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# Retinal energy demands control vascular supply of the retina in development and disease: The role of neuronal lipid and glucose metabolism

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## 1. Introduction

Neuronal energy demands are met by a tightly coupled and adaptive vascular network that supplies nutrients and oxygen. The retina is one of the highest energy-consuming organs, exceeding the metabolic rate of the brain; blood vessels grow and regress in reaction to changes in these high demands (Ames et al., 1992b; Anderson and Saltzman, 1964; Yu and Cringle, 2001). Reduced nutrients and reduced oxygen availability instigate compensatory albeit misguided pathological neovascularization in proliferative retinopathies (Chen and Smith, 2007; Sapieha et al., 2010). Conversely, impaired retinal ganglion cell (RGC) and photoreceptor survival are correlated with abrogated vascular development (Pennesi et al., 2008) and as neurons degenerate, the retinal vasculature atrophies to match the reduced metabolic requirements (Wang et al., 2000). In mice, genetic ablation of retinal ganglion cell neurons suppresses the inner retinal vascular development (Sapieha et al., 2008). Ablation of amacrine interneurons also prevents the development of the intermediate vascular plexus (Usui et al., 2015), while photoreceptor degeneration is associated with thinning of the choroid and inner retinal blood vessels (Ayton et al., 2013; Dhoot et al., 2013; Toto et al., 2016). Thinning of the choriocapillaris, in turn, may exacerbate retinal degeneration (Bird, 2010; Whitmore et al., 2015). However, the specific mediators that link neuronal metabolism with retinal angiogenesis in the developing eye and retinal disease remain largely unknown. Conditions such as diabetic retinopathy, vaso-proliferative retinopathy of prematurity and neovascular age-related macular degeneration (AMD) have been characterized as diseases of the vasculature. However, it is becoming more evident that the metabolic needs of the neural retina profoundly influence blood vessel supply in development and in disease.

Retinal oxygen sources and the vaso-proliferative response to low oxygen levels have been well characterized. However, understanding the specific fuels used in the retina to generate ATP and supply building blocks for biosynthesis, as well as understanding the vaso-proliferative response to the lack of fuel are also key to neurovascular development.

The metabolic and energy needs of the retina have been assumed to be met by glucose, as the retina is part of the CNS, and the brain relies almost exclusively on glucose (Mergenthaler et al., 2013). There are two primary pathways that cells can use to generate ATP from glucose, glycolysis and oxidative phosphorylation. However, Cohen and Noell concluded in 1960 that a substantial portion of the energy produced through oxidation by the retina (around 65%) was not derived from glucose (Cohen and Noell, 1960). We recently showed that the retina (photoreceptors) can also oxidize lipid through fatty acid  $\beta$ -oxidation to produce ATP, accounting for the energy gap noted by Cohen (Joyal et al., 2016). Little is known about lipid versus glucose fuel substrate preference and its importance during retinal development and pathology. Here we review the neuronal energy demands of the retina, describing both glucose and lipid metabolism as forces that shape the vascular supply of the eye in development and in vaso-proliferative eye diseases.

## 2. Evolving architecture of the retinal vasculature during development

Two vascular networks supply the mature retina, the inner retinal vasculature and the choroid. The inner retinal vasculature provides nutrients and oxygen to the inner two-thirds of the retina and forms three distinct vascular layers originating from branches of the central retinal artery. Murine inner retinal vascular development begins after birth, making it useful for preclinical studies of preterm retinal vascular development. The superficial vasculature forms first and emerges from the optic nerve, migrating radially to reach the periphery of the retina within 7–10 post-natal days (P10; Fig. 1a–e). As photoreceptors increase their metabolic demand, vessels then penetrate the inner retina to form the deep vascular layer, delineated by the outer plexiform layer (OPL), immediately adjacent to photoreceptors (P8–12) (Fig. 1d). The intermediate vascular layer forms last with further maturation of the inner neural retina (P14–20; Fig. 1d). Vascular development is completed shortly before full-term birth in humans and at about P21 in mice

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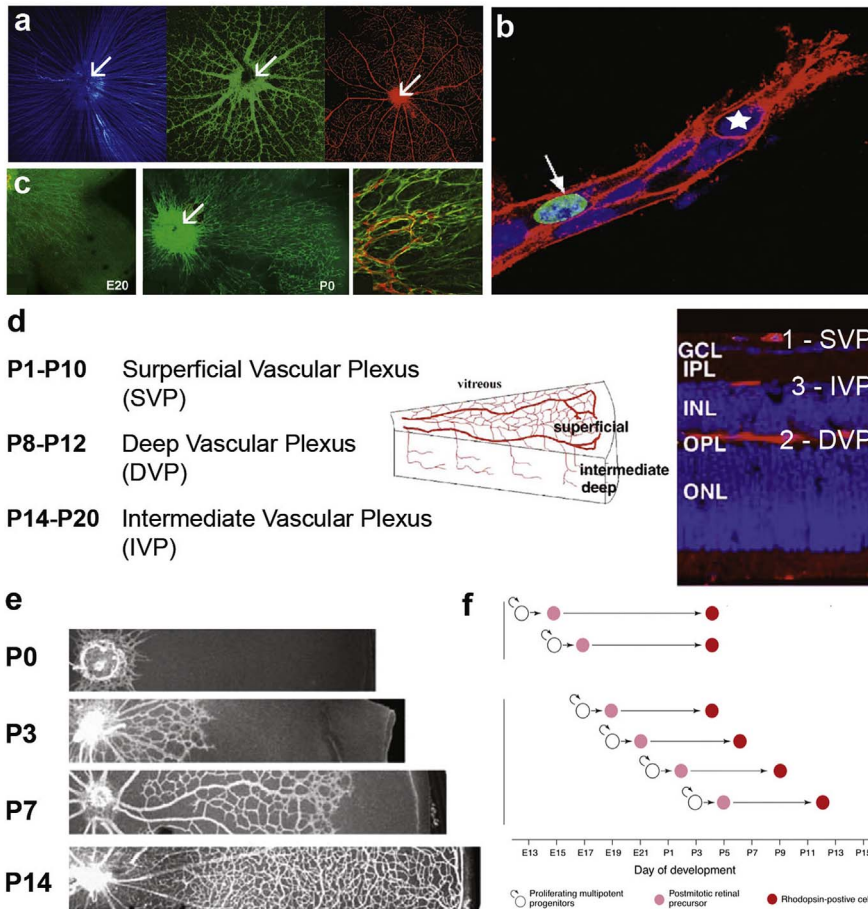
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**Fig. 1. Retinal vascular development follows neuronal maturation.** (a) Similar radial distribution of neurons (RGCs marked with leptin; blue), glia (astrocytes labeled with GFAP; green) and blood vessels (Dextran; red). Arrow points to the optic nerve. (b) Migratory tip cell, which does not proliferate (star; nucleus) with adjacent proliferating stalk cell in cellular division (phospho-histone-H3, green with arrow). (c) Astrocytes lay down a path for growing vessels, starting before birth (E20, left) and completed at birth (P0); vessels are red (right). (d) Inner retinal vascular development begins with the formation of the superficial vascular plexus (1 and e). Formation of the deep vascular plexus (2) corresponds temporally to the maturation of photoreceptors (f). The intermediate vascular plexus forms last (3), likely from vessels of the superficial vascular plexus. (f) Timeline of photoreceptor development from the terminal mitosis of rod precursors to the onset of rhodopsin expression. Mice open their eyes between P12–15, and the outer plexiform layer has developed by P14. Although some rod activity is detectable at P12, photoreceptor outer segments continue to mature until P19–25. Figure modified, with permission, from (Dorrell and Friedlander, 2006; Gariano and Gardner, 2005; Gerhardt et al., 2003; Morrow et al., 1998).

(reviewed by (Fruttiger, 2007; Ruhrberg and Bautsch, 2013; Ståhlberg and Bengtsson, 2010)). The inner retinal circulation is characterized by low blood flow and a high oxygen extraction; arteriovenous  $pO_2$  differences are  $\sim 40\%$  (Wangsa-Wirawan and Linsenmeier, 2003). Retinal vascular endothelial cells are not fenestrated and form tight junctions that create a blood-retinal barrier; this barrier is reminiscent of the blood-brain barrier and ensures selective exchanges between the circulation and the neural retina.

The retinal vascular development follows a highly stereotyped trajectory that suggests a carefully regulated and guided process (Dorrell and Friedlander, 2006). Neurons guide and attract blood vessels, which explains the anatomical coupling and functional crosstalk between these two major networks. Hence, superficial retinal vessels do not physiologically invade the adjacent vitreous body where they would obstruct incoming light to photoreceptors and create a shearing force on the retina. If shared guidance receptors are lost in neurons, such as VEGFR2 (Okabe et al., 2014), or the secretion of Vegf by neurons is aberrant (Mukouyama et al., 2002), the coupling is lost causing misdirected angiogenesis (Witmer et al., 2003). The deep and intermediate layers of inner retinal vessels are also strictly limited to a well-defined neuronal plane, which is violated only in disease states. Under conditions of normal vascular development, growth factors from the organizing neural cells guide vascular positions (Dorrell and Friedlander, 2006; Fantin et al., 2009; Klagsbrun and Eichmann, 2005). The formation of the outer vascular plexus in humans (25–26 weeks of gestation) (Hughes et al., 2000) coincides with the first appearance of neuronal activity (visual evoked potentials), with a corresponding and significant increase in oxygen and fuel consumption by retinal neurons (Cringler et al., 2006; Medrano and Fox, 1995; Yu and Cringler, 2001).

The choroidal vascular plexus arises from posterior ciliary arteries and branches of Sinn's circle, located around the optic disc, which

breaks up into fan-shaped lobules of capillaries (Hardy et al., 2000; Kiel and van Heuven, 1995). The choriocapillaris is a high-flow capillary network that provides nutrients and oxygen to the retinal pigment epithelium (RPE) and the metabolically demanding photoreceptors of the outer retina. Since the choriocapillaris is lined by fenestrated endothelial cells, the RPE instead comprises the entire outer blood-retinal barrier between the choroid and the neurosensory retina. Photoreceptors also interact with the inner retinal vasculature to some extent because mice with photoreceptor loss from retinal degeneration do not develop vascular proliferation in the inner retina in models of proliferative retinopathy (de Gooyer et al., 2006a; de Gooyer et al., 2006b).

### 2.1. Metabolic needs of maturing neurons sculpt the developing retinal vessels

Neuronal development drives the formation of the mature vasculature. In murine models, differentiation and maturation of the seven retinal neural cell types occur between embryonic day 9 (E9) and postnatal day 7 (P7). The cell types develop in an evolutionarily conserved order, although multiple cell types are generated simultaneously at any given developmental stage. Cones are created early in development, and rods are formed later (Altshuler, 1991; Morrow et al., 1998) (Fig. 1f). In both man and rodents, the vascular network that supplies the eye reorganizes dramatically as this neural development occurs, as an initial fetal network is replaced by the mature vasculature.

During the earliest phase of eye development, the embryonic optic cup is first vascularized through the fetal fissure by the hyaloid artery. With further differentiation and increasing metabolic maturity of the developing cells, the hyaloid artery extends from the optic nerve through the vitreous to form a branching network around the lens (the tunica vasculosa lentis) and extends branches to the retina to supply the

inner eye. The choroidal vessels are formed in the second and third months of gestation in man, before the development of retinal vessels (Lutty et al., 2010). The hyaloid system regresses as the retinal vasculature develops (Saint-Geniez and D'Amore, 2004; Zhu et al., 2000), a process that coincides with increasing differentiation and metabolic demands of the maturing neural retina. The metabolic shifts from aerobic to anaerobic metabolism in the differentiating lens fibers correlate with the loss of organelles including mitochondria, making the lens transparent (Ash and Overbeek, 2000; Gogat et al., 2004; Mitchell et al., 1998; Shui et al., 2003). Regression of the hyaloid vessels is controlled by retinal neurons, astrocytes, and macrophages via oxygen-sensing and Wnt pathways (Kurihara et al., 2010; Lobov et al., 2005; Rao et al., 2013). If hyaloid vessels do not entirely regress, the persistence of the hyaloid vasculature is associated with poor retinal development and poor vision in neonates (JONES, 1963; Liu and Nathans, 2008; Saint-Geniez and D'Amore, 2004). The regression of the hyaloid vessels normally occurs in humans around mid-gestation and mice after birth.

## 2.2. Migratory tip cells and proliferating endothelial stalk cells guide vascular growth

The primary navigating cell of the vascular front is the endothelial tip cell, which probes and senses chemo-attractive and repulsive environmental cues (Gerhardt et al., 2003 (Gerhardt et al., 2003)). Stalk cells follow just behind tip cells and proliferate to elongate the vessels (Gerhardt et al., 2003), then form a vascular lumen (Iruela-Arispe and Davis, 2009) (Fig. 1b). Tight junctions expressed on stalk cells maintain the integrity of the nascent vessel (Dejana et al., 2009). The final maturation of vessels involves pruning of excess vasculature (Ishida et al., 2003) as metabolic and oxygen needs are met and by recruitment of mural cells (Das and McGuire, 2003).

## 3. Neuronal growth factors and guidance cues that shape the vascular network

### 3.1. Vascular Endothelial Cell Growth Factor (VEGF)

Neurons with changing energy requirements signal for corresponding adaptations in vascular supply by shared neural and vascular guidance cues, of which a prime example is vascular endothelial growth factor (VEGF) (Robinson et al., 2001). The radial development of the superficial retinal vasculature with an expanding circular wave of hypoxia (and likely energy fuel deficits) corresponds to a wave of VEGF production in front of the developing vasculature and maturing neurons (Chan-Ling et al., 1995; Stone et al., 1995). Endothelial tip cells respond to VEGF by forming motile filopodia enriched in VEGFR2 and Nr1p (Fig. 1b), and also other guidance receptors such as Unc5b and Eph (Adams et al., 1999; Klagsbrun and Eichmann, 2005; Wilson et al., 2006), which respond to directional cues (Gerhardt et al., 2003). Proliferating stalk endothelial cells responding to VEGF, follow behind tip cells to elongate the vessels (Gerhardt et al., 2003). Posterior to the front of vascularization, the increased oxygen and nutrient supply from newly formed vessels suppresses VEGF expression, while anterior to the front, hypoxic maturing neurons, and glia without a vascular supply cause an increase in VEGF, proceeding in an expanding wave until vascularization is complete. In the maturing retina, neurons are an important source of VEGF to control the development of the superficial and deep vascular layers (Sapieha et al., 2008), more than astrocytes and Müller glial cells as was previously believed (Scott et al., 2010; Stone et al., 1995; Weidemann et al., 2010).

The oxygen-dependent regenerative vascular program is mediated in large part by the hypoxia-inducible factor 1- $\alpha$  (HIF1A). HIF1A is a transcription factor for a number of growth factors, including VEGF, that is stabilized by hypoxia and readily degraded by prolyl hydroxylases in the presence of oxygen (Boulahebel et al., 2009; Dor et al.,

2001; Fraisl et al., 2009; Pugh and Ratcliffe, 2003; Schofield and Ratcliffe, 2004; Semenza, 2007; Stolze et al., 2006). Regression of the rudimentary hyaloid vessels is also mediated by the suppression of HIF1A and VEGF in the presence of higher retinal oxygen (Kurihara et al., 2011). After the vascular development of the retina is complete, autocrine VEGF expression in endothelial cells (Lee et al., 2007) and neurons (Ruiz de Almodovar et al., 2009) is required to maintain tissue homeostasis. In the outer retina, production of VEGF by RPE is also essential to sustain the viability of the choriocapillaris (Kurihara et al., 2012).

VEGF also plays a critical role in pathologic angiogenesis. While hypoxia stimulates angiogenesis, excess oxygen suppresses VEGF and vascular growth. In preterm infants, supplemental oxygen exposure is a significant risk factor for retinopathy of prematurity, a disease associated with suppression of retinal vascular development (Chan-Ling et al., 2017; Hansen et al., 2017). As the now avascular neural retina matures, increasing its metabolic demands, VEGF is secreted by the hypoxic neuroglia giving rise to pathological neovascularization (Joyal et al., 2012, 2015; Pierce et al., 1995; Pierce et al., 1996; Scott et al., 2010; Sitaras et al., 2015; Stone et al., 1995; Weidemann et al., 2010). Microglial cells also contribute to VEGF secretion and pathological angiogenesis, which is reviewed elsewhere (Binet and Sapieha, 2015; Guillonnet et al., 2017). Similarly in the outer retina, excessive expression of VEGF by photoreceptors and RPE contributes to the sub-retinal neovascularization that characterizes wet AMD, discussed in more detail later (Campochiaro, 2015; Grisanti and Tatar, 2008; Joyal et al., 2016; Ohno-Matsui et al., 2002; Witmer et al., 2003).

