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Platinum nanoparticles therapeutic strategy for the prevention of oxidative stress in retinal dystrophies

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Abstract

Neurodegenerations are a complex pool of diseases united by a progressive loss of neuronal cells. Retinal neurodegenerations cause several visual impairment conditions worldwide, burdening the healthcare systems and lowering patients' quality of life. Several retinal degenerations are associated with reactive oxygen species overproduction, a well-known condition that, in the long term, has a detrimental effect on the degeneration progression.

While the neuronal cells loss is irreversible, several therapeutic approaches are currently used to prevent retinal degeneration, with contained and heterogeneous effects depending on both disease and patient. In addition to the preventive therapies available in clinical practice, the scientific community has investigated several therapeutic and preventative solutions, from gene therapy to dietary supplements. Nanomedicine has excellent potential for retinal degeneration prevention. Nanoparticles with ROS scavenging capability, such as gold or cerium-oxide nanoparticles, have been proven effective as neurodegeneration counteractors, leading to intensive research to find more efficient materials for nanoparticles core. Thanks to its high antioxidant activity, solubility and stability, platinum has proven to be a valuable candidate.

In this thesis, we demonstrate that citrate capped platinum nanoparticles, produced by seeded-growth approach and stabilized with an RSA corona preserve their catalytic activity, without inducing neuronal death *in vitro*. Furthermore, *in vivo* experiments enlightened a preservation effect on retinal electrophysiology, without altering the morphology or the inflammation levels in the retina. These data together suggest that PtNPs are safe to use *in vivo* and have the potential to be used as ROS scavengers in cases of degenerative diseases of the retina.

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I. Introduction

1 THE LIGHT PATHWAY

Vision begins in the retina, a highly specialized nervous tissue located at the bottom of the eye. Light has to cross both the anterior and the posterior segment of the eye before arriving to the retina. Moreover, photoreceptors (PRs), the cells deputed to the conversion of light into the electrochemical signal, are placed in the outer portion of the retina, meaning that light has to cross all the inner layers to be captured.

1.1 Eye bulb

The eye is the organ deputed to convert environmental light into electrochemical impulses. It converges photons from the surrounding environment through an optical system, regulating their amount with a muscular diaphragm, focusing it with a system of adjustable lenses and converting it into an electrochemical signal. In the eye, visual information undergoes a first elaboration before being sent to the higher portions of the brain.

The human eye is an irregular sphere, with the anterior part characterized by a smaller radius than the posterior one, which can be divided into three coats, different in both function and morphology. The outermost layer is called the fibrous tunic, having both roles as a protector from mechanical stress and shell shaping the eye. It is composed frontally by the cornea, a transparent, highly innervated and non vascularized tissue, and the sclera, an opaque fibrous layer that forms the rear portion of the bulb. The middle layer is the vascular tunic, otherwise called the uvea, divided in (Figure 1):

- the iris, a pigmented circular muscle placed behind the cornea. It works as a shutter for the incoming light that contracts or relaxes thus modifying the pupil diameter and consequently the amount of light entering the eye;
- the ciliary body, a contractile tissue that sustains and stretches the lens through a series of ligaments, called suspensory ligaments. Our eye can stretch the lens to change the focus of the light and adapt the image on the retina depending on the distance of the object we are focusing our attention on;

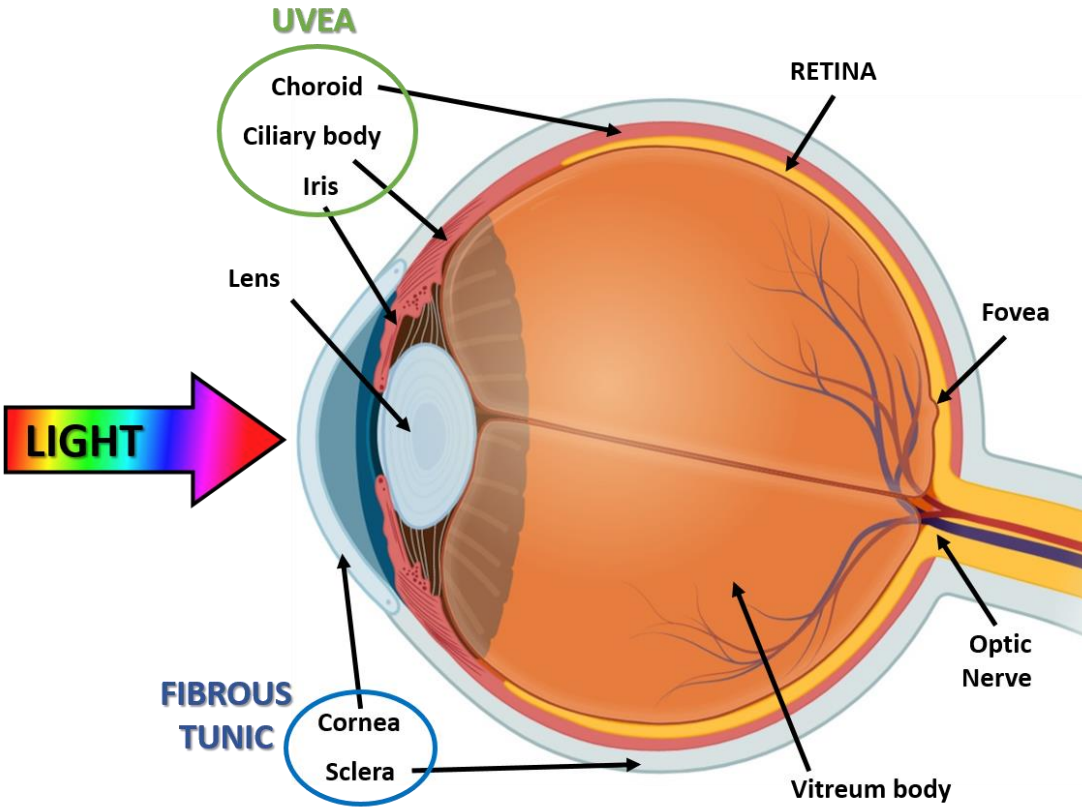

Figure 1. *Anatomy of the eye*. The figure shows a schematic representation of a human eye.

- the choroid coat, rich in blood vessels. Its role is to provide oxygen and nutrients to the inner layer and remove toxic waste.

The innermost layer is composed of the retina, the nervous layer responsible for the transduction of light into electrical signals, and the retinal pigment epithelium (RPE), a single-layer epithelium essential to PR physiology, as explained in the following sections.

The eye is internally divided into anterior and posterior compartments. The former can be further divided into an anterior chamber, which is delimited by the cornea and the iris, and a posterior chamber, formed by the rear part of the iris, the lens, and the suspensory ligament of the lens. These compartments are filled with aqueous humour, a transparent fluid poor in proteins with oxygenating and nutritional roles. The posterior segment of the eye occupies two-third of the total volume and contains the vitreous body. It is confined by the lens and the suspensory ligament of the lens frontally and the retina posteriorly.

1.2 Crossing the retina

The transduction of the photons into electrical signals occurs in the PR, placed in the outermost part of the retina.

A fundus image of the human retina enlightens two main structures called the optic disk and the macula (Figure 2). The optic disk appears as a circle placed in the nasal portion of the retina and it is the only part where the eye opens to the rest of the body. The blood vessels flowing in the eye eventually pass through the optic disk as the neuronal axons forming the optic nerve. The macula is a small area of 5.5 mm diameter localized in the centre of the retina, about 5 mm in the nasal direction from the optic nerve. This area has a higher concentration of PRs and creates the high-resolution central part of our optical field. It can be divided in:

- the foveal avascular zone, the central portion of 1.5 mm diameter where PRs are directly exposed to the posterior chamber. This structure allows photons to reach the PRs without being distorted by either the blood vessels or the innermost layers of the retina. Here, the thickness of the retina reaches its lower peak. In its center, a small area of less than 0.35 mm called foveola, cones are the only PRs we can find, highly packed in a hexagonal shape. Lacking vascularization from the inner layers, the only source of nutrients and oxygen for this area is from the choroid.
- the foveal rim, where all the downstream neurons receiving signals from the PRs present in the foveal avascular zone are located. It is the thickest part of the retina.

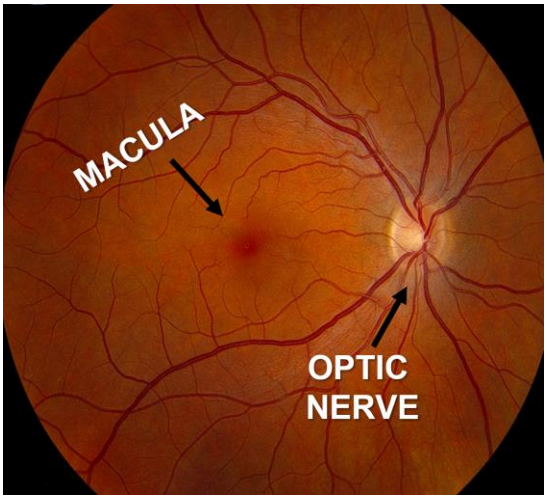

Figure 2. *Main structure of the human retina*. Image of the human ocular fundus. The macula, in the center, appears as a dark area, while the optic nerve, on the right side, appears as a lighter disk.

The retina is composed of different cell types (Figure 3A), which are highly organized into anatomically and functionally different layers as detailed in the next sections. Briefly, following the light path, the retina is composed of (Figure 3B):

- the ganglion cell layer (GCL), consisting of neurons that indirectly collect the processed information from the outer part of the retina. The axons of these cells group together forming the optic nerve, which leaves the eye and it is responsible for transferring visual information to the following stages of image processing of the visual system;
- the inner plexiform layer (IPL), where the synapses between the ganglion cells and the upstream neurons are located;
- the inner nuclear layer (INL) contains the bodies of three different neuronal cells types, bipolar cells (BCs), amacrine cells (ACs) and horizontal cells (HCs). These cells allow signal transmission and its elaboration between the outer layer and the ganglion cells in the GCL;
- the outer plexiform layers (OPL), which contains the synapses between PRs, bipolar cells and horizontal cells;
- the outer nuclear layer (ONL), which contains the body of PRs.

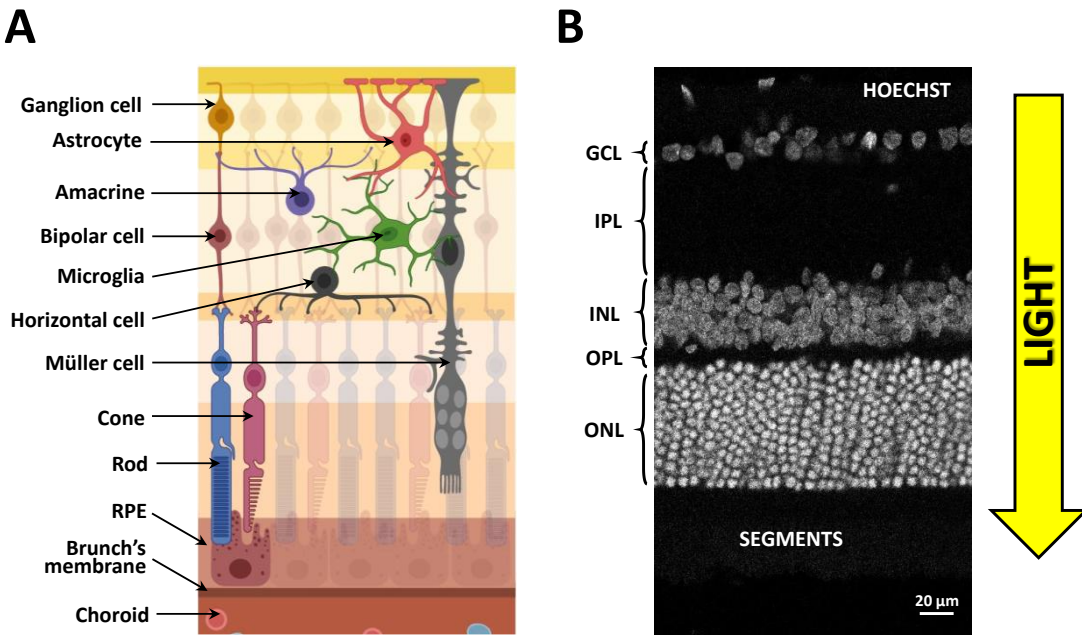

Figure 3. *Retinal layers and cellular types*. **A**) Retinal section stained with Hoechst to enlighten the nuclear and the plexiform layers. The yellow arrow on the right indicates the direction of the impinging environmental light. **B**) Schematic representation of retinal layers.

2 THE INFORMATION PATHWAY

2.1 Photoreceptors

PRs are a specialized type of neuroepithelial cells capable to perform phototransduction, a process that transforms visible electromagnetic radiation, light, into biochemical signals able to trigger a change in membrane potential, a type of information that the nervous system can elaborate.

PRs (Figure 4A) are composed of a central body, containing the nucleus and most of the organelles, an inner portion, a synapse that brings the information to the inner retina, and an external part, further divided into an inner segment (IS) and an outer segment (OS), separated by a connecting cilium. The IS, rich in mitochondria and ribosomes, is responsible for energy production and the proteins necessary to the phototransduction process. Moreover, at the level of the connecting cilium, the IS continuously creates the disks, membranous structures containing several intermembrane proteins necessary to trigger the phototransduction process. Those disks are piled together in the OS, the part of the PR where photons are captured and chemically transformed into a change in membrane potential. This electrical signal travels inwards until it reaches the synapses in the OPL.

The OS (Figure 4B) goes under continuous renewal (Young 1967; Young and Droz 1968). Disks move from the base of the OS towards its apex, while their molecules continuously activate phototransduction. At the tip of the OS, the disks shed and get engulfed by the RPE cells (Young and Bok 1969). The rate between formation and disposal of the disks is constant under healthy physiological conditions, while any unbalance may lead to shortening or lengthening of the OS, causing malfunction and disease. For example, mutation in the receptor-tyrosine kinase c-mer gene (Mertk), encoding a membrane receptor that trigger phagocytosis of the shredded disks by the RPE, lead to Retinitis pigmentosa (D'Cruz et al. 2000; Gal et al. 2000), one of the most diffuse retinal degeneration in humans that causes patchy visual alteration and progressive loss of vision (Webster 1878).

PRs can be divided into rods and cones, different for physiology and morphology. Rods compose 95 % of the total PRs, with an estimated 120 million cells in the human retina reaching their higher concentration in the periphery (Figure 4C, top). Thanks to their converging downstream network and high sensitivity, rods are responsible for the scotopic vision and saturate above certain luminances. Rods display an absorption spectrum that overlays cones' spectra (Figure 4C, bottom), peaking at 497 nm (Bowmaker and Dartnall 1980), and have a slower response rate with a threshold at 15 Hz (Hecht and Shlaer 1936). Furthermore, rods have a thinner

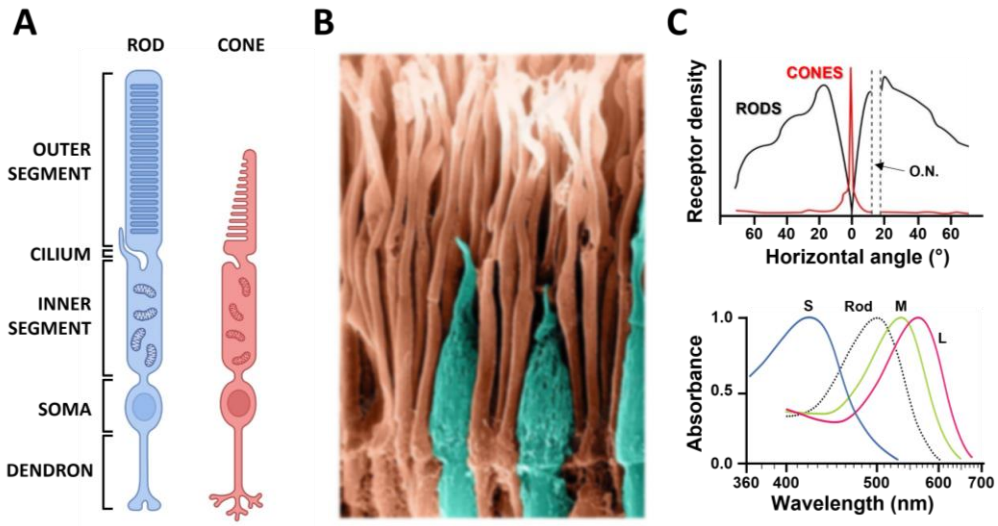

Figure 4. *The photoreceptors*. **A**) Schematic representation of a rod (left) and a cone (right). **B**) SEM image of the segments of both types of PRs. Rods are coloured in orange, while cones are represented in green. (Credit RALPH C. EAGLE, JR. / SCIENCE PHOTO LIBRARY) **C**) Top: graph showing the density of rods and cones in the retina on the visual field angle. The cone density is represented in red while the rod density is in black (after Österberg, 1935). In the bottom panel, a graph showing the spectrum of absorbance of different PR pigments is depicted (dotted-grey for rods; blue for S-cones, green for M-cones and red for L-cones).

elongated shape with longer OSs and disks piled up as independent units inside the OSs membrane. On the opposite, cones are much fewer in number, around 6 million in the human retina, densely packed in the fovea, while their number decreases moving towards the periphery (Figure 4C, top). Additionally, their low sensitivity, coupled with high adaptability at different luminance ranges, makes them responsible for daylight vision. Besides, cones can respond at a higher frequency, giving the retina the maximum response frequency of 60 Hz (Hecht and Shlaer 1936), and are responsible for colour vision. Indeed, we can divide cones into three types, depending on the pigment they produce (Figure 4C, bottom). The S-cones, which absorb blue light peaking at 420 nm; the M-cones, which absorb green light peaking at 533 nm; and the L-cones, which absorb red light peaking at 562 nm (Bowmaker and Dartnall 1980). Finally, cones are much shorter and thicker than rods, with a shorter cone-shaped OS, which membrane fuses in continuity with the disks membrane. Interestingly, cones have a direct connection with output cells in the fovea, which exquisitely increases the resolution of this area.

2.1.1 Phototransduction

Most of our knowledge about the phototransduction molecular events comes from rod biological activity due to the greater ease of isolation and their sensitivity to single photons in a dark-adapted state (Baylor, Lamb, and Yau 1979).

Phototransduction starts with the excitation of the PR pigment by a single photon, which leads to a massive amplification through a biochemical cascade of reactions, whose purpose is to change the OS membrane potential (Figure 5). The photo-reactive molecule is formed by a polyene chromophore derived from vitamin A, the 11-cis retinal, covalently bound through Schiff's base linkage to a lysine residue in position 296 of a G-protein coupled receptor, called opsin, which has its transmembrane domain in the OS disk membrane (Lamb 1996). Opsin is produced by the PRs in different isoforms between rods, named rhodopsin, and cones, name cone opsin. Moreover, different subtypes of cones express different cone opsin, allowing our retina to perceive different colours (Yokoyama 2008).

When the 11-cis retinal is excited by a photon of the appropriate wavelength, the molecule isomerizes, changing its conformation to all-trans retinal and inducing a reduction of the rhodopsin molecule stability. This instability leads to a series of transformations of 11-cis retinal, conducting to a series of reactive byproducts. Between them, metarhodopsin II (MII) is crucial for the phototrasduction. Indeed. it diffuses in the disk membrane and induces the catalytic activation of the G protein transducin by exchanging a GDP molecule with GTP. MII is a peripheral membrane protein formed by three different subunits, α, β and γ, and its activation leads to a structural modification that ends with the dissociation of the Tα-GTP subunit from the T-βγ complex (Malinski and Wensel 1992; Wensel 1993). Every molecule of MII can activate hundreds of transducin molecules during its short life, leading to the first step of the amplification cascade.

As for MII, Tα-GTP has a transmembrane portion which allows its diffusion in the disk membrane. When Tα-GTP encounters the inactivated form of cGMP phosphodiesterase-6 (PDE6), it binds it, forming a complex that increases the PDE6 enzyme kinetics of about 1000 times (He et al. 2000). PDE6 is a peripheral membrane enzyme that transforms guanosine monophosphate (GMP) to cyclic-GMP (cGMP), a cytoplasmic messenger that transmits the signal from the disk membrane to the OS plasma membrane.

In the dark, cGMP concentration is maintained constantly high, in the order of several μM (Nakatani and Yau 1988), thanks to the balanced activity between two enzymes: the cytosolic guanylate cyclase (GCy) (Koch 1991; Shyjan et al. 1992),

which synthesizes cGMP from GTP (Yang and Wensel 1992), and the PDE6 (Miki et al. 1975; Wensel 1993; Yee and Liebman 1978). At this concentration, cGMP constantly binds the cGMP-gated cation channel (CNG) present on the OS membrane (Fesenko, Kolesnikov, and Lyubarsky 1985), keeping them open and allowing the entry of Na^+ and, as a consequence, the entry of Ca^{2+} through the Na^+/Ca^{2+} exchanger. This influx of positive charges in the OS is counter balanced by the outflow of K^+ in the IS. Together, these currents, called dark current (Baylor 1996), maintain rods resting potential around -40 mV, more depolarized than typical neurons (Gordon, Brautigan, and Zimmerman 1992). The result is a constant release of glutamate at the level of PR synapses with BCs.

The activation of the PDE6 brings to a rapid decline of the cGMP concentration, which leads to immediate closure of CNG. This induce a consequent influx decrease of both Na^+ and Ca^{2+}, followed by hyperpolarization of the OS membrane potential (Yau and Nakatani 1985). Consequently, the synaptic release of glutamate decreases, signalling to the downstream network the presence of light.

