Lipid-Based Antioxidant Systems in Photoreceptors and RPE Cells

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The retina, a multi-layered nervous structure in the back of the eye, detects light stimuli via specialised primary sensory neurons, named after their morphologies as retinal rods and cones. Rods and cones in vitro may retain their ability to respond to light for several hours by generating an electrical response. However, their viability and long-term operation require the functional interaction with retinal pigment epithelial (RPE) cells and a vascular system with peculiar features, the choroidal capillaries (ChC), whose specific form and shape support its function.

metabolism acid elovanoids DHA Elovl2

1. Photoreceptors' Anatomy and Eye Structure Afford Protection from Oxidative Damage

In photoreceptors, the sustained oxidative metabolism in darkness may lead to the generation of reactive oxygen species (ROS), such as the hydroxyl $^{\bullet}OH^{-}$ or superoxide $O_2^{\bullet-}$ radicals [1], and direct measurements in vivo with either newly-developed probes [2] or MRI [3] indicate the inner segment (IS) or the outer retina as the most active retinal site for ROS generation. The ROS may damage sensitive photoreceptor cell components, such as nucleic acids and OS lipids, threatening photoreceptors and retinal pigment epithelial (RPE) cells' viability.

The localisation of mitochondria at the IS, a cellular compartment distinct from the nuclear region, and the non-random distribution of mitochondria within the IS [4] may help attenuate the damage expected from highly reactive oxygen radicals, whose travel distance may fall short of the inner segment-nuclear distance. Indeed, the travelling distance of $^{\bullet}OH^{-}$ has been estimated as few Angstroms, while $O_2^{\bullet-}$ may travel several tenths of microns [5][6], although their charge may prevent these ROS from crossing both plasma and nuclear membranes. This analysis is consistent with the observation that high energy heavy ions ($Z \geq 6$) interaction with water may generate $^{\bullet}OH^{-}$, which may interact with lipids to generate peroxyl radicals. The annihilation of two peroxyl radicals leads to the emission of a visible photon (revised in [7]). Astronauts travelling in deep space report the perception of light flashes [8], indicating rhodopsin activation by photons triggered in response to $^{\bullet}OH^{-}$ generation by high energy heavy ions [9]. Consistent with the limited diffusion space of $^{\bullet}OH^{-}$, people on the Earth do not usually report light flashes in darkness, suggesting $^{\bullet}OH^{-}$ generated by oxidative metabolism may not diffuse up to OS, consistent with its limited diffusion space.

Despite the protection afforded by photoreceptor morphology toward genetic material and OS components, photoreceptors need additional factors to protect these sensitive molecular components from the blue light-

triggered mitochondrial generation of $O_2^{\bullet-}$ and lipid peroxidation of docosahexaenoic acid (DHA) and very long chain fatty acids (VLCFA), whose travelling distance may extend over IS, OS and nuclear regions. The cornea has high transmittance (around 90%) at wavelengths ranging from 400 to 480 nm, and the energy of sunlight in this spectral region at midday is slightly lower than in the green region of the spectra. Despite the variability linked to daytime, latitude, season, and humidity, the blue/green light ratio stays close to 0.9 at midday [10]. The anatomical organisation of the eye and its optics provide additional protection against blue light-induced oxidant generation. Due to the chromatic aberration of eye optics, blue light is expected to focus at a different axial position from green-red light stimuli. Human eye chromatic aberration analysis indicates an axial focus shift of about one diopter (1D) between 450 and 550 nm light [11]. Considering a 60 D refracting power for the not accommodated emmetropic eye, the −1D axial chromatic aberration will cause blue light to focus about 280 µm in advance of the green/red light (550 nm) focus (**Figure 1**).

