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Light, lipids and photoreceptor survival: live or let die?

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Abbreviations: AIF, apoptosis inducing factor; AMD, age-related macular degeneration; CNTF, ciliary neurotrophic factor; DHA, docosaexaenoic acid; FGF, fibroblast growth factor; GDNF, glial derived neurotrophic factor; Gpx, glutathione peroxidase; NPD1, neuroprotectin D1; OS, outer segments; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RPE, retina pigment epithelium; RXR, retinoid X receptors; S1P, sphingosine-1-phosphate; SOD, superoxide dismutase.

Abstract

Due to its constant exposure to light and its high oxygen consumption the retina is highly sensitive to oxidative damage, which is a common factor in inducing the death of photoreceptors after light damage or in retina inherited degenerations. The high content of docosahexaenoic acid (DHA), the major polyunsaturated fatty acid in the retina, has been suggested to contribute to this sensitivity. DHA is crucial for developing and preserving normal visual function. However, further roles of DHA in the retina are still controversial. Current data support that it can tilt the scale either towards degeneration or survival of retina cells. DHA peroxidation products can be deleterious to the retina and might lead to retinal degeneration. However, DHA has also been shown to act as, or to be the source of, survival molecule that protects photoreceptors and retinal pigment epithelium cells from oxidative damage. We have established that DHA protects photoreceptors from oxidative stress-induced apoptosis and promotes their differentiation *in vitro*. DHA activates the retinoid X receptor (RXR) and the ERK/MAPK pathway, thus regulating the expression of anti and pro-apoptotic proteins. It also orchestrates a diversity of signaling pathways, modulating enzymatic pathways that control the sphingolipid metabolism and activate antioxidant defense mechanisms to promote photoreceptor survival and development. A deeper comprehension of DHA signaling pathways and context-dependent behavior is required to understand its dual functions in retina physiology.

Too much of a good thing…: Light and photoreceptor degeneration

The human eye is constantly exposed to light, either sunlight or artificial lightning, as a necessary requisite for visual perception and the retina, at the back of the eye, is essential for this perception. The preservation of its unique, highly stratified organization is crucial for the retina to detect light, transform it in an electrical impulse and transmit it to the brain. Diverse environmental insults can alter retina homeostasis and lead to visual dysfunctions. Photoreceptor cells, rods and cones, the main light detecting cells in the retina, are exquisitely adapted for light reception and can function over a wide range of ambient light conditions, from near darkness to bright light. However, too much light, due to an intense or prolonged exposure can threaten vision, by damaging both photoreceptors and retinal pigment epithelium (RPE) cells. This damage can then be reversed to recover visual function, or progress to photoreceptor death and vision loss. In spite of intense research, a comprehensive understanding of the mechanisms involved in this death is still missing.

Oxidative stress has long been linked to retina neurodegeneration, both arising from environmental insults or from genetic alterations. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell, and is a consequence of either an excessive production or an insufficient removal of ROS. Its constant exposure to light makes the retina particularly prone to oxidative stress and its high oxygen consumption adds up to this sensitivity. Photo-oxidative stress can trigger both acute and chronic retinal damage. The damaging effect of bright intense light or a prolonged light exposure has long been known to lead to retinal damage, causing the loss of visual and RPE cells [1]. Light-degeneration in rodents has been widely used for over 50 years as a model of photo-oxidative stress-induced retinal degeneration. Retinal degeneration occurs in rodents continuously exposed to normal light levels for more than 5 days and within 1 to 2

days after being exposed to bright fluorescent light for 1 hour; ultraviolet, blue, or white light can induce irreversible lesions in the central retina [2;3].

Photoreceptor cells play a central role in the transduction from a light to an electrical signal. In these cells, ROS can be generated as byproducts of photochemical reactions or as a result of cellular metabolism. Opsins are the visual pigments of rods and cones, and 11-cis-retinaldehyde, their most frequent chromophore, is a chief mediator of oxidative damage. Rhodopsin bleaching triggers both visual transduction and phototoxic effects in photoreceptors [2]. In mice lacking rhodopsin, the retina cannot be injured by intense white light and the rate of regeneration of rhodopsin is a crucial determinant of the threshold for light-induced damage [4]. After light absorption,11-*cis*-retinaldehyde isomerizes to all-*trans* retinal and the reactions involved in the visual cycle for its regeneration also contribute to ROS formation [5]. High levels of all-*trans* retinal released from photo-activated rhodopsin upon acute light damage might, when accumulated, act as mediators of light damage in the retina [6;7]. In the presence of oxygen, all-*trans* retinal generates ROS, such as singlet oxygen, hydrogen peroxide and superoxide upon photoexcitation, [8]. A small fraction of all-*trans* retinal molecules spontaneously condenses to form photo-toxic products, such as all-*trans*-retinal dimers or diverse potentially toxic bis-retinoids, which accumulate in RPE cells following disc phagocytosis [7;9]. Many of these compounds are very deleterious since they generate ROS [7] and are not easily degraded by lysosomal enzymes, being major constituents of lipofuscin in RPE cells. Drugs that sequester all-*trans* retinal contribute to the prevention of light-induced retinal degeneration [10]. The relevance of minimizing all-*trans* retinal accumulation is highlighted by the finding that mutations in virtually all the enzymes of the visual cycle lead to retinal degenerations [6].

The particularly active aerobic metabolism of photoreceptors, resulting in an O_2 consumption much higher than in the inner retina, contributes to their sensitivity to oxidative damage. The high mitochondrial activity participates in the formation of ROS, including free

radicals, such as superoxide anion, hydroxyl radical), and species with their full complement of electrons in an unstable or reactive state, as singlet oxygen and hydrogen peroxide. These molecules will readily react with lipid, protein, and nucleic acids, affecting cell function or leading to cell death [11].

The daily renewal of rod photoreceptor outer segments (OS) and the phagocytosis of their tips by RPE cells are the physiological mechanisms for removing oxidized proteins and lipids and making this physiological damage essentially reversible [12]. However, these mechanisms can be overcome by intense light exposure, leading to photoreceptor damage, increasing the number of phagosomes found in the RPE, decreasing the length of rod OS and ultimately eliciting photoreceptor death [2;13].

How does the retina cope with oxidative damage?

As part of its successful adaptation to its function in light reception, the eye is well-equipped to protect itself from photo-oxidative damage. The cornea and the lens absorb the more reactive short wavelength radiations, below 400 nm, and also part of infrared radiation [14]. The wavelengths of light reaching the retina are between 400–760 nm, with blue light being particularly toxic for this tissue. In addition to mechanisms to restrict light input, ocular tissues have efficient antioxidant defenses, the levels of which vary according to the degree of exposure to light and the oxygen consumption of the ocular compartment [15]. Any substance that delays or inhibits the oxidation of a certain molecule can be defined as an antioxidant. The retina is endowed with effective antioxidant defense systems to counteract the consequences of too much light.