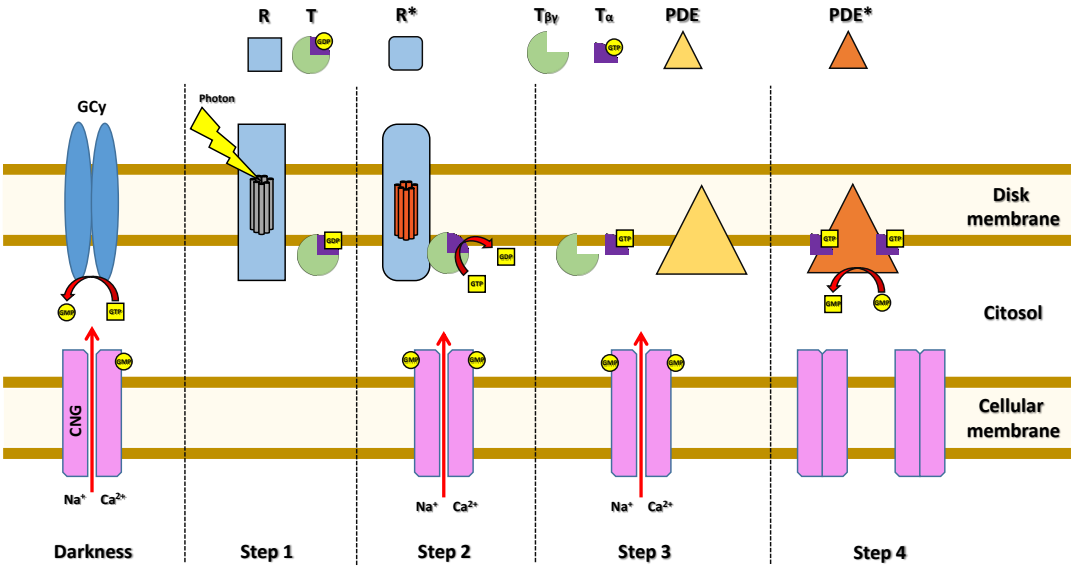

Figure 5. *The phototransduction cascade*. Schematic representation of the molecular cascade from the capture of a photon to the closure of CNG. In the *darkness*, GCy synthetizes cGMP from GTP, maintaining high its intracellular concentration and keeping open the CNG channel. *Step 1*, the photon (Ph) is captured by the rhodopsin (R). *Step 2*, rhodopsin becomes Metarhodopsin II (R*) and activates transducin (T), exchanging its GDP molecule with a GTP. *Step 3*, active transducin splits and the α-transducin (Tα) starts fluctuating in the disk membrane. *Step 4*, α-transducin binds PDE activating it (PDE*) and exponentially increases its catalytic activity. The conversion of cGMP to GMP by PDE* overcomes its formation by GCy. cGMP cytosolic concentration rapidly decreases. The CNG, normally open due to high concentration of cGMP molecules, loses most of the bounded cGMP and closes, blocking the entrance of Na^+ and Ca^{2+}.

Phototransduction process must be interrupted to allow the PR to return to its resting potential. This is necessary to increase the temporal resolution of the photoreceptorial activity. There are many mechanisms to shut down the activity of crucial molecules like MII:

1) the enzyme rhodopsin kinase (RK), located on the disk membranes, phosphorilates MII (Maeda et al. 2003), strogly reducing its affinity for transducin.
2) the protein arrestin bind to MII (Gurevich, Gurevich, and Cleghorn 2008; Palczewski 1994), blocking its residual activity.
3) Tα-GTP hydrolyzed either throught its intrinsic GTP-ase activity and by the protein complex called GTPase activating proteins (GAPs) (Cowan et al. 1998; He, Cowan, and Wensel 1998) Tα-GTP hydrolization decreases its affinity for PDE6 (Krispel et al. 2006; Pugh 2006), gaining back the ability to bind to the dimer T-βγ and return to the inactivate transducin.
4) the reduction of intracellular Ca^{2+} that follows the closure of CNG. In the dark, deactivates a calcium-binding protein of the calmodulin superfamily named GCAPs, which usually bind and silence the activity of GCy (Koch and Stryer 1988). This mechanism allows restoring cGMP basal level in about 100 ms, counteracting the action of PDE6.

Altogether, these mechanisms allow cGMP to return to its dark state concentration, closing the CNG channels and allowing both Na^{+} and Ca^{2+} to enter in the OS. The restablishment of PR resting potential is transmitted to the synapses between PRs and BCs, leading to the increase in glutamate release to the basal level. The photoreceptor can now capture new photons and begin a new cycle of phototransduction.

2.2 Vertical and horizontal retinal networks

While light has to cross all retinal layers outwards, the information acquired from the PRs follows an inwards path to be sent to the brain. Indeed, downstream to the PRs, other neurons cooperate in creating a vertical transmission pathwatwo, whose main actors are the BCs. These neurons lead the information to the GCL, influenced by two horizontal networks created by HCs in the outer retina and ACs, in the inner part, modulating the information in the surrounding areas.

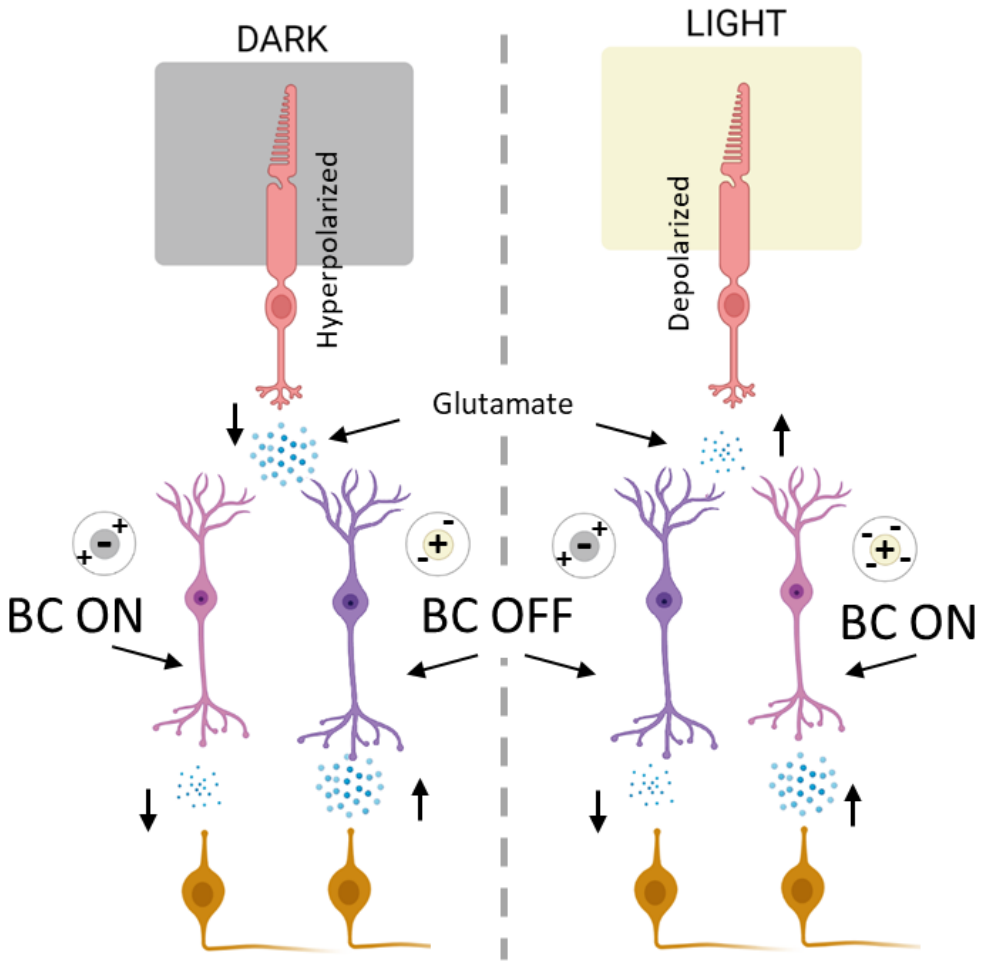

Figure 6. *Neuronal activation in the dark and in the light*. In the dark (left), PRs are in their resting potential (-40 mV) and release huge amount of glutamate in their synapses. Glutamate has different effects on BCs, depending on their nature. The ON BCs (pink) will react hyperpolarizing their membrane, therefore reducing the release of neurotransmitter to the RGCs, while the OFF BC (violet) will depolarize, increasing their neurotransmitter release on the RGCs. Upon illumination (right), PRs will start the phototransduction cascade and hyperpolarize the membrane. Its consequent reduction of glutamate release will induce hyperpolarization in OFF BCs, while depolarizing the ON BCs membranes and increasing neurotransmitter release at their synapses.

2.2.1 Vertical network

After phototransduction, the first to receive the visual information are the BCs that works as a bridge between PRs and the RGCs, which collect and integrate data from the surrounding portion of the retina (Figure 6). BCs have a radial distribution, starting their dendrites in the OPL, where they form synapses with PRs and collect inhibitory signals from HCs, and sending their axon in the IPL, where they

communicate with RGCs and ACs. Contrarily from other neurons, which use action potentials to transfer information along the axons, bipolar cells use graded potentials to send their signal (Saszik and Devries 2012).

More than 10 types of BCs have been observed in the retina (Ghosh et al. 2004; Wu, Gao, and Maple 2000). The first main difference has been observed by Santiago Ramón y Cajal in 1893, who recognized and described the different axonal stratification of BCs making synapses with either rods or cones (Cajal 1893). While only one morphological type exists for rod BCs, with a ramified dendritic tree and an axon that makes contact in lobulated terminals between IPL and GCL (Boycott, Dowling, and Kolb 1969; WäSsle et al. 1991), at least nine typologies of cone BCs have been identified, differing for number of cones they receive information from, their dendritic branch pattern and the stratification of their axon terminals in the IPL (Ghosh et al. 2004).

BCs can be further divided depending on their functionality, in ON and OFF BCs. The former present a metabotropic receptor for glutamate (mGluR6) that, when activated, triggers an intracellular signaling cascade. This ultimately leads to the closure of ionchannels for Na^+ and Ca^{2+}, inducing a hyperpolarization of its membrane (Tian and Kammermeier 2006). This mechanism maintains the ON BCs hyperpolarized in the absence of light, allowing their depolarization only when the release of glutamate from the PRs decreases. Contrarily, the OFF BCs can present either kainate receptors (DeVries and Schwartz 1999) or AMPA receptors (Brandstätter, Koulen, and Wässle 1997; Hack, Peichl, and Brandstätter 1999), both of which open the ion channels when bound to glutamate, inducing depolarization in the dark and hyperpolarizing it in response to PR activation (DeVries 2000).

2.2.2 Horizontal network

While BCs create a vertical pathway that brings the information from the outermost part of the retina to its innermost layer, HCs and ACs provide horizontal feedforward and feedback information between PRs and BCs in the OPL and between BCs and RGCs in the INL respectively. Both interneurons share the same inhibitory synaptic neurotransmitter, gamma-aminobutyric acid (GABA), but their functions are generally different.

HCs bring negative feedback to the PRs networks, measuring the average intensity of PRs activation in a local area while proportionally inhibiting sourounding PRs response. This mechanism, called centre-surrounding antagonism, is crucial for contrast enhancement and edge detection (Westheimer 2004).

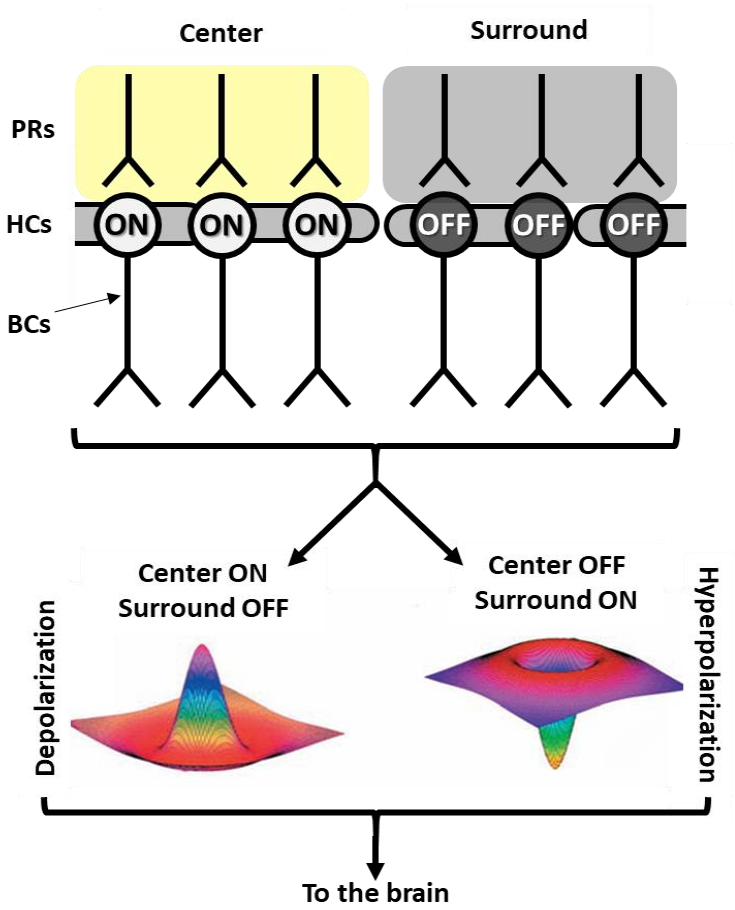

Figure 7. *Receptive fields of retinal ganglion cells.* A schematic representation of the receptive fields of both ON (left) and OFF (right) RGCs. ON-center RGCs depolarize in response to spots of light that hit their center, while Off-center RGCs depolarize when light hits only the periphery of their receptive fields. HCs convey antagonistic signals from their surrounding and send them to both BCs and PRs, indirectly influencing the RGC activity.

On the opposite, ACs are very different in morphology as well as in their functions. They lack the axon and can be classified based on their receptive fields in narrow-field (between 30 and 150 µm diameter), small-field (between 150 and 300 µm diameter), medium-field (between 300 and 500 µm diameter) and wide-field (larger than 500 µm diameter). They:

- send feedforward signals to RGCs, tuning their receptive fields spatial and temporal characteristics (Lee et al. 2016; Tien, Kim, and Kerschensteiner 2016).
- send feedback signals to BCs, both outlining their receptive field and refining their responses (Dong and Hare 2003; Flores-Herr, Protti, and Wässle 2001).
- sum inhibitory signals to neighbouring ACs to regulate their feedforward signals gain (Grimes et al. 2015).

An important type of ACs is the AII ACs, narrow field cells which connect the rods network to the rest of the retinal system (Trexler, Li, and Massey 2005).

2.3 Ganglion cells

Retinal ganglion cells are both the last stage of elaboration of the visual information in the retina and the messenger of this information to the higher parts of the brain.

Nowadays, thanks to exquisite works carried on by Steven Kuffler and Horace Barlow, we know how every RGC receives information from its receptive field (Figure 7), a small area of the retina from where the input information comes. Field sizes can go from few dozen to hundreds of μm, crossing other RGC's receptive fields to increase the precision and enlarge the types of information that a RGC can send to the brain (Barlow 1953; Kuffler 1953).

Ganglion receptive fields are composed of two concentric circular areas. When stimulated with a certain light pattern, which differs for each RGCs subtype, the inner circle will increase the firing rate of the RGC, while the outer area will decrease it. The former represents the area of the retina from which the RGC receives the information from BCs, while the latter corresponds to the information given by ACs about the periphery surrounding the inner field. Interestingly, RGC higher responses are obtained when the contrast of stimulation between the two areas of the retina is maximal, while a diffuse illumination between the fields will only give a slight variation of the firing rate (Kolb 2003).

RGCs can be divided, depending on the source of stimulus that induces their maximal response, into:

ON-RGCs, which fire with the maximum rate when the inner field is lightened and the outer field is in the dark;

OFF-RGS, which fire with the maximum rate when the inner field is kept in darkness while the outer field is in the light;

ON/OFF-RGC, which fire when stimulated by both light and darkness.

The receptive fields can be visualized as "Mexican hat" of excitation, a tridimensional gaussian with the positive peak in the inner field and a negative area representing the outer field for ON-RGCs, but reversed for the OFF-RGCs. Moreover, the size of the "hat" will be depending on the size of the receptive field, being larger for RGCs with large receptive fields and smaller for the one with small receptive fields (like RGCs in the fovea) (Kolb 2003).

2.4 Cells supporting retinal vision

2.4.1 Retinal pigment epithelium

The RPE is a single-layer epithelium composed of hexagonal polarized cells, closely apposed through tight junctions on their lateral surfaces (Miller and Steinberg 1977; Steinberg 1985). Their apical membrane faces the PRs, engulfing their OS with long microvilli, while the basal membrane faces the Brunch's membrane and the choroid (Bok 1993; Steinberg 1985; Strauss 2005). RPE is an essential component of the visual process and interacts with PRs in several ways (Figure 8).

First, RPE cells are rich in melanin, a dark pigment stored in melanosomes, also called pigmented granules (Loskutova et al. 2013), which absorbs the uncaptured light that crosses the PRs and avoids overexposure of the OS (Strauß 2016). This mechanism increases image resolution by absorbing the scattered light and protects PRs from excessive light, which generates oxidative stress (Noell et al. 1966).

Thanks to their close lateral relations, RPE cells form part of the brain-retina barrier (BRB), which is fundamental to isolate the outer retina from systemic interferences, to create the immune privilege of the eye and a mechanism of selective bidirectional transport with the blood. Indeed, RPE cells actively transport from the blood glucose, expressing the membrane transporters GLUT1 and GLUT3 (Ban and Rizzolo 2000; Deguchi et al. 1994), retinol transformed in 11-cis retinal and transported to the PRs (Pfeffer et al. 1986), and Ω-3 fatty acids essential for the formation of the OS disk membranes (Bazan, Gordon, and Rodriguez de Turco 1992; Bazan, Rodriguez de Turco, and Gordon 1994). At the same time, active transport to the blood is essential to eliminate metabolic water, through active Cl^- transportation (Hu et al. 1994; Hughes and Takahira 1996) and expression of aquaporin-1 channels (Hamann et al. 1998; Stamer et al. 2003), metabolic end products such as lactic acid (Hamann et al. 2003), and to maintain the homeostasis of the subretinal space through buffering the K^+ concentration in the subretinal space (Steinberg, Linsenmeier, and Griff 1983).

Moreover, RPE has a crucial role in visual function. Since PRs do not express re-isomerase, all-trans retinal needs to be delivered to the RPE, where it is re-isomerized before being sent back to the OS. This series of crucial steps for the renewal of the visual pigment is called the visual cycle (Saari 2016).

Furthermore, RPE preserves PRs morphology through the diurnal phagocytosis of exhausted disks of the OS (Steinberg 1985). Several crucial proteins are involved in this process, such as CD36, required for OS internalization, $\alpha_V\beta5$ integrin, that mediate the binding to the OS membrane, and Mertk (Feng et al. 2002; Finnemann and Silverstein 2001).

Besides, RPE communicates with the surrounding cells via secretion of several factors such as ATP, fibroblast growth factors (FGF), fas-ligand (fas-L), vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) (Strauss 2005). While some of them, like PEDF, which inhibits PRs apoptosis (Cayouette et al. 1999), and VEGF, which stabilizes choroidal endothelium (Witmer et al. 2003), are continuously secreted, the others are released only under stressful conditions, such as metabolic stress or hypoxia (Pan et al. 2006).

Finally, neurotrophic factors released by RPE, such as monocyte chemotactic protein-1 (MCP1) (Austin et al. 2009) and interleukin-8 (IL-8) (Relvas et al. 2009), together with the tight physical barrier formed by the epithelium, are essential for the creation of the immune privilege of the eye (Ishida et al. 2003; Streilein 1999; Zamiri et al. 2007).

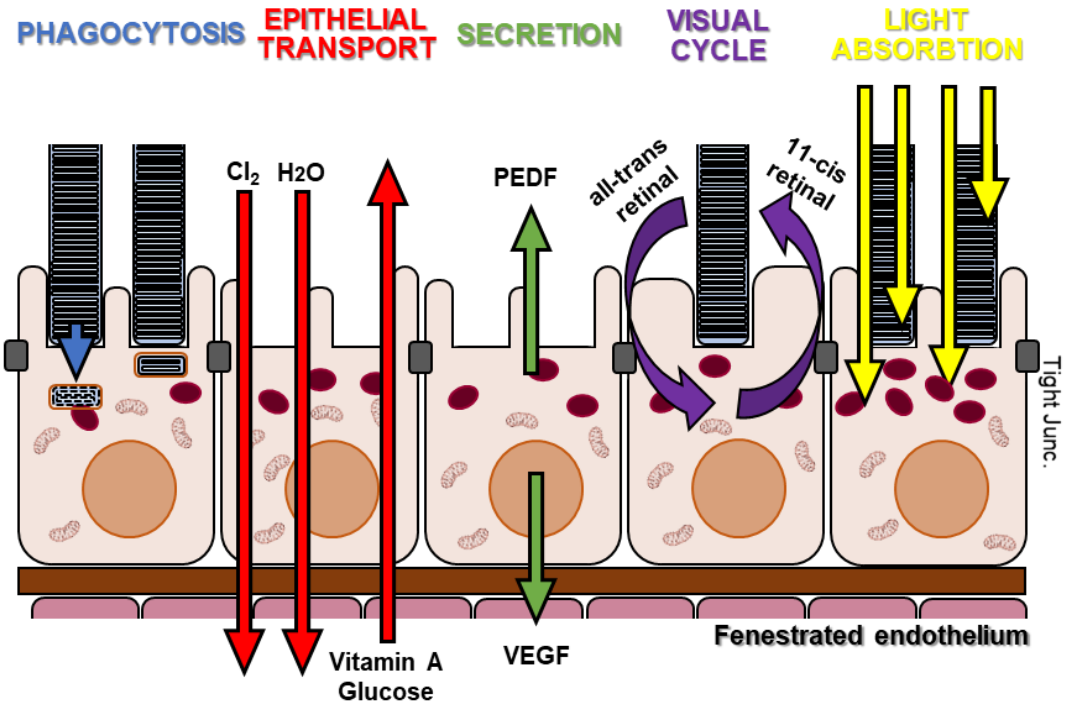

Figure 8. *Retinal pigment epithelium functions*. Schematic image representing the RPE cell functions and their relationships between each other and neighbouring cells.