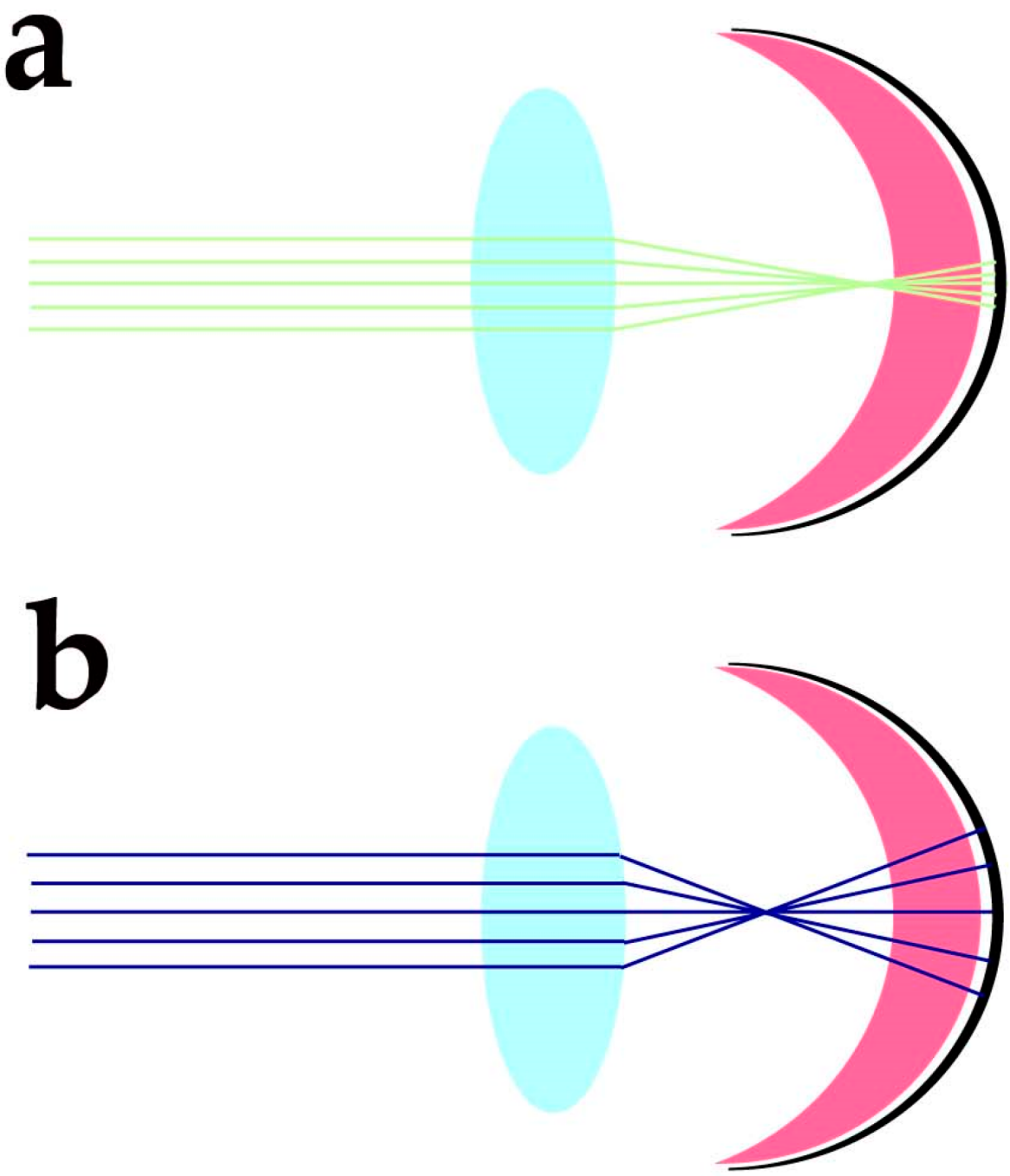

Figure 1. (**a**) Stray yellow lines represent green-yellow light being refracted by the eye optics represented by the cyan lens, with the image formed at the level of photoreceptors OS in the subretinal space; (**b**) blue lines represent blue light being refracted by the cyan lens, with the image formed at the level of photoreceptors OS in the subretinal space. The different focus positions for blue and green-yellow light indicate the chromatic aberration of the eye optics. As a result of chromatic aberration, the blue light will spread over a wider area of RPE than the green-yellow light.