The macular pigment has a relevant role, protecting the retina from photo-oxidative damage. Its filtering effect is thought to attenuate the amount of deleterious blue light reaching this tissue [11]. In addition, carotenoids such as lutein and zeaxanthin, the major diet-based compounds [16] of macular pigment, are versatile antioxidants and have been proposed to play an antioxidative role in the retina [11]. Epidemiological and genetic studies support their

protective effect in age-related macular degeneration (AMD) [17;18]. AMD, the most common cause of legal blindness among elderly individuals in industrialized countries [19;20], is a heterogeneous group of disorders with multifactorial causes and oxidative damage has a role in its pathogenesis [21;22]. Analysis of postmortem human retinas of patients with AMD evidences the apoptosis of both photoreceptors and RPE cells [23]. Lutein supplementation to diabetic rats reduces markers of oxidative stress in the retina and prevents alterations in the electroretinogram [24]; lutein or zeaxanthin supplementation protects primate fovea from blue light-induced damage [25]. Addition of lutein and zeaxanthin protects cultured rat photoreceptors from oxidative stress, preserving mitochondrial potential, and preventing cytochrome *c* release from mitochondria. [26]; They also protect ARPE-19 cells, a human RPE cell line, from photo-oxidative damage [27], whereas lutein prevents oxidative stress-induced apoptosis of ARPE-19 cells [28].

Glutathione and melatonin are potent antioxidants in the retina. Glutathione is released by Müller cells, the principal glial cells of vertebrate retina, in response to oxidative stress [29;30]. Glutathione protects the retina against ROS by direct scavenging of radicals and by acting as a cofactor of glutathione peroxidase (Gpx) to metabolize H_2O_2 and lipid peroxides [31]. Melatonin is synthesized in the retina and capable of protecting diverse retinal cells from oxidative damage. It decreases retinal damage in an animal model of diabetic retinopathy, diminishing lipid peroxidation and preventing the decrease in catalase activity [32]. Melatonin partially protects ARPE-19 from oxidative damage, acting both as an antioxidant and by receptor activation; this might be related to RPE cell damage in this disease, since its circulating levels are reduced in AMD patients [33;34].

Gpxs, superoxide dismutases (SODs), and catalase are among the principal enzymatic components of the antioxidant defense system. Upon intense light exposure, wild type mice upregulate the expression of Gpx1, hemooxygenase-1 and thioredoxin-1, whereas mice deficient in Gpx1 show increased lipid peroxidation [35]. Gpx protects premature retinas from

oxidative stress; lack of Gpx1 increases oxidative stress markers and exacerbated oxidative injury and retinal vascular injury in a mouse model of retinopathy of prematurity [36]. Gpx4, which directly reduces peroxidized lipids formed in cell membranes, appears to have an essential role both for survival and differentiation of photoreceptors. This enzyme is predominantly expressed in mitochondria [37]. Mice deficient in this enzyme are particularly sensitive to oxidative stress; inducing Gpx4 expression reduces oxidative stress damage in the retina whereas increased expression of Gpx4 protects the retina from oxidative damage [38;39]. Noteworthy, in photoreceptor-specific, Gpx4 conditional knockout mice, though photoreceptors differentiate as rods and cones, they exhibit few connecting cilia and a disorganized outer segment structure, and initiate a rapid apoptosis after day 12 [37], implying this enzyme is not only critical for its antioxidant capabilities but also required for photoreceptor maturation.

In many tissues, SODs are central players in antioxidant defenses, converting superoxide radicals to H_2O_2. SODs have a relevant role in protecting the retina from oxidative damage; mice deficient in SOD1 are more sensitive to hyperoxia or to a paraquat injection, showing increased apoptosis and oxidative damage in their retinas; a SOD1 transgene reduces the effects of this damage on ERG amplitudes [40].

Catalase is a cytosolic enzyme with greater capacity but lower affinity for peroxides than Gpx and thereby handles severe oxidative stress. Coexpression of catalase and SOD2 reduces cone cell death and oxidative damage in mouse models of retinitis pigmentosa [41]. Thioredoxin, an endogenous redox regulator, prevents light-induced damage; light exposure upregulates the levels of in the retina and RPE and its overexpression or intraocular injection with recombinant thioredoxin suppresses photo-oxidative damage in mice [42;43].

Countless additional relevant work, which is beyond our possibilities to list, demonstrates the diversity and complexity of the antioxidant defense system present in the retina, which provides this tissue with mechanisms to deal with the physiological levels of

oxidative stress it faces daily. The overpowering of these antioxidant defenses manifests as pathologies leading to visual dysfunction.

Photoreceptor apoptosis, a shared theme in retinal degenerations

Retinal degenerations, such as light-induced retina degeneration, AMD and retinitis pigmentosa, have multiple environmental and genetical causes leading to photoreceptor loss. However, they share a common outcome: the death of photoreceptor cells leads to vision loss and apoptosis is the chief pathway heading to this death in induced and inherited retinal degenerations in animal models [3;44;45] and probably in the related human pathologies. This led to the appealing hypothesis that targeting a common link would allow the prevention of apoptosis in these diseases. However, extensive research evidences that the apoptotic pathways leading to photoreceptor demise are not always the same and also other cell death mechanisms participate in this demise. A crucial divergence point are the proteases involved in this demise. While apoptosis is usually associated with caspase activation and chromatin fragmentation, rod cell death is characterized by chromatin fragmentation but the involvement of caspases appears to be age and disease dependent. Apoptosis during development seems to rely on caspase activation through the mitochondrial pathway, as evidenced by the high expression of pro-apoptotic Bcl-2 family members such as Bax, Bak, Bim, Puma, and that of Apaf-1 and caspase-3 in the retina at early developmental times [46;47], the detection of caspase-3 activity in rat retina photoreceptors during their early development *in vitro* [48]. No apoptotic photoreceptors are observed in retinas of Bax/Bak double knockout mice [49] and their apoptosis is effectively prevented by Bcl-2 overexpression [50]. This pathway seems to be turned down in the adult retina; photoreceptors become more resistant to proapoptotic stimuli and die through caspase-independent pathways [47]. Caspases play an important role in retinas expressing mutant rhodopsin [51-53], but are not the sole responsible for carrying on photoreceptor apoptosis in inherited degenerations. Multiple parallel pathways participate in photoreceptor death in animal models of retina degeneration [54]. Though activated caspase-3

expression has been detected in *rds/rds* photoreceptors [55] and caspase activity increases during photoreceptor apoptosis of *rd* and *rds* mice [54], their degeneration is delayed but not prevented by Bcl-2 overexpression [56] or by caspase inhibitors [57]. Calpain and cathepsin are activated during photoreceptor death in *rd* mice [46;54;58;59], and calpain inhibition cannot prevent this death [60]. Hence, autophagy, complement-mediated lysis and caspase-independent pathways also participate actively in the demise of photoreceptors in animal models of inherited retinal degeneration [46;54;60].