Diseases affecting RPE cells impair the functionality of the neuroretina, slowly leading to degeneration and vision loss. Among this category of pathologies, a famous example is the Leber Congenital Amaurosis type (Chung and Traboulsi 2009), for which a gene therapy treatment was recently approved on American and European markets. Other retinal diseases involving RPE include Stargardt disease (Lenis et al. 2018), age-related macular degeneration (Allikmets et al. 1997) and Gyrate atrophy of the choroid and retina (Takki and Milton 1981).

2.4.2 Retinal glia

Like the rest of our central nervous system, retinal functions and structure need the support of several specialized glial cells. Those cells are the Müller cells, Astrocytes and Microglia.

Müller cells (Figure 3A) share with PRs the same embryological progenitor (Turner and Cepko 1988). They are long radial cells that go through the whole retina, with the nucleus located in the INL. The human retina contains between 4 and 5 million Müller cells (Bringmann et al. 2006), which contribute in several ways to retinal functions:

- they form the inner and the outer limiting membrane, physical and functional supports of the retina, limiting the diffusion of substances from and to the outside of the retina (Bringmann et al. 2006). The inner limiting membrane (ILM) separates the retina from the vitreous body, while the outer limiting membrane, rich in tight junctions, is located at the level of the PR connecting cilium, between the IS and the OS;
- they perform in situ glycogen synthesis, glycogenolysis and anaerobic glycolysis, playing an essential role as active transporters of glucose through the BRB and adapting their metabolism to the needs of surrounding neurons (Puthussery et al. 2006);
- they uptake and inactivate both inhibitory and excitatory neurotransmitters, such as glutamate, GABA and glycine (Biedermann et al. 2004);
- they synthesize and release glutamine, the glutamate precursor, and carbonic anhydrase, essential to the regulation of both intra- and extracellular volume and pH through the conversion of CO_2 and water in bicarbonate (Linser, Sorrentino, and Moscona 1984; Nagelhus et al. 2005; Riepe and Norenburg 1977);
- they regulate the extracellular homeostasis by balancing the K^+ concentration in the extracellular fluid, redistributing it in different layers and the posterior chamber, contributing to the formation of several components of the

Electroretinogram, such as the b wave (Miller and Dowling 1970; Newman and Odette 1984) and the slow P3 (Karwoski and Proenza 1977).

Astrocytes (Figure 3A) do not originate from the retinal neuroepithelium, but migrate in the developing retina from the brain through the optic nerve (Stone and Dreher 1987). They play a crucial role in the neovascularization of developing retina, preserving the BRB integrity and vascular stability in adult retinas by endothelial proliferation inhibition (Luna et al. 2010).

In healthy retinas, astrocytes are localized in the innermost part of the retina, where there is a high vasculature, presenting a morphology that goes from symmetrical stellate to highly lengthened while moving from the periphery to the optic nerve (Reichenbach and Bringmann 2020). Moreover, they cannot be found in the fovea, probably due to the absence of vascularization of that area.

Finally, astrocytes function includes the formation of the BRB, surrounding capillaries and RGC axons with their branches, and a support role for nearby neurons, suppying both nutrition and regulation of the homeostasis of pH, potassium levels and neurotransmitters (Bay and Butt 2012; Newman 1988).

Microglia (Figure 3A) has a role in immuno-surveillance and immunological tolerance, having an anti-inflammatory phenotype crucial for the immune privilege of the retina (Zamiri et al. 2007). In humans, it can be divided into perivascular microglia, which controls the materials entering from the blood flow, and parenchymal microglia, a motile ramified cellular type that scouts the retinal matrix (Provis, Diaz, and Penfold 1996). Interestingly, microglial motility is increased under conditions of retinal stimulation, increasing with glutamatergic stimulation and decreasing after GABAergic neurotransmission (Fontainhas et al. 2011). Moreover, essential microglial functions include the removal of pathogens, toxic byproducts and cell debris, and the production of anti-inflammatory cytokines and neurotrophic factors (Bodeutsch and Thanos 2000; Langmann 2007; Mccarthy et al. 2013).

In a healthy retina, microglia appear as multipolar cells with irregular processes surrounding a small cell body, localized in inner retinal layers (Chen, Yang, and Kijlstra 2002). When activated, they modify their morphology withdrawing their branches, enlarging their cell body and migrating in the outer layers of the retina, like the ONL, where they begin to phagocytize PR and other cells residual bodies. Moreover, several molecules released by activated microglia, such as reactive oxygen species (ROS) matrix metalloproteinase, pro-inflammatory cytokines and prostaglandins, can induce chronic PR degeneration (Langmann 2007). They can also migrate as a precursor in the retina from the blood flow during inflammation,

increasing their number and activity (Langmann 2007). In some cases, if the insult persists, their action can be counterproductive due to massive phagocytosis of healthy neurons and overproduction of inflammatory cytokines.

Moreover, microglia containing PR debris has been found circulating in the body, working as antigen-presenting cells and finally activating immune responses against the retina (Raoul et al. 2008). Several retinal diseases, like age-related macular degeneration (AMD), glaucoma, and several forms of diabetic retinopathy often display an abnormal microglial activity (Fletcher 2020). Finally, with advancing age, microglia accumulates in the subretinal space, recruited by the lack of clearance of the PR wastes by the RPE (Buschini et al. 2011).

2.5 Electrophysiologica evaluation of retinal activity

Retinal electrical activity induced by light can be quantified to evaluate retinal health. A gold standard tool for the diagnosis and the follow up of several retinal pathologies is the Electroretinogram (ERG), which consist in a non-invasive analysis that deploy electrical potential variation following light stimulation. By using different light stimulus, it is possible to obtain information from radial extracellular currents elicited from different retinal components, such as PRs, inner retinal cells or even the RGCs.

Granit (1933) studies on cat's ERG proved how the ERG response can be divided in three components, P-I, P-II, and P-III (Granit, 1933). Granit's work paved the way for following pharmacological investigations that have revealed the physiological mechanisms behind the localization of the different components in different retinal layer. P-III is a negative part that can be further divide in a fast P-III, originated by PRs' hyperpolarization (Penn & Hagins, 1969; Sillman et al., 1969b), which compose the a wave, and a slow P-III (Sillman et al., 1969a), originated by MCs potassium buffering activity. The summation of P-II and P-III create a positive slower wave (Pepperberg et al., 1978) that forms the b wave. This part is produced by rise of potassium in the plexiform layers, induced by the ON-BCs (Dick & Miller, 1985), RGCs and ACs (Karwoski & Proenza, 1980; Dick & Miller, 1985). Finally, a third wave, called c wave, is created by P-I and P-II together, which is formed by the RPE cells (Noell, 1954; Steinberg et al., 1970). Other minor components, such as the early receptor potential (ERP), which originates from the dipole changes in the opsins and precede the a wave (Hodgkin & Obryan, 1977), and the oscillatory potentials (OPs), an oscillating wave originating in the IPL probably because of ACs, BCs and RGCs

activity (Heynen & Van Norren, 1985a, 1985b; Wachtmeister & Dowling, 1978; Yonemura & Kawasaki, 1979), contribute to the formation of the complex ERG wave.

Indeed, analyzing the amplitude of the two major components, the a, and the b wave, obtained from a single short flash (around 5 ms) on a dark-adapted retina, allows the physiologist to obtain crucial information on retinal physiology and consent the diagnosis of pathological condition even in an early state.

Moreover, ERG allow the isolation of retinal responses related to the cones circuitry, which are less incline to bleach their activity in photopic conditions compared to rods. This light-adapted response is slightly different from the dark-adapted one, with a smaller a wave in humans (almost absent in rodents) due to the smaller numbers of cones compared to rods. The amplitude of the positive peak measures the activity of the inner retinal network exclusively activated by cones, and therefore is a useful index of cone health and activity.

While the reduction of the ERG response amplitudes is characteristic of the progression of the pathology, the preservation of the amplitude over time in patients affected with neurodegeneration is a proof of the efficacy of the therapy and can be used both in preclinical and human studies (Chen et al. 2006; Fiorani et al. 2015; Maccarone et al. 2008; Piccardi et al. 2012).

3 OXIDATIVE STRESS IN THE RETINA

3.1 The stressful environment of the retina

The human retina is constantly under high stress due to environmental and intrinsic factors. First, the process of vision cannot exist without light, which is known to cause lipids and proteins oxidation due to a specific component, the ultraviolet (UV) light. UV light falls between the visible light and X-rays of the electromagnetic spectra, among 100-350 nm wavelength, and it is characterized by a high reactivity with biological tissues. It induces ROS formation in organic tissues by reducing catalase affinity for H_2O_2 (Heck et al. 2003), thus increasing the production of O_2^- (Deliconstantinos, Villiotou, and Stavrides 1996). The principal UV light source is sunlight, but our eyes can be exposed to artificial UV from fluorescent lamps, work-related exposures or medical treatments (de Jager, Cockrell, and Du Plessis 2017). Rhodopsin itself, when photobleached by light, become a source of ROS and cellular damages (Grimm et al. 2000).

Our eyes have developed different mechanisms to reduce the damage induced by light, like the pupillary reflex, which allows only the necessary light to pass across the pupil, and the presence of pigments in the RPE, which absorb the uncaptured light avoiding it to be a bounce-back on PRs. We also use artificial solutions to shield us from UV light, like sunglasses and UV filters for indoor exposition.

Retinal function requires a vast amount of oxygen and nutrition. PR's metabolism is mainly based on glucose reduction and does not differ from neuronal metabolism. Furthermore, their need for oxygen can be up to 4 times higher than any neuron in the cortex at its maximum activity (Stone et al. 2005; Yu et al. 2007) This astonishing oxygen consumption reaches its peak while the retina is in darkness (Stone et al. 2005), making it impossible to prevent. These surprising needs underline the PR's necessity for continuous blood flow, provided by the choroid. Unfortunately, oxygen is a double-edged sword for life. On one side, it allows the production of a vast amount of energy by the mitochondria (Li et al. 2013) but, on the other side, the same process creates a huge amount of ROS, which could damage the PR itself.

Continuous shredding of exhausted disk in the OS followed by their phagocytosis and disruption by the RPE can create a series of waste products, like lipofuscin or A2E, which are photo-reactive and induce lipid peroxidation and protein oxidation (Godley et al. 2005; Wassell et al. 1999).

Additionally, different diseases and unhealthy habits can negatively impact on retinal health. The major risk factor for retinal degeneration, such as AMD, is by far

cigarette smoke (Seddon 1996), which increases the onset prevalence up to 3 fold (Bird et al. 1995), reduces the avarage age of incidence of 10 years compared to non smokers (77 years) and speeds up the progression of degeneration (Mitchell 2002). Together with other risk factors, such as pollution, poor uptake of vitamins or omega-3 fatty acids (Lim et al. 2012), blue light from screens exposition (Lin et al. 2017), as well as diabetes (Montesano et al. 2021), obesity and cardiovascular diseases (Chakravarthy et al. 2010), smoking can dysregulate physiological retinal balance, increase ROS level and inflammation, leading to retinal degeneration.

3.2 Reactive oxygen species

ROS are very reactive molecules normally present in our organism. Their production is essential to regulate many physiological functions, such as gene expression, cells proliferation and immune response (Bardaweel et al. 2018; Dröge 2002). ROS can be divided into free radicals, like superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($_1O_2$), which are highly reactive due to their uncompleted pair of electrons, or non-free radicals, like ozone (O_3) and hydrogen peroxide (H_2O_2), which can indirectly induce ROS production (Wang, Chin, and Almeida 2021). Indeed, H_2O_2 has low reactivity per se but can easily penetrate membranes and form $OH^{\cdot}$ via Fenton's reaction (Turrens 2003).

$$H_2O_2 + Fe_2+ \rightarrow Fe_3+ + OH^- + OH^{\cdot}$$

Under physiological conditions, our retina is continuously fighting the production of ROS, thanks to the synthesis of antioxidants, molecules able to neutralize ROS before they can react with cellular structures, and through the continuous repairing and renewal of the damaged molecules (Nishimura et al. 2017). Antioxidants can be classified in enzymatic and non-enzymatic depending on their activity. The first line of cellular defense are the endogenous enzymatic antioxidants such as catalase, copper-zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2), glutathione reductase (GR) and glutathione peroxidase (GPx) (Dröge 2002). While SOD1 and SOD2 convert superoxide to oxygen and peroxide, catalase and GPx convert hydrogen peroxide in H_2O and O_2 (Wang et al. 2021). Furthermore, non-enzymatic antioxidants and small molecular weight, such as vitamin, C and E, flavonoids, carotenoids, curcumin, melatonin and bilirubin, are involved in the defense against ROS damages (Wang et al. 2021), but many of them cannot be produced by mammals and must be taken through the diet.

3.3 Oxidant/antioxidant unbalance

Unfortunately, due to pathological conditions, such as environmental stress or ageing (Jadeja and Martin 2021), the balance between ROS production and the cell defences eventually fail, leading to lipid peroxidation, DNA mutations, apoptosis and neurodegeneration (Evans, Dizdaroglu, and Cooke 2004). This can compromise retinal physiology, inducing the immune system to carry out a mild inflammatory response, described as a para-inflammation, to reestablish retinal homeostasis (Xu, Chen, and Forrester 2009).

Several proofs of oxidative stress involvement as a pathological agent of neurological diseases have been reported (Carocci et al. 2018; Picca et al. 2020; Yaribeygi et al. 2018). In this respect, the retina is particularly vulnerable to oxidative damage, being chronically exposed to light (B. Domènech and Marfany 2020), as demonstrated by the presence of oxidative products in Brunch's membrane of retinas from AMD donors (Beattie et al. 2010; Yuan et al. 2010).

Indeed, every retinal component showed damages or cell death under oxidative stress. *In vivo* experiments of exposure to white light in albino rats (Benedetto and Contin 2019) and induced hypoxia conditions in retinal degeneration 8 (rd8) mice (Lajko et al. 2017) have shown to increase the death of PRs, which display peroxidation and increased NADPH oxidase 4, both related to ROS overproduction (Hadziahmetovic and Malek 2021).

Moreover, the intense phagocytic activity, an energy consuming process that stresses mitochondria (Mao et al. 2014), together with the close proximity to the choroid (Alm and Bill 1970), the strong photo-oxidative stress coming from the apical side (Beatty et al. 2000) and accumulation of metabolic products, like A2E and lipofuscin (Sparrow and Yamamoto 2012) also put RPE under intense oxydative stress. Besides, a protective effect against phototoxic stress of the RPE has been shown *in vitro* after treatment with sulforaphane, an Nrf2 activator (Gao and Talalay 2004), glutathione (Sternberg et al. 1993; Yoon et al. 2011), vitamin E, vitamin C and beta-carotene (Kagan, Liu, and Hutnik 2012).

Microglia recruitment in the retina leads to augmented phagocytosis and pro-inflammatory factors concentrations, impacting the tissue's morphological integrity, therefore, its functionality. Microglia are also involved in the condition of unbalanced ROS production. Indeed, in the retina of mice exposed to bright light, retinal degeneration and PR death have been associated with the migration of microglial cells in the ONL (Wang et al. 2014).

3.4 Antioxidants as therapeutical agents

In the last decades, several treatments aimed at contrasting oxidative stress have proved to be effective, providing further evidence regarding ROS involvement in several forms of retinal degeneration.

The age-related eye disease studies (AREDS, AREDS2) unveiled the therapeutic potential of antioxidants against retinal degeneration, focussing on AMD. The AREDS study consists in dietary intake of vitamin C, vitamin E, beta-carotene, copper and zinc, showing a high variability in the results compared to the placebo, with a 85% reduction in the progression for some patients and a 300% increased risk of progression for others (Seddon, Silver, and Rosner 2016). This difference has been attributed to the genetic variability and the different aethiologies of the disease across patients (Vavvas et al. 2018). AREDS2 started few years later, exploring different compositions of antioxidants and adding omega-3-fatty acids, lutein and zeaxanthin. This study demonstrated an improvement in patients having beta-carotene replaced with lutein and zeaxanthin, with a reduction of the progression of dry AMD to the advanced stages up to 10% compared to the placebo (Chew et al. 2013).

Another proof of concept has been given by Resveratrol, a phenolic phytochemical produced by several plants against injuries and phatogens, able to suppress the UV induced H_2O_2 production in the OS (Calzia et al. 2015) and reduce the damage induced by A2E accumulation *in vitro*.

Moreover, Edaravone, a well-known free radical scavenger (Minnelli et al. 2019) has shown efficacy in *in vitro* and *in vivo* models of retinal degeneration, showing reduction of ROS, lipid peroxidation and endothelial cell proliferation induced by VEGF (Imai et al. 2010; Masuda et al. 2016; Shimazaki et al. 2011).

Unfortunately, despite the promising results, the patient's compliance has a crucial importance in determining the final results of these therapies.

4 AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is a neurological disease that affects the human fovea, causing a progressive decrease of central vision, in some cases, leading to total blindness (Coleman et al. 2008; Lim et al. 2012). In the industrialized countries, AMD is the leading cause of visual impairment and blindness in the elderly (Apte 2021), affecting around 10% of the total population over 65, with over 190 million people affected worldwide (Kawasaki et al. 2010; Klein, Klein, and Cruickshanks 1999; Wong et al. 2014). With an estimated global cost of more than 300 billion dollars every year (Apte 2021) and a predicted number of people that will suffer from this disease up to 300 million in 2040 (Wong et al. 2014), the economic interest in finding a cure is extremely high.

Moreover, AMD has a highly negative effect on patients' life. Indeed, people affected with AMD are more subjected to develop depression, report major life stress and have poor physical activity than healthy peers (Brody et al. 2001) or age-matched patients suffering of other serious chronic health diseases (Mitchell and Bradley 2006). Furthermore, it has been shown that AMD patients have an increased risk of negative outcomes in daily activities (Gopinath et al. 2014), often getting injured by either falling or other accidents with additional costs for the family and the healthcare system. Altogether, finding new therapies that may both reduce the financial burden and improve people quality of life.

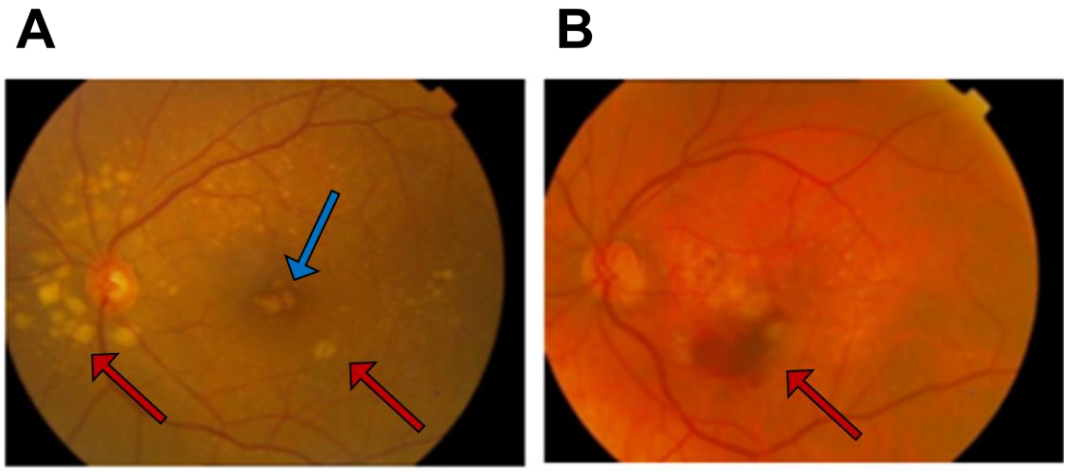

Figure 9. *Fundus of different AMD subtype*. **A**) Geographic (dry) AMD, characterized by macular degeneration (blue arrow) and presence of randomly dispersed large drusen (red arrows). **B**) Neovascular (wet) AMD is characterized by subretinal exudate, indicated with red arrows.

4.1 Aetiology

AMD is a complex multifactorial disease, and despite the intense research done in the last decades, most of the causes and the molecular mechanisms behind this pathology are yet to be revealed. Age is by far the major risk factor for the onset of AMD. Indeed, the vast majority of people affected are over 60 years and the prevalence rises along with ageing, reaching 13% of incidence in people over 85 (Smith et al. 2001).

Nevertheless, both environmental and genetic features have been proved to be significant risk factors for AMD onset. Smoking for more than 40 years has been associated with a 4-fold increased risk of AMD (Khan et al. 2006). Moreover, C57BI6 mice chronically exposed to cigarette smoke (5 h/day, 5 days/week for 6 months), displayed RPE apoptosis and abnormalities, such as increased intracellular vacuoles and basal laminar deposits, accompanied by Brunch's membrane thickening (Fujihara et al. 2008) together with an earlier onset of PR degeneration (Mitchell 2002; Smith et al. 2001). Moreover, an increased risk of AMD has been reported associated with uncontrolled hypertension (increased risk of progression in late neovascularAMD)(Klein et al. 2003), obesity (Seddon 2004), high fat rich diet (Uranga et al. 2010), alcohol consumption (Adams et al. 2012) and sunlight exposure (Taylor et al. 1990).