As a result, blue light in the range 415–455 nm, the most effective in generating hydrogen peroxide and the $O_2^{\bullet-}$ radical in RPE cells [12] and photosensitising A2E in photoreceptors and RPE cells, may still reach these

sensitive structures, but its energy will spread over a wider area than longer wavelengths (**Figure 1**), due to chromatic aberration.

2. Protection against Oxidative Damage Requires Tight Control over Docosahexaenoic Acid Synthesis

The DHA and DHA-derived molecules provide a second protective mechanism against oxidative damage. Although OS PUFA may undergo peroxidation in response to blue light, emerging evidence indicates that DHA in the retina appears to operate as a double-edged sword, with damaging effects resulting from its oxidation and a protective role due to its metabolism in photoreceptors (reviewed in [13]) and RPE cells [14][15].

The balance between these opposing effects may depend on DHA levels. In the transgenic fat-1 mice expressing a *Caenorhabditis elegans* desaturase able to convert ω6 PUFA into ω3 [16] by introducing a double bond, retinal DHA levels increased two to five times in all phospholipid classes compared to WT mice, whereas ω6 fatty acid levels decrease [17]. The increase in DHA also associates with C32 and C34 ω-3 pentaenoic and hexaenoic VLCFA in phosphatidylcholine and depletion of ω-6 VLCFAs. From a functional perspective, fat-1 mice have scotopic and photopic ERGs a- (photoreceptor response) and b-wave (bipolar cell response) responses of unusually high amplitudes and lower thresholds, suggesting an increase in OS length [17] and an increased sensitivity to light. The latter effect may reflect an increased membrane fluidity and rhodopsin diffusion coefficient. The OS elongation may, however, increase the metabolic burden on photoreceptors due to increased Na^+ and Ca^{2+} influx leading to increased O_2 consumption and the development of hypoxic conditions at the IS, causing glial fibrillary acidic protein (GFAP) expression in Müller cells and increased carboxyethylpyrrole (CEP, protein adducts produced from DHA oxidation) in photoreceptors [17]. In mice exposed to intense light, photoreceptors degeneration associated with enhanced lipid peroxidation increased with DHA levels [18], indicating a causal role for DHA in light-induced damage in photoreceptors. The observation of Müller glial cells activation, common during retinal degeneration, suggests that the increase in DHA leading to OS elongation may cause hypoxia and damage in photoreceptors and indicates the need for fine control over DHA levels in photoreceptors to prevent its adverse effects on retinal viability.

Photoreceptors may generate DHA from EPA [19] using the very long fatty acid elongase 2 (ELOVL2) (**Figure 2**a), which catalyses the conversion of EPA into docosapentaenoic acid (DPA) and then DPA conversion into tetracosapentaenoic acid, which is then converted into the DHA precursor tetracosahexaenoic acid by photoreceptor Δ6 desaturase [19] (reviewed in [20]).