### 3.2. Other growth factors and guidance cues

Other growth factors also govern vascular patterning. Astrocytes form an early superficial template that guides the initial vascularization of the inner retina. Neurons generate platelet-derived growth factor (PDGF) organizing the astrocytic network apposition, which expresses its corresponding receptor, platelet-derived growth factor receptor- $\alpha$  (PDGFRA). Tight junctions with the astrocyte bed (Fruttiger et al., 1996) (via R-Cadherin) and the graded production of VEGF by neuroglia (Stone et al., 1995) and RGCs (Joyal et al., 2014; Sapieha et al., 2008) on the retinal surface ensure directed growth of vessels (Okabe et al., 2014). Although Müller glia and astrocytes were previously believed to be the main source of VEGF, genetic ablation of VEGF in Müller glia and astrocytes does not impact retinal vascular development (Scott et al., 2010; Stone et al., 1995; Weidemann et al., 2010). Moreover, transgenic mice expressing a diffusible VEGF120 isoform present a disorganized astrocytic bed inferring a controlling role of VEGF, dominating that of PDGF, in orchestrating the neuro-glial relationship (Stalmans et al., 2002). Neurons are the most avid oxygen consuming cells in the retina. The recent demonstration of neurons' ability to liberate angiogenic factors (including VEGF) in response to metabolic needs suggests they may drive vascularization, while the astroglia likely play a more supportive role with amplification of their metabolic signals. Thus, localized regions of retinal hypoxemia or high energy need prompt VEGF, as well as erythropoietin (Epo) secretion (Alon et al., 1995; Chen et al., 2008, 2009; Chen and Smith, 2008), initiating the growth of neo-vessels. O<sub>2</sub>-independent factors, such as angiopoietins/Tie2 (Ramsauer and D'Amore, 2002) and insulin-like growth factor-1 (IGF-1) (Smith et al., 1999), also participate in the physiological vascularization of the retina. More discussion concerning the role of IGF-1 can be found in reviews (Hellström et al., 2013, 2016; Hellstrom et al., 2001; Liegl et al., 2016a; Liegl et al., 2016b; Lofqvist et al., 2009; Wu et al., 2010).

Both neurons and vessels appear to be guided by a combination of analogous attractant and repellent molecular cues. Of these conserved guidance proteins, the semaphorins, ephrins, netrins and slits and their cognate receptors the neuropilins (Nrp), Eph receptors, roundabout (Robo), and uncoordinated-5 (UNC5) respectively, play central roles in



both neuronal patterning and vascular growth. Semaphorin 3A (sema3A) in particular (Fantin et al., 2009; Joyal et al., 2011), ephrinB2 (Ehlken et al., 2011), and to variable extents Netrin-1a and Slit-2 (Wang et al., 2003; Wilson et al., 2006), generally repulse endothelial cells. Netrins and Slits may present opposing actions possibly due to stimulation of different receptor subtypes, cleavage of the original protein, or the modulation of their effects by pro-angiogenic factors (Klagsbrun and Eichmann, 2005). More in-depth discussion of the role of neuronal guidance cues in vascular development can be found in other reviews (Bussolino et al., 2006; Carmeliet and Tessier-Lavigne, 2005; Dorrell and Friedlander, 2006; Klagsbrun and Eichmann, 2005; Sapieha, 2012; Serini and Bussolino, 2004). Neurovascular crosstalk through shared guidance cues may, therefore, allow neuronal energy demands to signal for corresponding adaptations in vascular supply.

#### 4. Neuronal energy requirements to sustain retinal function

The metabolic demands of neurons that determine the vascular network that supplies oxygen and nutrients are intrinsically linked to neuronal structure and function. The primary energetic demands include light sensing via phototransduction and maintenance of electrical gradients, production of the molecules and structures (such as renewable outer segments) that allow vision and managing the oxidative stress arising from these processes.

##### 4.1. Energy cost of phototransduction and the maintenance of electrical gradients

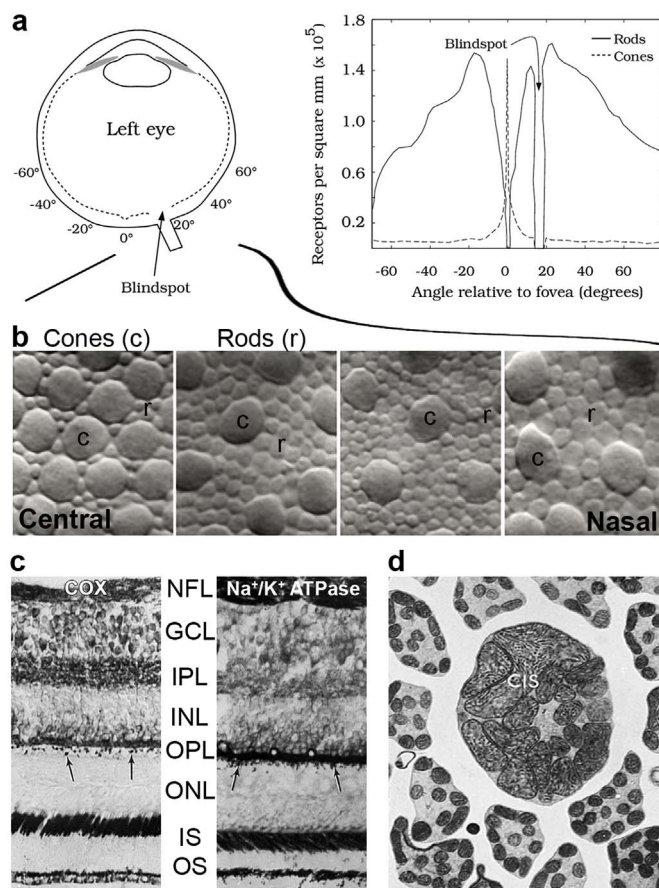
The primary site of phototransduction, which is energy intensive, is the specialized photoreceptors (Fig. 2). Unlike most neurons, photoreceptors signal in absence of stimuli and their neurotransmitter release is higher in the dark. In the absence of light, open channels allow a steady flow of ions in and out of the cell resulting in a cellular depolarization known as the 'dark current' (Stryer, 1991). Upon light stimulation, the ion channels are closed, neurotransmitters release is suppressed causing photoreceptor hyperpolarization, which leads to phototransduction. More than half of photoreceptor energy (ATP) consumption is due to  $\text{Na}^+/\text{K}^+$  ATPase ion pumps, which maintain ion levels in the cell (Hagins et al., 1970; Okawa et al., 2008). Concordantly,  $\text{Na}^+/\text{K}^+$  ATPase distribution corresponds to areas rich in mitochondria with expression levels proportional to neuronal activity (Fig. 2) (Ames et al., 1992b). Thus photoreceptors (seemingly paradoxically) consume more energy in darkness.

##### 4.2. Energy cost of managing oxidative stress

Dealing with the high oxidative stress experienced by the retina and particularly the photoreceptor is energetically costly. The light that stimulates phototransduction also leads to light-induced damage of lipids, proteins and nucleic acids which must be detoxified and replaced (Kagan et al., 1973). Furthermore, the high levels of oxygen needed for energy production (i.e. oxidative phosphorylation) increases oxidative stress, first by exposure of the retina to free oxygen from high blood flow and second from the reactive oxygen species generated in the mitochondria during oxidative phosphorylation. The retina spends additional energy to prevent, neutralize and repair the effects of oxidative damage.

##### 4.3. Energy cost of continuous replacement of outer segments

To avoid accumulation of light-induced oxidative damage to the non-replicating cell body of the neuron, photoreceptors have evolved a segregated and renewable outer segment, which is a lipid- and protein-rich structure housing light-sensing rhodopsin and cone opsins. Unlike the non-replicating inner segment, which contains the nucleus, the mitochondria, and biosynthetic machinery, the outer segment structure



**Fig. 2. Photoreceptors and mitochondrial distribution in primate retina.** (a,b) Distribution of cones (c) and rods (r) in the human retina, relative to the optic nerve. Cone density increases in the macula and peaks in the fovea. (c) Macaque retina stained for cytochrome c oxidase (COX) or  $\text{Na}^+/\text{K}^+$  ATPase, markers of mitochondria or areas of higher energy consumption in the retina. Inner segments (IS) of photoreceptors strongly express both markers. NFL: Nerve fiber layer, GCL: ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer, IS: inner segment, OS: outer segment. (d) Cone inner segments (CIS) contain much larger and more densely packed mitochondria than rod photoreceptor, by electron microscopy. Figure modified, with permission, from (Curcio et al., 1990; Kageyama and Wong-Riley, 1984; Wong-Riley et al., 1998).

is continuously built, shed, and then regenerated. Approximately 10% of the outer segment is discarded daily from the photoreceptor and phagocytized by the neighboring retinal pigment epithelium (RPE) (LaVail, 1976). The photoreceptors must continually synthesize or scavenge lipids and proteins to maintain the outer segment structure and function. While the anatomical partition of photoreceptors into non-replicating inner segments and replicating outer segments minimizes the risk of cumulative oxidative damage to the photoreceptor nucleus, there is still a high energy cost to ensure the continuous regeneration of outer segments.

#### 5. Pathways to energy production in the retina

How the retina meets its energy demands is not fully understood. There are two primary pathways that cells can use to generate ATP, glycolysis and oxidative phosphorylation (OXPHOS). Work over the past century has highlighted the need of the retina to have both a very high glycolytic and oxidative capacity. The following sections will review the relative contributions of these energy-producing pathways in the retina, as well as discuss the substrate utilization and ability of the retina to use lipids to fuel OXPHOS.

## 6. Oxygen consumption and oxidative phosphorylation

Oxygen consumption reflects the activity of the electron transport chain and the production of ATP by the mitochondria. The retina is one of the most oxidative tissues in the body, consuming more oxygen than the brain (Ames Iii, 1992) and has the equivalent expression of oxygen-carrying proteins as skeletal muscle (Schmidt et al., 2003). The outer retina, which consists mainly of photoreceptors with some Müller glial feet, is estimated to account for more than 60% of the oxygen consumption of the retina (Du et al., 2016; Medrano and Fox, 1995). This is a consequence of the very high density of mitochondria and oxidative enzymes in the photoreceptors. Approximately 60% of the total mitochondria of the retina are localized specifically to the photoreceptor inner segments (Lowry et al., 1956).

*In vivo* measurements of oxygen tension in the retina of various animal models have shown that oxygen levels are highest near the choroid and rapidly decrease moving towards the photoreceptors (Linsenmeier, 1986; Yu and Cringle, 2005) (recently reviewed (Linsenmeier and Zhang, 2017)). Indeed, the oxygen tension is lowest at the ellipsoid zone in the photoreceptors, correlating with the location of the mitochondria. This suggests that the mitochondria in the outer retina are actively consuming the vast majority of the available oxygen as it diffuses from the choroid. In vascularized retinas (those with a blood supply to the inner retina), the oxygen tension increases again after passing the photoreceptor mitochondrial layer towards the inner retina, indicating that the inner retina is supplied by these vessels. In the cat, the choroid provides 90–100% of fuel and oxygen to photoreceptors both in the dark and light and the inner retinal circulation supplies the inner retina with 100% of its supply in dark or light (Linsenmeier and Braun, 1992). Photoreceptors may derive oxygen from the inner retinal vasculature to some extent because photoreceptor loss from retinal degeneration alters the inner vasculature (de Gooyer et al., 2006a; de Gooyer et al., 2006b). This may be indirect though, since the activity of the second and third order neurons are dependent on the presence of the photoreceptors.

In addition to supplying ATP via oxidative phosphorylation, the mitochondrial TCA cycle is a hub for anabolic reactions that supply many of the building blocks needed for biosynthesis. It is fitting therefore that photoreceptors have a high oxidative capacity and high mitochondrial content to help meet their high ATP requirements as well as to meet the high biosynthetic demands of regenerating their outer segments.

## 7. Carbohydrate metabolism in the retina

### 7.1. Role of aerobic glycolysis

During glycolysis, glucose is oxidized to pyruvate, which can either be converted to lactate or transported into the mitochondria and fully oxidized, yielding substantially more ATP (Fig. 3a and b). When glucose is converted to lactate, approximately 15 times less ATP is generated than when glucose is used for OXPHOS. Nearly a century ago, researchers, including Otto Warburg, noted that a defining feature of retinal glucose metabolism was the rapid production of lactate (Cohen and Noell, 1960; Ng et al., 2015; Warburg et al., 1924). Canonically, high lactate production occurs when oxygen is limiting. However, in the retina, high lactate levels are produced even in the presence of oxygen and elevated mitochondrial respiration (as discussed; Fig. 3a and b). Lactate production in the presence of oxygen is referred to as aerobic glycolysis or the *Warburg effect* (Fig. 3b and c).

While there is substantial evidence that the retina primarily metabolizes glucose through glycolysis (discussed below), the benefit of relying on aerobic glycolysis over oxidative phosphorylation of glucose is not fully understood and remains controversial (Liberti and Locasale, 2016; Vander Heiden et al., 2009). Potential benefits may include faster ATP synthesis kinetics and increased production of carbon compounds

for biosynthetic pathways that feed off glycolysis. These branching pathways include glycan synthesis, serine biosynthesis and the pentose phosphate pathway (PPP). Many glycans are synthesized from the glycolysis intermediate, fructose-6 phosphate. Glycans are a diverse class of metabolites essential for numerous cellular functions including the glycosylation of many proteins including rhodopsin (Murray et al., 2009). The PPP and serine synthesis are both important for the reduction of NADPH, which is needed for fatty acid synthesis, maintaining redox homeostasis and for reducing all-*trans* retinal produced from photo-bleaching (Punzo et al., 2012). Both pathways can also contribute to increased nucleotide synthesis, which is necessary for highly proliferative cells, although differentiated photoreceptor nuclei do not replicate.

It is also thought that the high glycolytic demand of the retina may be due to the localization of the mitochondria to the inner segment, which forces the outer segment to rely on aerobic glycolysis (Ng et al., 2015). This idea is supported by the localization of lactate dehydrogenase (LDH) involved in glycolysis to the outer segment whereas mitochondrial enzymes are found in the inner segments (Lowry et al., 1956). However, this problem could potentially be overcome by the shuttling of energy to the outer segment, which is seen at synapses via phosphocreatine (Linton et al., 2010).

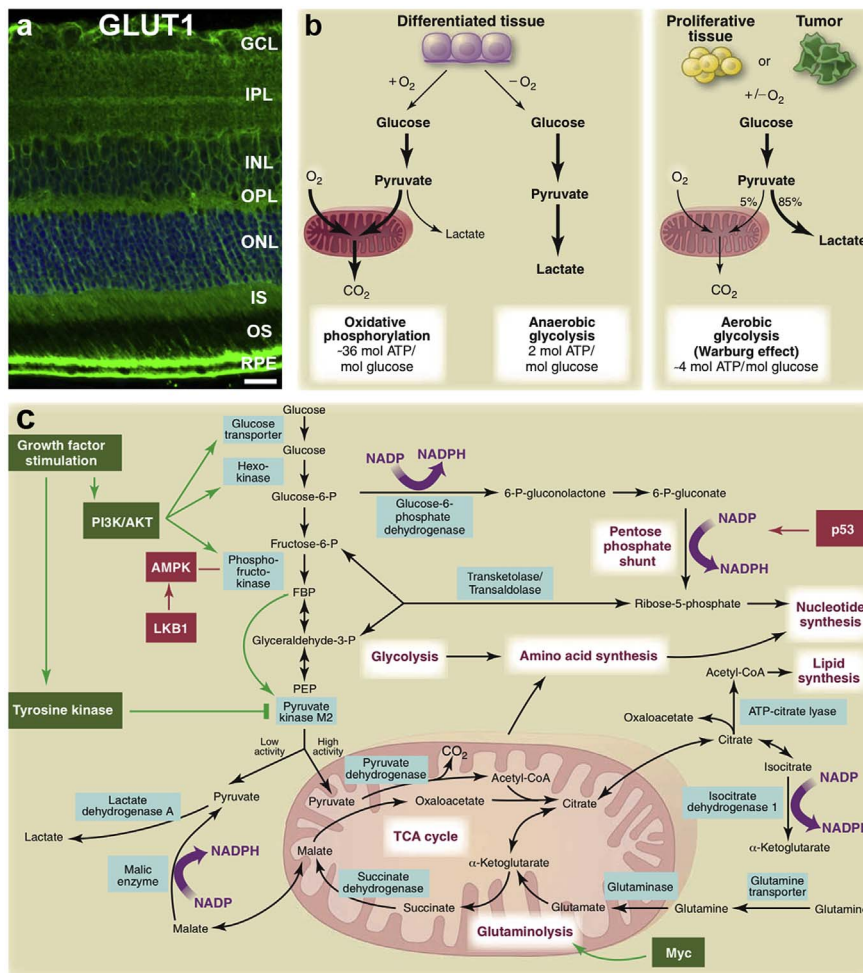
The flux through the PPP has been studied in *ex vivo* rat and rabbit retinas using glucose with radiolabeled carbons at specific sites and measuring the rate of labeled CO<sub>2</sub> production (Noell, 1952; Winkler et al., 1997). The PPP was calculated to account for between 1.5 and 10% of glucose oxidation under normoxic conditions (Noell, 1952; Winkler et al., 1997). However, when mitochondrial activity is blocked, the flow of glucose carbons through PPP increases (Noell, 1952; Winkler et al., 1997). Studies in rat retinal cell cultures have shown that under conditions where mitochondrial activity is blocked, blocking glycolysis or PPP leads to increased cell death, suggesting that the PPP is essential for retinal metabolism (Han et al., 2013).