The iris colour was the first genetic factor associated with increased AMD incidence (Smith et al. 2001). Nowadays, around 34 genetic loci correlated with increased AMD onset have been identified (Fritsche et al. 2016). Those genes codify for proteins involved in DNA repair, cell signalling, lipid metabolism, protein binding and collagen production (Apte 2021). However, the mutations associated with highest risk are the genes encoding complement factor H, like ARMS2 and HTRA1 (Strunz et al. 2020).

Traditionally, AMD (Figure 9) has been classified into two subtypes:

- geographic AMD, also called dry AMD (Figure 9A), is characterized by multifocal atrophy of the RPE and the neurosensory retina with demarcated borders, which may or may not involve the foveal centre (Ferris et al. 2013; Spaide, Fujimoto, et al. 2018). Moreover, RPE abnormalities, hyperpigmentation and choriocapillaris loss are also present (Malek and Lad 2014; Mullins et al. 2011). Dry AMD represents around 90% of the total cases, has a slow progression and leads to blindness only after decades;
- neovascular AMD (Figure 9B), or wet AMD, presents choroidal neovascularization able to penetrate the Brunch's membrane in the foveal area. It can lead to intraretinal fluid leakage or subretinal or sub-RPE haemorrhage, lipid exudates

and RPE detachment (Ferris et al. 2013; Spaide, Fujimoto, et al. 2018). The end-stage of wet AMD is often accompanied by "disciform" scars in the fovea, causing permanent loss of central vision (Ferris et al. 2013; Spaide, Ooto, and Curcio 2018). Even if this form represents only 10% of total AMD, its quick progression leads to blindness in a few years.

It is essential to underline that the two AMD forms are not mutually exclusive. Indeed, atrophic AMD may present neovascular lesions, while wet AMD can cause retinal atrophy (Hadziahmetovic and Malek 2021).

AMD can be classified into two main stages: early AMD and late AMD (Figure 10). Early AMD is characterized by drusen, deposits of heterogeneous material located between the RPE basal membrane and the inner collagenous layer of the Brunch's membrane (Green 1999), mainly present in the macula region. They are formed by

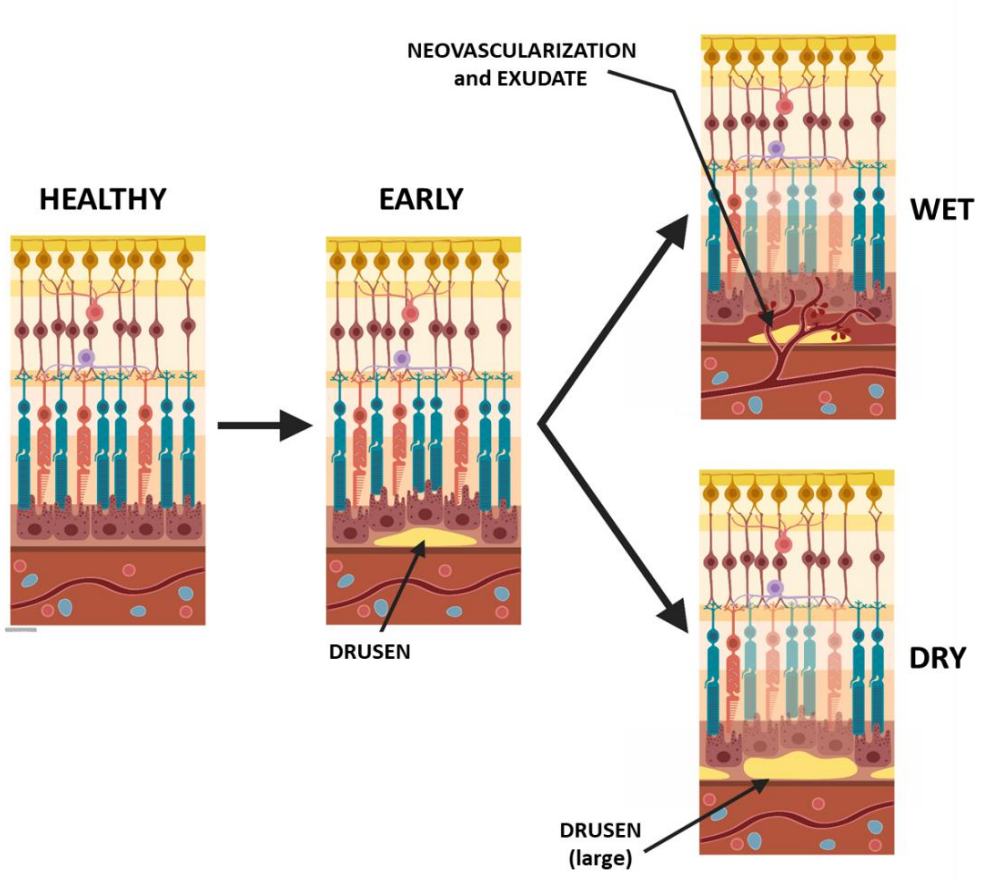

Figure 10. *Progression of age-related macular degeneration*. Schematic image representing various stages of AMD progression. On the left is shown a cartoon of the healthy retina. The early stage of the disease (middle) is characterized by drusen accumulation between the RPE basal membrane and the Brunch's membrane. The progression of the disease can lead to wet AMD (top right), characterized by neovascularization with exudate, or to dry AMD, characterized by enlargement of the drusen. Both forms of AMD lead to RPE cell and PR death.

cholesterol (more than 40%) (Li et al. 2007), zinc and iron ions (Curcio et al. 2009), and a plethora of different proteins (129 identified nowadays), including β-amyloid, several types of apolipoproteins and proteins of the complement system (Curcio et al. 2009; Rudolf et al. 2008). Drusen can be classified in small (less than 63 μm diameter), medium (between 63 and 125 μm), or large (more than 125 μm) (Curcio et al. 2013). Only medium drusen are specific of the early stages of AMD because small drusen could form during ageing without leading to the pathology (Coleman et al. 2008). Moreover, their size, shape and density are determinants for the progression of the disease (Davis et al. 2005), with soft, large and confluent drusen associated with a higher risk of progression to the late stage of either dry or wet AMD (Hadziahmetovic and Malek 2021).

The importance of the choriocapillaris network integrity has been proven for both forms of the advanced form of AMD (Chirco et al.), observed as choriocapillaris density dropout and choroid thinning in humans retinas (Mullins et al.; Choi et al.). Moreover, while in dry AMD the RPE loss seems to precede the choriocapillaris damage (Mcleod et al.), in the wet form the choriocapillaris loss occurs in regions where the RPE is still intact (Moreira-Neto et al.).

4.2 Therapeutic strategies

Before the year 2000, being diagnosed with wet AMD meant blindness, because no treatments were available to counteract neurodegeneration. The first hope was given to patients when, in 2004, FDA approved Macugen, an anti-angiogenic drug developed by Eye Tech Pharmaceuticals, able to slow down the progression of the disease. Since then, the discovery of many new and more effective medications has followed. Three treatments are available nowadays for wet AMD, all sharing the same principle of action, focusing on the blockage of the vascular endothelial growth factor (VEGF) (Hadziahmetovic and Malek 2021). In 2006, FDA approved Ranibizumab (Lucentis, Genentech), which is a recombinant humanized antibody fragment (Fab) able to bind, therefore inhibit, all forms of VEGF-A (Brown et al. 2006; Rosenfeld et al. 2006). Five years later the recombinant antibody Aflibercept (Regeneron Pharmaceuticals) was approved by FDA. It is created by combining the constant region of human immunoglobulin G with the extracellular domain of the VEGF receptor 1, working as a decoy to reduce the soluble VEGF in the extracellular fluid before it can activate angiogenesis. The greater benefit of this drug is the long half-life of the protein, which reduces the frequency of intravitreal injections (Schmidt-Erfurth et al. 2014). The third drug in use for wet AMD treatment is Bevacizumab

(Avastin, Genentech), a full-length humanized monoclonal antibody able to bind VEGF-A and reduce neovascularization, having a longer half-life than all the other antiangiogenic drugs (Ferrara et al. 2004; Ferrone et al. 2014; Rofagha et al. 2013; Schmidt-Erfurth et al. 2014; Wang et al. 2004).

In the last years, a plethora of new medications were studied either targeting VEGF-A, such as Brolucizumab, a recombinant antibody targeting all the three major isoforms of VEGF-A (Dugel et al. 2020), and Lumitin, which inhibits also placental growth factor (PlGF)(Călugăru and Călugăru 2019; Liu et al. 2019), or acting as combination therapies to improve anti-VEGF-A treatments, as OPT-302, which targets VEGF-C and VEGF-D (Al-Khersan et al. 2019). All of them require frequent injections, creating an enormous burden for the patient and the healthcare system. Moreover, frequent injections are associated with higher risk of cataract formations, retinal detachment and vitreous haemorrage (Huang and Chau 2019).

Gene therapy has the potential to improve the quality of life of people affected by AMD, mostly because either only one or a few injections may be sufficient to induce a permanent rescue from the disease. Gene therapies encoding for an antibody fragments able to inhibit VEGF and ADVM-022, or an Aflibercept gene therapy product, are actually under development (Al-Khersan et al. 2019). Modern gene therapies are based on adeno-associated viral (AAV) vectors (such as RGX-314), able to transduce efficiently both RPE and photoreceptors. Unfortunately, their relatively small payload does not allow to pack large molecules of DNA, therefore limiting the number of diseases treatable with this vector (Daya and Berns 2008; Vandenberghe and Auricchio 2012). Non-viral vectors like nanoparticles can be prepared in order to achieve entry in both photoreceptors and RPE cells and have larger DNA payloads than AAV vectors. Finally, it is essential to mention the non-invasive strategy that exploits the use of eye drops to deliver a VEGF-2 receptor antagonist, Pazopanib (PAN-90806 from PanOptica)(Al-Khersan et al. 2019; Hussain and Ciulla 2017; Patra et al. 2018), which may lead the future for crucial technological improvement.

A controversial contraindication of VEGF inhibitors is that VEGF-A overexpression is a retinal survival mechanism induced by the overproduction of ROS. Indeed, a SOD-/- mouse model, developed by Imamura and collaborators (Imamura et al. 2006), shows human-like neovascularization similar to AMD. This finding, together with the ineluctable visual worsening in about one-third of the patients treated with VEGF inhibitors (Bhisitkul et al. 2015; Moutray and Chakravarthy 2011; Rofagha et al. 2013), may suggest the possibility that finding new strategies able to either support

anti-VEGF therapies or act upstream of the VEGF pathway may promote more beneficial effects in blocking the neurodegeneration.

5 LIGHT DAMAGE ANIMAL MODEL

5.1 Rodent visual system

Rodents' eyes are very different from other species from anatomical and physiological points of view, reflecting different evolution demands from their vision. For example, rats' eyes present a substantial difference in their lens, which is thicker and less elastic, providing them with a worse long-distance focus than human sight.

Moreover, rats' evolution as nocturnal animals resulted in a higher density of rod photoreceptors (4-6 fold higher in all retinal areas) than other mammals (Wang et al. 2021). Therefore, their retinas present no macula with a lower density of cones throughout the whole retina (Baylor 1996). In addition, rats possess only two types of cones, the G cones, with a similar absorption spectrum of its human counterpart, and the S cones (Szél et al., 1992; Szél & Röhlich, 1992), which peak sensitivity is shifted towards a shorter wavelength, allowing them to see in the UV spectrum (Jacobs et al., 1991). The result is a much lower visual acuity under daylight conditions, reflected in the photopic ERG response. Indeed, Sprague Dawley (SD) rats show no a wave (an index for cones activity evaluation) in the photopic electroretinogram compared to monkeys, having the closest visual system to humans, with little difference in the b wave amplitude (Lei, 2003).

Nevertheless, rodents are a raw but good model for the investigation of several human retinal pathologies. The Royal College of Surgeons rat provides an exquisite example of the similarity between rats and human retinal physiology. RCS rat is a vastly used animal model of retinal degeneration. Indeed, defective phagocytosis of the exhausted OS, caused by a mutation of the gene *Mertk* (D'Cruz et al., 2000), induces PRs degeneration by an abnormal elongation of the OS, with consequent loss of function. Likewise, a mutation involving the human orthologue gene *MERTK* (Gal et al., 2000). A mutation of this gene induces a form or retinitis pigmentosa in humans characterized by the same OS phagocytosis defect, which cause the elongation of PRs' OS that eventually led to blindness through an analogous PRs death pattern.

Importantly, the absence of a macula or a similar structure in the rodent's retina can limit the study of AMD in mouse models. However, peripheral areas of the human macula present a lower density of cones than the areas in the same eccentricity in the rodent's retina. Since peripheral degeneration with rod loss is part of the pathogenesis of AMD, the mouse rodent retina can still represent an acceptable model of the same pathogenic processes (Wang et al. 2021). Moreover, exposure to

light in dim light reared rodents (e.g., rats) has shown localized degeneration with morphological and molecular similarity to human macular degeneration.

5.2 Light damage

The damage induction in the retina through exposure to high-intensity light, called light damage (LD), has been widely investigated in translational medicine to understand the mechanism beyond PR degeneration and test the efficacy of new therapeutic approaches *in vivo*.

Nowadays, even if the exact processes behind retinal LD are not understood, we know that this induced neurodegeneration is a multifactorial process involving both genetic and environmental factors, many of which seem to be shared between species (Organisciak and Vaughan 2010). Anyway, the intensity, the localization, and the type of morphological alteration in the retina induced with LD can differ considerably depending on several factors.

The mechanisms beyond cellular responses and LD-induced morphological changes seem to differ among different species. Nocturnal animals, like rodents, appear to be less resistant to light, therefore showing an increased alteration in retinal morphology when exposed to high luminances than other animals (Noell et al. 1966). Moreover, the absence of pigment (e.g., in albino animals) is a crucial factor for the efficacy of light in the induction of cell death.

It has been demonstrated that animals reared in total darkness present a more severe damage in the RPE than in the PR layer, while animals raised in dim light show minor damage to the RPE at the cost of PR alteration (Noell 1980). In any case, due to the tight metabolic relationship between the two cell types, the functional alteration of one of them will lead to the degeneration of the other (Organisciak and Vaughan 2010).

Nocturnal rodents have shown damages mostly confined to the ONL following LD, with a gradual reduction in the thickness that peaks in rat's retina around 1-2 mm from the optic nerve in the dorsal retinal hemisphere (HOTSPOT) (Rapp and Williams 1980). This different sensitivity to light damage between superior and inferior hemisphere of the retina seems to be attributed to the direct exposition to light of the inferior retina during the critical period, which increase the resistance to light induced stress in the ventral retina, and the direction of the light source used for the LD. Indeed, in 1999, Stone and collaborators proved that if the source of illumination in the animal facility is moved from the ceilings to the walls, the characteristic hotspot does not form (Stone 1999) , leading to a spread PRs death along the retina.

Several hypotheses have been formulated on both the molecular and anatomical differences below this asymmetrical reactivity to light. Indeed, unbalance in OS length, different rhodopsin levels in the OS (Battelle and LaVail 1978; Penn, Naash, and Anderson 1987), improved circulation of the inferior retina and/or higher production of a neuroprotective factor (Li, Cao, and Anderson 2003; Liu et al. 1998) seems to play a role in the LD asymmetry.

5.3 Etiology of the light-induced neurodegeneration

It has been shown by Organisciak, Faber and their colleagues, that rats reared in the dark present increased levels of rhodopsin and transducin and a reduced level of arrestin, which might be the reason for their increased susceptibility to LD (Farber, Danciger, and Organisciak 1991). It is well known that the rhodopsin activation spectrum is identical to the spectrum of light needed to induce LD neurodegeneration (Williams and Howell 1983), proving that rhodopsin activation is one of the triggers of PR damage in LD (Grimm et al. 2000; Humphries et al. 1997; Noell et al. 1966). Moreover, the reduction of arrestin expression, which can impair the ability of PRs to shut down rhodopsin signaling, has been confirmed to be another factor to increase LD susceptibility (Xu et al. 1997).

Another variability that may change the animal sensitivity to LD is the time of LD within the circadian rhythm. It has been shown that increased damage is induced on animals placed under intense light in the middle of the dark shift of their circadian rhythm, compared to those subjected to LD at the beginning of the day shift. Moreover, LD was scarcely effective in the middle of the day cycle, proving a circadian dependency of the susceptibility (Penn et al. 1992; Rapp and Williams 1980; Vaughan et al. 2002).

Many scientific reports point out the possible involvement of ROS in the induction and the perseveration of LD neurodegeneration. Transgenic mice carrying a mutated SOD, a crucial endogenous antioxidant able to eliminate O_2^-, incurred in stronger LD compared to mice having normal SOD (Mittag, Bayer, and La Vail 1999). Moreover, the preventive effect of different antioxidants both organic (such as L-stereoisomer of N-acetyl-cysteine (Busch et al. 1999; Tanito et al. 2002), N-nitro-arginine methyl ester (Donovan, Carmody, and Cotter 2001; Goureau et al. 1993; Káldi et al. 2003), dietary supplement of saffron (Maccarone, Di Marco, and Bisti 2008), Ginkgo biloba extract (Ranchon et al. 1999)) and inorganic (Cerium-oxide nanoparticles (Chen et al. 2006; Fiorani et al. 2015)), support the hypothesis of a key role of ROS in light-induced neurodegeneration in the LD model. While some experiment shows that the

maximum efficacy of antioxidants is obtained only when they are administered before LD induction (Organisciak et al. 2000; Vaughan et al. 2002), other studies showed the efficacy of those treatments even when administered a few hours after the end of the LD (Chen et al. 2006).

Retinal LD not only represents a good model for understanding common basic mechanisms of degeneration between rats and humans, simulating AMD with the presence of the hotspot (Marc et al. 2008), but has also a translational significance in predicting the efficacy of many treatments for eye diseases (Falsini et al. 2010; Piccardi et al. 2012).

6 NANOPARTICLES

A relatively new field in biomedicine is nanotechnology. Thanks to the unique properties of nanoparticles (NPs), nanotechnology is revolutionizing the concept of modern medicine, from the diagnostic to the therapeutical fields. NPs have higher stability than endogenous enzymes at high temperatures, extreme pH and can resist electromagnetic radiation. Moreover, NPs have at least one dimension below 100 nm (Vert et al. 2012), similarly to endogenous proteins, which may allow their internalization when in contact with cells (Sabella et al. 2014). NPs have a high surface-to-volume ratio that maximizes the material reactivity and reduces the concentration necessary to trigger a chemical reaction. Due to their high free energy, NPs get covered with proteins in the biological environment, allowing the formation of a protein layer called corona, which reduces the free energy and promotes NP dispersion (Monopoli et al. 2012). This corona can be engineered to recognize some receptors to reach a precise target or to cross the blood-brain barrier (BBB) (Baimanov, Cai, and Chen 2019). The ability of some kinds of NPs to carry drugs and molecules in their core, opens many chances for NPs to be used for drug delivery. Finally, NPs can be created in different shapes, sizes and even more than one material, opening a world of possibilities for their engineering.

6.1 NP classification

NPs can be classified based on their core material being organic or inorganic. Organic NPs are biodegradable, non-toxic, can cross, or fuse with, biological membranes but are sensitive to high thermal and electromagnetic radiation (Tiwari, Behari, and Sen 2008). Moreover, some organic NPs can carry molecules in their empty core or lipidic structure, making them perfect for drug delivery. Common organic NPs are ferritin, micelles, polymeric and hydrogel-based liposomes and dendrimers (Ealias and Saravanakumar 2017).

Inorganic NPs have a non-organic core, usually metals and metal oxide or semiconucting materials. Oxidation might be used to change the properties of the respective metal base NPs for a variety of applications, such as increasing their reactivity. In the shape of NPs, metals obtain higher reactivity to environmental factors, increased volume/surface ratio, surface-charge and surface charge density (Nikam, Prasad, and Kulkarni 2018). Only a few metals cannot be synthesized as NPs (Salavati-Niasari, Davar, and Mir 2008).

6.2 NP synthesis

NPs sharing the same core can have different properties depending on the synthesis process used to create them. Indeed, NPs size and morphology can be easly influenced by varying the concentrations of precursors, reducing agents, capping agents, or by modyfing pH and temperature (Jeyaraj et al. 2019).

The protocols used to synthesize NPs can be grouped into two main approaches, bottom-up or top-down. The former protocol, also called constructive methods, consists of building the NPs up from single atoms, using reducing or capping agents to stop the reaction in order to obtain precise shapes and sizes. Most commonly used bottom-up techniques are seeded-growth, self-assembly of monomer/polymer molecules, chemical or electrochemical nanostructural precipitation, sol–gel processing, laser pyrolysis and bio-assisted synthesis (Dhand et al. 2015). The latter protocol, also called destructive method, generates NPs through mechanical milling, laser ablation or nanolithography (Ealias and Saravanakumar 2017).

NPs synthesis protocols can be further divided based on the principle exploited for the synthesis, in physical, chemical and biological methods. Physical methods involve the application of mechanical forces, radiation, temperature or other physical properties of the materials to create NPs. It requires intense energy and produce considerable wastes, but the final product is free from toxic contaminants (Dhand et al. 2015). On the opposite, chemical methods mostly involve reduction of cation precursors to trigger formation of metal monomers. Those methodologies have low cost, and display easy functionalization, high yield, size control, thermal stability and reduced dispersivity. Despite these advantages, NPs obtained with these methodologies have low purity and the use of toxic products is an hazard for their bio-applications (Jeyaraj et al. 2019). Finally, biological methods, also called biomolecule-assisted synthesis, are receiving an increasing interest in the last years. NPs produced with biological synthesis are soluble, cost effective, sustainable and eco-friendly, while being highly biocompatible. Unfortunately, with those methods is difficult to control shape and size, stability and presence of endotoxins, while being time-consuming due to the purification process of the biological reagents. Bacteria, fungi, plants and small biomolecules are used to produce NPs, but unfortunately these processes are limited by the biological properties of the reagents, limiting their use to cores and shapes of the NPs (Dhand et al. 2015).

