PN15 and PN17 in far peripheral, mid peripheral and central retina in the ONL of wild type, rd1 and progesterone-treated rd1 mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

-Nº of rows in the ONL

Far Peripheral	**PN11**	**PN13**	**PN15**	**PN17**
Wt	11,972± 0,260	11,667± 0,167	12,917± 0,186	9,944± 0,250
rd1	12,389± 0,159	10,111± 0, 419*#	6,306± 0,18*#	4,306± 0,225*#
rd1 treated	12,528± 0,169	11,056± 0,281*#	9,667± 0,648*#	4,708± 0,269*#

Mid Peripheral	**PN11**	**PN13**	**PN15**	**PN17**
Wt	12,611± 0,310	12,611± 0,164	13,25± 0,412	11,194± 0,161
rd1	12,583± 0,268	9,444± 0,391*#	5,403± 0,102*#	3,139± 0,668*#
rd1 treated	12,083± 0,191	10,166± 0,281*#	7,056± 0,166*#	3,361± 0,144*#

Central retina	**PN11**	**PN13**	**PN15**	**PN17**
Wt	13,25± 0,294	13,139± 0,117	13,417± 0,297	11,361± 0,145
rd1	12,861± 0,159	8,944± 0, 419*	4,806± 0,180*#	2,459± 0,225*#
rd1 treated	12,138± 0,199	9,194± 0,371*	5,986± 0,744*#	2,736± 0,097*#

Table 10. Means ± error standard of number of TUNEL positive cells at PN11, PN13, PN15 and PN17 in far peripheral, mid peripheral and central retina in the ONL of wild type, rd1 and progesterone-treated rd1 mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

-% POSITIVE TUNEL CELLS/AUA

Far Peripheral	**PN11**	**PN13**	**PN15**	**PN17**
wt	2,50E-05± 7,9E-06	2,15E-05 ± 6,99E-06	2,63E-05 ± 4,87E-06	1,23E-05 ± 5,23E-06
rd1	3,5E-03± 2,2E-04*#	3,6E-03± 1,04E-04*#	3,56E-03 ± 2,2E-04*#	2,6E-03 ± 1,6E-04*#
rd1 treated	9,81E-04± 1,8E-04*	2,35E-03 ±8,04E-05*	1,93E-03 ± 1,04E-04*	1,65E-03 ± 1,7E-04*

Mid Peripheral	**PN11**	**PN13**	**PN15**	**PN17**
wt	3,1E-05± 1,2E-05	1,7E-05± 6,8E-06	4,9E-05± 2,6E-05	2,9E-05± 1,7E-05
rd1	4,6E-03±9,7E-04*#	4,8E-03 ±4,9E-04*#	3,2E-03 ± 5,8E-04*#	2,5E-03 ± 1,3E-04*#
rd1 treated	1,4E-03 ±7,07E-04*	2,5E-03 ± 2,9E-04*	1,94E-03 ± 4,55E-04*	1,6E-03 ± 5,3E-04*

Central retina	**PN11**	**PN13**	**PN15**	**PN17**
wt	*3,2E-05 ± 1,03E-05*	*2,77E-05 ± 6,3E-06*	*3,40E-05 ± 1,51E-05*	*2,29E-05 ± 2,99E-06*
rd1	*4,23E-03 ± 3,7E-04*#*	*5,27E-03 ± 2,19E-04*#*	*3,13E-03 ± 2,62E-04*#*	*2,40E-03 ± 2,13E-04*#*
rd1 treated	*1,56E-03 ± 392E-04**	*2,32E-03 ± 2,9E-04**	*1,87E-03 ± 1,85E-04**	*1,36E-03 ± 6,13E-05**

Table 11. a- and b-waves amplitude (µV) and a- and b- latency (ms) values of groups studied (wild type, rd1 and progesterone-treated rd1 mice) at different post-natal days (PN13, PN15 and PN17) (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