The role of serine synthesis and serine metabolism in the retina is largely unknown. Retinal metabolism is often compared to cancer metabolism given that both have high biosynthetic demands (cancer cells need to supply building blocks for rapid proliferation, and the retina needs to continuously synthesize outer segments) (Ng et al., 2015). Furthermore, like the retina, cancer cells also rely heavily on a large glycolytic flux despite high OXPHOS capacity. More recent work in cancer indicates the dependency of many cell lines on serine metabolism (Yang and Vousden, 2016). Serine is a building block for neurotransmitters, sphingolipids, and ceramide and can be converted to glycine in a reaction that provides carbon for folate-mediated one-carbon metabolism. It is therefore possible that the energetics of the retina might also be reliant on serine metabolism. Supporting this idea, it has recently been shown that the rare macular degenerative and neovascular disease, macular telangiectasia type II, (Mactel), has genetic associations linked to serine and glycine metabolism, as well as decreased serine and glycine levels in patient serum (Scerri et al., 2017).

### 7.2. Balance of oxygen consumption (OXPHOS) and glycolysis in the retina

Both oxygen consumption (OXPHOS) and aerobic glycolysis have been shown to be essential for retinal function and vision. In humans, if the retinal circulation and therefore oxygen delivery is blocked, vision is lost within ~5 s. The visual function can be prolonged if oxygen is provided, indicating that O<sub>2</sub> is a limiting factor (Ames et al., 1992a; Carlisle et al., 1964). Work by Noell in the 1950s showed that injecting iodoacetate (IAA), (which blocks glycolysis by inhibiting GAPDH), very rapidly and profoundly impairs vision while having no other observable effects on the animals, suggesting that the retina is also particularly sensitive to impairment of glycolysis (Noell, 1952).

The relative contributions of aerobic glycolysis versus oxidative phosphorylation to retinal energetics have been assessed using several



**Fig. 3. Glucose metabolism and the Warburg effect in the retina.** (a) Distribution of GLUT1, the main glucose transporter of the retina. (b) Schematic representation of oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). In the presence of oxygen, differentiated tissues metabolize glucose to pyruvate via glycolysis. Pyruvate is then metabolized in mitochondria by oxidative phosphorylation. The electron transport chain requires oxygen to completely oxidize glucose. When oxygen is scarce, cells redirect pyruvate away from mitochondria to produce lactate (anaerobic glycolysis). Anaerobic glycolysis allows glycolysis to continue by cycling NADH back to NAD + but limits ATP production compared to oxidative phosphorylation. Warburg observed that cancer cells convert most glucose to lactate, irrespective of the presence of oxygen (aerobic glycolysis). The ‘Warburg effect’ is also observed in non cancerous tissues, such as the retina, which, though non proliferative have continuous replacement of outer segments and behave metabolically as “proliferative” cells. Aerobic glycolysis which is less energy efficient may provide building blocks required for growth. In proliferating cells, glucose is in part diverted to biosynthetic pathways upstream of pyruvate production. (c) This schematic summarizes glucose metabolism including glycolysis, oxidative phosphorylation, the pentose phosphate pathway, and glutamine metabolism in “proliferating” cells. Growth factor signaling, such as VEGF, leads to both tyrosine kinase and AKT/PI3K activation. In doing so, growth factors promote glucose uptake and flux through the early part of glycolysis, while inhibiting the late steps; they force glycolytic intermediates towards biosynthetic pathways of macromolecules essential for cell “proliferation” and NADPH production. Metabolic pathways are labeled in purple, and the enzymes controlling critical metabolic steps are shown in blue boxes. Figure modified, with permission, from (Gospe et al., 2010; Vander Heiden et al., 2009).

ex vivo models. The rates of lactate production and oxygen consumption are monitored as a readout of glycolytic flux and oxidative phosphorylation respectively. The electrical activity of the retinal explant can also be monitored in response to light stimuli to indicate phototransduction maintenance. From these types of experiments, we have learned that for optimal electrical activity the retina requires both glucose and O<sub>2</sub> (Ames et al., 1992b; Winkler, 1981). Reducing O<sub>2</sub> leads to a reduction in energy production and visual function is compromised (Ames et al., 1992b). Similarly, when glucose is removed or glycolysis is chemically blocked, phototransduction, though measurable, is severely reduced (Winkler, 1981). These experiments, as well as *in vivo* studies, have shown that the vast amount of glucose is metabolized through aerobic glycolysis with ~80% of glucose being converted to lactate rather than oxidized through oxidative phosphorylation (Cohen and Noell, 1960; Wang and Bill, 1997; Wang et al., 1997a, 1997b). However, this reaction accounts for less than 20% of the ATP production in the retina, indicating that mitochondrial OXPHOS generates the vast majority of ATP using only a small portion of the glucose taken up. Therefore, alternative carbon substrates such as lipids can contribute to OXPHOS and ATP production (Joyal et al., 2016).

### 7.3. Metabolic shift in response to light

As discussed, maintaining the dark current is very energetically expensive. Sustaining the electrical gradient through Na/K ATPase activity accounts for more than 50% of the energy (ATP) demand in the retina (Ames et al., 1992b). This implies that retinal energetics are different in the light versus dark. Studies in ex vivo retinas found that light adapted retinas have a reduced oxygen consumption rate (OCR)

(Medrano and Fox, 1995). Ames et al. also found that while O<sub>2</sub> consumption is reduced 10–30% in response to light, lactate production does not change, indicating that OXPHOS is responsible for supplying the energy needed for the dark current. They also found that light flashes that increase neurotransmission lead to increased lactate production, suggesting that glycolysis supplies the energy needed for neurotransmission (Ames et al., 1992a). However, in an *in vivo* porcine model, where lactate and O<sub>2</sub> levels were measured in the arterial blood and venous blood supplying the outer retina, constant light causes a marked decrease in lactate production that exceeds the reduction of OXPHOS (Wang et al., 1997b). So, while it seems clear that OXPHOS is increased in the dark and likely fuels the dark current, changes in glycolysis in response to light appear to vary depending on the method, light exposure, and model.

### 7.4. The metabolic shift in the developing retina

The metabolic demands of the retina change during development. Work in both rabbit and frog retinas indicate that young retinas use very little oxidative phosphorylation and rely more on aerobic glycolysis. As development proceeds and the retina differentiates, there is a shift to higher oxygen consumption. Compared to the young retina, the adult retina increases lactate production by 25% but nearly triples oxygen consumption (Cohen and Noell, 1960). While this may reflect an increase in glucose consumption, it also suggests that as the retina differentiates alternative substrates are used to fuel OXPHOS. Agathocleous et al. have shown that the switch to oxidative phosphorylation only occurs with terminal differentiation and that retinal metabolism is intrinsically linked to development, with the acceleration of



differentiation stimulating an earlier shift to oxidative phosphorylation. The young retina also appears to have a greater endogenous energy store and blocking glycogen use also causes the retina to increase oxidative capacity (Agathocleous et al., 2012).

## 8. Lipid metabolism in the retina

Although the prevailing dogma has been that glucose is the only fuel substrate of the neural retina, as noted above, pioneering work by Cohen and Noell in the 1960s, implied that this was not the case. They reported that almost 65% of the CO<sub>2</sub> produced from the TCA cycle by retinas is not derived from glucose (Cohen and Noell, 1960). These results imply that the oxidation of non-carbohydrate carbons is used to meet the retinal ATP demand. In the retina, one might rationalize that the use of both lipids and glucose as fuel would be beneficial in periods of high fuel need or nutrient deprivation.

In this section, we focus on fatty acids' newly discovered function as fuel in photoreceptors. Lipids diverse roles as signaling molecules have been previously reviewed (Bazan, 2003; Giusto et al., 2010; Marrache et al., 2005; Shimizu, 2009) and will not be explored here. It is not yet known which lipids can be used as fuel so we will also discuss the general lipid composition of the retina as well retinal lipid metabolism, including fatty acids biosynthesis in the endoplasmic reticulum and lipid degradation and oxidation in peroxisomes and mitochondria.

### 8.1. Use of lipids as fuel: FA $\beta$ -oxidation in the retina

In the eye, FA  $\beta$ -oxidation disorders are associated with retinopathy (Fletcher et al., 2012b; Roomets et al., 2008; Tyni et al., 2004). Conversely, glucose-uptake deficient patients with GLUT1 deletions, the main retinal glucose transporter, develop intractable seizures but have normal vision (De Vivo et al., 2002; Klepper, 2008; Klepper et al., 2001). Hence, there is strong evidence that the oxidation of FAs is a major contributor to retinal function.

Fatty acids, like glucose, can be oxidized in mitochondria to acetyl-CoA and enter the Krebs cycle to produce energy (Houten and Wanders, 2010). Lipid through FA  $\beta$ -oxidation is an alternative energy source to glucose in organs with high metabolic rates, such as the heart and skeletal muscle (Lopaschuk et al., 2010). These organs are rich in very low-density lipoprotein receptor (VLDLR), which facilitates fatty acid (FA) uptake. Photoreceptors and RPE express high levels of VLDLR, but lipid metabolism has been mostly explored in the retina from the standpoint of membrane biosynthesis, because of the high turnover of photoreceptors outer segments. Functional FA  $\beta$ -oxidation enzymes have been identified in Müller glia, RPE and photoreceptors (Aotsuzawa et al., 2010; Oey et al., 2005; Tyni et al., 2002, 2004). Moreover, VLDLR mutations and mutations resulting in mitochondrial deficiency and changes in trifunctional proteins (TFP) that metabolize long-chain FA, result in progressive retinopathies (Fletcher et al., 2012a; Lawlor and Kalina, 1997; Roomets et al., 2008).

We showed that mouse retina (and specifically photoreceptors) can use FAs to produce energy Fig. 4e–h. VLDLR deficient mice have reduced uptake of fatty acids and decreased fatty acyl intermediates of  $\beta$ -oxidation (Fig. 4d,g,h) resulting in early vascular changes, secondary to energy deficits (Joyal et al., 2016). VLDLR deficient retinas exposed to palmitate increase their oxygen consumption, which is prevented by blocking fatty acid beta oxidation (by inhibiting CPT1 with etomoxir; Fig. 4e and f). Our findings were confirmed in a photoreceptor cell line (661W cone photoreceptors). FA  $\beta$ -oxidation is, therefore, an important metabolic energy pathway in the retina that is only beginning to be explored.

### 8.2. Peroxisomes and mitochondria in lipid oxidation

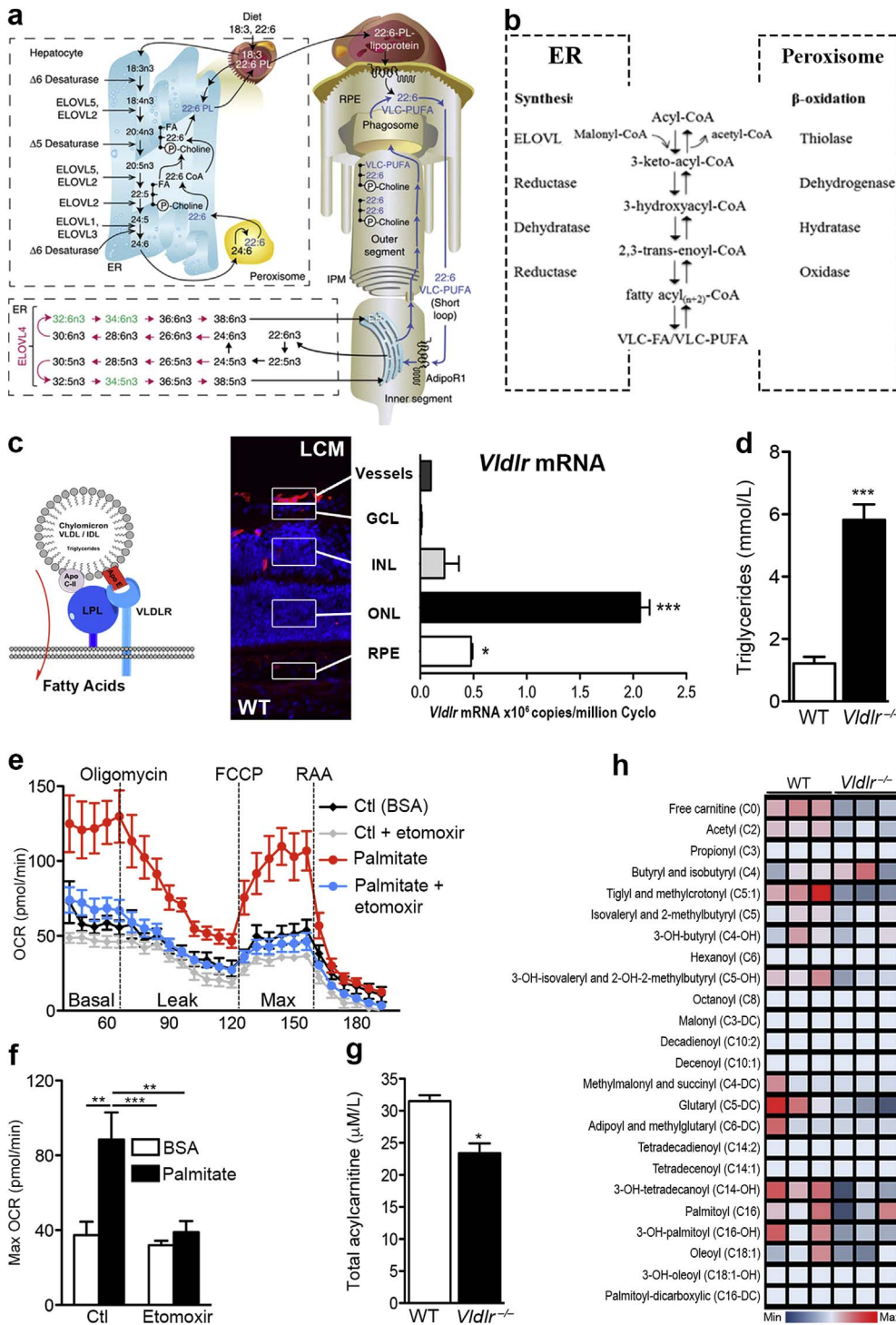
Peroxisomes play complementary roles to mitochondria in lipid metabolism. As discussed later, mitochondrial diseases can present with

retinal dysfunction. Similarly, peroxisomal disorders such as Zellweger syndrome, adrenoleukodystrophy and Refsum's disease cause severe retinal degeneration (Braverman et al., 2016), suggesting that both organelles are vital for retinal function.

Peroxisomes were initially described by Christian De Duve as 'microbodies' with oxidase and catalase activity able to metabolize hydrogen peroxide. Several lines of evidence point to their unique contribution of metabolizing long-chain fatty acids to shorter chains that can be further oxidized in the mitochondria (Wanders et al., 2010). Anatomically, peroxisomes are in close juxtaposition to lipid droplets seen in most living cells which are an intracellular source of energy for growth or use during starvation (Novikoff and Novikoff, 1982). Lipid droplets also prevent cellular exposure to high levels of free fatty acids that can be readily oxidized. As seen in yeast, peroxisomes extend pexopodia, or little foot processes, which are rich in peroxisomal  $\beta$ -oxidation enzymes, into the core of lipid droplets (Binns et al., 2006). When energy is required, VLC-FA stored as triglycerides can readily be oxidized by peroxisomes. Conversely, peroxisomes can synthesize and store neutral lipids in droplets creating a bidirectional energy stream for intracellular lipid storage and utilization adapting fuel supply to the cellular metabolic demands.