Several protocols require capping agents to stabilize NPs and avoid aggregation (Pedone et al. 2017), which leads to another variability in an already very busy literature. This variability has generated conflicting results on the toxicology profile

of NPs both *in vitro* and *in vivo* (Baati et al. 2012; Matsuda et al. 2011; Park et al. 2008). Therefore, it is essential to develop strategies for the synthesis, purification and stabilization of NPs that will allow them to be safely used in biomedicine, while avoiding negative ecological impact.

Altogether, it is difficult to understand if the observed characteristics of NPs, such as toxicity, are due to NP components themselves or to the production method (Pedone et al. 2017). Unfortunately, physical methods require huge amount of energy and may release radiation, while most of the chemical methods use toxic chemicals and release toxic wastes, which may create both biohazards and ecological problems (Jeyaraj et al. 2019).

6.3 Protein corona

A crucial aspect for NP biological applications is the formation of the protein corona that covers the NP surface. This is due to the NP surface high free energy, which leads to a spontaneous protein adsorption onto their surface. In biological fluids, NPs acquire a protein layer that modifies their interaction with biological tissues, increases their mass and size and reduces the available surface for reactions (Baimanov et al. 2019). The corona properties strictly depend on the nature of the interaction between NPs and biological fluids, therefore on the strength and typology of the surface charge of NPs and its hydrophobicity or hydrophilicity (Sund et al. 2011).

The corona can be divided into a hard corona, an inner layer made by the first proteins that bind NPs, and the soft corona, an outer layer whose composition may change while the NPs navigate through biological fluids with different protein composition (Walczyk et al. 2010). The hard corona is tightly bound to the NP surface and can be removed only with an artificial process, carrying a memory of the first biological solution or fluid NPs encountered. On the opposite, the soft corona has a variable composition that can modify the biodistribution of the NPs in the body and their dynamic of internalization. Therefore, the corona composition differs depending on the way of administration and may alter the diffusion and internalization characteristics *of NPs* (Monopoli et al. 2012).

6.4 NPs in medicine

A crucial aspect for NP biological applications is the formation of the protein corona that covers the NP surface. This is due to the NP surface high free energy, which

leads to a spontaneous protein adsorption onto their surface. In biological fluids, NPs acquire a protein layer that modifies their interaction with biological tissues, increases their mass and size and reduces the available surface for reactions (Baimanov et al. 2019). The corona properties strictly depend on the nature of the interaction between NPs and biological fluids, therefore on the strength and typology of the surface charge of NPs and its hydrophobicity or hydrophilicity (Sund et al. 2011).

6.5 Platinum NPs

Platinum (Pt) is a rare noble metal characterized by low reactivity compared to other metals, high corrosion resistance, stable electrical properties and high solubility in water. Moreover, Pt has robust catalytic activity (Hamasaki et al. 2008; Horie et al. 2011) and its synthesis as NPs can be easily controlled by changing pressure, temperature and reagents ratio. Furthermore, many platinum-based compounds, such as cisplatin, carboplatin and oxaliplatin, have been extensively used as therapeutic agents in chemotherapy (Wheate et al. 2010). Thus, the interest around Pt as core material for NPs grew in the last decade due to its possible applications for hypertermia and photoablation terapies, targeted drug delivery, bioimaging, biosensing and as antimicrobial and anticancer agents (Jeyaraj et al. 2019).

Nevertheless, Pt has been poorly investigated as a possible core material for NPs applications in biomedicine. Indeed, several *in vitro* studies enlightened positive effects of PtNPs (Hosaka et al. 2014; Pedone et al. 2017; Yoshihisa et al. 2010), but the contradictory results on adverse effects, induced by the release of Pt^{2+} ions, known to be toxic for the DNA integrity, prevented Pt nanozymes use in several biomedical applications (Pedone et al. 2017).

Assuming that the cytotoxicity of the PtNPs was observed in some published experiments, Moglianetti and collaborators used a non-toxic synthesis method and particular care to avoid contaminations to prove that PtNPs toxicity had to be attributed to residual contaminants from the NP synthesis (Moglianetti et al. 2016). These NPs capped with citrate to increase their stability in the solution, were safe *in vitro,* causing no alteration in mitochondrial metabolism, or damage to membranes or DNA in all the cultures tested. Moreover, cc-Pt-NPs showed much higher activity as ROS scavengers than endogenous peroxidase, catalase and SOD (Moglianetti et al. 2016)

II. AIMS

PtNPs are highly effective against ROS dangerous activity, a common feature shared by most neurodegenerations, including retinal degenerations. Nanoparticles with similar catalytic activity have displayed strong preventative effects against neurodegeneration progression, but without achieving a resolutive effect. Unfortunately, the controversial data on PtNPs safety, which has been ascribed to the heterogeneous pool of protocols used for NPs creation, is an obstacle to their application as preventative therapy.

We hypothesize that PtNPs could be safe as core material for NPs and effective in retinal neurodegeneration prevention if produced with proper methods.

This thesis proposes 5 nm citrate-capped Pt-NPs, produced by seeded-growth approach, as an intravitreal injectable treatment to prevent retinal degeneration. A suitable protein corona functionalization has been engineered for the NPs to fit the ocular environment and allow a good dispersion in physiological media, as characterized by TEM size distribution, dynamic light scattering, agarose electrophoresis, and H2O2-TMB Chromogenic Assay techniques. Furthermore, *in vitro* experiments involving propidium iodide and fluorescein diacetate were used to assess neuronal viability after PtNPs exposition, while DCF assay was used for ROS scavenging ability evaluation *in vitro*.

The biocompatibility of PtNPs in vivo and their efficacy as a preventive agent for retinal neurodegeneration were tested in a rodent model of light-induced retinal degeneration, in which the particles were injected intravitreally ahead of the LD inducing degeneration. Electrophysiological measurements in vivo and post-mortem analysis of retinal tissues allowed for a characterization of the biocompatibility of the particles and of their efficacy in ameliorating PR degeneration.

III. RESULTS

1 NANOPARTICLE SYNTHESIS AND CHARACTERIZATION

Most NPs tend to aggregate in physiological media due to their high free surface energy. Besides, proteins spontaneously adsorb onto NPs surfaces, defining their biological identity and interactions with cells both *in vitro* and *in vivo*, thus altering their fate and physiological effects. Hence, understanding the interaction between NPs and proteins is crucial for nanomedicine. Here, the fabrication and characterization processes of PtNPs are described, with particular emphasis on the engineering of a protein functionalization corona to allow for a suitable dispersion of the NPs in the intravitreal compartment of the eye and the inner retinal layers.

1.1 Synthesis and morphological characterization

Given the choice of a rat model of retinal degeneration as target for the intravitreal treatment, endogenous rat serum albumin (RSA) was selected as a stabilizer, also due to its high concentration in both the humour vitreous (around 293 ± 18 $\mu g/cm^3$ (Clausen et al. 1991)) and the blood. The goal was to create a protein corona on the NPs to increase their solubility in physiological solution and avoid aggregates after injection without activating the immune system. Therefore, a characterization of the ccPtNPs coated with RSA corona (ccPtNPs-RSA) was needed to evaluate the stability and catalytic efficiency (Figure 11).

ccPtNPs were synthesized and characterized in collaboration with Nanobiointeractions and Nanodiagnostics Lab in the Italian Institute of Technology (IIT) with a seeded-growth approach, starting from platinum salt (H_2PtCl_6) and adding two reducing agents, sodium borohydride ($NaBH_4$) and the stabilizer sodium citrate (Na_3Ct). ccPtNPs were characterized using TEM (Figure 11A), which allows the observation of the single mono-disperse NPs, ensuring that no aggregation occurred during the synthesis, and the measure of their diameters. By sampling over 100 NP diameters per batch of produced NPs using ImageJ (Figure 11B), we obtained an average diameter of 5.21 ± 0.03 nm (mean ± sem), in line with data obtained in previous works by Moglianetti and collaborators (Moglianetti et al. 2016).

1.2 Stabilization with a protein corona

As shown in Figure 11C, ccPtNPs were incubated with RSA (10 mg/mL) for 30 minutes at 37 °C to allow the protein corona formation and then sedimented with

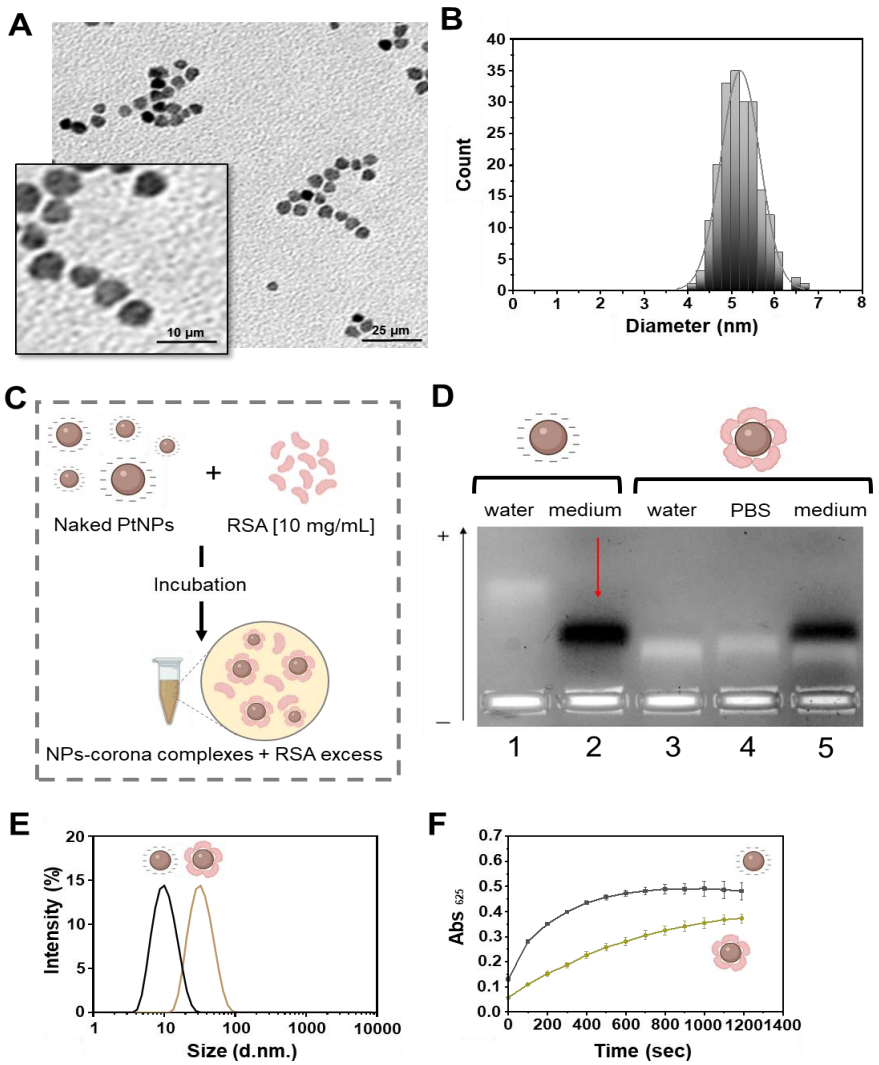

Figure 11. *PtNPs characterization and stabilization.* A) Representative TEM micrograph of 5 nm cc-PtNPs and B) statistical size distribution calculated over 100 NPs with ImageJ. C) Cartoon of the stabilization of 5 nm PtNPs with 10 mg/mL of rat serum albumin (RSA). Proteins spontaneously adsorb onto the NPs surface, improving their stability in physiological media. D) PtNPs stability in cell culture medium assessed by agarose (3.5%) gel electrophoresis: **Lane 1 -** "Naked" PtNPs in water (white band); **Lane 2 -** "Naked" PtNPs in medium: only the black band related to the proteins is visible, indicating NP aggregation in the well; **Lane 3 -** PtNPs-RSA in water; **Lane 4 -** PtNPs-RSA in PBS; **Lane 5 -** PtNPs-RSA in medium. The presence of the white band in all PtNPs-RSA columns indicates good stability. E) DLS measurement for "naked" PtNPs (black) and PtNPs-RSA (gold), confirming the increase of size due to protein corona. F) Catalytic activity evaluation using the H_2O_2-TMB chromogenic assay for "naked" PtNPs (black) and PtNPs-RSA (gold). The corona slightly decreases cc-PtNPs-RSA catalytic activity covering part of the NP surface area. In all experiments, the excess of RSA was washed out by ultracentrifugation. Results from: Valentina Mastronardi, Luca Boselli, and Giulia Tarricone (NanoBD group)

ultracentrifuge to wash out the protein excess. The pellet was then suspended in saline solution and stored at +4 °C.

Further experiments were designed to evaluate ccPtNPs-RSA stability in physiological conditions and cell culture media, which could influence their distribution, intracellular uptake, and scavenging capability. The medium used for the *in vitro* culture of primary neurons, consisting of Neurobasal (NB) supplemented with B27 (2 %), Glutamax (1 %) and penicillin-streptomycin (1 %), was used to test NP stability. NPs were added to the culture medium at 10 µg/ml and incubated for 48 hours. This medium has been used for the *in vitro* culture of primary neurons.

To ensure the presence of an RSA corona, we performed agarose (3.5%) gel electrophoresis. The agarose concentration was chosen to create pores sized to maximize the difference in the migration speed between different NPs. Indeed, the protein corona's presence on the NPs reduces their surface charge and the velocity of migration of the ccPtNPs-RSA compared to the "naked" NPs. As shown in Figure 11D, we compared "naked" ccPtNPs, dissolved either in ddH20 (lane 1) or in culture medium (lane 2), with ccPtNPs-RSA, dissolved in either ddH20, PBS or culture medium. The NPs will appear as white bands. It is possible to notice how NPs dissolved in water (lanes 1 and 3) showed a faster migration velocity, as compared with the slowdown of ccPtNPs-RSA due to the larger size given by the corona. Furthermore, no difference in migration speed was observed between ccPtNPs-RSA dissolved in water (lane 3) or PBS (lane 4), demonstrating a preserved stability in physiological media. Interestingly, when NPs were incubated in culture medium, whose proteins appear as a dark band in the gel, it was possible to observe NP migration only for the ccPtNPs-RSA, proving the increased stability induced by the protein corona.

Additionally, ccPtNPs-RSA displayed a significant shift in the hydrodynamic radius measured with dynamic light scattering (DLS) compared to naked PtNPs (Figure 11E), providing further proof of the presence of a stable protein corona after incubation with RSA.

1.3 Effects of the corona on NP kinetics

Unfortunately, the presence of the protein corona also reduces the NPs surface available for the catalytic reaction. The ccPtNPs catalytic activity was assessed to ensure that the corona did not obliterate NPs scavenging capability using the tetramethylbenzidine (TMB) assay. The formation of the OH• radical on the surface of ccPtNPs oxidizes the TMB substrate, releasing water and forming the colored TMB

radical (Pedone et al. 2020). The shift of the absorbance created by the reaction is an index of the redox reaction catalyzed by the PtNPs. As displayed in Figure 11F, the presence of the corona reduced the NP catalytic activity by 20% compared to the naked ccPtNPs. All the characterization confirmed that citrate capped PtNPs coated with RSA corona were more adapt to both *in vitro* and *in vivo* applications, although

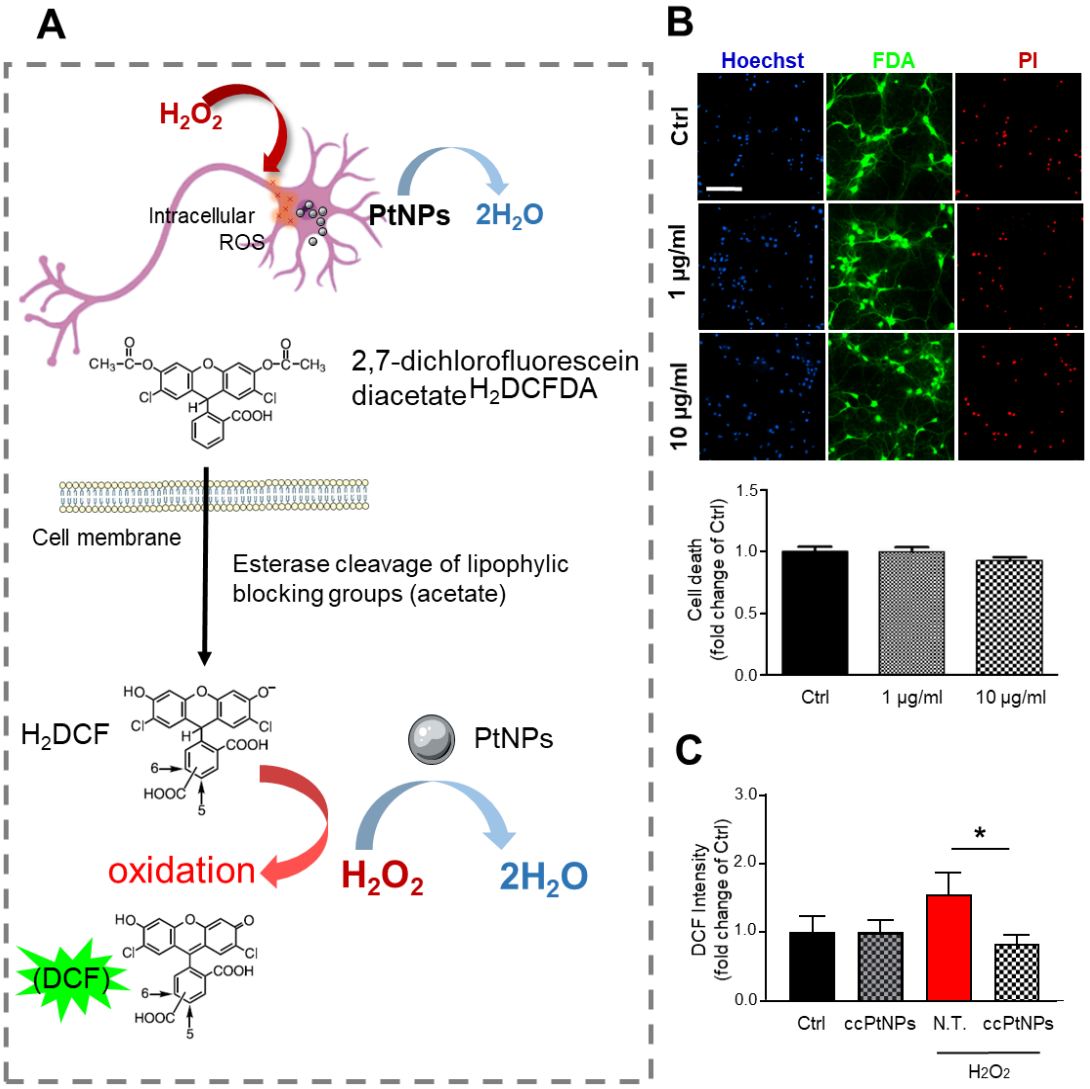

Figure 12. *Protein coated PtNPs effects in vitro.* A) Schematic of the intracellular ROS scavenging activity of PtNPs measured H2DCFDA. After intracellular esterase cleavage, H2DCFDA turns fluorescent after being oxidized by H_2O_2. B) Viability of primary rat cortical neurons exposed to different concentrations of PtNPs (1 and 10 µg/mL), assessed by fluorescein diacetate (FDA; viable cells) and propidium iodide (PI; necrotic cells). Nuclei are stained using the Hoechst dye. C) ROS scavenging activity of PtNPs in primary rat cortical neurons. Neurons were preincubated with 10 µg/mL PtNPs for 24 hours and exposed to 5 mM H_2O_2 for 5 minutes. The presence of PtNPs alone did not induce any ROS increase. Neurons pre-treated with PtNPs showed a significant decrease in ROS compared to the untreated controls. Results obtained in collaboration with Valentina Castagnola, Mattia Bramini and Giulia Borgonovo.

they have a slightly reduced catalytic effect. Therefore, all further experiment will be performed using ccPtNPs-RSA, which will be reported as PtNPs only.

2 *PTNP EFFECTS IN VITRO AND THEIR BIODISTRIBUTION IN THE RETINA*

Once assessed that the RSA protein corona increased PtNPs stability, it was necessary to confirm the biocompatibility and the preventive effects of the PtNPs on ROS-induced damage *in vitro* (Figure 12). A crucial molecule used to assess cell viability and PtNPs scavenging activity is 2,7-dichlorofluorescein diacetate (H_2DCFDA), a probe able to permeate cellular membrane (Figure 12A). When in the cytosol, H_2DCFDA is deacetylated in H_2DCF by endogen esterase, losing its lipophilic groups and remaining trapped into the cell. H_2DCF can be oxidized in the fluorescent molecule dichlorofluorescein (DCF) either by ROS or by endogenous enzymatic activity (Kolthoff, Lauer, and Sunde 1929; Wolfe and Rui 2007). Therefore, a higher presence of ROS induces a fluorescence increment, allowing the indirect quantification of intracellular antioxidant activity (Wolfe and Rui 2007).