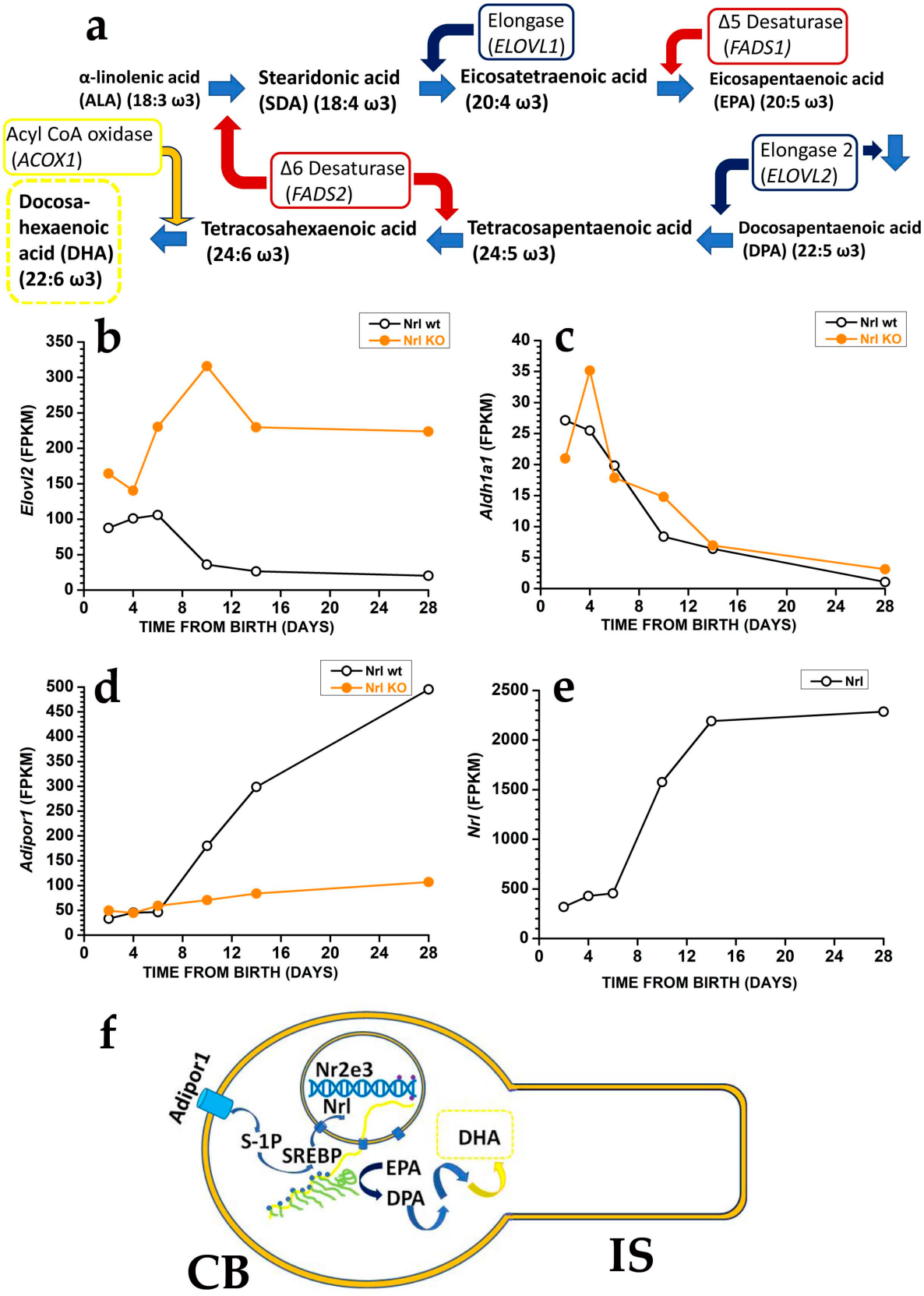

Figure 2. (**a**) Enzymatic pathway converting essential fatty acid alpha-linolenic acid to EPA and DHA. Elongases are enclosed in blue squares, and desaturases are in red squares. Note that ELOVL2 converts EPA into DPA. The yellow boxes indicate the peroxisomal compartment, where tetracosahexaenoic acid conversion into DHA by ACOX1 occurs; (**b**–**d**) Circles plot expression in rod precursors as fragments per kilobase exon per million