Light damage also leads to photoreceptor apoptosis, the onset of which depends on light exposure [13;61]. The mechanisms involved in photoreceptor apoptosis after light damage seem to be animal model specific and to differ upon the intensity of the light stimuli [62] and caspase involvement is controversial. Increases in the mRNA, activity and levels of several caspases upon light damage have been reported [2;63]. Caspase-3 mRNA was elevated in rat retina after 6–12 hours of light, but its enzymatic activity was not increased [64]. Caspase-3 levels and activity were not increased after bright light-induced retina degeneration [65;66] whereas calcium-dependent calpains were active and calcium channel blockers inhibited photoreceptor apoptosis [46]. In a canine model of autosomal dominant retinitis pigmentosa, a brief white light exposure causes ultrastructural alterations in both the outer and inner segments and initiates cell death, inducing caspase-12 transcription and calpain activation, but not that of caspase-3 [67]. Increased activities of caspase-3, and also of cathepsin and lyzozime, involved in autophagic death, have been found after moderate light damage [54]. Upon light stress, caspases, calpain 2 and cathepsin D were activated in 661W, a cone photoreceptor cell line [68].

As a whole, the evidence implies that multiple cell stressors simultaneously activate different death pathways in photoreceptors. Moreover, the data showing that separate inhibition of the different proteases is not enough to prevent photoreceptor death suggest that these pathways might concur to lead to photoreceptor demise.

Oxidative stress, a common topic in photoreceptor death

Being photoreceptor death a multi-factorial process, the search for a common triggering step is crucial for devising therapeutic strategies for the treatment of retinal degenerations. Oxidative stress emerges as a shared theme, involved in promoting the onset of this death in multiple pathologies, irrespective of their origin [11;46;54;69-71]. An early and sustained increase in intracellular ROS accompanies photoreceptor apoptosis *in vitro* and scavenging of these ROS can prevent this apoptosis [69]. Rod and cone photoreceptors in retinal explants generate ROS when deprived of trophic support [72].

Oxidative stress has been related to cone cell death in retinitis pigmentosa [73]; oxidative stress markers increase in animal models of retinitis pigmentosa and in light damage [54] whereas an increased expression of oxidative stress-related genes is observed during the progression of retinal neurodegeneration [74]. Antioxidants can ameliorate the progression of retinal neurodegeneration [75;76], rescue photoreceptors and decrease cone cell death in animal models of retinitis pigmentosa [77-79].

Oxidative stress appears to be an early event in light-induced damage in the retina, particularly in photoreceptors. ROS increase in isolated rods minutes after light exposure and rhodopsin activation is sufficient for their production, with no further downstream steps of visual transduction being required [80]. In contrast, cultured ganglion cells require over 2 days of intense light to evidence mitochondrial oxidation and apoptosis [81]. Transgenic mice with a mutated SOD are more severely affected by light damage than non-transgenic animals with a normal SOD [82]. Reduced expression of SOD1, SOD2, catalase and Gpx in a rat model of light-induced degeneration increases oxidative stress [83]. Several natural and synthetic antioxidants provide effective protection from light damage [2]. The rapid induction of oxidative stress after light exposure might explain the finding that antioxidants appear to be more effective *in vivo* when given prior to light exposure and not immediately after [2].

Substantial evidence supports the involvement of oxidative stress in AMD. Mice deficient in SOD1 show features typical of human AMD, such as drusen, thickened Bruch´s membrane and coroidal neovascularization; moreover, excessive exposure to light of these mice increases drusen formation [84]. Different markers of oxidative stress were detected in serum and aqueous humor of wet AMD patients [85;86]. Epidemiological studies suggest that oxidative stress contributes to the onset and the progression of AMD and supplementation with antioxidant vitamins and supplements might slow the progression of intermediate and late forms of the disease [71;87-89]. The ability of smoking to promote oxidative stress might contribute to it being a major risk factor for this disease. Cigarette smoke exposure has been shown to induce oxidative stress and complement activation in RPE cells, resulting in endoplasmic reticulum stress, and both oxidative stress and complement might act synergistically in AMD pathogenesis [90].

Antioxidants have been extensively tested as a therapeutic tool for treating retina degenerations and have proved to be quite useful in decreasing ROS formation and hence, oxidative damage. However, it should be taken into account that in addition to their deleterious effects, ROS have also been proposed as intracellular signaling molecules, regulating proliferation, survival, and differentiation [91-93]. ROS modify signaling pathways through the redox inactivation of tyrosine phosphatases, due to the oxidation of key cysteine residues in their active sites, and the concurrent activation of tyrosine kinases, thus tuning the duration and amplification of the phosphorylation signal [94;95]. Hence, a limitation of antioxidant therapies is that essential pathways might also be affected [96-98]. The identification of molecules able to potentiate locally the antioxidant defense mechanisms of photoreceptor cells would provide a useful alternative approach to these therapies.

Survival factors for photoreceptors

With apoptotic death of photoreceptors as a common cause of retinal degenerations, the search for photoreceptor trophic factors and investigating whether they can prevent

photoreceptor death in these diseases has been an area of upmost interest in the field for more than two decades. Photoreceptors need several trophic factors for their survival and development, such as glial derived neurotrophic factor (GDNF), basic fibroblast growth factor (FGFb), ciliary neurotrophic factor (CNTF), pigment epithelium derived factor (PEDF), neurotrophin-3 (NT-3) and others [99-103]. The distribution of trophic factor receptors changes in the retina during development and upon different injuries and the loss of trophic support induces neuronal apoptosis in the retina and plays a relevant role in the pathogenesis of AMD and RP [103;104]. We have proposed that docosahexaenoic acid (DHA), a 22-carbon ω-3 fatty acid with six double bonds, also acts as a trophic factor for photoreceptors, promoting their survival and differentiation by activating different signaling pathways and protein transcription in these cells [48;105-112].

Inhibition of apoptotic degeneration and maintenance of retinal homeostasis have been for the last two decades the major proposed functions of trophic factors in the treatment of retinal degenerations. The recent progress in cell transplantation in the retina to restore lost neurons, has broadened their application, since they might improve conditions of cell transplantation by expanding and preserving cell populations *in vivo* [103].

The yin and the yang of DHA

The high content of polyunsaturated fatty acids in the retina has been proposed to contribute to provide a permissive environment for oxidative stress. DHA is the most abundant polyunsaturated fatty acid (PUFA) in the retina [113]. It amounts to 25% of total fatty acids in this tissue and over 50% of those esterified in the phospholipids that form the outer segments (OS) of photoreceptors [114], the disk structures were rhodopsin is concentrated. Phospholipids surrounding rhodopsin have two ω-3 PUFAs esterified at sn-1 and sn-2 positions of the glycerol backbone, and at least one of them is DHA [115;116]. Hence, DHA is a major determinant of the biophysical properties of the membrane environment in which

rhodopsin and its associated proteins are embedded and where they experience the conformational changes that make phototransduction possible.