2.1 PtNP effects on cells viability and scavenging activity in vitro

A live/dead assay based on Fluorescein Diacetate/Propidium Iodide was performed on 14 DIV primary rat cortical neurons incubated with PtNPs for 48 hours (Figure 12B) to assess cytocompatibility. The top panel in Figure 12B displays representative images of the viability assay with H_2DCFDA (FDA, green) and propidium iodide (PI, red) in neurons untreated (top row) or incubated for 48 h with either 1 µg/ml (middle row) or 10 µg/ml (bottom row) of PtNPs. In blue are shown cell nuclei stained with Hoechst. While the intensity of the FDA fluorescence is a direct index of the enzymatic activity of the cell, therefore their vitality, the PI fluorescence is an index of the cell death. Indeed, PI is a hydrophilic molecule that, when it binds the DNA, increases its quantum yield up to 30 times. When a cell is alive, its membrane is intact keeping PI in the extracellular fluid. On the opposite a dying/damaged cell allows the entrance of PI, which can reach the DNA and bind it, drastically increasing its fluorescence. As shown in the graph in Figure 12B (bottom panel), dead cells in the plates incubated with both concentrations of PtNPs tested were similar to the untreated control, showing no cytotoxicity effects of the NPs, even at the highest concentration.

To assess PtNPs scavenging activity, cellular antioxidant activity (CAA) assays were performed (Figure 12C). This technique allows the quantification of the antioxidant activity in the cytosol of live cells when exposed to a stressful environment. After

48-hour incubation with 10 µg/mL of PtNPs, cells were exposed to 5 nM of H_2O_2 for 5 minutes, followed by fluorescence quantification. Interestingly, PtNPs alone did not alter the physiological level of oxidation in the cytosol, while they were able to restore physiological oxidation levels in cells exposed to H_2O_2. These results prove an excellent biocompatibility together with the ability of NPs to induce significant antioxidant effects in live cells.

2.2 PtNP biodistribution in the eye

Since the protein corona did not alter the PtNP capability to restore physiological ROS levels in the presence of H_2O_2 *in vitro*, we preliminarily examined whether NPs were able to cross the ILM, a tight physical barrier between the posterior chamber of the eye and the retina and were internalized by PRs. This passage is crucial because the most dangerous effects of ROS overproduction are experienced by inner retinal structures, such as PR's mitochondria, disks and organelle (B. Domènech and Marfany 2020). Due to their dimension, PtNPs do not interact with light. Moreover, the conjugation of fluorescence dyes with the NPs may alter the diffusion kinetics through the tissues. These considerations make the visualization of NPs at the light microscope very challenging. Due to their high electron density, we proceeded to visualize PtNPs in the retinal tissue by electron microscopy. We performed a qualitative TEM analysis of retinas explanted from animals injected with PtNPs to prove their ability to cross the ILM and reach the PRs in the outer retina.

We injected 3 healthy adult Sprague-Dawley rats with 2 µl of a 100 µg/ml suspension of PtNPs and sacrificed them at different time points (1 hour, 1 and 7 days) after the injection. After euthanasia, the diopter system was quickly removed from the bulb, the eye divided in 4 pieces and immediately fixed for TEM imaging.

Figure 13 shows 60–70 nm thick retinal sections observed at different magnifications at the TEM. is displayed A representative retinal section stained with hematoxylin and eosin, showing the different layering of the retina is shown Figure 13A (left). The yellow rectangle indicates the retinal portion shown in the TEM images on the right at various magnifications. Figure 13B shows a higher magnification of the basal portion of the inner segments. It is possible to appreciate the presence of nanoparticles internalized in the cells and stored in intracellular vesicles. Thus, intravitreally injected PtNPs can cross the ILM, reach the outermost portion of the retina, and be internalized by PRs, where the Pt-driven catalytic effect takes place.

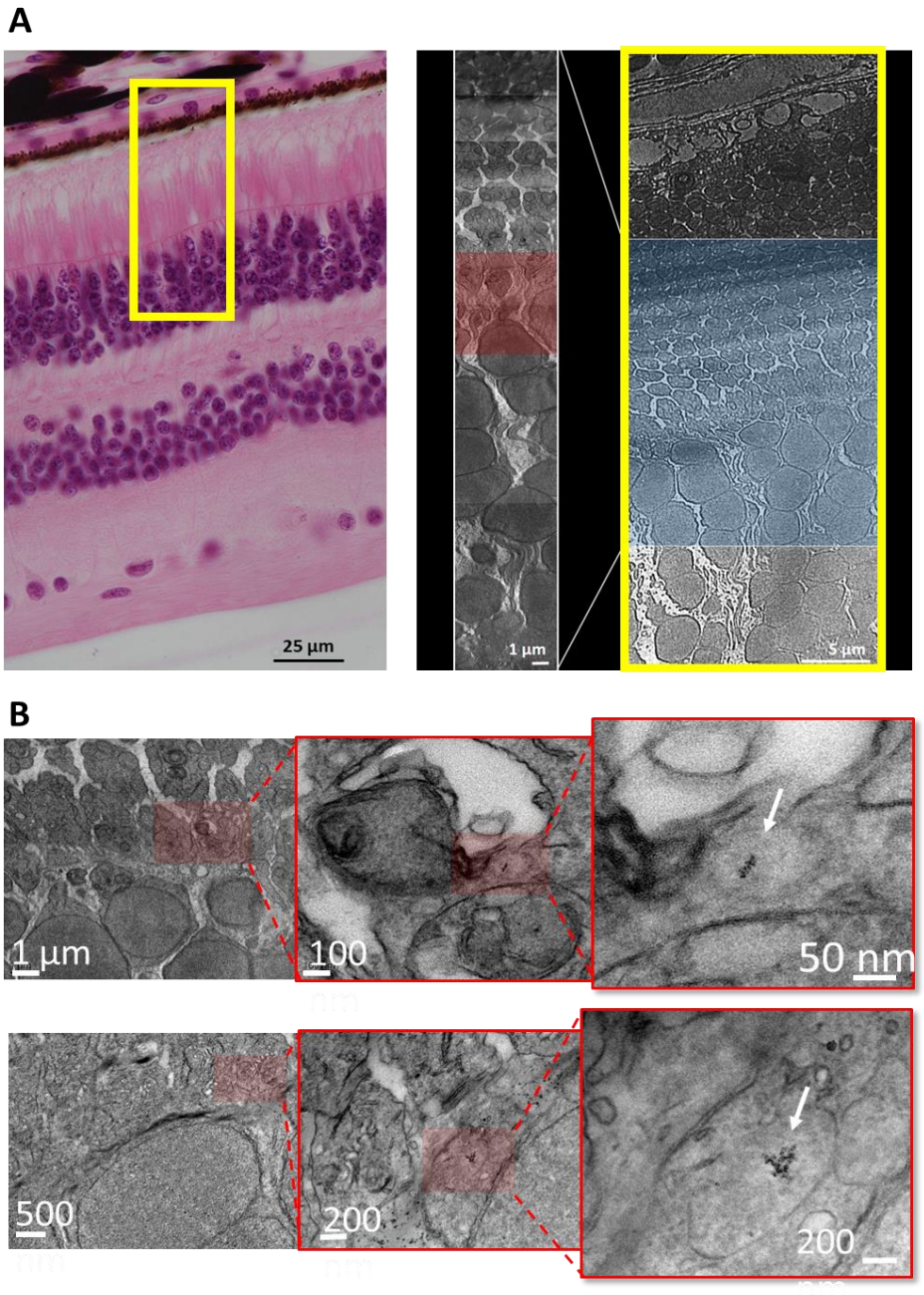

Figure 13. *PtNPs distribution in the retina.* A) Retinal section with hematoxylin-eosin staining (left) and TEM micrographs of the area in the yellow rectangle (right). TEM micrographs presented on the right are reconstructions based on multiple acquisitions in adjacent areas. B) Representative TEM micrographs with increasing magnification show PtNPs localized in intracellular vesicles. The images are taken seven days after the intravitreous injection of 100 µg/mL PtNPs. Images obtained in collaboration with Valentina Castagnola

3 THE LD ANIMAL MODEL

Once assured that our NPs could reach the ONL after crossing the ILM and being internalized by PRs, we proceeded to develop the light-induced animal model of retinal degeneration. For *in vivo* experiments, we used SD rats that, lacking the pigments responsible for the scattered light absorption, are highly susceptible to LD.

3.1 Timeline of the in vivo experiments

The experiments began at p60, when the rats were adult (2 months-old), as shown in Figure 14A. Animals were dark-adapted for not less than 12 hours during the dark phase of the circadian rhythm and anesthetized with a ketamine and xylazine cocktail, as described in the Materials and Method section. Animals were subjected to electroretinogram (ERG) in dim red light to maintain the dark adaptation, followed by the intraocular injection of either vehicle RSA or PtNPs. Seven days after the injection, we performed the LD, as described in the Materials and Methods. Then, animals were placed back in the housing cabinet for other seven days to let degeneration make its course, at the end of which they were anesthetized and subjected to a second ERG analysis. At the end of it, animals were sacrificed following the ethical procedure as indicated in the approved ethical protocol, for histochemical analysis.

3.2 Evaluation of different LD protocols

Several protocols of LD are efficient models to simulate macular degeneration in rodents (Organisciak and Vaughan 2010). We isolated the two most promising models which were already used to test the efficacy of NPs with different core materials. The first, used by Chen and collaborators, consisted in 6-hour exposure to 2700 lux (Chen et al. 2006), while the second, used by Fiorani and colleagues, consisted in 24-hour exposure to 1000 lux (Fiorani et al. 2015). Both protocols obtained the same average extent of damage, evaluated with both electrophysiological and morphological analysis. For the rest of the study, we adopted the second protocol that was more reproducible in term of LD (data not shown).

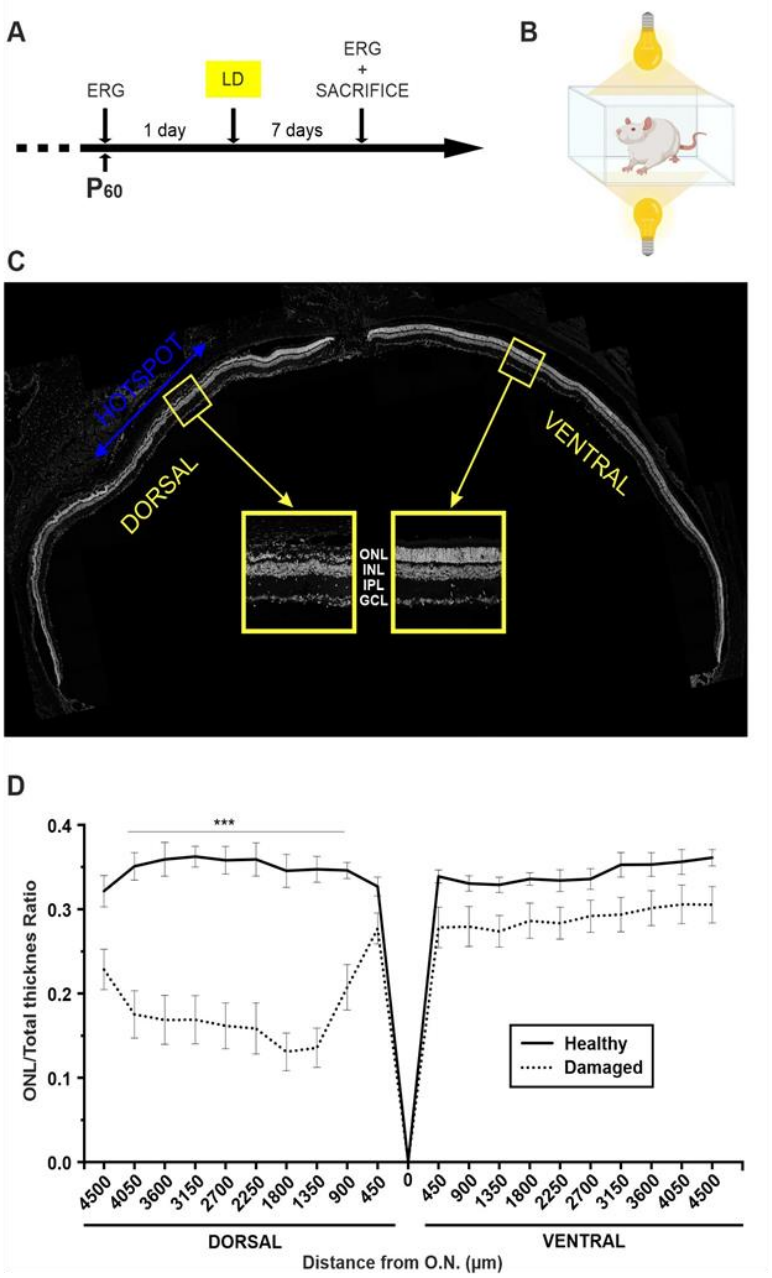

Figure 14. *Morphological alteration induced by light damage in albino rats*. A) Timeline of the *in vivo* experiments. B) Schematic representation of the LD induction. C) Morphological alteration occurring seven days after LD. In blue, the position of the hotspot in the dorsal part of the retina. Yellow squares depict the disrupted ONL in the hotspot (left) in comparison with the corresponding point in the ventral retina. D) Reduction of ONL thickness/total thickness along the retinal section in damaged animals 7 days after LD (dotted line) compared to healthy retinas (solid line). Statistical analysis shows the effectiveness of the LD in reducing the retinal thickness ratio after seven days of recovery. Both groups: n = 6 eyes. All data are mean± s.e.m. U test, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$.

3.3 Characterization of the LD model

Even if the light damage model is well characterized in literature, the high variability between different protocols requires refined experimental setting to ensure high reproducibility. At the beginning of my Ph.D. project, our laboratory possessed poor knowhow in both the animal model setting or the intravitreal injection. Therefore, the reproducibility of the model had to be set before starting with the PtNPs *in vivo* effects evaluation.

As shown in Figure 14A,B, to induce the LD 2 months-old animals housed in dim light (<10 lux) were placed in transparent cages and subjected to 24 hours of high-intensity light stimulation at 1000 lux, measured at the middle of the cage, from above and below. To mimic Ganzfield-like light stimulation during LD, the walls surrounding the cages were partially reflective. The animals received food and water during the LD, avoiding as much as possible to create any shadow in the cage. Temperature, humidity, and ventilation were controlled to respect animal welfare values. Animals were selected to have equal sex distribution between groups. The experimental groups used for the characterization of the LD model were:

- Healthy, n = 6 animals, no injection or LD.
- Damaged, n = 3 animals, no injection, retina exposed to LD.

Animals were sacrificed for most-mortem morphological and immunostaining analysis after electrophysiological characterization 7 days after LD. Figure 14C shows a single z-stack of a retinal section of the eye of an animal subjected to LD. The image was obtained by slicing the eyes on the dorsal-ventral equator (left to right) passing through the optic nerve, stained with Hoechst, and acquired with confocal microscopy. It is possible to notice the presence of the typical hotspot of PRs damage in the dorsal hemisphere (blue arrow). The yellow squares, placed in the center of the image, show the surrounding field's magnification in both the dorsal (left square) and ventral (right square) retinal hemisphere. The strong reduction of the ONL thickness in the dorsal square compared to the ventral one is visible, while the other layers' thickness seems not to be affected by the light-induced neuronal degeneration. It is possible to notice the presence of the typical hotspot of photoreceptor damage on the dorsal hemisphere, enlightened with the blue arrow.

3.4 Morphological alterations

The graph in Figure 14D shows the average ratio between ONL thickness and total retinal thickness along the whole retinal length. The measurement has been taken by dividing dorsal (D) and ventral (V) hemispheres in 10 fields of about 450

µm in length and averaging the measurement of three ratios for each field. The morphological analysis obtained in the damaged group (dotted line) clearly shows a reduction in thickness when compared to the healthy control (solid line), in accordance with previous literature (Fiorani et al. 2015; Maccarone et al. 2008; Di Marco et al. 2019). The two groups showed a statistically significant difference in most of the dorsal fields, with a p value between 0.005 and 0.0004, at 4050 and 900 µm from the O.N., respectively, where the hotspot results to be deeper. Interestingly, the ventral hemisphere showed a constant, but not statistically significant, trend for a reduction of the thickness in all the fields, showing a non-localized neurodegeneration even in the ventral hemisphere. This proves that light was able to reach uniformly every portion of the retina during LD. Taken altogether, these results show a successful model of retinal degeneration at the morphological level.

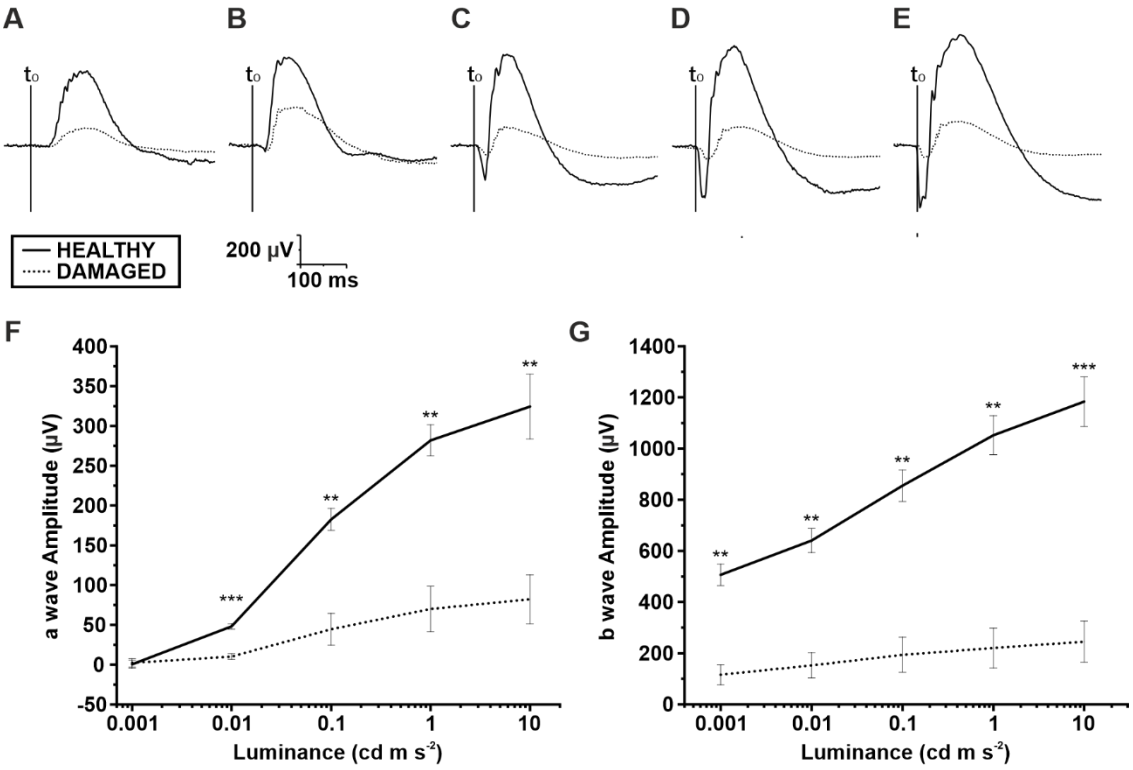

Figure 15. *Scotopic ERG responses induced by light damage.* A-E) Representative waves obtained at 0.001 (A), 0.01 (B), 0.1 (C), 1 (D) and 10 (E) cd s m^{-2} before (straight line) and seven days after (dotted line) the LD. F, H) Reduction in the scotopic "a" wave (F) and "b" wave (H) amplitude seven days after the LD (dotted line) with respect to the amplitudes recorded before LD (straight line). Significant differences are observed at all luminances for both "b" wave and "a" wave, except for for the 0.001 cd s m^{-2} where the PR activity is too weak to be consistently recorded. Both groups: n = 6 eyes. All data are means ± s.e.m. Mann-Whitney's *U*-test, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$.

3.5 Electrophysiological evaluation

The morphological results have been confirmed with the electrophysiological analysis showed in Figure 15. With ERG we evaluate retinal neuronal activity by recording the rapid potentials generated from the retina when stimulated with light. ERG is an essential tool to diagnose a variety of retinal disorders, evaluating the pathological progressions and assess either the efficacy of therapies or the damages induced by toxics or injuries. An alteration of the ERG responses is predictive for several pathologies onset, like retinitis Pigmentosa (RP) or macular degenerations, sometimes years before the symptoms emergence.