fragments (FPKM) at different times from birth of wt (black circles) and Nrl-KO (orange circles) mice for (**b**) *Elovl2*; (**c**) *Aldh1a1*; (**d**) *Adipor1*; (**e**) Circles plot Nrl expression in rod precursors in FPKM at different times from birth of wt mice; (**f**) Scheme summarising the control over *Elovl2* transcript (yellow line crossing the nuclear membrane) and protein (green) by nuclear transcription factors Nrl and Nr2e3, by the transmembrane protein Adipor1 (cyan transmembrane cylinder) via sphingosine-1 phosphate (S-1P) and the sterol regulatory element binding protein1 SREBP1, and by DNA methylation (purple circles). Panel data (b-e) have been redrawn using values downloaded from the RetSeq database at https://retseq.nei.nih.gov/ (accessed on 29 January 2023).

In mice, controlling DHA synthesis from EPA starts in rod precursors via the modulation of *Elovl2* expression. The *Elovl2* expression is already present two days after birth, i.e., before OS generation, but its levels decrease after postnatal days six, i.e., just before rods start developing an OS. As shown in **Figure 2**b, the reduction in *Elovl2* expression may depend on the rod-specific transcription factor neural retina leucine zipper (Nrl), as Nrl-KO mice have an increased *Elovl2* expression [21] (see also the RetSeq database at https://retseq.nei.nih.gov/ (accessed on 29 January 2023)). Vitamin A (all-trans-retinol) derivatives all-trans and 9-cis retinoic acid (RA) promote *Nrl* expression [22] in photoreceptors. There is evidence that during the early phase of mouse retinal development, both RPE cells and rod precursor express *Aldh1a1*, a gene coding for aldehyde dehydrogenase family 1 subfamily a1 (**Figure 2**c) to generate at-RA from at-RAL [21] (see also the RetSeq database at https://retseq.nei.nih.gov/ (accessed on 29 January 2023)).

The orphan receptor transcription factor Nuclear receptor subfamily group E member 3, coded by *Nr2e3* and whose expression is promoted by Nrl, may also contribute to *Elovl2* downregulation [23]. These data may indicate that during the earlier retinal maturation, rods keep *Elovl2* expression high to promote DHA synthesis and boost OS formation. However, rods reduce *Elovl2* expression when OS appears, possibly to avoid their overgrowth that would translate into an increased at-RAL load for RPE cells. It is important to note that also *Aldh1a1* expression drops at about the same time, which would translate into a reduced Nrl activation by at-RA. Reduced Nrl activation by RA would upregulate *Elovl2* expression and DHA synthesis, thus promoting OS growth, and reduced *Aldh1a1* expression may also prevent at-RAL detoxification in at-RA [24]. Recent evidence indicates that in the adult retina, at-RAL detoxification via its conversion into at-RA is carried out by two different enzymes, coded by *Cyp26a1* expressed in Müller glial cells and by *Cyp26b1* expressed in RPE cells [24]. It is relevant that *Cyp26a1* expression in Müller cells starts after postnatal day 4 [25], i.e., when *Aldha1* expression declines in rod precursors, indicating that the two enzymes operate over different temporal windows.

The finding that rods have lower *Elovl2* expression than cone-like cells of Nrl KO mice [26], while DHA levels are higher in rods than in cones, indicates a mismatch between the expression levels of the gene coding for a critical enzyme in DHA biosynthesis and DHA concentration in rods and cones OS. Although the mismatch cause is unclear, the different DHA turnover between rods and cones may be a possible reason. In rods, DHA is mainly used in phospholipids to increase disk membrane fluidity, increase rhodopsin diffusion coefficient, and amplify the first step in phototransduction, and its turnover is low. On the other hand, cones may have lower requirements for amplification in their phototransduction. They may instead mainly use DHA as a substrate for generating antioxidant molecules, whose increased turnover requires the higher synthetic rate provided by higher *Elovl2*

expression. Although this explanation sounds reasonable, it is important to stress that direct experimental support is lacking.