Fatty acid oxidation occurs in both mitochondria and peroxisomes, but peroxisomal fatty acid oxidation does not result directly in ATP synthesis. Insight into the complementary role of peroxisomes emerged from peroxisomal disorders where increased levels of branched and very long chain fatty acids accumulate in serum (Brown et al., 1982; Poulos et al., 1986). Branched-chain fatty acids, such as phytanic acid, must first undergo oxidative decarboxylation ( $\alpha$ -fatty acid oxidation) in peroxisomes to become adequate substrates for further metabolism by  $\beta$ -oxidation in either peroxisome or mitochondria. Interestingly, FA  $\beta$ -oxidation of very long chain FA ( $\geq 26$  carbons) occurs exclusively in peroxisomes, whereas shorter FA can be oxidized by either organelle (Wanders et al., 2010). FA oxidation is, however, less energetically favorable in peroxisomes (with no ATP production) compared to mitochondria, and the process is not carried to completion. Hence, peroxisomes shorten VLC-FA by oxidation, which can then be fully metabolized in mitochondria or utilized for membrane biosynthesis.

Fatty acid  $\beta$ -oxidation, in both peroxisomes and mitochondria, can be summarized by four consecutive enzymatic steps: dehydrogenation, hydration, second dehydrogenation and thiolytic cleavage. The first dehydrogenation step is catalyzed by peroxisomal straight-chain acyl-CoA oxygenase, followed by hydration and dehydrogenation, both of which are catalyzed by the bifunctional enzyme D-bifunctional protein. Lastly, 3-ketoacyl-CoA thiolase carries out the thiolytic cleavage reaction, forming a new acyl CoA molecule that is shorter by two carbons. Carnitine acyltransferase expressed in peroxisomes converts acyl CoAs to acylcarnitines so that they can be transferred to mitochondria for further oxidation (Wanders, 2013). Shorter chain fatty acyl CoA uptake across the double mitochondrial membranes requires a carnitine exchange shuttle, consisting of carnitine palmitoyl transferases (CPT) and the transporter protein carnitine-acylcarnitine translocase (CACT). Located on the outer mitochondrial membrane, CPT1 exchanges CoA for carnitine, and CACT transports the FA carnitine inside the mitochondrial matrix, where it is converted back to fatty acyl CoA by CPT2. FA oxidation is regulated transcriptionally in both organelles by PPAR $\alpha$  (Aoyama et al., 1998), but mitochondrial oxidation is also tightly regulated by malonyl-CoA and CPT1 levels (McGarry et al., 1977, 1978). Fatty acid oxidation, therefore, requires complementary contributions from both peroxisomes and mitochondria. Interestingly, peroxisomes have unique genetic forms of enzymes common to mitochondria (Lodhi and Semenkovich, 2014). However, specifically in the retina, very little is known about the role of the peroxisome and much work remains to determine why peroxisomal disorders result in retinal degeneration.



**Fig. 4. Very long-chain fatty acids and lipid metabolism in the retina.** (a) Schematic of the formation of VLC-PUFAs. The elongation steps catalyzed by ELOVL4 are unique to photoreceptor inner segments (in red). Shed photoreceptor apical disks are phagocytosed by RPE. DHA (22:6) and VLD-PUFAs are recycled back to photoreceptor inner segments. C, carbons; ELOVL, elongase of the very long-chain fatty acids; ER, endoplasmic reticulum; IPM, interphotoreceptor matrix; PL, phospholipid; RPE, retinal pigment epithelium. (b) Fatty acid elongation and β-oxidation pathways. Each round of elongation involves four successive steps in the endoplasmic reticulum. Elongated fatty acyl-CoA product may undergo further rounds of elongation, be released for use in the cell, or β-oxidized in the peroxisome. (c) VLDL receptors bind triglyceride-rich chylomicrons and VLDLs that express Apo-E, allowing lipoprotein lipase (LPL) to release long-chain fatty acids. VLDLR is highly expressed in photoreceptors (outer nuclear layer; ONL) by laser capture microdissection (LCM and qRT-PCR). GCL: ganglion cell layer, INL: inner nuclear layer, RPE: retinal pigment epithelium. (d) Circulating plasma triglyceride levels in WT and *Vidlr*<sup>-/-</sup> mice. *P* < 0.0001. (e) Oxygen consumption rate (OCR) and (f) maximal OCR of wild-type (WT) retinas provided with long-chain fatty acid (FA) palmitate or control (Ctl: bovine serum albumin or BSA) in the presence or absence of FA oxidation inhibitor, etomoxir (40 μM). *n* = 6–8 retinas. (g) Total acylcarnitine levels (*P* = 0.0108) and (h) metabolite array of FA β-oxidation, measured by LC/MS/MS. *n* = 3 animal retinas. Figure modified, with permission, from (Joyal et al., 2016; Rice et al., 2015).

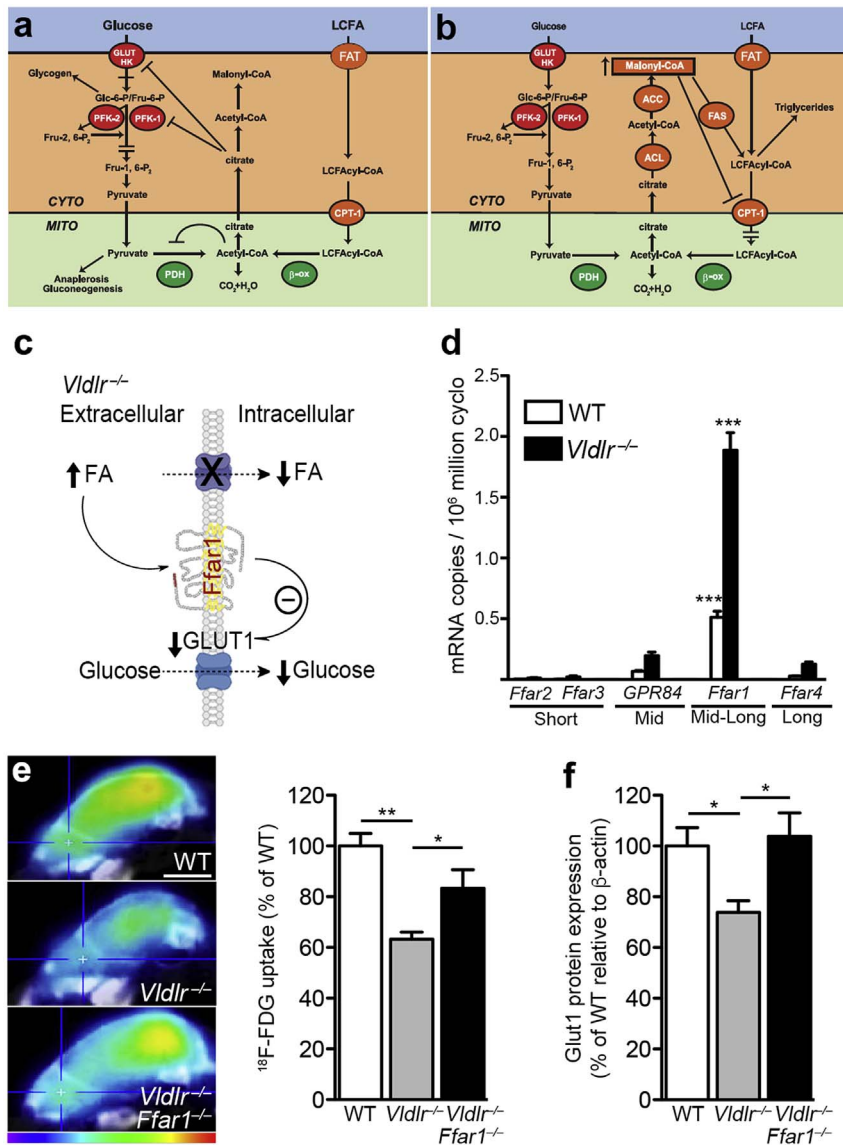
## 9. Lipids versus glucose as fuel: the Randle cycle and fatty acid receptors

Adapting fuel utilization to match nutrient availability might improve metabolic efficiency in the retina as in other tissues. Hormones, such as insulin and glucagon, help to control the relative abundance of fuel substrate in circulation but different mechanisms are needed at the cellular level to determine which substrates are used. Randle and colleagues first proposed a mechanism for fuel selection by tissue, independent of hormonal control (Fig. 5a and b). Tissues that use lipids to produce energy, curb glucose uptake during starvation (Cahill, 1970; Ferrannini et al., 1983; Owen et al., 1979). The glucose-fatty acid cycle,

or Randle cycle, describes a graded inhibition of various enzymes of glycolysis (mostly pyruvate dehydrogenase) mediated by the accumulation of acetyl-CoA and NADH resulting from fatty acid oxidation (Fig. 5a). Preferential FA oxidation, therefore, reroutes glucose towards glucose-dependent tissues, such as the brain, as well as glycogen synthesis or gluconeogenesis.

Subsequently, a general molecular explanation was also offered for the converse inhibition of FA oxidation by glucose (Collier et al., 1993; Sidossis and Wolfe, 1996) (Fig. 5b). Glucose through glycolysis, which also contributes to the production of acetyl-CoA in mitochondria, is oxidized by the citric acid cycle to citrate. Increased citrate levels promote transportation of citrate back to the cytosol where it





**Fig. 5. Choosing between lipids and glucose as fuel: the Randle cycle and fatty acid receptors.** (a) Randle cycle: inhibition of glucose utilization by fatty acid oxidation. Accumulation of acetyl-CoA and NADH from FA oxidation inhibits pyruvate dehydrogenase (PDH), whereas cytosolic citrate regulates 6-phosphofructo-1-kinase (PFK) activity. Glucose uptake regulation is not fully explained by the Randle cycle. (b) Randle cycle: inhibition of fatty acid oxidation by glucose. Malonyl-CoA, which is produced by ACC when glucose is abundant, governs the expression of CPT1, hence regulating the entry of long-chain FA into the mitochondria. This effect re-routes fatty acids toward esterification and storage. CYTO: cytosol; MITO: mitochondria; GLUT: glucose transporter; HK: hexokinase; Glc-6-P: glucose 6-phosphate; Fru-6-P: fructose 6-phosphate; Fru-2, 6-P<sub>2</sub>: fructose 2,6-bisphosphate; PFK-1: 6-phosphofructo-1-kinase; PFK-2: 6-phosphofructo-2-kinase; ACC: Acetyl-CoA carboxylase; ACL: ATP-citrate lyase; FAS, fatty acid synthase. (c) Elevated circulating fatty acid levels, as seen in *Vldlr*<sup>-/-</sup> retina, activate fatty acid receptors (such as Ffar1) that suppress GLUT1 expression and glucose uptake when lipids are abundant. (d) FA sensing receptors are expressed in WT and *Vldlr*<sup>-/-</sup> retinas (qRT-PCR). ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. n = 3 animal retinas. (g) Glucose uptake (<sup>18</sup>F-FDG, scale: 4 mm) and Glut1 protein expression of WT and *Vldlr*<sup>-/-</sup> mice compared to littermate *Vldlr*<sup>-/-</sup>/*Ffar1*<sup>-/-</sup> mice (P16). Figure modified, with permission, from (Hue and Taegtmeier, 2009; Joyal et al., 2016).

regenerates acetyl-CoA, and ultimately forms malonyl-CoA. Malonyl-CoA is a molecular switch that inhibits CPT1, preventing entry of long-chain FA in mitochondria (McGarry et al., 1977, 1978). Hence, excess FA are redirected towards storage in lipid droplets locally or adipocytes systemically. If the glucose-fatty acid cycle helps elucidate preferential substrate metabolism inside the cell based on relative abundance, it fails to adequately explain how nutrient uptake is regulated at the cell surface to redirect unwanted fuel to distant storage locations, such as liver or adipocytes. The control mechanisms for substrate selection in the retina have not yet been fully determined although we found that high circulating lipids signal through Ffar1 to suppress glut 1 and glucose uptake in the retina (Joyal et al., 2016).

**9.1. Lipid-sensing G-protein coupled receptor control of glucose uptake**

Fatty acids are key endocrine regulators of lipid and carbohydrate metabolism, in part through the activation of lipid-sensing G-protein coupled receptors (GPCRs) or free fatty acid receptors (FFAR). GPCRs are plasma membrane environmental sensors, and several are specialized in the detection of nutrients, including amino acids, glucose, and lipids (Ichimura et al., 2009; Kraakman et al., 1999; Wauson et al., 2013). Free FA receptors Ffar2 and Ffar3 are activated by short-chain fatty acids (Brown et al., 2003), Ffar1 is activated by medium- and long-

chain fatty acids (Briscoe et al., 2003; Costanzi et al., 2008; Oh et al., 2010; Poitout, 2003), while Ffar4 is primarily activated by long-chain FA, such as ω-3 FA (DHA and EPA) (Oh et al., 2010).

Ffar1 is abundantly expressed in the CNS (Boneva et al., 2011; Briscoe et al., 2003; Ma et al., 2007, 2010; Nakamoto et al., 2012; Yamashima, 2008) and is expressed in the retina, where its function has only recently been investigated (Joyal et al., 2016). First discovered in the pancreas, Ffar1 regulates insulin secretion (Itoh et al., 2003). In β-islet cells, circulating FA and Ffar1 agonists (GW9508 and TAK-875) (Briscoe et al., 2006; Burant et al., 2012; Leifke et al., 2012; Naik et al., 2012; Tsujihata et al., 2011; Yashiro et al., 2012) release insulin in the presence of glucose; it was explored as a target to treat type II diabetes. Interestingly, Ffar1 over-expression that mimics long-term high FA exposure eventually decreases insulin secretion leading to overt diabetes. Chronic Ffar1 signaling inhibits GLUT2 expression, a constitutive glucose transporter in the pancreas (Steneberg et al., 2005). As a result, lower intracellular glucose reduces insulin secretion (Itoh et al., 2003; Salehi et al., 2005; Schnell et al., 2007; Steneberg et al., 2005). Ffar1 signaling in the presence of high FA uptake also activates PPARα, an enhancer of FA β-oxidation; this metabolic switch may also inhibit glycolysis via the Randle cycle (Steneberg et al., 2005). Therefore, by sensing circulating FA nutrients, Ffar1 in the pancreas determines whether glucose or FA will be used as fuel, in part by regulating glucose

uptake. Similarly, in the retina, we found that Ffar1 is expressed in photoreceptors where it regulates glucose uptake (Fig. 5c and d). High serum palmitate levels or pharmacological agonists of Ffar1 suppress GLUT1 and retinal glucose uptake, which is corrected in Ffar1 deficient mice (Joyal et al., 2016). Lipid sensing plasma membrane GPCRs may, therefore, govern glucose uptake. We speculate that long-term suppression of glucose entry by Ffar1 in photoreceptors (perhaps secondary to that by increased levels of circulating lipids) might contribute to age-related mitochondrial dysfunction in AMD or MacTel. The retinal effects of Ffar1 agonists, which are currently being considered for the treatment of type 2 diabetes, should be carefully monitored, particularly in older individuals who are at increased risk for AMD. Lipid metabolism in the eye is, therefore, an essential area of research, both because of the unique biosynthetic composition of the retina and because of its energy requirements, which may become dysregulated with aging and under pathological conditions.

## 10. Retinal lipid composition

As we do not yet know what lipids are used as fuel in the retina we will review lipid composition and lipid metabolism in the retina.

### 10.1. Long-chain polyunsaturated fatty acids (LC-PUFA)

The retina is rich in lipids derived from essential  $\omega$ 3 and  $\omega$ 6 long chain polyunsaturated fatty acids (LC-PUFA), which are critical for many retinal functions. Since humans lack key enzymes to synthesize  $\alpha$ -linolenic acid ( $\omega$ 3) and linoleic acid ( $\omega$ 6), they must be obtained through dietary sources (SanGiovanni and Chew, 2005). Longer chain FA are then synthesized from these essential FA by iterative steps of desaturation (by insertion of double bonds) and elongation (by adding 2 carbons) in the endoplasmic reticulum of liver and to a lesser extent, *in situ* in retina (Bazan, 1989a,b; Li et al., 2001). Fatty acid structural nomenclature describes the number of carbons, double bonds and the position of the first double bond relative to the methyl terminal ( $\omega$ ) of the acyl chain.  $\alpha$ -LLNA (or C18:3 $\omega$ -3) therefore has 18 carbons, 3 double bonds, and the first unsaturated double bond is inserted at carbon 3.  $\alpha$ -Linolenic acid (C18:3 $\omega$ -3) is the dietary precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whereas linoleic acid (C18:2 $\omega$ -6) is the dietary precursor to arachidonic acid (AA). Pro-inflammatory prostaglandins and leukotrienes are derived from the  $\omega$ -6 AA. Conversely, the anti-inflammatory neuroprotectins and D-series resolvins originate from the  $\omega$ -3 DHA and the E-series resolvins from EPA. The molecular basis for the health benefits of  $\omega$ -3-PUFAs is thought to occur primarily through the direct integration of EPA and DHA in membrane phospholipids, replacing  $\omega$ -6-LC-PUFAs such as linoleic acid (18:2 $\omega$ -6) and AA. The biological impact of altering lipid intake is thought to be primarily through modification of membrane microdomain composition and specific receptors. Dietary  $\omega$ 3 to  $\omega$ 6 fatty acids may, therefore, impact retinal function and retinal neovascularization (Connor et al., 2007; Fu et al., 2015; Gong et al., 2015, 2016a, 2016b, 2017; Hård et al., 2013; Rezende et al., 2014; Sapieha et al., 2011, 2012; Shao et al., 2014; Stahl et al., 2010). There is no evidence these essential fatty acids are used for fuel, and without adequate synthetic machinery, it would be surprising if they were not used primarily in other capacities.