We performed ERG recordings with scotopic white flashes in 2 months-old rats, before and seven days after LD treatment. The top section of Figure 15 (A-E) shows the difference between representative ERG waves obtained in dark-adapted animals in response to single flashes under scotopic conditions at 0.001 (Figure 15A), 0.01 (Figure 15B), 0.1 (Figure 15C), 1 (Figure 15D) and 10 (Figure 15E) $cd*s*m^{-2}$, respectively. Healthy (straight line) and light-damaged (dotted line) animals were compared to appreciate the reduction of the ERG signal after LD treatment. The vertical line, marked with t_0, denotes the time in which the 5 millisecond-flash starts, while the trace recorded before t_0 represents the baseline trace used as reference for the evaluation of the wave amplitudes. The graphs at the bottom (Figure 15F, G) display the average amplitude of the two main components of the single scotopic flash wave, the "a" wave (Figure 15F) and the "b" wave (Figure 15G), in the same animals recorded before (solid line) and seven days after (dotted line) LD. The "a" wave amplitude is an index of PR activity reflecting their health and physiological reactivity, while the "b" wave is a more complex component mostly due to the activity of second order neurons. The "b" wave amplitude gives in fact an indication of both the information transfer activity from PRs to BCs and the information processing in the inner retina. In Figure 15F we can observe that in both low luminance (from a 0.01 $cd*s*m^{-2}$; $p = 0.00027$, Mann-Whitney's *U*-test), at which the a wave is mostly due to the activation of the rods, and high luminances (1 and 10 $cd*s*m^{-2}$; $p = 0.0051$ and 0.01487 respectively, Mann-Whitney's *U*-test), at which the amplitude is mostly due to cones activation, there is a significant difference between the wave amplitudes obtained from the two groups of treatments. It is important to mention that, at very low luminances the "a" wave is not present due to the very small number of activated rods. Therefore, this value will not be shown in the next "a" wave result. Together, these data confirm the results obtained from the morphological analysis, indicating that the electrophysiological response in LD

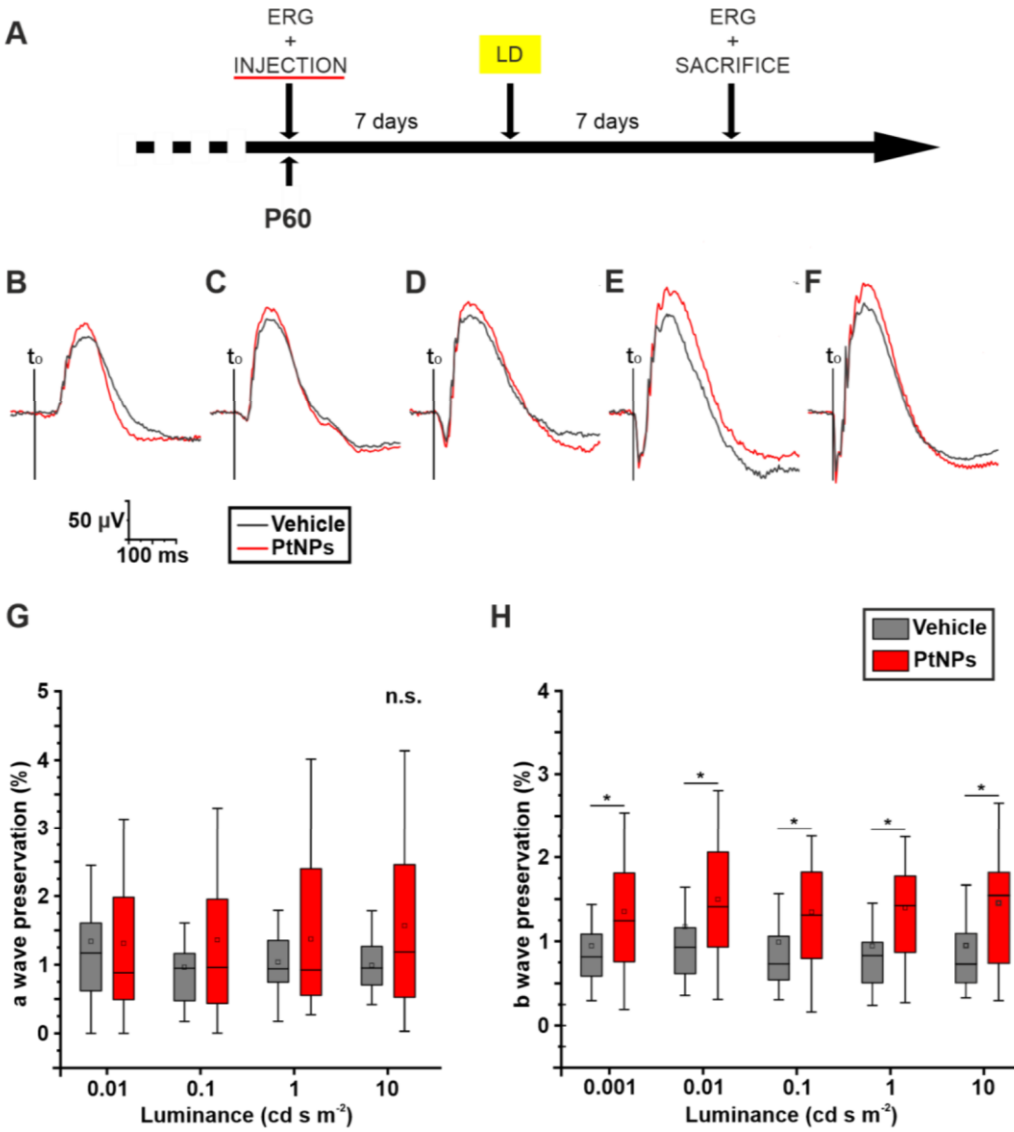

Figure 16. *PtNPs effects on scotopic ERG amplitudes.* A-E) Representative waves obtained at 0.001 (A), 0.01 (B), 0.1 (C), 1 (D) and 10 (E) cd s /m^2 seven days after LD in animals injected with PtNPs (red) or vehicle (black). F,H) Preservation of the wave amplitudes obtained with scotopic flashes at different luminances in animals injected with PtNPs (red) or vehicle (black). No significant differences were observed in the "a" wave preservation (F), while a significant difference was present in the "b" wave preservation at all luminances analyzed. PtNPs: n = 22 eyes, Vehicle: n = 24 eyes. Bars show 25% and 75% of the median. The whiskers display outliers, the square indicates the mean, the horizontal line the median. Mann-Whitney's *U*-test, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$.

animals are significantly reduced compared to healthy controls, due to PR death together and morphological alterations of the inner retinal matrix.

4 PRESERVATION OF VISUAL FUNCTION AFTER INTRAVITREAL INJECTION OF PTNPs

Once developed a suitable photoreceptor degeneration model, we started injecting PtNPs in adult albino rats to assess their efficacy against the neurodegeneration. A saline solution containing 1 mg/ml RSA (vehicle) was always employed as sham injection to verify that the surgical procedure itself did not affect the visual performance. We used two experimental groups of 2 months-old Sprague-Dawley rats, both subjected at LD for 24 hours by exposure to 1000 lux seven days after the microinjection:

- PtNPs n = injected with of 2 µl of 100 µg/ml ccPtNPs suspended in 1 mg/ml of RSA.
- Vehicle n = injected with 2 µl of 1 mg/ml RSA.

4.1 Electrophysiological evaluation

4.1.1 Scotopic ERG

Figure 16 shows the results obtained from single scotopic flashes in animals adapted to the dark for more than 12 hours, 7 days after the LD in PtNPs injected animals (red) and sham-injected animals (black). Figure 16A display the timeline of the experiments. Figure 16B-F shows the representative waves of both groups recorded respectively at 0.001 (Figure 16B), 0.01 (Figure 16C), 0.1 (Figure 16D), 1 (Figure 16E) and 10 (Figure 16F) $cd*s*m^{-2}$. To quantify the PtNPs effect on the scotopic ERG we calculated the *preservation* of ERG wave amplitude after LD. The preservation is an index that shows, in percentage, how much of the amplitude of a wave (either "a" or "b" wave) is preserved after LD as compared to the respective amplitude recorded in the same animal before any manipulation. These values were then normalized to the average preservation of each group of experiment to account for litter/batch variability. Figure 16G and H displays the normalized average amplitude preservation of "a" and "b" waves obtained at the different luminances (PtNPs n = 22 eyes, Vehicle n = 24 eyes). Despite the absence of significant differences (Figure 16G), the increased "a" wave preservation in PtNPs-injected animals throughout the luminance dose-response clearly suggest a potential preservation of rods activity.

This can be appreciated especially if we compare the means (square symbol) of the two groups at 0.1, 1 and 10 cd*s*m^{-2}. A much stronger effect was obtained in the "b" wave part analysis (Figure 16H), in which the difference between the NPs-treated animals and the control group was significant at each luminance examined (p = 0.0226, 0.0411, 0.0471, 0.0212, 0.0367, respectively for 0.001, 0.01, 0.1, 1 and 10 cd s /m^2). These data show how PtNPs induce a persistent preservation of the electrophysiological activity in the inner retina at all luminances that is attributable to increased PR survival. The lack of significant differences in the "a" wave may be due either to the short time given to the PRs to recover after LD, as showed from recently published data (Riccitelli et al. 2021), or to the smaller amplitude of the "a" wave component compared to the "b" wave. Moreover, a longer recovery could have included the effects of NPs on the long-term ROS activity associated with the para-inflammation that establishes following LD (Xu et al. 2009).

4.1.2 Photopic ERG

A confirmation to the previous hypothesis is provided by the photopic ERG (Figure 17), in which light adaptation before flashes bleaches rod responses and isolates the cone response. The protocol was performed on the same animals described before immediately after the scotopic protocol. Figure 17A shows representative traces

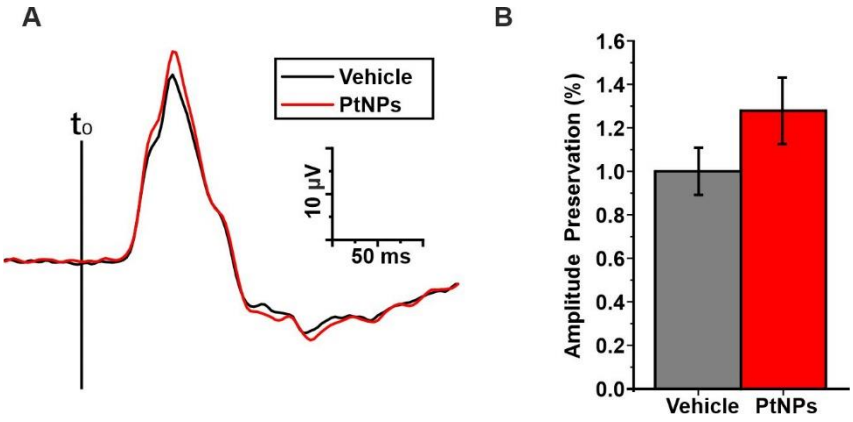

Figure 17. *Photopic ERG stimulation shows no significant effects.* A) Representative waves obtained with photopic flash stimulation at 3 cd s /m^2 seven days after LD from animals injected with either PtNPs (red) or vehicle (black). The perpendicular line marks the time of the flash stimulus. B) Normalized amplitude preservation obtained with photopic flash stimulation seven days after LD from animals injected with PtNPs (red) or vehicle only (black). The difference in the preservation of cone activity is not statistically significant. PtNPs: n = 22 eyes, Vehicle: n = 24 eyes. All data are means ± s.e.m. Mann-Whitney's *U*-test.

obtained from single flash photopic stimulation at 3 cd*s*m^{-2} in PtNPs- (red) and sham- (black) injected animals 7 days after the LD. As expected, no "a" wave signals were detected, confirming the cone selectivity of the illumination protocol. Figure 17B shows that the normalized amplitude preservation in the animals injected with PtNPs is slightly higher than the sham-injected group only, even if with no evidence of statistical significance (p= 0.2163; PtNPs n = 22 eyes, Vehicle n = 24 eyes). The lack of significance in the effect could be due to the low density of cones in the rat retina compared to rods. Therefore, starting from a very small pool of cells, the amplification of the "b" wave component could not be enough to reach significance.

4.2 Morphological evaluation of the effect of PtNPs on retinal thickness

After sacrificing the animals, the eyes were processed, as previously described in the methods section. Figure 18 displays the results of the morphological analysis on retinal sections from both vehicle- and PtNPs-injected animals seven days after the LD and electrophysiological investigations. Figure 18A shows representative images of the field located 900 μm from the O.N. in the dorsal retina for both experimental groups (vehicle, left; PtNPs, right). No clear difference in morphology was revealed, as confirmed by the quantification shown in Figure 18B. The graph in Figure 18B displays the measurement of the thickness ratio in both PtNPs (n = 20 eyes, red) and vehicle (n=18 eyes, black) animals collected along the retinal length, as previously described in Figure 14D. No statistical significance was observed, while a slight tendency in preserving ONL morphology was present in animals injected with PtNPs, especially for the external fields of the hotspot. This might be due to the moderate effects of NPs in preventing the neurodegeneration and, mostly, its progression. This mild effect on the morphology may explain why we did not observe a significant difference in the preservation of the "a" wave. On the other side, the amplitude preservation of the "b" wave, might have been boosted by the amplification of the inner retina, resulting in a significantly higher preservation in PtNPs-injected animals not withstanding a very similar retinal morphology.

4.3 Inflammatory response preservation after PtNPs injection

Figure 19 shows the results obtained from immunohistochemistry analysis performed on retinal sections (PtNPs n = 29 eyes, Vehicle n = 33 eyes) collected as previously described in the Methods section. We addressed the possibility that the injection of NPs could prevent inflammatory effects by analyzing GFAP and Iba-1

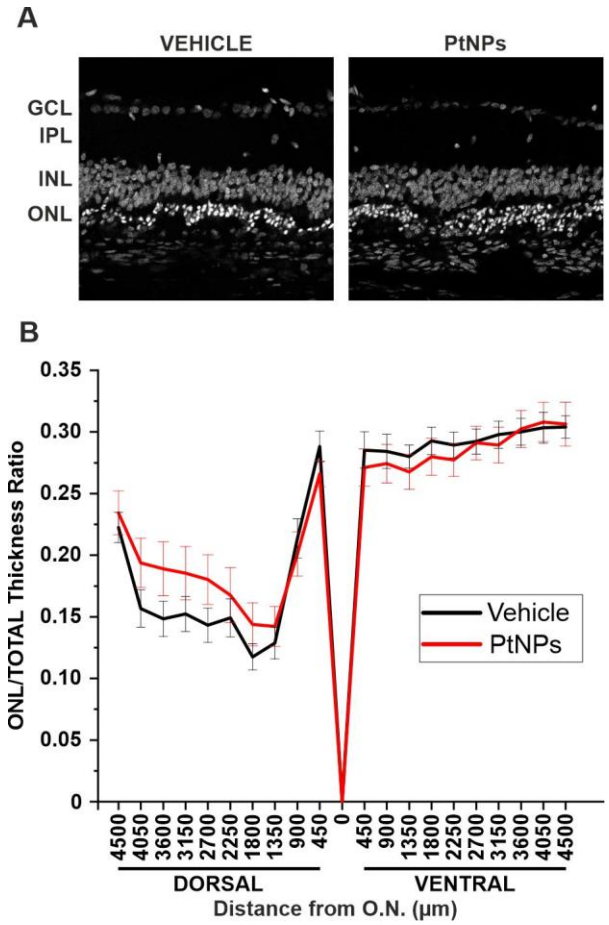

Figure 18. *The retinal structure is partially preserved by PtNP injection*. A) Representative images showing the difference in ONL thickness induced by PtNPs injection (right) as compared to the sham-injected animals (left) in the field D3. B) Comparison of the average [ONL thickness/total retinal thickness] ratios along the retinal section between PtNPs (red) and vehicle (black) injected animals. PtNPs: n = 20. Vehicle: n = 18. All data are means ± s.e.m. Mann-Whitney's *U*-test showed no significant differences between the groups.

immunoreactivities as markers of astrocyte/Muller cell gliosis and microgliosis, respectively. Figure 19A reports representative images of the immunofluorescence staining. As shown in Figure 19B, the analysis was performed on four different fields of the sections, two in the dorsal hemisphere (marked with "D"), one of which on the deepest portion of the hotspot (marked with "int") and one on a more external field of the hotspot (called "ext") respectively, and other two exactly specular fields in the ventral portion (marked with "V"). Figure 19A displays representative confocal images of retinal sections of healthy (top) and LD animals injected with either vehicle (middle) or PtNPs (bottom), taken in the V_{ext} field of the dorsal retina. GFAP expression is shown in red, IBA-1 positive cells are in green, and nuclei stained with Hoechst in blue. It is well known that the retina reacts to a variety of insults by

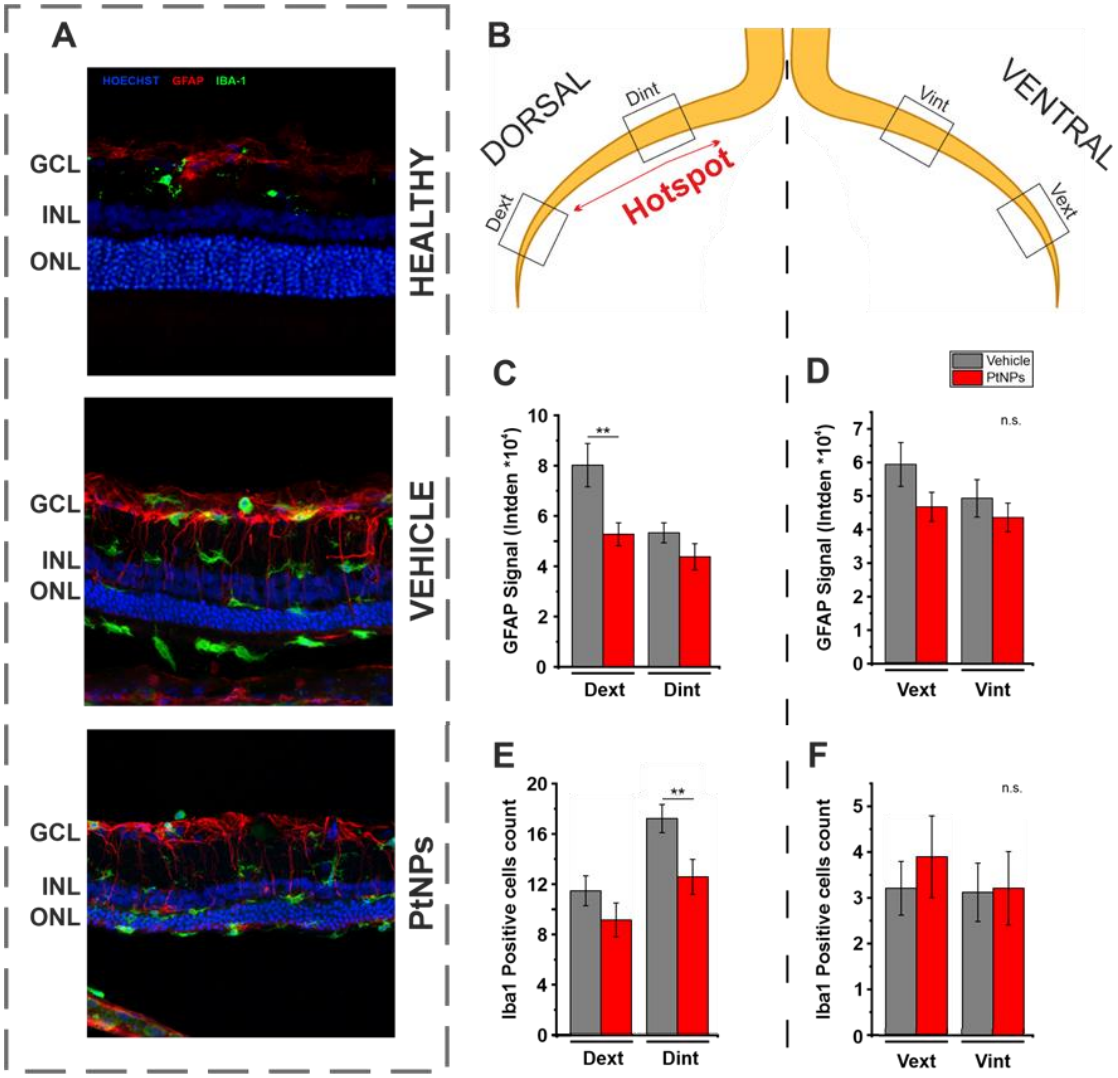

Figure 19. *GFAP quantification and IBA1 positive cells counting.* A) Representative images of the immunohistochemical results. The GFAP signal is red, the microglia cells are stained in green while the nuclei are in blue. From the top to the bottom, we have a healthy retina, the vehicle control and the cc-PtNPs group. B) Schematic representation of a retinal section to enlighten the fields taken in consideration for the analysis. C,D) Histograms that show the quantification of GFAP signals in both dorsal (B) and ventral (C) hemispheres of the retina, showing a significant difference between the groups of treatment in the external field ($p = 0.0083$), where the progression of the degeneration is advancing. E,F) Histograms showing the average numbers of IBA-1 positive cells in both dorsal (E) and ventral (F) retina, showing how PtNPs can significantly reduce the infiltration of the microglia in the outer retina of the hotspot ($p=0.0097$). PtNPs n = 29 eyes, Vehicle n = 33 eyes. All data are means ± s.e.m. U test, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$.

modifying the expression and/or the localization of several proteins to re-establish physiological conditions. GFAP is known to be in the GCL layers in the healthy retina, forming a homogeneous plexus (Figure 19A, red-top). After an insult, GFAP expression increases and the proteins are quickly radially relocated from the inner layer towards the outer retina (Figure 19A, red-middle and bottom). This pattern of expression of GFAP is observed after retinal detachment (Erickson et al. 1987), blood flow impairments (Block and Sontag 1991) and other insults (Eisenfeld, Bunt-Milam, and Sarthy 1984), with no exception made for LD retinas (de Raad et al. 1996).

Moreover, along with inflammation, neurodegeneration can trigger macrogliosis (Li, Eter, and Heiduschka 2015), another well-known mechanism of defense of the nervous system. Under healthy conditions, microglia cells are normally present in the inner retina, between the IPL and INL (Figure 19A, green-top). The activation of microglia not only leads to the modification of its functions and morphology, preparing itself to phagocytosis and ROS and cytokines release, but also retrieves macrophage precursors from the blood to help fighting against the insult (Langmann 2007) (Figure 19A, green-mid and bottom).

4.3.1 ccPtNPs induce GFAP preservation

Densitometric analysis of GFAP (Figure 19C,D) in the dorsal (Figure 19C) and ventral (Figure 19D) retinal hemisphere was performed using Image J threshold tools (Gallego et al. 2012), as described in the Material and Methods section. The results showed that GFAP expression was massively increased in the external portion of the hotspot, where neurodegeneration was still progressing, showing a significant difference ($p = 0.0083$, Mann-Whitney's *U*-test) between control retinas (black) and retinas injected with PtNPs (red). Interestingly, the absence of any difference in the deepest portion of the hotspot may suggest that the damage has severely progressed, bypassing the retinal mechanisms of defense.