The levels of DHA increase in the rat retina during the first month of postnatal life, in parallel with retina development, and this fatty acid is then tenaciously retained [117;118]. This avid uptake and retention of DHA has recently been shown to involve adiponectin receptor 1 [119]. DHA is an essential fatty acid in mammals. It is derived from the diet or it is synthesized from 18:3 ω-3 and longer chain precursors largely in the liver, which is the main responsible of its provision to the brain and retina [120;121]. DHA is rapidly esterified in phospholipids to form hexaenoic and dipolyunsaturated molecular species that undergo an active synthesis and turnover in the retina [107;122;123]. Isolated photoreceptors actively take up and esterify DHA in phospholipids [107]; after leaving the endoplasmic reticulum in the inner segments, DHA-containing phospholipids associate with rhodopsin in the Golgi to form the new disk membranes that are vectorially transported to the OS [124]. MALDI imaging mass spectrometry analysis has recently suggested that DHA-enriched glycerophosphatidylcholine and –ethanolamine are only found in photoreceptors [125]. The tips of these outer segments are daily phagocytized by the RPE cells, which have efficient mechanisms to recycle DHA back to photoreceptor cells [126;127].

DHA plays dual roles in retinal physiology and pathology. It has long been known that DHA has an essential role in retinal function. Provision of DHA to human infants is required for an adequate development of the visual system [128-132] and sustained dietary deprivation of this fatty acid or of its metabolic precursors markedly affects proper visual function in rats, guinea pigs and monkeys [133-139]. Adequate levels of DHA are essential for rhodopsin conformational changes and to optimize the early steps during visual signal transduction [140;141]. The high affinity binding of DHA to interphotoreceptor-retinoid binding protein (IRBP) and its modulation of the interactions of 11-*cis* retinal and all-*trans*-retinol with IRBP

has suggested a role for DHA in directing the flux of these retinoids during the visual cycle [142;143].

On the other hand, the effect of DHA in retina degenerative diseases is controversial. The finding that DHA levels are decreased in plasma and outer segments of animal models and patients with retinal degenerations [144-149] suggests a relationship exists between the reduced DHA levels and retina degeneration. This relationship is still puzzling and several hypotheses have been proposed in this regard: 1) DHA decrease causally contributes to retina degeneration; 2) DHA decrease is an adaptive response to the increased metabolic/oxidative stress in the diseased retinas, in order to reduce the likelihood of increased peroxidation and toxic product formation; 3) DHA decrease results from the increased peroxidation in the affected retinas [146]. None of them can be ruled out with the existing data. Increasing DHA levels in animal models by dietary supplementation or by genetic manipulation has no effect on preventing or slowing retina degeneration [150-153]. However, the finding that impaired DHA uptake in photoreceptors in adiponectin receptor 1 knockout mice precedes their progressive degeneration [119] supports a connection exists between adequate DHA levels and photoreceptor survival.

DHA effects in retina degeneration in humans are still under debate. DHA supplementation studies have either shown beneficial effects for subgroups of patients with retinitis pigmentosa in clinical trials, with no effect on the majority of the patients in the studies [154-156] or have shown no clear effect [156;157]. Several studies have shown DHA-enriched diets to be associated with a reduced risk of AMD in animal models [158]. Large-scale human studies on AMD suggest a reduced likelihood of having or progressing to neovascular AMD among people with highest dietary intakes of omega-3 long-chain polyunsaturated fatty acids [159-164], whereas a recent report suggests no significant effect on reducing risk of progression to advanced AMD [17]. Diets enriched in ω-3 fatty acids effectively reduce pathological retinal neovascularization in a mouse model of oxygen-induced retinopathy, and a DHA metabolite,

4-hydroxy-docosahexaenoic acid (4-HDHA) has been proposed to inhibit endothelial cell proliferation and to sprout angiogenesis [165;166].

Conflicting data exist regarding the role of DHA upon oxidative damage in the retina [167]. Its high number of double bonds makes DHA the most easily oxidizable among retina PUFAs, which together with its abundance in the retina contributes to it being associated to retina sensitivity to oxidative injury [168-171]. Bright light exposure causes the oxidation of retina PUFA in rat retinas in vivo, and DHA, and not ω-6 PUFAs, has been shown to be the main source of reactive oxidation products that induce protein modifications [172]. These aldehydes react with rod OS proteins after light exposure and antioxidants can inhibit their formation upon light damage [172;173]. Increased levels of DHA in retinas of transgenic animals with the *fat-1* protein, which converts ω-6 to ω -3 PUFA, correlate with an increased peroxidation of ω-3 PUFA, and with higher retina and photoreceptor vulnerability to photo-oxidative damage, which increases the amount of apoptotic photoreceptors and reduces the amplitude of electroretinograms [174]. Interestingly, the increase in DHA levels does not appear to have the same effect in animal models of inherited retinal degenerations. The amount of DHA can be increased in animal models by dietary manipulation [150] and even doubled in the retina, as observed in transgenic mice expressing mutant rhodopsin or mutant human *ELOVL4* and *fat-1* [152;153], but this does not increase the rate of degeneration, as would be expected to result from increased peroxidation of DHA. This suggests that the deleterious effect of increased DHA might depend on the animal model or the intensity of the damage.

DHA can also be damaged by oxidative stress generating a seven carbon oxidation fragment, which forms protein adducts found in higher levels in plasma and outer retinas of AMD patients than in controls [175]. These adducts have been shown to produce immune responses and AMD-like lesions in mice, thus linking DHA oxidation with the initial steps of this disease [176;177].

Noteworthy, a different product of DHA oxidation, Neuroprotectin D1 (NPD1) is neuroprotective for RPE cells. Oxidative stress rapidly activates the synthesis of NPD1 in RPE cells to protect them from apoptosis and several neurotrophins induce NPD1 synthesis and apical release in RPE cells [178;179]. It has been proposed that disruption of RPE cell polarization might contribute to retinal neurodegeneration not only through the secretion of pro-inflammatory signals but also due to the loss of NPD1 protection [180]. Puzzingly, DHA supplementation elicited deleterious effects on RPE cells when exposed to intense light, increasing the formation of ROS and altering cell function [181]. Again, this emphasizes that DHA effects are not unique, but rather depend on the type, or intensity, of the injury. A relatively mild injury might activate DHA neuroprotection, whereas very toxic injuries, as those induced by intense bright light, might overcome the protective pathways activated by DHA or its metabolites, tilting the scale to its neurotoxic effects, due to the accumulation of toxic peroxides and oxidation fragments.

Multiple data support a neuroprotective role of DHA in the stressed retina. DHA at low concentrations is protective for bovine retina endothelial cells upon oxidative stress, whereas it induces oxidative stress at high concentrations; it has opposite effects on cytosolic Gpx activity, slightly reducing it at low concentrations [182]. Dietary intake of DHA prevents retina degeneration in rats injected with N-methyl-N-nitrosourea [183]. DHA supplementation prevents cell loss in the ganglion cell layer in an animal model of experimental diabetic retinopathy [24]. DHA ameliorates the activation of oxidative stress enzymes, and of calpain and caspase-3, thus preventing oxidative stress and apoptosis in 661W, a cone cell line, stressed by an increase in cGMP levels [184]. 661W cells have been recently shown to increase the synthesis of NPD1 from DHA when under light stress and both DHA and NPD1 protect them from light-induced degeneration [185]. DHA and NPD1 pools increase in ARPE-19 cells during photoreceptor OS phagocytosis and this phagocytosis markedly attenuates oxidative stress-induced apoptosis of these cells; both DHA and NPD1 further increase during oxidative stress

challenge, suggesting they have a role in the crosstalk between RPE and photoreceptor cells, essential for their survival [179]. Dietary supplementation with DHA in the retinoschisin (Rs1h)-deficient (Rs1h(-/Y)) mouse model of inherited retinal degeneration prevents the activation of microglia and enhances photoreceptor survival [186].