### 10.2. Phospholipids in membranes

LC-PUFA are incorporated predominantly into phospholipids in the neural retina (Bazan et al., 1997). Four types of phospholipids constitute structural elements of retinal membranes; phosphatidylcholine (PC; 40–50%) forms the outer leaflet, while phosphatidylethanolamine (PEA; 30–35%) and phosphatidylserine (PS; 5–10%) localize mostly on the cytoplasmic leaflet of membranes. Phosphatidylinositol (PI; 3–6%) is concentrated in membrane signaling domains (Bazan et al., 1997).

Disc membranes of photoreceptor outer segments have the highest concentrations of DHA in the body (30% of total retinal fatty acids) (Neuringer, 1993). DHA comprises 20% of outer segment PC and 30% for both PEA and PS. Half of all PC and PEA fatty acids are saturated (30% and 10% palmitic acid; 20% and 36% stearic acid, respectively), compared to a third of PS (28% stearic acid (Anderson, 1970; Fliesler and Anderson, 1983). While on average the phospholipids throughout the retina contain a PUFA in one of the two acyl positions, the phospholipids in the outer segments predominately have PUFAs in both acyl positions. (Avelldano and Sprecher, 1987; Choe and Anderson, 1990; Choe et al., 1990; Wiegand et al., 1991). The high concentration of PUFAs in the outer segments improve rhodopsin activation and phototransduction in model membrane systems (Fliesler and Anderson, 1983; Litman and Mitchell, 1996). Fliesler and Anderson (1983) provide a detailed review of the chemistry and metabolism of lipids in the vertebrate retina. In short, biophysical and biochemical properties of DHA and longer chain fatty acids govern membrane function in photoreceptors and impact vision.

### 10.3. Lipid uptake of LC-PUFA and processing to very low-density lipoproteins (VLDL)

Retinal uptake of preformed long-chain (LC)-PUFA is much more efficient than *in situ* retinal biosynthesis (Su et al., 1999; Wetzel et al., 1991). The liver is a key site for LC-PUFA biosynthesis (Bazan, 1989a,b; Li et al., 2001) (Fig. 4a). Dietary lipids absorbed by the gut form chylomicrons and very low-density lipoprotein (VLDL), which are either readily metabolized to produce energy or stored in the liver or adipose tissues (Scott and Bazan, 1989). They are secreted into the lymphatic system and reach the blood circulation via the thoracic duct. Levels of fuel reserve in adipocytes are gauged by secretion of adipokines, such as leptin and adiponectin, which signal to the brain and peripheral tissues and regulate energy homeostasis. Adipose tissue through cycles of lipolysis and re-esterification ensures fatty acid availability for oxidative tissues. During periods of starvation, adipocytes liberate FA to fuel tissues. The liver is an essential homeostat for transient energy fluctuation, storing excessive FA from circulation following postprandial elevated triglyceride levels, as benign less reactive triacylglycerol (TAG), and secreting VLDL following the peak lipid load (Bordin et al., 1998; Nenseter et al., 1992; Nestel, 2000; Vasandani et al., 2002). Oxidative tissues capable of using lipid as fuel, such as heart, skeletal muscle, and retina, express VLDL receptors, which increase FA uptake. Indeed, deletion of VLDLR or LPL prevents efficient lipid uptake and  $\beta$ -oxidation in the heart (Augustus et al., 2006; Niu and Evans, 2011; Perman et al., 2011).

Once in the liver, essential FA, such as  $\alpha$ -linolenic, are released in hepatocytes and form a complex with fatty acid synthase in the presence of malonyl coenzyme A (CoA). FA are elongated and desaturated in the endoplasmic reticulum (ER) to form DHA-CoA, which is then esterified to phospholipids (Fig. 4b). Apoproteins and phospholipids are transported in vesicles to the Golgi, where very low-density lipoproteins (VLDL) are assembled before being secreted (Bazan, 1990). DHA of hepatic origin is transported with dietary DHA as VLDL and chylomicrons to the choriocapillaris. Uptake of radiolabeled DHA in photoreceptors is highly efficient, beginning 1 h post-ingestion and peaking at 24 h (Li et al., 2001). Lipoprotein lipase (LPL) expressed in retinal choriocapillaris hydrolyzes chylomicron remnants and VLDL, liberating free fatty acids. Free fatty acids may subsequently form non-covalent bonds with albumin in blood plasma for tissue delivery.

Passage of fatty acids from blood to retina may proceed by facilitated transport or diffusion (Kawamura et al., 2003; Kelley et al., 1987; Matsugi et al., 1997; Wu et al., 2003). Transport of fatty acids from vessels to the retinal pigment epithelium (RPE), and to outer and inner segments of photoreceptors appears to be mediated by a high-affinity receptor (Bazan et al., 1997). Fatty acids are hydrophobic and therefore require specialized cytoplasmic transport systems, binding

proteins, and receptors to reach photoreceptors. Since photoreceptors are not in direct contact with the vascular supply, adjacent cell types (RPE, astrocytes, and Müller cells) intercede in the process (Bazan et al., 1997). Fatty acid transport proteins (FATP)-1, FATP-4 and CD36 are the predominant transporters expressed on blood-brain barrier endothelial cells (Mitchell et al., 2011). Very low-density lipoprotein receptor (VLDLR) (Donati et al., 2006), FATP-1 and 4 (Chekroud et al., 2012) are also expressed in RPE and photoreceptors. Thus, fatty acid transport to the retina is inferred although more evidence is still needed. Organs with high metabolic rates that are capable of using lipids as an energy substrate, such as the heart and skeletal muscles (Lopaschuk et al., 2010) are rich in VLDLR to help FA uptake (Fig. 4c and d). VLDLR anchors ApoE of triglyceride (TG)-rich chylomicrons and VLDL, enabling the cleavage of long-chain FA from TG by lipoprotein lipase (LPL) (Fig. 4c). VLDLR also participate in the transcytosis of active LPL across endothelial cells (Obunike et al., 2001). VLDLR may, therefore, facilitate the delivery of TG-derived free FA across capillary beds to fuel tissues (Beisiegel and Heeren, 1997; Takahashi et al., 1995).

#### 10.4. Very long-chain fatty acid biosynthesis and function in retina

*In situ* biosynthesis of very long chain (VLC)-PUFAs is a distinctive feature of the neural retina (Fig. 4a and b). ELOVL4, a condensing enzyme, is currently the only known elongase responsible for the synthesis of VLC fatty acids ( $\geq 22$  carbons). ELOVL4 is highly expressed in photoreceptors inner segments (Kunye et al., 2014), and to a lesser extent in brain, testis, and skin (Agbaga et al., 2008), but not in liver (Zadavec et al., 2011). ELOVL4 elongase sequentially attaches two-carbon units to the acyl backbone of shorter long chain (LC)-PUFAs (McMahon and Kedziński, 2009). Interestingly, n-3 VLC-PUFAs are predominantly synthesized from EPA, and less from DHA despite their retinal abundance (Suh and Clandinin, 2005). Major retinal VLC-PUFA end products are 32:5n-3 and 34:5n-3 (Agbaga et al., 2008), but their specific function remains essentially unknown.

The recent discovery of EVOLV4 mutations in Stargardt macular dystrophy type 3 (STG3) highlights the integral role of VLC-PUFAs for vision. STG3 is an autosomal dominant genetic disorder with deficient VLC-PUFAs associated with macular dystrophy and loss of central vision, suggesting a regional importance of these lipids in the macula. VLC-PUFAs are presumed to improve membrane fluidity and packing density, potentially stabilizing the unique folding pattern of outer segment discs, which may allow adequate shedding of rods and cones photoreceptor outer segments (Agbaga et al., 2010; Suh et al., 2000, 2009). Aging eyes are substantially depleted in VLC-PUFA, and their levels are further reduced in age-related macular degeneration (AMD) (Liu et al., 2010). The retinal lipid composition and *in situ* biosynthesis of VLC-PUFA might, therefore, contribute to the onset of AMD, and may offer a therapeutic target to address age-related retinal diseases.

### 11. Retinal cell-specific metabolism

*In vivo* or *ex vivo* measurements of retinal metabolism is the sum of the activity of multiple cell types. These cells have distinct metabolic activities and potentially compartmentalized and opposing metabolic reactions. In this review, we will focus on the metabolism of cells of the outer retina, namely photoreceptors, Müller glia, and RPE.

#### 11.1. Photoreceptors

The majority of both retinal OXPHOS and glycolysis occurs in photoreceptors. As previously noted, in accordance with their high energy demand, photoreceptors have over 60% of retinal mitochondria (located in the photoreceptor inner segments) (Cohen, 1961; Hoang et al., 2002), as well as the highest electron transport chain enzyme Cytochrome C oxidase activity (Giulian et al., 1989; Kageyama and Wong-Riley, 1984). Based on *ex vivo* analysis with selective inhibitors,

the outer retina (photoreceptors) is estimated to have 2–3 times greater oxygen consumption compared to the inner retina (Medrano and Fox, 1995). *In vivo* analysis finds that the inner retina has comparable O<sub>2</sub> consumption in the light (when less ATP is needed for the Na<sup>+</sup>/K<sup>+</sup> ATPase ion pumps to maintain gradients) but in the dark the outer retinal (photoreceptor) O<sub>2</sub> consumption increases where the inner retina is unchanged, supporting that OXPHOS fuels the dark current in photoreceptors (Wang et al., 1997a, 1997b). Concerning lactate production during aerobic glycolysis, the outer retina (photoreceptors) accounts for the vast majority with very little detected in the inner retina (Wang et al., 1997a, 1997b). Wild-type rat retinas have > 50% more lactate production and O<sub>2</sub> consumption than dystrophic retina lacking photoreceptors (Graymore, 1959; Graymore and Tansley, 1959). Combined, these data suggest that the photoreceptors are the primary consumers of energy in the retina and likely the site of the majority of OXPHOS and aerobic glycolysis as discussed below.

Photoreceptors (both rods and cones) have a unique mitochondrial localization and morphology (Winkler et al., 1997) with a high concentration of mitochondria in the ellipsoid region of the inner segment, near the ciliary junction to the outer segments (Goldberg et al., 2016). As noted, maintaining the sodium gradient in the photoreceptors is a crucial physiological feature that requires substantial energy derived from ATP hydrolysis (Hagins et al., 1970). The sodium pumps are most dense in the ellipsoid region of the photoreceptor inner segment and as noted earlier (when comparing light and dark metabolism) are likely fuelled via oxidative phosphorylation rather than through glycolysis (Ames et al., 1992b).

#### 11.2. Photoreceptors are the primary site for glycolysis

Enzymes for aerobic glycolysis localize to photoreceptors, indicating that they are the primary site of aerobic glycolysis in the retina. Photoreceptors express high levels of enzyme isoforms that favor the conversion of pyruvate into lactate, including hexokinase II (HKII), the pyruvate kinase M2 isoform (PKM2) and lactate dehydrogenase subunit A, LDHA (Ait-Ali et al., 2015; Chinchore et al., 2017; Casson et al., 2016; Lindsay et al., 2014; Rajala et al., 2016; Reidel et al., 2011). Hexokinase catalyzes the first reaction of glycolysis via the phosphorylation of glucose. The HKII isoform localizes to the mitochondrial membrane and specifically to the ATP transporter (VDAC) providing the enzyme an ample supply of the ATP needed (Mathupala et al., 2006; Rueda et al., 2016). The preferential expression of HKII, compared to the more widely expressed HKI isoform, is associated with aerobic glycolysis (and increased growth in cancer cells) (Wolf et al., 2011).

Pyruvate kinase mediates the final reaction in glycolysis converting PEP to pyruvate. The M2 isoform, as opposed to the M1 isoform, is allosterically regulated between a tetramer with high affinity for PEP and a less active dimeric form. The less active dimer leads to reduced pyruvate formation and a build-up of glycolytic intermediates and potentially leads to increased anabolic reactions from these intermediates (reviewed in (Ng et al., 2015)). This enzyme is also preferentially expressed in rapidly dividing cells including cancer cells. While some controversy exists regarding the expression of PKM1 in photoreceptors, with some reporting the absence of PKM1 and others observing it (Chinchore et al., 2017; Casson et al., 2016; Lindsay et al., 2014; Rajala et al., 2016; Rueda et al., 2016), PKM2 is indisputably the dominant isoform in photoreceptors.

Lactate dehydrogenase (LDH) exists as a tetramer comprised of A and B subunits. A tetramer consisting solely of A subunit favors the conversion of pyruvate to lactate and is highly expressed in cancer cells, whereas the tetramer of B subunit favors the reverse reaction (reviewed in (Vander Heiden et al., 2009)). Graymore first noted that photoreceptors might be the site of LDHA expression in the retina (Graymore, 1964). Since then many groups have noted the high expression of LDHA in the retina and specifically in photoreceptors, in a pattern matching the expression of PKM2 (Chinchore et al., 2017; Casson et al., 2016;



Rueda et al., 2016).

### 11.3. Glycolysis is required for photoreceptor outer segment synthesis

There is increasing evidence connecting light stimulation in photoreceptors to the regulation of aerobic glycolysis. Rajala et al. observed PI3K-mediate phosphorylation of PKM2 with light stimulation, leading to enzyme inhibition, suggesting a reduced production of pyruvate with light and potentially a build-up of glycolytic intermediates leading to anabolic reactions (Rajala et al., 2016). Chinchore et al. have recently shown that impairing glycolysis specifically in rods with a variety of methods (interfering with the activity of LDHA, PFK, and PK) leads to shorter rod outer segments (OS), which is thought to be the result of reduced OS synthesis (Chinchore et al., 2017). Their data suggest that high glycolytic flux and the production of lactate is necessary for the biosynthesis of OS. Interestingly, while reducing PKM2 led to shorter OS, overexpression of PKM1 had a similar effect. The observation that OS are shorter with both reduced glycolytic flux and increased flux suggests that the ability of the photoreceptor to precisely regulate the glycolytic rate is crucial to the OS length (Chinchore et al., 2017). Supporting the hypothesis that glycolytic intermediates are needed for biosynthesis during light exposure, keeping animals with impaired glycolytic function in the dark rescued OS length (Chinchore et al., 2017). However, it remains to be shown directly if there is an altered flux through anabolic reactions derived from glycolytic intermediates (such as PPP or serine biosynthesis) in response to light and if these pathways are necessary for OS biosynthesis.

### 11.4. Metabolic differences between cones and rods

The function and morphology of rods and cones are different, leading to differences in metabolic needs and photosensitivities. While rods are highly sensitive to light and able to respond to single photons, cones are less sensitive but respond and recover more quickly. The OS of a rod also differs from that of a cone, consisting of enclosed membrane discs in the outer segment as opposed to a continuous membrane in the cone. Cones and rods also differ in the number of mitochondria per cell. In mice, cones have twice the number of mitochondria, whereas in primates it is estimated to be ~10 times more than rods (Perkins et al., 2003). Furthermore, cones contain more ATP than rods (Scarpelli and Craig, 1963) and potentially cones have glycogen stores while rods do not (Nihira et al., 1995). It is possible that increased OXPHOS (mitochondrial) energetic capacity in cones is merely a response to an increased energetic demand compared to rods. Cones may also have increased energy capacity to allow increase resistance to metabolic insult and apoptosis. Rods and cones also differ with respect to their reliance on aerobic glycolysis. Noell reported that monkey cones survive an injection of iodoacetate, which blocks glycolysis, while rods rapidly die, suggesting that rods rely more on glycolysis than cones (Noell, 1952). Rod function is impacted by subtle increases or decreases in glucose levels, whereas cones maintain normal function with similar glucose changes and only show defects when glucose levels are severely reduced (Macaluso et al., 1992). In accord, in diabetes, rod function appears to be affected early as diabetic patients have difficulty seeing in the dark (Bailey and Sparrow, 2001).

There is metabolic cross-talk between rods and cones. Rods help support the metabolism and survival of cones through the release of RdCVF (rod-derived cone viability factor) (Ait-Ali et al., 2015). RdCVF is released from rods and increases glucose uptake in cones (Ait-Ali et al., 2015). When rods die and no longer secrete RdCVF cones are secondarily impacted.