In keeping with the relevance of control over retinal DHA synthesis, recent evidence indicates additional mechanisms controlling *Elovl2* expression. Adiponectin, a hormone produced by the adipose tissue, regulates insulin sensitivity and glucose levels and has been reported to have antioxidants and anti-inflammatory actions [27]. A reduced *Elovl2* expression in mice lacking both alleles coding for the adiponectin receptor 1 *(Adipor1*) was associated with reduced DHA retinal levels and photoreceptor degeneration by three weeks of age [28][29], indicating the relevance of *Adipor1* in photoreceptors viability. Evidence shows that loss of *Adipor1* in RPE also associates with photoreceptor degeneration [30]. As shown in **Figure 2**d, Nrl is required to promote *Adipor1*, and the increase in *Adipor1* follows the upregulation of *Nrl* expression (**Figure 2**e). Therefore, Nrl appears to reduce *Elovl2* expression while promoting *Adipor1* expression, which increases *Elovl2* expression. The Nrl may fine-tune *Elovl2* expression acting via two separate, unknown pathways, although recent evidence points to sphingosine 1-phosphate involvement in signalling via Adiponectin receptors [31]. Adiponectin receptors may not sense adiponectin [32] and instead operate as sensors of membrane fluidity (discussed in [33]), underscoring the importance of controlling membrane fluidity in photoreceptors.

An additional level of control over DHA synthesis has recently been reported via the increased methylation of the *Elovl2* promoter, which leads to its decreased expression during ageing [34]. In mice, the decreased *Elovl2* expression with ageing may be reversed by intravitreal treatment with 5-Aza-2′-deoxycytidine, which increases *Elovl2* expression and reverses visual function decline [34]. Intriguingly, the *Elovl2* variant C23W disrupting ELOVL2 activity is associated with an early increase in lipofuscin deposition in the mouse retina [34], a sign of early ageing and an indication of DHA in controlling A2E formation.

Figure 2f summarises the multiple control operating on *Elovl2* expression in rod photoreceptors, based on the above evidence, which supports the control of DHA synthesis in photoreceptors at the level of *Elovl2* transcription. However, as shown in **Figure 2**a, the last step in DHA synthesis requires the translocation of its precursor, tetracosahexahenoic acid, from the smooth endoplasmic reticulum to the peroxisome. Recent data indicate that the selective disruption of the central peroxisomal β-oxidation enzyme in photoreceptors and bipolar cells does not affect photoreceptors' length and number up to one year of age [35]. As mice lacking this enzyme in all body cells undergo an early loss of photoreceptors, photoreceptors may receive DHA from RPE cells [36], in general agreement with the metabolic ecosystem notion. However, the mechanisms controlling *Elovl2* expression in RPE cells remain unknown (reviewed in [37]).

3. Docosahexaenoic Acid and Docosahexaenoic Acid-Derived Very Long Chain Fatty Acids Exert Protective Effects toward Oxidative Stress by Turning on Specific Transduction Pathways

In response to oxidative stress generated by either H_2O_2 or the herbicide paraquat, able to generate lipid peroxides and other ROS, DHA may activate the ERK/MAPK pathway [38] to prevent caspases activation via the regulation of Bcl-2 and Bax. The ERK/MAPK activation requires the retinoid X receptor (RXR), as shown by the inhibition of DHA protection in the presence of RXR antagonists [39]. The pathway activated by RXR appears specific, as tyrosine receptor kinase (Trk) antagonists do not suppress DHA protection. Moreover, RXR nuclear localisation in rods and the finding that phospholipase A2 inhibition prevents DHA protection suggest that DHA must be released from membrane phospholipids to activate the transcription of specific genes via RXR.

Upon release from membrane phospholipids by phospholipase A2, DHA could be processed by the enzyme 15-lipoxygenase (15-LOX)-1 to generate the protectin 10,17 docosatriene or neuroprotectin D1 (NPD1). This is the stable di-hydroxylated derivative of the short-lived hydroperoxy DHA. The presence of NPD1 has been shown to protect against oxidative stress, inflammation, and apoptosis following stroke by inhibiting cyclooxygenase-2 and the NFkB pathway [40].