Group	***a-wave***		***b-wave***	
	Amplitude (µV)	***Latency (ms)***	***Amplitude (µV)***	***Latency (ms)***
wt PN13	19,27 ± 2,7	25,86 ± 3,75	36,36 ± 3,47	65 ± 7,2
rd1 PN13	17,63 ± 0,56	22 ± 2,08	33,17 ± 1,69	59,33 ± 1,76
rd1 treated PN13	14,17 ± 2,13	24,4 ± 3,18	21,68 ± 4,19	56,3 ± 4,59
wt PN15	15,28 ± 2,53	22,75 ± 3,26	63,93 ± 8,92	72,25 ± 5,53
rd1 PN15	7,01 ± 0,47	17,8 ± 3,67	9,62 ± 0,82*	45,2 ± 5,49*
rd1 treated PN15	11,60 ± 2,41	14,29 ± 1,46	19,67 ± 4,74*	30,14 ± 2,66*#
wt PN17	21,98 ± 2,22	23,88 ± 2,11	56,81 ± 6,53	67 ± 4,11
rd1 PN17	4,30 ± 1,67*	16 ± 3,56*	15,58 ± 3,42*	52,25 ± 9,13*
rd1 treated PN17	12,28 ± 2,21	17,14 ± 1,88*	21,59 ± 3,85*	41,57 ± 3,41*

Table 12. Glutamate concentrations at PN11, PN13, PN15 and PN17 in the retinas from wild type, rd1 and progesterone-treated rd1 mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

Glutamate	*PN11*	*PN13*	*PN15*	*PN17*
Wt	*166,981 ± 27,31*	*162,661 ± 27,57*	*83,351 ± 5,69*	*155,809 ± 9,92*
rd1	*387,112 ± 28,31*#*	*177,140 ± 17,08#*	*252,620 ± 20,62*#*	*203,53 ± 17,8*#*
rd1 treated	*114,871 ± 18,42*	*109,720 ± 8,18*	*103, 401 ± 3,9**	*106,942 ± 3,03**

Table 13. Cysteine (Cys) concentrations at different post-natal days (PN11, PN13, PN15 and PN17) in the retina from wild type, rd1 and progesterone-treated rd1 mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

Cysteine	*PN11*	*PN13*	*PN15*	*PN17*
Wt	*75,270 ± 4,32*	*60,695 ±3,96*	*51,321 ± 4,27*	*76,711 ± 9,19*
rd1	*69,074 ± 9,69*	*71,388 ± 4,69#*	*67,164 ± 6,65*	*62,369 ± 3,21*
rd1 treated	*61,993 ± 16,76*	*30,650 ± 2,99**	*51,680 ± 5,75*	*69,221 ± 4,73*

Table 14. Glutathione (GSH) concentrations at different post natal days (PN11, PN13, PN15 and PN17) in the retina from wild type, rd1 and progesterone-treated rd1 mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

GSH	*PN11*	*PN13*	*PN15*	*PN17*
Wt	*33,545 ± 3,15*	*28,110 ± 3,71*	*23,802 ± 2,52*	*21,491 ± 3,11*
rd1	*29,106 ± 3,34*	*22,169 ± 2,55*	*14,456 ± 1,26*#*	*42,980 ± 5,15**
rd1 treated	*34,799 ± 6,24*	*25,077 ± 2,17*	*30,454 ± 2,24*	*48,586 ± 9,06**

Table 15. Glutathione disulfide (GSSG) concentrations at different post-natal days (PN11, PN13, PN15 and PN17) in the retina from wild type, rd1 and progesterone-treated rd1 mice (* p< 0.05 vs wt; # p< 0.05 vs treated rd1).

GSSG	*PN11*	*PN13*	*PN15*	*PN17*
wt	*3,216 ± 0,20*	*3,005 ± 0,50*	*3,902 ± 0,71*	*3,802 ± 0,64*
rd1	*3,490 ± 0,51*#*	*2,773 ± 0,43*#*	*3,110 ± 0,89*#*	*6,131 ± 0,64*#*
rd1 treated	*15,501 ± 2,05**	*5,863 ± 0,53**	*12,409 ± 2,26**	*15,318 ± 1,99**