### 11.5. RPE metabolism

The retinal pigment epithelium (RPE) is a pigmented monolayer of cells that comprises the entire blood outer blood-retinal barrier

between the choroidal vascular plexus and the neurosensory retina. Photoreceptors depend heavily on the RPE to maintain homeostasis; there is a constant molecular exchange between these two cell types and their metabolism is tightly linked. Indeed, loss of RPE function in mice leads to photoreceptor degeneration and loss or dysfunction of the RPE in humans occurs in many diseases, which comprise leading causes of vision loss, including age-related macular degeneration, retinitis pigmentosa, and diabetic retinopathy.

In many ways, the metabolic demands of photoreceptors translate to metabolic requirements for the supportive RPE. The RPE phagocytize the shed outer segments, detoxifying and degrading the light-damaged components and recycling the usable fatty acids, particularly DHA and the restored retinal photosensitive pigments back to the photoreceptors (Strauss, 2005). The RPE cells are responsible for vectorially transporting H<sub>2</sub>O, ions, energy substrates and other nutrients between the photoreceptors and choroid (Strauss, 2005). Oxidative stress further increases the metabolic burden on the RPE. The RPE is exposed to high photo-oxidative stress from direct light exposure. Additional sources of oxidative stress include photoreceptor light-damaged outer segment lipid, protein and photopigment components before detoxification (Winkler et al., 2008) as well as direct exposure to oxygen from the high rate of blood flow of the choroid (Bill et al., 1983). To deal with the excessive oxidative stress, RPE invests energy into increasing the anti-oxidative capacity (Strauss, 2005). The RPE cells have an unusually high rate of reductive carboxylation, whereby glutamine enters the TCA cycle and is carboxylated to yield citrate. Increased flux through this pathway appears to enhance the redox potential of RPE and feed lipid synthesis (Du et al., 2016). Despite the substantial investment into antioxidant capacity, age correlates with a decrease in mitochondrial number, reduced ATP levels and increased apoptosis in RPE cells. This phenomenon is exacerbated in patients with AMD, suggesting long-term stress leads to RPE dysfunction and ultimately degeneration of photoreceptors (Bhutto and Lutty, 2012; Feher et al., 2006; Zhao et al., 2011a).

RPE have high mitochondrial activity and also appear to have high aerobic glycolysis in cell culture models (Adjianto and Philp, 2014; Kurihara et al., 2016). While cell culture models suggest high glycolytic rates in RPE, it is thought that RPE preferentially passes glucose to the photoreceptors (Strauss, 2005). Supporting this notion, the RPE express low levels of hexokinase, which would allow glucose to pass through as opposed to entering glycolysis to be consumed in the RPE (Lowry et al., 1961; Wang et al., 2016). Kurihara et al. have shown that increasing glycolytic rates in RPE may lead to photoreceptor degeneration. Inducing hypoxia or hypoxia-signaling in RPE eventually results in photoreceptor cell death (Kurihara et al., 2016). In response to hypoxia, oxidative phosphorylation is reduced, and glycolysis is increased in RPE cells, and glucose transport is reduced which might be mostly responsible for the photoreceptor degeneration (Kurihara et al., 2016).

In addition to transporting glucose, the RPE might also produce additional energy substrates for the photoreceptors. Adjianto et al. have found that RPE oxidizes lipids to yield and secrete ketone bodies. They propose a cycle in which lipids are taken up by the RPE during outer segment recycling and are oxidized to produce ketones, which are then returned to the photoreceptors and enter the TCA cycle to produce ATP and amino acids (Adjianto et al., 2014).

### 11.6. Müller glia metabolism

The Müller glia span the retina. The processes are highly branched and have arbors that extend into all of the retinal layers. The function and the metabolism of these cells are still enigmatic (Reviewed in (Hurley et al., 2015)).

Similar to a lactate shuttle proposed in the brain, it has been proposed that Müller glia constitute a significant site of aerobic glycolysis in the retina. This hypothesis suggests that the Müller glia take up glucose to produce and secrete lactate that is then used by the

photoreceptors for oxidative phosphorylation. Supporting this idea, Poitry-Yamate et al. showed that freshly isolated Müller glia provide high levels of lactate and when Müller glia were isolated in combination with photoreceptors the lactate release was reduced, suggesting that the photoreceptors were consuming the lactate (Poitry-Yamate et al., 1995). Winkler et al. supported this idea by showing that in cultured Müller glia, essentially all glucose is used to produce lactate and there is little oxidative phosphorylation of glucose (Winkler et al., 2000). It is important to note that isolation of Müller glia intact and without other cell parts attached is very difficult.

Conversely, it has also been proposed that Müller glia lack the ability to produce lactate and instead use lactate and amino acids produced by the photoreceptors to generate energy and metabolites via the TCA cycle (Lindsay et al., 2014). As described earlier, there is substantial evidence supporting the idea that photoreceptors have high levels of aerobic glycolysis and secrete lactate. The lack of expression of various glycolytic enzymes in Müller glia further suggests that these cells do not have high glycolytic rates *in vivo*. Several groups have noted the lack of pyruvate kinase (PKM1 or PKM2) in Müller glia in the rodent retina (Casson et al., 2016; Lindsay et al., 2014; Rajala et al., 2016). It has also been noted that Müller glia do not express LDHA (Casson et al., 2016) but may express LDHB (Chinchore et al., 2017). Lindsay et al. suggest that Müller glia can utilize lactate and aspartate generated by the photoreceptors as energetic and anaplerotic substrates (Lindsay et al., 2014).

Most evidence for Müller glia metabolism comes from cultured cells, which are imperfectly isolated from the retina, and are unlikely to reflect Müller glia metabolism *in vivo* in the absence of physiological cell/cell interaction. Given the cellular morphology of Müller glia (spanning the retina with multiple cellular interactions), it is likely that the metabolic pathways vary in various parts of the cells depending on their location and their cellular neighbors. Further work determining the *in vivo* metabolic properties of Müller glia and their relationship to photoreceptors is needed.

## 12. Neuroglial mitochondria: nutrient and oxygen sensors that drive angiogenesis

Elaborate mechanisms preserve the critical homeostasis between the vascular supply of nutrients and oxygen, and the neuronal energy demands fuelled by mitochondria, the cell's powerhouse. Major mitochondrial energy pathways including the TCA cycle, fatty acid  $\beta$ -oxidation, and oxidative phosphorylation require nutrients and oxygen to produce energy. Glucose, amino acids and fatty acids fuel the Krebs cycle by generating acetyl-CoA. Nutrient deficiency may, therefore, decrease acetyl-CoA synthesis and downstream metabolites of the Krebs cycle, such as  $\alpha$ -ketoglutarate.

The incomplete reduction of oxygen in mitochondria by complex III of oxidative phosphorylation leads to the generation of reactive oxygen species (ROS) (Guzy and Schumacker, 2006). Many heme-containing enzymes, such as prolyl hydroxylases (PHDs) are tightly regulated by oxygen and ROS. The important Krebs cycle metabolite  $\alpha$ -ketoglutarate is also a co-factor of prolyl hydroxylase domain-containing protein (PHD), required for the hydroxylation and degradation of hypoxia-inducible factor 1 (HIF1). Hence, a nutrient shortage that decreases Krebs cycle metabolites, hypoxia, or ROS will inhibit PHD and stabilize HIF1 $\alpha$ , by preventing its immediate degradation (Pouyssegur and Mechta-Grigoriou, 2006). HIF1 $\alpha$  is a critical transcription factor for more than 60 genes, including VEGF, which triggers cytoprotective adaptation and compensatory angiogenesis during ischemia (and during fuel shortages) (Joyal et al., 2016).

Oxygen is also directly coupled to mitochondrial oxidative phosphorylation (Johnson and Hansford, 1975). Complex IV of the electron transport chain (ETC) reduces oxygen to water, enabling the flow of electron and the reduction of flavin (FAD) and nicotinamide (NAD) nucleotide, which is then used by enzymes of the Krebs cycle (King

et al., 2006). Though the Krebs cycle does not directly use oxygen, it can only take place when oxygen is present, since it relies on by-products of the ETC. Hypoxia, therefore, inhibits both the ETC and the Krebs cycle leading to the accumulation of key metabolites, particularly succinate which is used by both metabolic pathways (Folbergrová et al., 1974; Hoyer and Krier, 1986). Moreover, HIF and succinate dehydrogenase activity are intertwined, such that during hypoxia, HIF is stabilized and succinate dehydrogenase levels are decreased (Strumilo, 2005); the latter increases HIF stabilization through reactive species generation (Pouyssegur and Mechta-Grigoriou, 2006; Tretter and Adam-Vizi, 2005), HIF, in turn, can directly suppress levels of succinate dehydrogenase 'B' (Dahia and Consortium, 2006), and together amplify succinate accumulation. Physiological functions for the Krebs cycle intermediates beyond their traditional roles as metabolites of this pathway were only recently described (Bénil et al., 2014). However, several indicators suggest their potential roles in ischemic adaptations, such as vasomotor regulation and angiogenesis. Succinate administration after an ischemic event improves brain recovery (Cannella et al., 1989; Gurvitch et al., 1997) and dysfunctional mutations in succinate dehydrogenase (which converts succinate to fumarate) lead to succinate accumulation, which is associated with tumorigenesis (Gottlieb and Tomlinson, 2005; King et al., 2006).

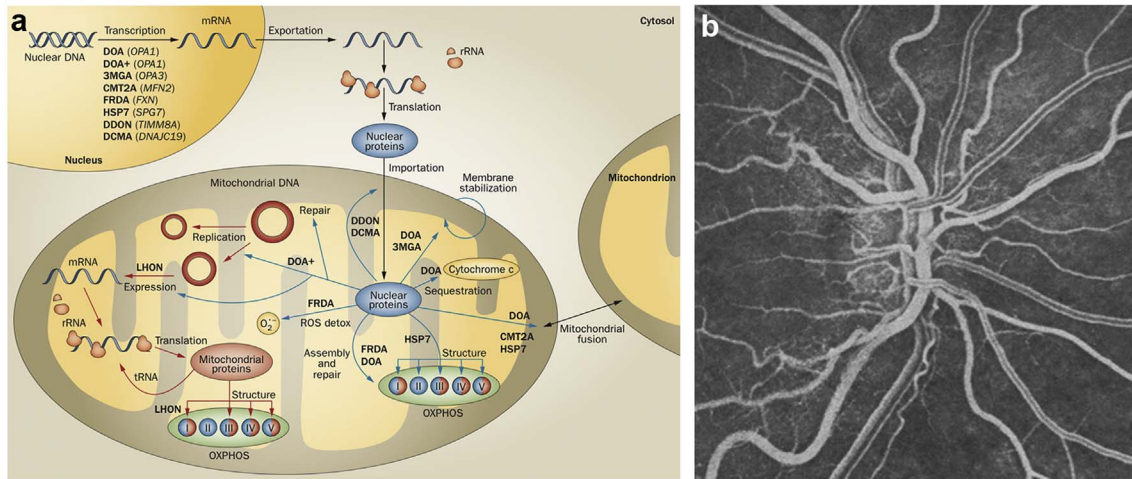
Although succinate has been studied for over 60 years, the discovery of the G-protein coupled receptor GPR91 (previously designated as an orphan receptor) that specifically binds succinate implied biological functions beyond energy production. GPR91 is predominantly expressed in highly vascularized tissues such as the kidney, placenta, liver and the retina (He et al., 2004; Wittenberger et al., 2001) suggesting a possible sensitivity to hypoxia. GPR91 is a purinergic-like receptor with ~53% homology to P2 purinoceptors. As in the case of Krebs cycle intermediates, metabolic products of purines (ATP, ADP, AMP, and adenosine) accumulate during hypoxia and activate purinergic receptors that may participate in neovascularization (Linden, 2005). But whether they act directly or through the release of proangiogenic factors is controversial (Grant et al., 1999; Wakai et al., 2001).

In the retina, GRP91 is almost exclusively expressed in retinal ganglion cells (RGCs) where it senses hypoxic stress during retinal vascular development and disease. These ischemic neurons through succinate production and GPR91 signaling secrete angiogenic factors, particularly VEGF, known for its chemotactic properties (Sapieha et al., 2008). RGCs shape their microvascular environment to re-instate metabolic equilibrium.

## 13. Pathological angiogenesis in disease as a marker of retinal energy failure

Vessels supplying oxygen and nutrients to neurons, continually adapt to neuronal energy requirements. Vascular remodeling is, therefore, an early sign of changes in retinal neuron metabolism, possibly driven by energy needs. Hence, diseases involving mitochondria may present a unique vascular signature.

Mitochondrial ocular diseases are categorized as either primary or secondary. Primary retinal disorders result from direct impairment of mitochondrial functions by mutations in either mitochondrial DNA (mtDNA) or nuclear genes coding for mitochondrial proteins (which contribute the largest number involved in mitochondrial function). Mitochondrial diseases, therefore, reflect the contributions of two genomes, mitochondrial and nuclear, in addition to environmental influences. Many mitochondrial gene mutations may be lethal, but of those mutations that survive, the heterogeneous, slowly progressive and often late presentations of primary mitochondrialopathies highlight the complexity of the compensatory mechanisms in place to palliate energy failure in the retina. Acquired (or secondary) mitochondrial dysfunction is believed to contribute to the development of diabetic retinopathy (from adaptation to hyperglycemia and dyslipidemia) and age-related macular degeneration (AMD) (from mitochondrial aging leading to a



**Fig. 6. Mitochondrial disorders and vascular adaptation.** (a) Cellular homeostasis is under the dual control of nuclear (blue) and mitochondrial genomes. Mitochondrial diseases (bold text), many of which impact vision, arise from mutations in genes (italic text) from either genome. Mitochondrial DNA encodes 13 structural subunits of the electron transport chain (complexes I, III, IV, and V) and the RNA needed for gene translation. All other mitochondrial proteins are encoded by the nuclear genome. Abbreviations: CMT2A, Charcot-Marie-Tooth disease, type 2A; DCMA, dilated cardiomyopathy with ataxia; DDON, deafness, dystonia and optic neuropathy; DOA, dominant optic atrophy; DOA+, dominant optic atrophy-plus syndrome; FRDA, Friedreich ataxia; HSP7, hereditary spastic paraplegia, type 7; LHON, Leber hereditary optic neuropathy; OXPHOS, oxidative phosphorylation; ROS detox, reactive oxygen species detoxification; rRNA, ribosomal RNA; tRNA, transfer RNA; 3MGA, 3-methylglutaconic aciduria, type III. (b) Fluorescein angiography of fundus during the acute stage of LHON, showing tortuous vessels and telangiectasias. Energy metabolism has a direct impact on the retinal vascular phenotype. Figure modified, with permission, from (Newman, 2012).

dysfunction of photoreceptors and support cells). These are the most common causes of blindness in adults. We will describe the presentations of classical mitochondrial retinal diseases to illustrate the importance of angiogenesis as a marker of energy failure.

#### 14. Primary mitochondrial pathologies and ocular diseases

Neuro-ophthalmic mitochondrial disorders can be broadly divided into four groups: (1) bilateral optic neuropathies; (2) pigmentary retinopathies that affect the inner and outer retina respectively; (3) ophthalmoplegia with ptosis; and (4) retrochiasmal visual loss (Fig. 6a). The extra-retinal ocular manifestations and are reviewed elsewhere (Biousse and Newman, 2003; Newman, 2012).

##### 14.1. Leber's hereditary optic neuropathy (LHON): paradigm of the inner retina

Leber's hereditary optic neuropathy is the most common mitochondrial ocular disease. The condition was initially described by Von Graefe in 1858 (von Graefe, 1858a; Von Graefe, 1858b), but was only coined as a formal clinical entity by Leber in 1871 (Leber, 1871a, b). LHON was the first maternally inherited (Erickson, 1972) mitochondrial disorder of the eye (Wallace, 1999) to be discovered. Three different point mutations in mitochondrial DNA account for more than 90% of cases (Wallace et al., 1988). However, if the presence of a mitochondrial mutation is necessary for the pathogenic expression, it is not sufficient. The co-existence of both mutant and normal mitochondria (heteroplasmy) in a given tissue is often offered as an explanation for the variation in penetrance of mitochondrial disease. However, many carriers of mitochondrial point DNA mutations without significant heteroplasmy will never suffer visual loss. Other factors, genetic and environmental, therefore modify the expression of mitochondrial disorders (Carelli et al., 2015; Giordano et al., 2013).