We have found that DHA postpones the apoptosis of photoreceptors in rat retina neuronal cultures. In the absence of trophic factors photoreceptors start degenerating after 4 days *in vitro* through the mitochondrial apoptotic pathway; addition of DHA delays the onset of this apoptosis [105;106]. DHA also promotes the exit of neuronal progenitors from the cell cycle in retina neuronal cultures, increasing the levels of the cyclin-cdk inhibitor p27Kip1 [187]. When cultured in the absence of their trophic factors, development of photoreceptors seems to be arrested; they develop as round cells with a small cell body and a short cilium [105], but usually lack the high opsin levels and characteristic outer segments found in photoreceptors *in vivo*. DHA also protects photoreceptors in culture from apoptosis after oxidative stress induced with paraquat and H_2O_2 [110;188].

Sphingolipids have crucial roles in regulating cell death, survival and development; ceramide and sphingosine induce cell death and their phosphorylated derivatives, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate promote survival in diverse cell types [189]. We established that ceramide and sphingosine mediate the apoptosis of photoreceptors induced by oxidative stress and DHA prevents this apoptosis by modulating the expression or the activity of enzymes of the sphingolipid metabolic pathways to decrease ceramide and sphingosine levels [48;190;191].

DHA also advances the differentiation of cultured photoreceptors [105;107;111], which is consistent with its tight association with rhodopsin and its effect promoting rhodopsin delivery for outer segment biogenesis [192;193]. S1P has been shown to stimulate photoreceptor differentiation, targeting rhodopsin to the OS [112;194]. DHA upregulates the levels of

sphingosine kinase 1, the enzyme involved in S1P synthesis, to promote the synthesis of S1P, which then acts as an intracellular mediator to enhance the formation of OS [112].

A lesson from evolution

In view of the conflicting experimental data showing both benefic and harmful effects of DHA, it seems advisable to take a look at the evolutionary mechanisms operating on living organisms in our planet. Living species have the evolutionary pressure to become adapted to their environments; they eventually become un-adapted, and even risk extinction, when the environmental conditions change. In general, when a structure or molecule becomes detrimental for a given species, accumulative changes are added to that structure or molecule, to allow the organism to become better adapted to the environment. When this does not happen, the disadvantage negatively affects the reproductive index and the species, eventually becomes extinct.

The Earth surface has been exposed to light since its origin and the current oxygen concentration in the atmosphere has remained stable for the last billion years. Most organisms have ended up successfully adapting to live under these conditions. Evolution allowed the retina of vertebrate species to accumulate high levels of a highly unsaturated and easily oxidizable molecule as DHA, probably because it was the best suited for establishing the biophysical conditions required for phototransduction. To minimize the risks posed by this fatty acid, most organisms, including humans, count with a repertoire of molecules and mechanisms in the eye that prevent the accumulation of the deleterious products that can originate from this fatty acid, under normal, physiological conditions. In this context, the preservation of high DHA levels in the retina underscores its relevance for visual function. It would be unlikely for evolution to allow DHA to be retained for near a billion years in the retina of vertebrates if it were more noxious than benefic for a sense as critical as vision.

DHA activates intracellular signaling pathway to prevent photoreceptor death

Work from several laboratories evidence that both the ERK/MAPK and the PI3K signaling pathways participate in DHA effects. The ERK/MAPK pathway has a central role during retina development and for photoreceptor survival. In mouse retina, ERK expression is distributed throughout the whole retina at P0, and decreases in a time-dependent manner in the outer nuclear layer, although it is still detectable in adult retina [195]. In the neonatal retina, activation of ERK by cytokines is developmentally regulated, and is mediated by its upstream kinase, MEK1/2; increased pERK levels in response to cytokine signals are found both in retinal proliferating progenitors and in posmitotic photoreceptor precursors, in which it might influence cell fate specification and promote differentiation and survival, respectively [196]. The ERK/MAPK pathway participates in the protective response to bright light damage in the retina [197]. Different trophic factors such as FGF2 and CNTF activate this pathway to promote photoreceptor survival and prevent light induced damage after optic nerve section [196;198;199], whereas BDNF, protects retinal neurons via the TrkB/ERK/MAPK pathway in a model of diabetic retinopathy [200]. Underscoring the relevance of the ERK/MAPK pathway for photoreceptor survival and development, we have shown that DHA enhances ERK phosphorylation in rat retina photoreceptors in culture, whereas inhibiting this pathway completely blocks DHA anti-apoptotic effect and its stimulatory effect on differentiation [109]. The ability of DHA to stimulate this pathway has also been reported in human neuroblastoma SH-SY5Y cells, in which DHA enhances neurite growth by inducing sustained ERK1/2 phosphorylation, while a MEK specific inhibitor abolishes this effect [201]. Hence, DHA promotes the survival and differentiation of photoreceptors by activating the same signaling pathways triggered by peptidic trophic factors in these cells.

DHA also activates the PI3K/Akt signaling pathway. This pathway is an important regulator of several important cellular processes, including apoptosis, survival, proliferation, and metabolism. Akt regulates caspase-9 activity via phosphorylation and inhibits apoptosis [202], whereas inhibition of the PI3K/Akt pathway blocks increased nuclear accumulation of

Akt, inducing apoptosis [203]. High-glucose has been shown to increase the level of tumor necrosis factor α (TNF-α) and IL-6, inducing oxidative stress and apoptosis in hippocampal neurons in vitro; DHA supplementation reduces oxidative stress and TNF-α expression and protects these neurons by increasing Akt phosphorylation and decreasing expression of caspase-3 and -9 [204].