4.3.2 ccPtNPs prevent microglial cell infiltration in the ONL

Counting of IBA-1 positive cells was performed considering the layers included between the GCL and the IPL as inner retina, while only the IBA-1 positive cells having their body in the ONL was counted as located in the outer retina (Figure 19A top). A significant difference ($p = 0.0097$, Mann-Whitney's *U*-test) was observed in the outer layer of the hotspot area between retinas treated with PtNPs (red) and those injected with vehicle (black) ($p = 0.0057$ Mann-Whitney's *U*-test) (Figure 19E) but not in the ventral retina (Figure 19F), suggesting an effect of NPs in preventing microglia migration from the blood flow, preventing inflammation. This may be due

to the delayed effect of NPs that, thanks to their scavenging ability, reduced ROS-induced cellular damage, and thereby microglia migration.

IV. DISCUSSION

Many retinal neurodegenerations involve the overproduction of ROS, due either to a reduction in the balance between oxidation and endogenous antioxidant molecules (Jadeja and Martin 2021), genetic mutations or environmental exposition to disruptive agents (Leveziel et al., 2011; Mainster et al., 1983; Ni Dhubhghaill et al., 2010). Unlikely to other organs, neurological tissues cannot regenerate, and modern technologies are not able either to revert this ineluctable fate or restore a lost in sight. Therefore, a preventative treatment able to arrest the degeneration appear to be the best strategy to avoid the worsening of patients vision. Several approaches involving antioxidant has been proven rather effective in slowing down retinal deteriorations (Chen et al., 2006; Maccarone et al., 2008; Mandal et al., 2009; Mitra et al., 2014), but new therapeutical approaches are in need to ameliorate millions of people's quality of life.

PtNPs possess an enormous potential for their use in several fields, included medicine. Nevertheless, their applications as therapeutic agent has been not widely investigated due to controversial data about their safety (Pedone et al., 2017). In this work, we demonstrated how PtNPs, when properly synthetized, are safe both *in vitro* and *in vivo* on neurons, possess an intense catalytic activity as antioxidant and may prevent retinal degeneration induced by light. A seeded-growth synthesis approach associated with citrate as stabilizer, has already proven to create PtNPs that are non-toxic in HeLa, MCF7 and Caco-2 cell lines, do not release Pt^{2+} ions after internalization, and mimic endogenous antioxidant enzymes (Moglianetti et al., 2016). Considering that citrate is a week stabilizer, we improved PtNPs solubility creating an RSA protein corona on the PtNPs, which is invisible to the immune system and prevent aggregation in physiological condition. Those preliminary steps are crucial to avoid aggregation in biological tissues, as showed with the agarose gel electrophoresis experiment, where the presence of inorganic ions, normally present in physiological solution, modify the ionic strength and induce aggregation (Mukherjee & Weaver, 2010).

Furthermore, the protein corona reduces about 20% only the catalytic activity properties of the PtNPs. Considering the astonishing results obtained by Moglianetti about the cc-PtNPs catalytic activity as endogenous antioxidant mimetic *in vitro* [Moglianetti], we proved that the protein corona does not alter the PtNPs activity as antioxidant enlightened with the "naked" PtNPs. Moreover, their application on primary cortical neurons shows no alteration in both viability and cell death ratio compared to untreated cells. A crucial point is that the incubation was done for 24

hours only, right before the assays, while the time of exposition of the cells to PtNPs *in vivo* is much longer. Moreover, there are no data about non-lethal damages that the PtNPs may induce in non-essential organelles, which could cumulate in time and bring to serious alterations. Therefore, more *in vitro* experiments may be performed, not only to evaluate PtNPs safety and activity, but also to deeply investigate the mechanisms of protection on the cc-PtNPs.

Before starting with the investigation regarding the therapeutical activity, we assessed the biodistribution of the PtNPs *in vivo*, observing how the NPs were able to cross the ILM, the major physical barrier to the retina, and being internalized in the photoreceptors. This internalization occurred quickly after the injection, and the NPs were observed in the lysosomes even a week after the administration. It is not clear how many of the PtNPs reached the ONL, were able to be internalized by the photoreceptors or if they can leave the eye through the blood after the LD, which cause a disruption of the BRB (Tisi et al., 2021). A quantification of the PtNPs in both the full eye and the isolated retina (and maybe a ratio between those values to assess the distribution from the vitreum to the retina), both before and after LD, may be helpful to understand the dynamic of the biodistribution. Furthermore, an investigation regarding the presence of the PtNPs in other organs, such as liver, kidney, spleen, and lungs, followed by both a quantification and histological evaluation (to identify eventual alteration) could complete the information of both biodistribution and safety.

To evaluate the PtNPs antioxidant activity *in vivo* against retinal neurodegeneration, we induced a degeneration in an albino rat by overexposing them for 24 hours to high intensity (1000 lux) light. Our results clearly show a good efficacy of the NPs in preserving the electrophysiological activity in the inner layer of degenerated retinas. Considering the absence of a morphological preservation in the ONL thickness in the treated animals compared to the control group, it is not clear wherefore these effects are evident at all luminances tested in the b wave amplitude preservation in scotopic conditions, without showing significant influence in the a wave at the same experimental condition, nor following photopic stimulation. One hypothesis is that the higher noise/signal ratio of the a wave obtained as consequence of the strong reduction in its amplitude in the damaged animals for both groups increase the variability of the preservation value, therefore leading to a loss of the eventual positive effects of the NPs. A detail supporting this hypothesis is revealed with the a wave preservation means, which are very different for both groups at 0.1 ,1 and 10 $cd*s/m^2$ luminances (1.39, 1.42, 1.51 for PtNPs and 0.98, 1.18, 1.03 for vehicle respectively). Moreover, the distribution of the data sets in the treated group is much

wider, suggesting a possible action of the NPs in preserving the PRs activity, even if with no significant effects with these experimental settings.

Moreover, PtNPs could act on the inner layers either better preserving cells that are less stressed by light compared to PRs or by positively modulating the transmission efficacy of those retinal components, for example by downregulating trophic factors as FGF2, known to reduce the synaptic transmission efficiency (Gargini et al., 2004). Indeed, a wider spectrum observation of the retinal sections at the TEM may enlighten an eventual internalization of the PtNPs in BCs and MCs, which are responsible for the generation of the b wave (Dick et al., 1985, 1985; Sillman et al., 1969), rather than PRs. Furthermore, additional immunohistochemistry assays, looking to b-FGF2 expression (Gargini et al., 2004; Valter et al., 2005) or PKC positive cells organization, coupled with Multi Electrode Array (MEA) and biochemical analysis, may unveil the mechanisms behind PtNPs protection.

Interestingly, PtNPs seems to have statistical effects in the reduction of two major inflammatory actors induced during degeneration, the activation of the MCs, measured through GFAP expression quantification (Bringmann et al., 2006; Lewis et al., 1995; Madigan et al., 1994), and the migration of the microglia from the inner retina to the ONL (Thanos, 1992). Indeed, an analysis of the microglia morphological differentiation, thought a Sholl analysis, and the ratio between a proinflammatory M1 type, usually associated with neurodegeneration (Tang & Le, 2016), and the positive M2 type, with biochemical or histological assays, could provide further details about the mechanisms behind PtNPs action as anti-inflammatory agent.

Finally, a combined observation on the ventral hemisphere of the morphological alteration, GFAP expression and IBA-1 positive cells migration, displayed no differences between the eyes treated with PtNPs and the vehicle only. Even if the time of exposition of the retinas to the PtNPs were rather short (15 days) the absence of any visible alterations suggests the biocompatibility of those NPs in the retina, paving the way for further investigations.

Our results proved that PtNPs are non-toxic *in vivo* and could be safely use for further application. Moreover, they proved effective in preserving retinal function, with special focus on the inner layers, but showing little effect in both preserving the morphology and the activity of PRs. Even if those results are mild compared to other NPs tested on similar models of retinal degeneration (Chen et al., 2006; Maccarone et al., 2008; Mitra et al., 2014), the comparison of the damage depth in the dorsal hemisphere of the retina in our experiments seems to be more extended compared to the literature. This may suggest that by inducing a weaker damage, a more similar condition to the chronical degeneration that occurs in most of the retinal

neurodegenerations, may enlightened stronger results of the PtNPs as therapeutic agents against photoreceptorial degeneration.

V. Material and Methods

1 *NANOPARTICLE SYNTHESIS*

PtNPs were synthesized by collaborators at the Nanobiointeractions and Nanodiagnostics Lab (IIT), adding 160 µL of H_2PtCl_6 0.5 M (BioXtra grade, Sigma-Aldrich) and 192 µL of sodium citrate 0.5 M (BioUltra grade, Sigma-Aldrich) to 79.65 mL of MilliQ water. Under vigorous stirring, $NaBH_4$ 0.06 M was added to the reaction vessel dropwise. Then, while still under stirring, the solution was brought to 75 °C for 30 minutes. After being cooled to room temperature, PtNPs were extensively washed using 10 K Amicon filters with 2 mM sodium citrate solution.

2 NANOPARTICLE CHARACTERIZATION

2.1 Size Distribution

The NPs physiochemical properties are crucial for nanozymes peroxidase mimetic activity. Indeed, ensuring reproducible NP size and shape in different batches is essential (Sobaniec et al. 2007). Therefore, PtNPs were characterized by transmission electron microscopy (TEM) using a JEOL JEM 1011 microscope. 3 µl of NPs suspension were placed on a 150 mesh Cu grid coated with ultrathin holey carbon film. The statistical size distribution analysis was performed using ImageJ, by measuring at least 100 NPs from at least 5 different images.

2.2 Dynamic Light Scattering

Particle size was evaluated by Dynamic Light Scattering (DLS) using a Zetasizer Nano Range instrument (Malvern, Worcestershire, UK). The reported values are an average of three independent measurements (each consisting of 11 runs).

3 STABILIZATION IN BIOLOGICAL MEDIA

PtNPs (1 mg/ml) were incubated with 10 mg/ml of rat serum albumin (RSA, A6272, Merk) for 30 minutes at 37 °C. Albumin was selected as it represents the major protein component in vitreous fluid (Chen and Chen; Hawkins).

3.1 Agarose Electrophoresis

The RSA excess in the PtNPs preparation was washed through ultracentrifugation steps (25,000 rpm for 30 minutes, Optima™ MAX-XP Ultracentrifuge, Beckman Coulter), followed by suspension in 0.9% NaCl solution.

To assess PtNPs-RSA stability in the cell culture medium, samples and controls were loaded on a 3.5% agarose gel assay and let run for 30 minutes upon application of 90 V. This agarose concentration allows stable dispersion of PtNPs to enter the gel, while blocking the entrance of aggregated samples. The presence of RSA coating increases the size and modify the total charge with a subsequent reduction of the electrophoretic mobility.

3.2 H_2O_2-TMB Chromogenic Assay

The ability of PtNPs to catalyze the redox between hydrogen peroxide and the chromogenic substrate TMB was assessed for both "naked" and RSA-coated PtNPs. In details, 1 µl of PtNPs pre-diluted stock (125 pM) was mixed with 99 µl of sample (milliQ water or 0.9 % NaCl) and incubated for 5 minutes. The sample was then diluted 1:10 in the reaction mix, composed by 10 mM sodium acetate buffer (CH3COOH/CH3COONa) pH 4.5, 200 mM hydrogen peroxide and TMB Substrate Reagent Set RUO (BD OptEIA) final dilution 1:10. TMB oxidation was detected by UV–Vis spectrophotometry.

4 PTNPs IN PRIMARY NEURONAL COLTURES

4.1 Primary Neuronal Cultures

Primary cortical cultures were obtained from 20 days-old SD rat embryos (Charles River). Pregnant rats were sacrificed by CO_2 inhalation followed by cervical dislocation. The embryos were removed immediately by cesarean section. Briefly, cortices were dissociated from the surrounding tissue by enzymatic digestion, with trypsin-EDTA 0.125 % for 30 minutes at 37 °C, and a mechanical titration, thought repeated pipetting. Trypsin activity was inhibited by adding Neurobasal medium supplemented with 2 % B27, 1 % GlutaMax, 1 % penicillin/streptomycin (complete medium), and 10 % fetal bovine serum. After digestion, neurons were washed in complete medium, centrifuged at 700 RPM for 5 minutes and resuspended in complete medium at the desired concentration. All products were purchased from Life Technologies.

4.2 Cell Viability Assay

At 17 days *in vitro* (DIV), primary rat cortical neurons were exposed to Pt-NPs (1 and 10 µg/ml) for 48 hours before performing the viability assay. Cells were then live stained for 3 minutes at room temperature (RT) with fluorescein diacetate (2 µM) for cell viability, propidium iodide (PI) (1 µM) for cell death and Hoechst 33342 (1 µM) for nuclei visualization. Cell viability was quantified at 20X (0.5 NA) magnification using a Nikon Eclipse-80i upright epifluorescence microscope (Nikon, Tokio, Japan), with random sampling of 5 fields per coverslip (n=3 coverslips/sample, from 3 independent cell preparations). Image analysis was performed using the ImageJ and the Cell Counter plugin.

4.3 DCF Assay

Primary rat cortical neurons cells were exposed to 1 µg/ml Pt-NPs for 48 hours before performing the dichlorofluorescein (DCF) assay. Briefly, neurons at 17 DIV were washed with 0.1 M phosphate buffered saline (PBS, pH 7.4), incubated with 5 µM 2 H2-DCFDA (2', 7'-dichlorodihydrofluorescein diacetate, Sigma) in phosphate buffered saline (PBS) for 30 minutes at 37 °C, and washed with PBS again. The DCF fluorescence intensity was measured by using a plate reader (Infinite F500, TECAN, Switzerland). The excitation and the emission filters were set at 485 nm and 535 nm, respectively. The results were expressed as fold-increase over control (untreated neurons). To verify PtNPs ROS scavenging activity, prior to DCF assay primary cortical neurons were treated with 2 mM H_2O_2 for 5 minutes after the 48 hours incubation with PtNPs.

5 TEM ANALYSIS ON RETINAS

Retinas were extracted, cut in quarters, and a standard fixation procedure was employed. CO_2 anesthesia and cervical dislocation were performed to euthanize the animals. The eye and retina dissection were realized under dim red light. Eyes were enucleated and transferred to carboxygenated Ames' medium (Sigma Aldrich). After enucleation, eyes were immersed for 2 hours in a 2 % glutaraldehyde solution in 0.1 M Sodium Cacodylate (SC) buffer at pH 7.4. Subsequently, eyes were washed in 0.1 M SC buffer and incubated for 1 hour with 2 % OsO_4 solution in 0.1 M SC buffer. Retinas were washed in SC buffer and then with milliQ water. A 45-minute incubation in 1 % uranyl-free solution in water and progressive dehydration washes completed

the fixation procedure, together with a final step of overnight incubation in propylene oxide and EPON epoxy resin. Finally, the inclusion was cured by leaving samples in EPON for 48 hours at 60 °C. These samples were sliced at the ultramicrotome at thicknesses ranging from 1000 to 70 nm. The slicing of the samples was guided by a toluidine blue staining of thicker slices to foresee retinal orientation upon transfer of the samples to the metallic TEM grids. Retina explants were imaged using a JEOL 1011 transmission electron microscope operated at 100 kV.

6 ELECTROPHYSIOLOGICAL RECORDINGS

6.1 Dark adapted Electroretinogram

ERG was recorded in SD rats (p60, weight 200g /300g females/males) breaded in dim light subjected to a dark-adapted condition in response to a single white light flash of increasing intensity (scotopic and photopic conditions), delivered by a standard Ganzfeld Stimulator (CSO, Pisa Italy). All the operations were performed in the dark, using only a dim red light when necessary and covering the ERG system to avoid to screen light reaching the animals. Rats were anaesthetized with intraperitoneal injection of a Ketamine/Xylazine cocktail (33 mg/100 g and 1.2 mg/100 g body weight) and mounted on a stereotaxic apparatus. The body temperature of the animal was maintained at 36.5 °C (± 0.5 °C) for the whole duration of the experiment. Lidocaine drops were used to anesthetize the eye and 5 µl drop of atropine was applied to maximize pupil diameter. Both eyes were simultaneously recorded with platinum electrode loops (2 mm diameter) gently

Table 1. *Protocol of ERG stimulation*. The table displays the different luminances used for the dark-adapted ERG, the number of flashed averaged to reduce the noise, the recovery time between flashes in the same test and between the last flash of a test and the first of the successive one.

		Recovery time	
Luminance	Flashes	Between flashes	Between exams
cd·s·m^-2	n°	sec	sec
0.001	5	20	120
0.01	5	60	120
0.1	3	60	180
1	3	120	180
10	2	240	240

placed on the cornea. Reference and ground electrodes were subcutaneously placed near the eye and in the anterior scalp, respectively. The responses were recorded at increased light intensities as described in **Table 1**.

At the end of each recording session, traces were band-pass filtered between 0.1 and 300 Hz. Both a- and b-waves component's amplitude (μV) for each luminance of the scotopic ERG was calculated as maximal amplitude.

The evaluation of the photopic response was performed at the end of the scotopic analysis. The anesthetized animals were light adapted at 10 $cd*s*m^{-2}$ for 5 minutes, followed by a stimulation of 20 flashes at 0.5 Hz of 3 $cd*s*m^{-2}$ intensity, given on top of a 10 $cd*s*m^{-2}$ background light. At the end of it, swipes were mediated to reduce the noise on the tracks.

The animals resulting with cataract at the end of the experiment were eliminated from the experiment.

7 INTRAVITREAL INJECTION

For intravitreal injections, we used a 100 μg/ml PtNPs and 1 mg/ml RSA in 0.9 % NaCl for the treated group and 1 mg/ml RSA in 0.9 % NaCl for the vehicle group (similar to physiological albumin concentration in the vitreous) (Hawkins 1986).
Microinjection were performed immediately after ERG recordings, while the animals were still anesthetized. After ocular exophthalmos, 2 μl of either the PtNPs preparation or RSA solution (both previously mentioned) were injected in both eyes using a Hamilton syringe under sterile conditions. At the end of the surgery, ophthalmic antibiotics were applied on the corneas to prevent infections. The animals were placed back to their cages and monitored daily for the whole duration of the experiment to ensure good health conditions and absence of ocular alterations.

8 ANIMAL HOUSING

Animals were housed in ventilated Cabinet BIO-C36" (TECNIPLAST S.p.A., 21020 Buguggiate (VA) Italy), to ensure a constant light intensity exposure of the cages. The light source was positioned above the cage, at equal distance from the lid of every cage. The light intensity was kept between 5-10 lux, measured at the top of the cages, for the entire animal lifespan. Food and water were given *ad libitum*.

9 LIGHT EXPOSURE

At the end of the daily phase of the circadian cycle, the animals were placed in individual transparent plexiglass cages. Following 12 hours of dark adaptation, the animals were exposed to bidirectional white light, from both top and bottom of the cage, at 1000 lux for 24 hours. The LD started at the beginning of the light phase of the circadian cycle. Temperature, humidity, and ventilation were controlled. The health of the animals was assessed several times during the light exposure. Food and water were strategically placed to avoid the formation of shadow spots in the cage. At the end of the procedure, animals were placed back in their breeding cage and monitored daily for the presence of distress.

10 MORPHOLOGY

Animals were euthanized, eyes enucleated and fixed with 4% paraformaldehyde solution for 2 hours at room temperature. After several washes in PBS, eye-cup processing was performed. The eye underwent a cryoprotective procedure of dehydration as a result of the immersion in rising concentrations of sucrose (15% for 30 minutes and 30% overnight) dissolved in PBS. Then, eyes were embedded in OCT (optimum cutting temperature; Sicigen Scientific Gardena, CA) and frozen in dry ice. Using a cryostat, 25 µm-thick slices were made for each retina and collected onto gelatin/poly-L-lysine-coated slides. Slices were taken in a superior - inferior direction, selecting those which presented the optic nerve emergence in order to obtain a topographic comparison between the various samples.

11 IMMUNOSTAINING

Cryosections were also used for immunostaining. A pap-pen (Sigma Aldrich) was used on the slices to create a hydrophobic edge around the retinal sections. When dried, slices were placed in a humid chamber, an opaque box with soaking paper at the bottom to keep them in the dark and avoid dehydration. Three washes of 10 minutes with PBS were gently done to avoid mechanical stress. Next, slices were incubated with a blocking solution, composed of 5% bovine serum albumin (BSA) 5% normal goat serum (NGS) and 0.3% Triton-X-100 (all from Sigma Aldrich) in PBS, at room temperature for 2 hours. Slices were incubated overnight at 4 °C with primary antibodies against two molecular markers of retinal inflammation, ionized calcium-binding adaptor molecule 1 IBA-1 (1:1000; Wako Pure Chemical Industries, Japan) and GFAP (1:200; Sigma) in a solution composed of 1% bovine serum

albumin (BSA), 1% normal goat serum (NGS) and 0.3% Triton-X-100. Slices were then washed 3 times with PBS before incubation with the fluorescent secondary antibodies Alexa Fluor 488 and Alexa Fluor 564 (1:500; Molecular Probes, Invitrogen, Carlsbad, CA), together with bisbenzimide nuclear dye 33342 (1 µM; Hoechst), in 1% bovine serum albumin (BSA), 1% normal goat serum (NGS) and 0.3% Triton-X-100 for 2 hours at room temperature. Finally, the slides were washed 3 times with PBS and mounted with glass coverslips and Mowiol (Sigma Aldrich). Slices images were acquired using a confocal microscope (SP8, Leica Microsystems GmbH, Wetzlar, Germany) with 20X (NA 0.5) and a 40X (NA 1.3) magnification lens. The image analysis was performed with ImageJ.

12 STATISTICAL ANALYSIS

The statistical analysis are described in the figure legends. Data are given as means ± SEM for n = sample size. To assess for normal distribution of data, the D'Agostino-Pearson's normality test was used. To compare data between two groups that were not normally distributed, we used the non-parametric Mann–Whitney's *U*-test. Statistical significance was set at p-value < 0.05, using the GraphPad Prism statistical software 8.0.0

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VII. REFERENCES

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