LHON presents as an acute or subacute loss of central vision that generally progresses within a few months, and affects mostly young males (Hwang et al., 2017). Almost 50% of affected men will suffer significant vision loss compared to only 10% of females (Hwang et al., 2017). The reason for this gender bias is suggested to be related to estrogen effects on mitochondrial DNA (Pisano et al., 2015). In affected patients, color and visual acuity fades progressively in one eye, rapidly

followed by a similar involvement of the contralateral eyes days to months later (rarely years). Visual field defects mainly involve central vision, creating a large central absolute scotoma, while preserving peripheral vision. Preferential loss of central high acuity color vision is also a generalizable characteristic of many mitochondrial disorders, possibly correlating geographically with an area of higher retinal energy demands of the cone-rich central retina. Interestingly, earlier onset of visual loss (younger than 15 years) and a subacute time course, have a better visual prognosis (Nakamura and Yamamoto, 2000). Rarely patients, mostly carrying the 14484 mtDNA mutation, will experience significant vision recovery, which suggests the presence of yet unknown rescue mechanisms for energy-depleted neurons. Finally, LHON patients usually do not have symptoms until early adult life, when some ill-defined trigger, endogenous or environmental, is believed to initiate acute vision loss. A large case-control study of LHON patients did not show, however, an association between usual environmental culprits of mitochondrial stress, such as tobacco or alcohol consumption, and the onset of visual loss although other studies suggest an association (Kerrison et al., 2000). LHON illustrates that environmental triggers result in tissue-specific energy failure and that compensatory mechanisms may alleviate the energy crisis (Carelli et al., 2004).

The pathogenesis of the selective damage to the optic nerve (retinal ganglion cells) in LHON remains uncertain. Unmyelinated prelaminar retinal ganglion cell axons are mitochondria-rich and have shown a high degree of mitochondrial respiration (Pan et al., 2012). This is, therefore, an area of high metabolic energy requirements for the retina. Moreover, the acute-angle turn made by axons as they enter the optic nerve may represent a 'choke-point' for axoplasmic mitochondrial transport, associated with an inherent metabolic vulnerability. Mutations of the electron transport chain complexes, as observed in LHON, may result in abnormal oxidative phosphorylation (respiration), decreased ATP production, and more ROS production, together resulting in damage to ganglion neurons and their axons. Mitochondrial biogenesis appears to be more active in carriers of mitochondrial mutations that do not develop the disease, suggesting that more mitochondria, albeit defective, may collectively better maintain the energy requirement of ganglion cells above the optic nerve degeneration threshold (Giordano et al., 2013). Similarly, estrogen receptors were recently shown to trigger mitochondrial biogenesis providing a compelling explanation for the gender bias observed in LHON (Pisano et al.,



2015). A mouse model of complex I deficiency presents histopathological features of LHON (Lin et al., 2012), highlighting the inherent vulnerability of retinal axons and their dependence on mitochondrial respiration. Further exploration of mitochondrial diseases in mouse models will be key to explore the pathogenesis of human mitochondrial disorders.

Vascular remodeling in LHON is an early sign of the underlying mitochondrial disorder (Fig. 6b). Microangiopathy may be seen for years before the onset of the optic neuropathy and is also seen in asymptomatic disease carriers (Wallace et al., 1988). During the active phase of the disease, however, retinal artery branches become acutely dilated and tortuous, forming arteriovenous shunts and telangiectasia. Hyperemia of the optic disc is associated with occasional peripapillary hemorrhages (Nikoskelainen, 1984; Nikoskelainen et al., 1983, 1984). Eventually, as neurons atrophy and their energy demands fall, the microangiopathy completely disappears. Optic disc pallor observed in the late phase of the disease correlates with loss of ganglion cells and a paucity of vessels in the affected central retina. Closer inspection of the endothelium and smooth muscle of microangiopathy lesions suggest an accumulation of morphologically aberrant mitochondria (Wallace et al., 1988). Whether angiogenesis directly contributes to the disease etiology or is a consequence of neuronal energy demands is unknown. Similar proliferative vascular changes are noted surrounding necrotizing lesions in Leigh and MELAS syndrome, mitochondrial disorders affecting the central nervous system more severely (Grönlund et al., 2010; Isashiki et al., 1998; Uziel et al., 1997). It is therefore plausible that increases in neuronal energy demands compel an increase in angiogenesis to reinstate energy homeostasis. Conversely, as neurons degenerate and their energy demand fall, so may the drive to increase vascular supply leading to neurovascular atrophy.

#### 14.2. Outer retina (photoreceptor) bioenergetics and pigmentary retinopathies

Retinal pigment epithelium (RPE) and photoreceptors constitute a bioenergetic unit fuelled primarily by the choroidal vasculature. Disorders that impair energy production often present with pigmentary changes of the retina, sometimes referred to as salt-and-pepper retinopathy, secondary to aberrant migration of RPE. Unaffected carrier relatives of patients with a more severe mitochondrial disease often have isolated pigmentary retinal changes. Degeneration of RPE, and sometimes degeneration of rod and cone photoreceptors, contributes to (usually mild) vision loss, affecting roughly 50% of patients with known mitochondrial mutations (Bhatti, 2006; de Crecchio et al., 2006; Durlu et al., 1997; Lowes, 1975; McDonald, 2003).

Pigmentary retinopathy is a diagnostic criterion for Kearns-Sayre Syndrome (KSS) (Kearns and Sayre, 1958). KSS is the result of duplication or deletions in mitochondrial DNA and is often associated with chronic progressive external ophthalmoplegia (CPEO). Mitochondrial encephalopathy, lactic acidosis and stroke-like episode (MELAS), is also a disease of mitochondrial genes, which often presents with pigmentary retinal changes (Isashiki et al., 1998). A specific mutation associated with MELAS (3243) may also give rise to more severe macular dystrophy (Isashiki et al., 1998). Mitochondrial mutations in the *ATPase-6* gene, which account for approximately a third of patients with Leigh's syndrome is often associated with a pigmentary retinopathy and sometimes the loss of photoreceptors. Leigh syndrome, also called subacute necrotizing encephalomyelopathy, is a disorder of aerobic energy production characterized by early childhood encephalopathy, lactic acidosis, and central hypoventilation (Uziel et al., 1997). Progressive pigmentary retinopathy is also noted in some cases of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD: EC1.1.1.211), a defect in mitochondrial fatty acid  $\beta$ -oxidation (Tyni et al., 2002). In short, mitochondrial disorders that impair energy production often present with pigmentary retinopathies, with some degree of photoreceptor atrophy, reflecting the energy dependency of

the outer retina.

### 15. Secondary mitochondrial pathologies and ocular diseases

Acquired (or secondary) mitochondrial dysfunction is likely involved in the development of diabetic retinopathy, and age-related macular degeneration (AMD). AMD is primarily caused by aging changes, which include aging mitochondria, while fuel shifts induced by hyperglycemia and dyslipidemia contribute to DR. Both diseases are associated with secondary energetic dysfunction of photoreceptors and vessels. Retinal degenerative disease, such as retinitis pigmentosa, often also present signs of secondary mitochondrial dysfunction that likely exacerbate disease progression.

#### 15.1. Retinitis pigmentosa: cone degeneration after rod loss

Genetically determined photoreceptor degeneration in retinitis pigmentosa (RP) is associated with changes in cellular energetics and vascular attenuation (Ayton et al., 2013; Campochiaro and Mir, 2017; Dhoot et al., 2013; Toto et al., 2016). RP is used to describe any inherited monogenic disorder associated with photoreceptors and frequently, pigmentary degeneration. RP is characterized initially by progressive loss of night vision due to many different mutations affecting primarily rod function (Hartong et al., 2006). Peripheral visual fields are lost first as the peripheral retina primarily consists of rods, leaving only a small island of central vision due to surviving macular cones. Secondary cone loss follows in most RP subtypes (Punzo et al., 2009); Punzo and Cepko found that cone loss may be a result of cone starvation (as well as oxidative stress). They assessed four mouse models of photoreceptor degeneration secondary to mutations in rod-specific genes. Alterations in the insulin/mammalian target of rapamycin pathway (mTOR) aligned with the activation of autophagy during cone death. Suppression of endogenous insulin caused an increase in cone loss and exogenous insulin increased cone survival. These data suggest that cone death in retinitis pigmentosa could, at least in part, be a result of the starvation of cones, a bystander effect of rod loss.

There is other evidence suggesting the involvement of photoreceptor energetics in RP. The function of many genes implicated in photoreceptor degeneration, highlight two converging themes: bioenergetic dysfunction and the accumulation of reactive oxygen and nitrogen species (RONS). Photoreceptors have a narrow cilium connecting the inner segment mitochondria and biosynthetic machinery to the continuously regenerating membranes of the light-sensing outer segments. Cilium dysfunction compromises cargo delivery from the inner segment to the outer segment of photoreceptors, and impairs lipid membrane regeneration, and energy utilization, which increases the vulnerability to oxidative damage. Mutations involved in ciliary transport accounts for 25% of genetically determined photoreceptor degenerations, such as Usher syndrome. Mutations affecting lipid metabolism, phototransduction and the visual cycle, all associated with cell metabolism, together account for another 25% of photoreceptor degenerations (Wright et al., 2010).

The RP visual cycle mutations affect energy metabolism, lipid metabolism, and regeneration of outer segments. *Photo-excitation* of rhodopsin initiates the visual cycle, converting 11-cis retinal to all-trans retinal, liberating singlet oxygen in the process. All-trans retinal released from rhodopsin is then conjugated to lipid phosphatidylethanolamine and transported to the cytoplasm by ATP-binding cassette, subfamily A, member 4 (ABCA4). More than 180 mutations in the ABCA4 gene have been implicated in photoreceptor degeneration, including autosomal recessive Stargardt macular dystrophy, fundus flavimaculatus, autosomal recessive retinitis pigmentosa, cone-rod dystrophy and somewhat controversially for age-related macular degeneration (Michaelides et al., 2006; Molday et al., 2009; Tsybovsky et al., 2010; Zhong and Molday, 2010).

Lipid oxidation of the very long-chain polyunsaturated fatty acid (PUFA) abundant in the outer segment can cause a build-up of oxidized lipids that must be cleared by RPE phagocytosis, which processes 10% of photoreceptors outer segment each day. Disorders of PUFA synthesis can also result in photoreceptor degeneration, as seen with ELOVL4 autosomal dominant mutations, a subtype of Stargardt disease (STG3) (Agbaga et al., 2008).

Altered bioenergetic functions may also directly result in photoreceptor demise. Mutation in NAD-dependent mitochondrial isocitrate dehydrogenase 3 (IDH3), a critical enzyme of the Krebs cycle, occurs in a familial form of RP through metabolic dysfunction (Hartong et al., 2008). Finally, as photoreceptors degenerate and retinal ATP demands fall, more oxygen may be delivered to the retina than can be reduced to H<sub>2</sub>O by mitochondrial oxidative phosphorylation. Excess tissue oxygen is partially reduced by the mitochondrial electron transport chain (ETC), generating more RONS and potentially increasing retinal toxicity. Despite the genetic heterogeneity of disease mechanisms, mitochondrial dysfunction and oxidative damage are common denominators of photoreceptor degeneration. Similar to inner retina mitochondrial diseases, the choroid plexus atrophies as the energy demand of degenerating photoreceptors declines, confirming the intimate coupling between vascular supply and retinal energy demands.

Vessels in RP regress as photoreceptors degenerate (Rezaei et al., 2017); 13 patients with RP at various stages were imaged by optical coherence tomography angiography. The resulting optical microangiograms provide detailed visualization of retinal and choroidal vascular networks within the retina and choroid. All patients with moderate to severe RP showed abnormal microvasculature in both deep retinal and choroidal layers. Images of patients with only peripheral abnormalities demonstrated relatively normal vasculature networks. Microvascular changes in the retinal and choroidal vasculature correlate with structural changes in IS/OS junction to RPE layer.

### 15.2. Secondary mitochondrial pathologies and common ocular diseases: DR and AMD

Mitochondrial dysfunction may be accelerated in certain diseases, such as diabetic retinopathy and age-related macular degeneration (Feher et al., 2006; Mueller et al., 2012). Mitochondria-rich retinal neurons exposed to light (and oxygen) generate ROS. Antioxidant enzyme defenses, such as superoxide dismutase (SOD), glutathione (GSH), and catalase (found only in peroxisomes) help protect the retina against excess ROS in a highly oxidizing microenvironment (Dorrell et al., 2009). The process of mitochondrial DNA repair previously believed non-existent, is in fact highly regulated and can be impaired by oxidative stress (Berneburg et al., 2006; Liu and Demple, 2010). Hence, mitochondrial mutations may accumulate over time, leading to age-acquired mitochondrial dysfunction (Ceriello, 2012; de Zeeuw et al., 2015; Giacco and Brownlee, 2010; Ihnat et al., 2007).

#### 15.2.1. Diabetic retinopathy

Diabetic retinopathy is the leading cause of vision loss in working-age adults and is the most prevalent microvascular complication of diabetes. Diabetic retinopathy primarily affects vessels in the inner retina. Within 15 years of diagnosis, almost 50% of patients with type I diabetes and 10% of patients with type II diabetes develop microvascular complications, classified into two phases (Antonetti et al., 2012). Non-proliferative diabetic retinopathy (NPDR) is associated with capillary drop out, leading to retinal ischemia, loss of nutrients and loss of waste removal. This initial retinal insult eventually triggers compensatory but ultimately pathological neovascular proliferation. This second phase of the disease, proliferative diabetic retinopathy (PDR), is associated with more severe vision loss. Macular edema can develop in both phases from increased vascular permeability (Antonetti et al., 2012; Stitt et al., 2016).

Hyperglycemia-induced metabolic abnormalities coincide with and

likely cause mitochondrial dysfunction and oxidative damage. Advanced glycation end products (AGEs), increased activity of the polyol pathway and protein kinase C signaling are involved in the progressive metabolic dysfunction seen in diabetic retinopathy, together mediating oxidative stress (Geraldes et al., 2009). Free radicals are generated by mitochondrial respiration and are necessary for normal cellular function (Chouchani et al., 2016; Divakaruni and Brand, 2011; Spiegelman, 2007). Excessive suppression of free radicals impairs mitochondrial function (Chouchani et al., 2016) as does the excess production of free radicals (Gonzalez-Lima et al., 2014; Scatena, 2012; Shi and Gibson, 2007). Oxidative stress results from the accumulation of RONS secondary to the inability to sufficiently scavenge excess free radicals, either because of excessive production or impaired removal of RONS (Barot et al., 2011; Bell and Guarente, 2011; Qiu et al., 2010).

Mitochondria are the most abundant source of endogenous superoxides, peroxynitrites and hydroxyl radicals (Balaban et al., 2005). Antioxidant scavengers that detoxify excess levels of these naturally occurring free radicals may be suppressed in diabetic retina and high-glucose cultured retinal mitochondria (Jarrett et al., 2008). Indeed, elevated levels of lipid peroxides, superoxide, and hydrogen peroxide, together with suppression of SOD and glutathione reductase are reported in murine diabetic retinopathy models (Giacco and Brownlee, 2010; Lamoke et al., 2015). Increased oxidative stress is therefore widely regarded as pathogenic in diabetic retinopathy. Changes in mitochondrial permeability from lipid membrane oxidative damages may explain in part the mitochondrial swelling observed in diabetic mice (Hammes, 2005). Ultimately, severe mitochondrial damage releases cytochrome c from mitochondria to the cytosol, initiating apoptosis in diabetic retinal capillaries (Caldwell and Slapnick, 1989). However, the exact mechanism by which high-glucose mediates changes in mitochondria redox state and the proliferative vascular signal likely governed by metabolism remains ill-defined. Our limited mechanistic understanding of proliferative DR is in part due to the lack of proper animal models, since diabetic mice present with capillary dropout (NPDR) (Lutty et al., 1997), but these models fail to develop the proliferative neovascular disease (PDR). We can however infer from primary mitochondrial pathologies that surviving neurons faced with reduced vascular supply from capillary dropout will signal a need for energy, driving vaso-proliferative DR.

#### 15.2.2. Age-related macular degeneration

Age-related macular degeneration (AMD) is a progressive outer retinal neurodegenerative disease of the central retina and macula and the leading cause of vision loss in aging adults. More than 20% of our aging population is expected to develop AMD (Lim et al., 2012). Drusen deposition and areas of hyper- or de-pigmentation are early signs dry AMD, which may progress to photoreceptors and retinal pigment epithelium atrophy, sometimes referred to as geographic atrophy. Neovascular or wet AMD is associated with the invasion of neovessels into the photoreceptor layer, which is often associated with vision loss. Pathological neovessels in AMD may originate from the choroid (85–90%), or inner retinal vessels (10–15%). This latter sub-type of AMD is also called retinal angiomatous proliferation (RAP) (Bottoni et al., 2005; Donati et al., 2006), and resembles to some extent, macular telangiectasia (MacTel), a rare multifactorial inherited disease of the macula (Shukla et al., 2012; Toy et al., 2012; Yannuzzi et al., 2012).