The PI3K pathway might also play a role in DHA effects in axonal development in photoreceptors. DHA stimulates neurite outgrowth in rat retina neuronal cultures [111], in hippocampal neurons and in human neuroblastoma cells [201;205]. In the absence of insulin and in insulin receptor knockout mice, photoreceptors have an abnormal morphology with a wide axon cone and a disorganized cytoskeleton. DHA restores normal axonal outgrowth in insulin-lacking cultures [206], implying that insulin and DHA activate at least partially overlapping signaling pathways that are essential for the regulation of actin cytoskeletal organization in developing photoreceptors. Light-induced tyrosine phosphorylation of insulin receptor generates 3′-phosphoinositides that regulate the reorganization of the actin cytoskeleton [207]. Light induces the activation of retinal insulin receptors in rod OS, and these receptors regulate PI3K and Akt activation in photoreceptors and OS [207-210]. Rajala and col. [206] speculated that the activation of this pathway results from changes in DHA levels in the OS membranes; consistently, they find higher amounts of DHA in light adapted OS. Though they do not observe any DHA-induced direct activation of insulin receptor *in vitro*, DHA may be indirectly activating the insulin receptor signal transduction pathway. In line with this hypothesis, DHA promotes the survival of neuroblastoma cells upon serum deprivation by facilitating the translocation and activation of Akt, without stimulating PI3K activity; activation of this pathway is nevertheless required for DHA protection, as PI3K inhibitors block the effect of DHA on neuroblastoma cell survival [211]. Inhibitors of the PI3K signaling pathway do not affect DHA protection of cultured rat retina photoreceptors, though they increase the apoptosis of amacrine neurons, which depend on this pathway for their survival

[212]. Hence, DHA promotes the survival of rat retina photoreceptors at early culture times and upon oxidative stress exclusively through the activation of the ERK/MAPK pathway, without stimulating PI3K [109]. This suggests that DHA activation of distinct signaling pathways is cell type-dependent or, for a specific cell type, DHA differentially activates signaling pathways for modulating diverse cellular outcomes.

DHA modulates the apoptotic pathway in photoreceptors

DHA protection is closely related to the preservation of mitochondria membrane polarization. Mitochondria have a central role in the regulation of apoptotic cell death [213-215]. The loss of mitochondrial membrane potential is involved in photoreceptor apoptosis during early development in vitro and upon oxidative damage and DHA prevents mitochondrial depolarization in photoreceptors [109;188]. Inhibiting the ERK/MAPK pathway blocks DHA protection, simultaneously inducing mitochondria depolarization, suggesting DHA activates a signaling pathway that blocks an early event in apoptosis induction, upstream of mitochondria depolarization [109].

Multiple evidences supports a role of the Bcl-2 family in the regulation of apoptosis at early developmental times, which decreases in mature and degenerating retinas [46;47]. No Bcl-2 expression is found in photoreceptors in adult rat retinas [216], and conflicting data exist on the role of Bcl-2 in retinal degeneration. Although Bcl-2 overexpression has been shown to enhance photoreceptor survival in several animal models of retinal degeneration [56;217], other studies show either little or no protection [218;219].

Bax is activated during degeneration of P23H, *rd1*, and Rho knockout retinas and this activation is regulated by calpains and cathepsin D in the last two animal models; activated Bax in the photoreceptor cell inner segment is only detected in the degenerating mutant retinas [220]. Bax is only partially regulated by this cascade of proteases in dominant mutations of Rho and both *rd1* and P23HTg retinas show a significant downregulation of Bcl-2 levels [220]. In rat retina photoreceptors *in vitro*, Bax levels noticeably increase during time in culture

and upon oxidative damage [188], whereas the Bcl-2/Bax ratio decreases, accompanying the increase in apoptosis. DHA increases Bcl-2 expression in photoreceptors, and prevents the increase in Bax expression, leading to an increase in the Bcl-2/Bax ratio that is probably related to DHA preservation of mitochondrial functionality at this early developmental time. As development proceeds, DHA fails to keep a high Bcl-2/Bax ratio and this decrease accompanies mitochondrial depolarization and photoreceptor apoptosis. Interestingly, at a later time *in vitro*, GDNF, another photoreceptor trophic factor, enhances Bcl-2 levels, restoring an adequate Bcl-2/Bax ratio to promote survival. These results suggest that photoreceptors depend on two, or perhaps more, trophic molecules acting in a concert to prevent apoptosis. DHA protection from oxidative stress similarly involves the up-regulation of Bcl-2 levels and down-regulation of those of Bax in cultured photoreceptors [188], and an enhancement of Bcl-2 and Bcl-xL expression in ARPE-19 cells [178]. GDNF is unable to protect cultured photoreceptors from oxidative stress because it does not increase Bcl-2 expression at the time of the oxidative challenge [188]. Thus, the effectiveness of DHA as a survival molecule for photoreceptors *in vitro*, is closely related to its ability to maintain high Bcl-2 levels, emphasizing the relevance of the relative levels of Bcl-2 and Bax in preserving mitochondrial polarization and, hence, photoreceptor survival during early development.

As mentioned above, caspase involvement in retinal cell death is still a matter of considerable controversy; conflicting results exist regarding its involvement in photoreceptor death during development and in animal models of retinal degeneration [47;51;221-224]. DHA down-regulates caspase-3 activity in neuroblastoma cells [225] and in hippocampal neurons exposed to high-glucose [204]. DHA partially decreases caspase- 3 activity in cultured photoreceptors at day 6 *in vitro* [109]. This suggests that regulation of caspase-dependent pathways is involved in DHA protection.

Bax and Bak activation lead to caspase activation in various systems; in *rd1* mutant photoreceptors Bax activation is linked to the translocation of apoptosis inducing factor

(AIF), a death activator, from the mitochondria to the nucleus [220;226]. Oxidative stress induces cytochrome C release from rat retina photoreceptors *in vitro* [26]. We investigated whether AIF might also be released from mitochondria and participate in photoreceptor apoptosis during development *in vitro*. As shown in Fig. 1, in 6-day retina neuronal cultures lacking photoreceptor trophic factors, where about 60% of photoreceptors were already apoptotic, nuclear translocation of AIF (thin white arrows in A) was observed in photoreceptors that evidenced an advanced stage of apoptosis (condensed or pyknotic nucleus, thin white arrows in C) and showed mitochondrial membrane depolarization (thin white arrows in B). Nuclear translocation of AIF was substantially reduced by DHA, consistent with the reduction in the amount of apoptotic photoreceptors. These results suggest that DHA protects photoreceptors from apoptosis by preventing mitochondrial depolarization, thus inhibiting AIF release from the mitochondria intermembrane space.

Both caspase activation and AIF translocation in the mitochondrial pathway are closely dependent on mitochondrial outer membrane permeabilization [214;215]. DHA preservation of mitochondrial membrane barrier properties leads to its reduction of caspase-3 activity and AIF nuclear translocation. As a whole, our data suggest an active involvement of the mitochondrial pathway in the apoptosis of photoreceptors during development *in vitro*; the decrease in Bcl-2 expression and the increase in Bax expression during time in culture lead to mitochondrial depolarization, with the subsequent release of death factors such as cytochrome c and AIF, and caspase-3 activation. DHA protection involves a partial reduction of caspase-3 activation and AIF release, which are consistent with the inability of DHA to completely prevent photoreceptor death, but rather to postpone it during early development *in vitro*.

DHA and retinoid X receptors, a key for photoreceptor survival?