NPD1 has been shown to suppress the generation of inflammatory cytokines in response to oxidative stress and the activation of apoptotic mechanisms, indicating a role in counteracting inflammation and apoptosis in photoreceptors and RPE cells (reviewed in [41]). In RPE cells, NPD1 reduces apoptosis triggered by A2E by promoting the expression of members of the Bcl-2 family of antiapoptotic factors and reducing caspase-3 activity in response to oxidative stress [14]. Intriguingly, the neurotrophin Pigment Epithelium Derived Factor (PEDF) promotes NPD1 synthesis and release at the apical membrane of RPE cells facing photoreceptors [14], suggesting NPD1 has both autocrine (to RPE cells) and paracrine (to photoreceptors) actions. The PEDF acts synergistically with DHA to promote NPD1 synthesis and release to protect RPE cells from A2E-induced oxidative stress and damage. In keeping with the notion that photoreceptor and RPE cells organise in a metabolic ecosystem providing mutual benefits, photoreceptor OS phagocytosis by RPE cells has been known to increase their viability, although the underlying mechanisms remained undefined. Both free DHA, i.e., released from membrane phospholipids, and NPD1 increased during OS phagocytosis, suggesting their increase may afford protection against oxidative stress [15]. The notion agrees with the observation of polystyrene microspheres phagocytosis failing to afford neuroprotection against oxidative stress-induced apoptosis and to increase DHA and NPD1. These data suggest that phagocyted OS phospholipids represent the source of DHA required to promote NPD1 synthesis and protect RPE cells and photoreceptors from oxidative damage [15] (reviewed in [13]).

A new class of homeostatic lipid mediators has been identified in the brain as the product of a reaction catalysed by the enzyme elongation of very long-chain fatty acids 4 (ELOVL4) using EPA and DHA as substrates [42], although EPA appears as the preferred substrate [43] (reviewed in [44]); these very long fatty acids (N > 28) are indicated as elovanoids (ELVs) [45]. The first ELVs characterised so far against uncompensated oxidative stress and oxygen/glucose deprivation are ELV-32 and ELV-34 [45]. A difference between DHA and ELVs is their different positions in membrane phospholipids. While DHA forms an ester bond with the alcoholic group in position 2 of membrane phospholipids, such as phosphatidylcholine, the ELV bond occurs at position 1 [45].

In a mouse model of Stargardt disease 3 (STGD3) with conditional KO of *Elovl4* in rods and cones [46], the near complete absence of very long chain fatty acids with nearly normal DHA levels did not lead to photoreceptor loss. On the other hand, transgenic mice expressing the mutation causing STGD3 in patients had reduced DHA and very long-chain fatty acid and retinal degeneration. This finding may indicate that the loss of ELOVL4 in photoreceptors does not reduce their viability. Indeed, the adverse effect of the *ELOVL4* mutation associated with STGD3 in patients may result from protein misrouting due to the loss of the dilysine endoplasmic reticulum retention motif [47]. However, human RPE cells (ARPE-19) also express *ELOVL4* and generate Elovs, suggesting they may provide ELV-32 and ELV-34 to photoreceptors lacking ELOVL4 [48]. Application of ELV-32 and ELV-34 to RPE cells exposed to uncompensated oxidative stress (H_2O_2) and inflammatory stimuli (TNF-α) protect the cells from apoptotic death by promoting the expression of pro-survival proteins Bcl2 and Bcl-xL, while suppressing pro-apoptotic Bax [48]. The protective action of ELVs appears independent from the increase in NPD1 due to 15-LOX-1 activation by oxidative stress, as ELVs still exert their protection against oxidative stress in the presence of an inhibitor of 15-LOX-1 [48]. The role of ELOVL4-derived elovanoids in photoreceptors and RPE cells has recently been reviewed [49].

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