The pleiotropic effects of DHA can be explained by its generating diverse metabolites and by the fact that PUFAs are transcriptional regulators acting through multiple interactions with different nuclear receptors [227-229] and transcription factors [230-232]. DHA is an endogenous

ligand for retinoid X receptors (RXR) in mouse brain [227], acting in the range of 5–10 μM [233]. RXR signaling is essential for normal development and involved in nervous system development [234;235]. RXR are members of the steroid/thyroid receptor superfamily, and bind to DNA response elements only after the formation of either RXR:RXR homodimers or heterodimers with other members of this receptor family, including retinoic acid receptors (RAR) [236]. In addition, DHA activates the steroid coactivator receptor 1 (SCR-1) and Nurrl/RXR suggesting that changes in RXR heterodimerization partners might affect DHA response [227;237].

DHA acts as a natural RXR agonist in diverse cell types. In a human placental choriocarcinoma cell line, BeWo cells, RXR-antagonists, PA451 and HX531 inhibit DHA induction of adipose differentiation-related protein (*Adrp)* mRNA and ADRP protein accumulation, suggesting DHA activates RXR to increase ADRP levels [238]. Neuroprotectin D1, the DHA metabolite, activates another nuclear receptor, the peroxisome proliferator-activated receptor γ (PPAR γ) to protect neurons in a model of Alzheimer disease [239].

RXR have different isoforms. Mice with mutated genes for each of the RXR isoforms show these receptors regulate several developmental processes, including neurogenesis. Some redundancy exists in the function of all isoforms, but each isoform also shows certain specific functions. The RXRα isoform is the most relevant during eye development as RXRα null mutants exhibit ocular abnormalities; these mutants die after E14.5, making it impossible to study its function at later developmental times [240]. Expression and localization of these receptors has been studied in rodent and chicken retina. RXRα is uniformly distributed in the embryonic retina before lamination, and then decreases, being undetectable in the adult retina. RXRβ is ubiquitously distributed throughout the retina and RPE, both during development and in adult retina; no abnormalities are found in the eye of the RXRβ null mutant mice. The RXRγ isoform is expressed in rodent retinal neuroblasts, and is found later in ganglion cells and in cone photoreceptors [241-244]. RXRγ null mutant mice show no alterations in retina

histology or in the number of photoreceptors [244]. On the contrary, null knockout mice for combinations of retinoid receptors (RXR with RAR) show a deep ocular abnormality, including the absence of the ventral retina [245;246]; double knockouts evidence a somewhat disorganized retinal histology and a failure in photoreceptor differentiation [247]. Other RXR heterodimers also have important roles in this differentiation; RXRγ and Trb2 are necessary to establish the dorsal-ventral gradient of S-opsin, suppressing its expression in a subset of dorsal cones. RXRγ regulates expression of S-opsin, but not of M-opsin, whereas Trb2 upregulates M-opsin expression [248]. Another member of the RXR family involved in the differentiation of photoreceptor type (rods and cones) is NR2E3 (PNR, photoreceptor specific receptor); mutant mice lacking this receptor have a greater number of S-cones and mutations in this receptor underlie S-cone syndrome, a human inherited disease [249;250]. These results evidence a crucial role of RXR/RAR during eye morphogenesis and in the regulation of photoreceptor development and differentiation.

In rat retina neuronal cultures RXR are expressed in photoreceptors, with an almost exclusive nuclear localization, whereas they are heavily concentrated in the cytoplasm in amacrine neurons, with only sparse aggregates in the nuclei [110]. This is consistent with the findings in rodent and chick retinas, evidencing that RXR have specific and dynamic patterns of distribution in ocular tissues during development *in vivo*. We have demonstrated that DHA requires the activation of RXR to promote the survival of photoreceptors; the addition of RXR antagonists completely blocks the protective effect of DHA on photoreceptor apoptosis at early culture times or after oxidative stress, induced with two different oxidants, paraquat and H_2O_2 [110]. Remarkably, two synthetic agonists for RXR can also protect photoreceptors from oxidative damage, emphasizing the relevance of RXR activation for photoreceptor survival. DHA might directly bind and activate RXR to promote photoreceptor survival or alternatively, DHA-derived metabolites, such as NPD1, might also act as ligands for RXR, activating downstream survival pathways in photoreceptors. The hypothesis that DHA

directly binds and activates RXR in photoreceptors is supported by the evidence that it binds and induces the robust activation of RXR in brain [227;233]. DHA has to be released from phospholipids to trigger the mechanisms leading to photoreceptor protection. Phospholipids are the main storage sites for PUFAs in mammals, and their release is tightly controlled by phospholipase A2 [251]. To take part in RXR activation, DHA might remain as a free fatty acid in photoreceptor membranes or be stored in membrane phospholipids to be deacylated when required. DHA accumulation in membrane phospholipids, chiefly phosphatidylserine, is responsible for the protective effect of DHA in Neuro2A cells [225]. When added to neuronal cultures, DHA is efficiently taken up and esterified in phospholipids to reach the concentration found in adult rat retinas, only minor amounts remaining as free DHA [105;225]. The release of DHA, and thus the size of the free DHA pool, is tightly controlled, mainly by iPLA 2 [252]. The finding that inhibiting iPLA2 before inducing oxidative damage to retina neurons completely blocks the antiapoptotic effect of DHA on photoreceptors suggests that DHA is released from phospholipids on demand to exert its effect [110]. Ischemia and seizures elicit a rapid release of DHA in brain [253;254]; as has been shown for arachidonic acid [255], oxidative damage might elicit the activation of iPLA2 and the subsequent release of DHA, which would then lead to the activation of RXR to promote photoreceptor survival.

Further research is required to establish which of the above hypotheses is correct. Nonetheless, these data imply that DHA signaling through RXR is pivotal for orchestrating survival mechanisms in photoreceptors and provide a novel pathway for DHA effects in photoreceptors. DHA would be released from phospholipids upon cellular stress to induce activation of RXR and then trigger the ERK/MAPK signaling pathway to promote photoreceptor survival.

However, DHA release might turn out to be a double edged sword. Though the high content of DHA in retina photoreceptors is undoubtedly essential to establish the biophysical characteristics of OS membranes for modulating the conformational changes in rhodopsin

upon light exposure, a minimal release of DHA would be sufficient for its function as a signaling molecule, for its subsequent RXR activation and downstream signaling. A massive release of DHA would have catastrophic consequences for retina integrity. As described above, DHA is easily oxidizable, giving rise to oxidation products that become neurotoxic [256], after depleting cellular detoxification systems. Noteworthy, despite its sensitivity to peroxidation, DHA protects photoreceptors in culture from oxidative stress induced apoptosis [188]. DHA acts in a narrow window of concentrations in cultured retina neurons, and above 10 μM, it is deleterious for photoreceptors [105]. An adequate concentration of DHA and its proper chemical stability are crucial factors that might affect the outcome of clinical trials for retina pathologies.

The finding that protection of photoreceptors can also be achieved through activation of RXR by synthetic agonists [110] introduces versatility into the control of the survival of these cells and may provide new potential tools for treating diseases involving the death of photoreceptors. The local use of RXR agonists would restrain their effects to the site of interest, limiting these effects to those resulting from RXR activation. In addition to being more stable than DHA, there are already established therapeutic protocols for RXR agonists, which demonstrate their effectiveness in the treatment of other pathological processes [257-259] and would allow its rapid use in neurodegenerative diseases of the retina.

Does DHA activate antioxidant defense systems in photoreceptors?

Paradoxically, in spite of being easily peroxidized, DHA also actívates antioxidant enzymes, decreases ROS production and prevents the accumulation of oxidated proteins, thus protecting neural tissues [167;260-262]. Dietary supplementation with DHA and eicosapentaenoic acid (EPA), a metabolic precursor of DHA, increases SOD and Gpx activities in rats [263]. A similar diet lowers oxidative stress and increases the activity of antioxidant enzymes in a rat model of metabolic syndrome [264]. DHA induces the upregulation of hemooxygenase-1 in a

microglia cell line, which is attenuated by PI3K/AKT and MEK/ERK inhibitors [265]. Administration of DHA to diabetic rats, both with or without insulin, decreases oxidative stress and prevents changes in their electroretinograms [24]. DHA was shown to act as a radical scavenger in a ganglion cell line subjected to oxidative stress, preserving cell viability [266] and to decrease the production of ROS in 661W cells [184]. This implies that DHA might act as an antioxidant, either directly, by acting as a scavenger of ROS or indirectly, by promoting their removal through the activation or upregulation of enzymes of the antioxidant defense system.

Using retina neuronal cultures subjected to oxidative damage we explored whether DHA prevents photoreceptor apoptosis by stimulating the intracellular antioxidant mechanisms. We first evaluated if DHA reduced the formation of ROS induced by treating neuronal cells in culture with H_2O_2. (Fig. 2A). Cultures treated with DHA evidenced no increase in ROS formation compared to controls, whereas H_2O_2 induced almost a 3-fold increase in the amount of ROS. In cultures pre-treated with DHA, H_2O_2 only slightly increased ROS formation (140% compared to controls), suggesting DHA decreased the formation of ROS induced by this oxidant.

We then evaluated the effect of DHA addition on Gpx activity, as increased levels of this enzyme, and particularly of Gpx4, preserve retinal structure and function in transgenic mice subjected to oxidative stress, being more efficient than SOD in preventing cone death [39;41]. Gpx activity showed a two-fold increase, compared to controls in cultures treated with H_2O_2, evidencing that oxidative damage prompted an increase in this antioxidant enzyme activity. DHA addition doubled Gpx activity, even in the absence of oxidative damage, and increased it 4 times when cultures were supplemented with DHA before H_2O_2 addition (Fig. 2B). Hence, DHA increased Gpx activity, which would reduce H_2O_2 and consequently, ROS formation. Since DHA activates RXR to enhance photoreceptor survival [110], we cannot rule out that once activated and heterodimerized, this transcription factor might increase the expression of this enzyme in photoreceptors.

This result is consistent with evidence that increases in Gpx4, and to a lesser extent Gpx1, reduce oxidative stress-induced RPE cell damage; different antioxidants increase Gpx expression and reduce photoreceptor apoptosis in *rd1* [39;77]. In line with these findings, DHA has been shown to increase GSH levels and decrease accumulation of intracellular peroxides in murine macrophages [267]. The finding that DHA increases the activity of a key enzyme in photoreceptor antioxidant defense system supports the hypothesis that it regulates this system to prevent photoreceptor death. Interestingly, lack of Gpx4 in photoreceptors in a conditional knockout mouse does not affect photoreceptor acquisition of a rod or cone fate but impairs their further differentiation and leads to their apoptosis [37]. We have shown that once photoreceptors exit the cell cycle and acquire a rod fate, DHA is essential to advance their differentiation and promote their survival [106;107;111]; the ability of DHA to increase Gpx activity even in the absence of oxidative stress suggests that this enzyme might contribute to the effects of DHA in photoreceptor differentiation.

Concluding remarks

The evidence described here shows DHA as a puzzling and paradoxical molecule. In addition to being essential for a proper visual function, DHA can act or be the source of pro-survival molecules, rescuing photoreceptors from an untimely death or give rise to neurotoxic compounds, ready to execute these cells. DHA orchestrates a diversity of signaling pathways in retinal cells evidencing a Jekill and Hyde behavior, which emerges as being cell type, dose, injury and context dependent. In addition to available promising data, deeper understanding of DHA signalolipidomics is required to take advantage of DHA potential for preventing or slowing down the progression of retinal degeneration.

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Figure legends

Figure 1. Nuclear translocation of AIF during photoreceptor apoptosis.

Confocal fluorescence photomicrographs of rat retinal neurons cultured for 6 days in a chemically defined medium without photoreceptor trophic factors showing AIF, labeled with a specific monoclonal antibody (A), the preservation of mitochondrial membrane potential with the fluorescent probe MitoTracker (B), nuclear integrity, using the probe TOPRO (C) and merge image (D). Hollow white arrows indicate non-apoptotic photoreceptors that preserved their mitochondrial membrane potential and retained AIF in their mitochondria; note the co-localization of AIF and MitoTracker in non-apoptotic photoreceptors in D. Thin white arrows indicate apoptotic photoreceptors that showed the nuclear translocation of AIF and evidenced mitochondrial membrane depolarization.

Figure 2. DHA activates antioxidant defenses in photoreceptors

Three day neuronal cultures, with and without DHA addition at day 1, were treated with 10 µM H_2O_2. A. The percent increase of ROS levels compared to control conditions was analyzed by adding 10 µM Dichloro dihydro-fluoresceindiacetate (DCFDA) and incubating for 30 min at 37°C in the darkness. Cells were then lysed with dimetylsulphoxide (DMSO), proteins were quantitated and samples diluted with buffer phosphate saline to have the same protein concentration, and fluorescence was determined at 480-520 nm [268]. B. Gpx activity was evaluated using the Glutathione Peroxidase Assay kit (Cayman Chemical Company).

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Fig. 1

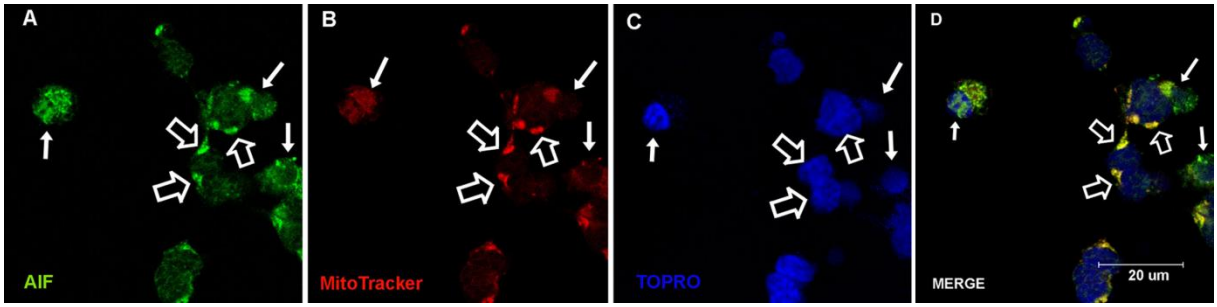

Fig. 2