Mitochondrial dysfunction is correlated with AMD. Mitochondrial DNA polymorphism (Mueller et al., 2012; Park et al., 2012; Udar et al., 2009) and variants of the age-related maculopathy susceptibility 2 protein (ARMS2) are powerful AMD predictors. ARMS2 by some reports is found in mitochondria (Fritsche et al., 2008; Kanda et al., 2007), although its localization is debated (Kortvely et al., 2010). Human induced pluripotent stem cells (hiPSCs) from AMD patients transformed into RPE, secreted more complement and inflammatory factors, which was exaggerated in cells from ARMS2/HTRA1 homozygous patients.

Table 1

– Animal models of neovascular age-related macular degeneration. Table adapted, with permission, from (Pennesi et al., 2012).

	CNV	RAP-Like	Reference	Comment
<b>Complement Factor Pathway</b>				
Transgenic C3 Overexpression mice		X	Cashman et al., 2011	
<b>Chemokines</b>				
Ccl2 $-/-$ and Ccr2 $-/-$ mice	X		Ambati et al., 2003	
Ccl2 $-/-$ mice1	–		Luhmann et al., 2009	1 - Results differ from Ambati et al., (2003).
Cx3cr1 $-/-$ mice	X <sup>2</sup>		Combadière et al., 2007	2 - Increased laser-induced CNV.
Ccl2/Cx3cr1 $-/-$ mice	X		Tuo et al., 2007	
<b>Oxidative Damage models</b>				
Ceruloplasmin/hephaestin $-/-$ mice		X	Hahn et al., 2004	
SOD1 knockout mice	X		Imamura et al., 2006	
NRF2 $-/-$ mice	X		Zhao et al., 2011	
OXYS Rat		X	Markovets et al., 2011	
<b>Glucose/Lipid Metabolism</b>				
APOE2/e4 transgenic mice $\pm$ high fat	X <sup>3</sup>		Malek et al., 2005	3 - Only on ApoE4 mice when on high fat.
Vldlr $-/-$ mice	X	X	Heckenlively et al., 2003, Chen et al., 2007	
<b>Other</b>				
Senescence accelerated mouse		X <sup>4</sup>	Takada et al., 1994	4 - Intrachoroidal NV
<b>Induced CNV</b>				
Matrigel induced CNV	X		Shen et al., 2006; Cao et al., 2010	
PEG-8 injection	X		Lyzogubov et al., 2011	
PEC-injected MCP mice	X		Jo et al., 2011	
Rat Subretinal lipid hydroperoxide injection	X		Baba et al., 2010	5 - intrachoroidal NV (VMD2/VEGF mice)
VEGF Transgenic mice	X <sup>5</sup>	X <sup>6</sup>	Multiple - see text	5 - CNV if subretinal injection 6 - rho/VEGF mice

Nicotinamide improved the AMD phenotype of hiPSCs-derived RPE cells (Saini et al., 2017). Retinal pigment abnormalities and RPE atrophy characteristic of early dry AMD are noted in 75% of individuals with mitochondrial mutation A3243G associated with MELAS syndrome (Primary mitochondrialopathies and ocular diseases) (Isashiki et al., 1998). Mitochondrial haplotypes have also been correlated with changes in the prevalence of AMD. RONS primarily produced by complex III of the mitochondrial electron transport chain (ETC), accumulate in the aging macula and correlate both geographically and temporally with AMD progression (Ting et al., 2009). Despite evidence converging on the role of energy deregulation, surprisingly little is known about the energy signals that may govern AMD. Few suitable animal models replicate all aspects of AMD, but several transgenic mice present some characteristic signs of the disease (Table 1) (Ambati et al., 2003; Baba et al., 2010; Cao et al., 2010; Cashman et al., 2011; Chen et al., 2007; Combadière et al., 2007; Hahn et al., 2004; Heckenlively et al., 2003; Imamura et al., 2006; Jo et al., 2011; Luhmann et al., 2009; Lyzogubov et al., 2011; Malek et al., 2005; Markovets et al., 2011; Shen et al., 2006; Takada et al., 1994; Tuo et al., 2007; Zeiss, 2010; Zhao et al., 2011b).

Dyslipidemia and other common cardiovascular risk factors are associated with AMD (Haines et al., 2006; Lim et al., 2012). Interestingly, human *VLDLR* deletions result in photoreceptor degeneration and a maculopathy (Boycott et al., 2009; Sarac et al., 2012). *Vldlr*<sup>-/-</sup> mice display some salient pathologic features that may inform us about causes of neovascular AMD, including dyslipidemia and macular telangiectasia reminiscent of retinal angiomatous proliferation (RAP) as well as choroidal neovascularization. Poor lipid uptake in *Vldlr*<sup>-/-</sup> mice results in high circulating triglycerides and FA levels (Goudriaan et al., 2004). Finally, RONS accumulate in *Vldlr*<sup>-/-</sup> photoreceptors (Dorrell et al., 2009; Zhou et al., 2011); also an essential aspect of AMD (Barot et al., 2011; Schrier and Falk, 2011). Therefore *Vldlr*<sup>-/-</sup> mice may be a relevant model of RAP in which to explore how retinal lipid energy metabolism impacts aberrant vessel growth. Very low-density lipoprotein receptor (Vldlr), which is present in photoreceptors (Dorrell et al., 2009; Joyal et al., 2016) and is expressed in other tissues with a high metabolic rate, facilitates the uptake of triglyceride-derived fatty acids (Goudriaan et al., 2004; Lopaschuk et al., 2010). In the retinas of *Vldlr*<sup>-/-</sup> mice with low fatty acid uptake (Goudriaan et al., 2004) but high circulating lipid levels, we found that *Ffar1* suppresses the

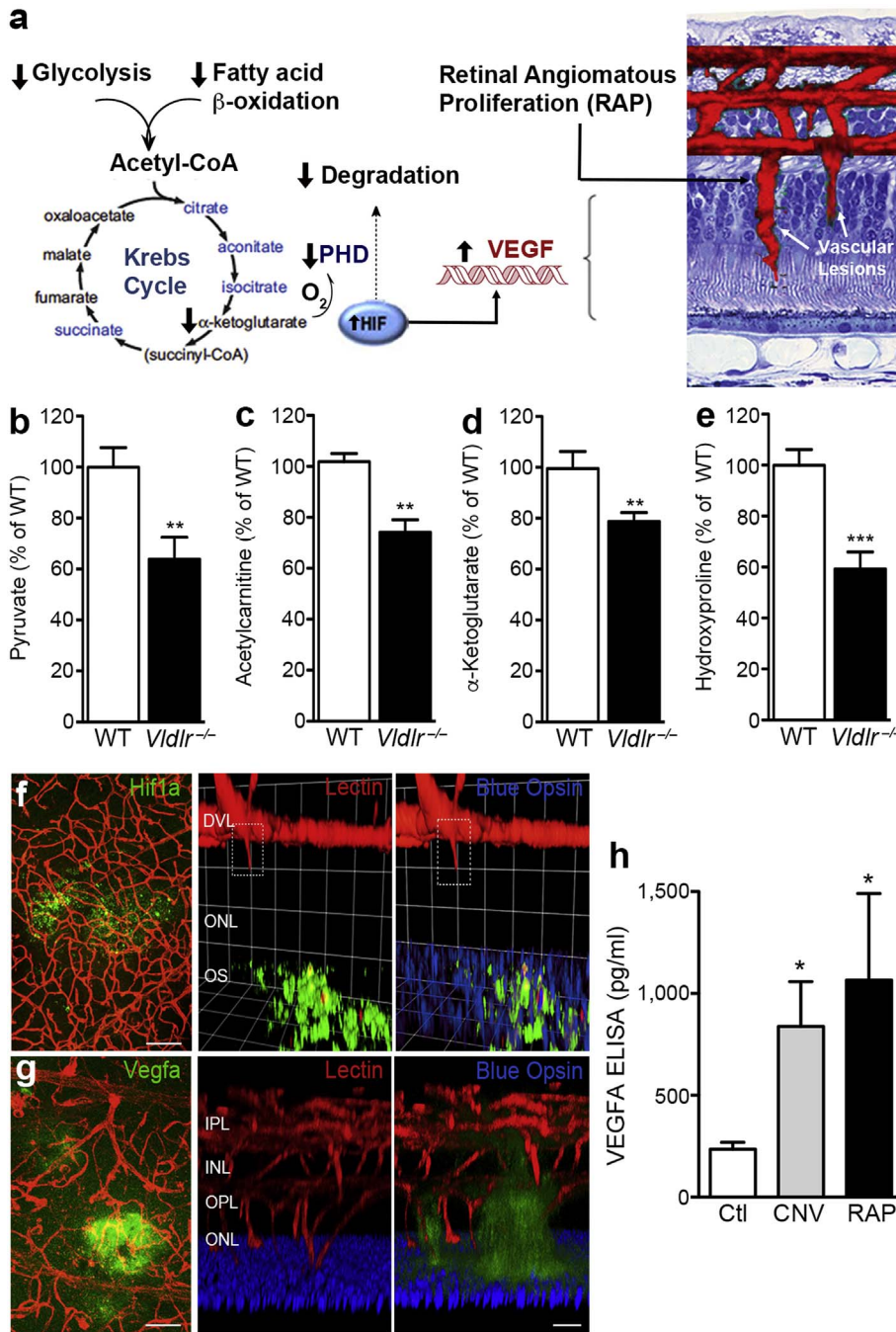
expression of the glucose transporter *Glut1* (Fig. 5c–f). Impaired glucose entry into photoreceptors results in a dual (lipid and glucose) fuel shortage and a reduction in the levels of the TCA cycle intermediate  $\alpha$ -ketoglutarate ( $\alpha$ -KG; Fig. 7a). Low  $\alpha$ -KG levels promote the stabilization of *Hif1a* and the secretion of *Vegfa* by starved *Vldlr*<sup>-/-</sup> photoreceptors, leading to neovascularization (Fig. 7b–g). We also confirmed the presence of high vitreous VEGFA levels in humans with AMD and RAP (Fig. 7h). Dysregulated lipid and glucose photoreceptor energy metabolism might be a driving force in neovascular AMD and other retinal diseases.

Mouse models used to investigate secondary proliferative retinopathies and AMD are described in Fig. 8.

## 16. Kinetics of photoreceptor degeneration and mitochondrial adaptation

Our mechanistic understanding of photoreceptor and neuronal decay due to bioenergetic failure and excess RONS would predict progressive damage accumulation, and accelerating cell death with aging (Clarke et al., 2000) (Fig. 9a). This conflicts with all investigated examples of inherited photoreceptor loss, where cell death kinetics is almost constant, and declines slightly in later stages of the disease (stretched exponential kinetics; Fig. 9b) (Clarke et al., 2000). Exponential decay of photoreceptors would, therefore, be proportional to the number of surviving photoreceptors, with a rate constant that differs according to disease severity. However, a decline in cell death rate as diseases progress may suggest a compensatory process. More importantly, the consistency of this paradoxical decay kinetics across many neurodegenerative disorders may suggest a universal mechanism of neuronal death and compensatory mechanisms. To reconcile the apparent discordance between mechanistic and kinetic data, it is argued that diverse mutations in photoreceptor functions have in common mitochondrial signaling, which would ultimately control neuronal survival. Hence, numerous cellular stressors would lead to a steady-state level of apoptotic and survival mitochondrial signals, reflecting the equilibrium between the cost of damage repair required for a neuron to survive versus the wasteful expenditure on a futile repair when survival is unlikely. Evolution of the biochemical functions of mitochondria in neurons is consistent with this theory of integrated damages and repairs leading to neuronal death or survival (Wright





**Fig. 7. Energy-deficient photoreceptors drive angiogenesis.** (a) Dual shortage of glucose (b, metabolized to pyruvate) and FA uptake reduces acetyl-CoA (c, estimated by measuring acetylcarnitine) and (d) TCA (Krebs) cycle intermediate  $\alpha$ -KG in *Vldlr*<sup>-/-</sup> retina (LC/MS/MS). Together with oxygen (O<sub>2</sub>),  $\alpha$ -KG is an essential co-activator of propyl-hydroxylase dehydrogenase (PHD) that tags HIF-1 $\alpha$  for degradation by proline hydroxylation (hydroxyproline). (e) Levels of hydroxyproline residues in WT and *Vldlr*<sup>-/-</sup> retinas measured by LC/MS/MS (n = 15 WT, 12 *Vldlr*<sup>-/-</sup> animal retinas, P = 0.0004). (f) Hif1a retinal expression in *Vldlr*<sup>-/-</sup> photoreceptor layer (P12 retinal flat mounts, Scale: 100  $\mu$ m; left: extended focus; middle and right panels: 3D confocal IHC, n = 3) where (g) Vegfa was then also secreted and localized (P16 retinal flat mounts, Scale: 100  $\mu$ m; left: extended focus; middle and right panels: 3D confocal IHC, n = 3 retinas). (h) Human subjects with AMD, either retinal angiomatous proliferation (RAP, n = 3) or choroidal neovascularization (CNV, n = 7) had higher VEGFA vitreous levels by ELISA compared to control subjects without pathologic neovessels (macular hole; n = 8). Results are presented as mean  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Figure modified, with permission, from (Joyal et al., 2016).

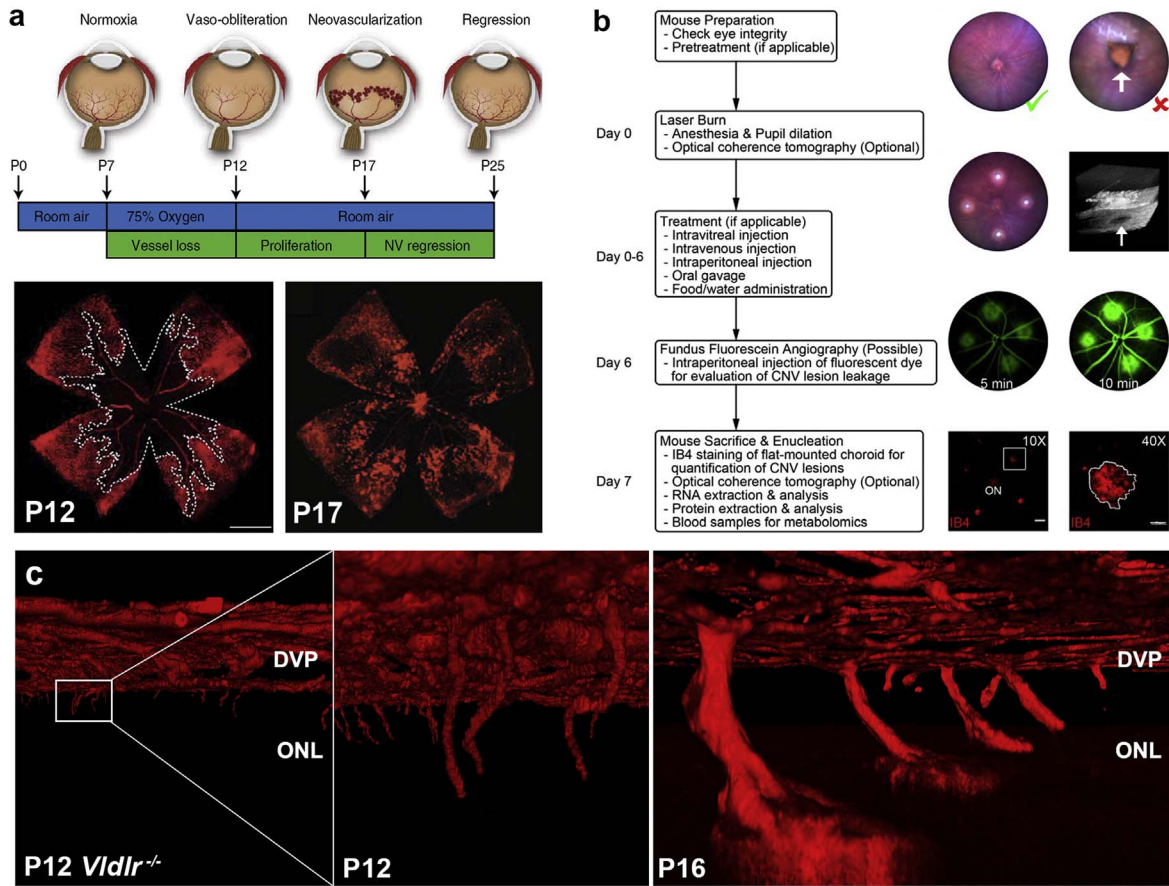
et al., 2010).

### 17. Conclusion and perspectives

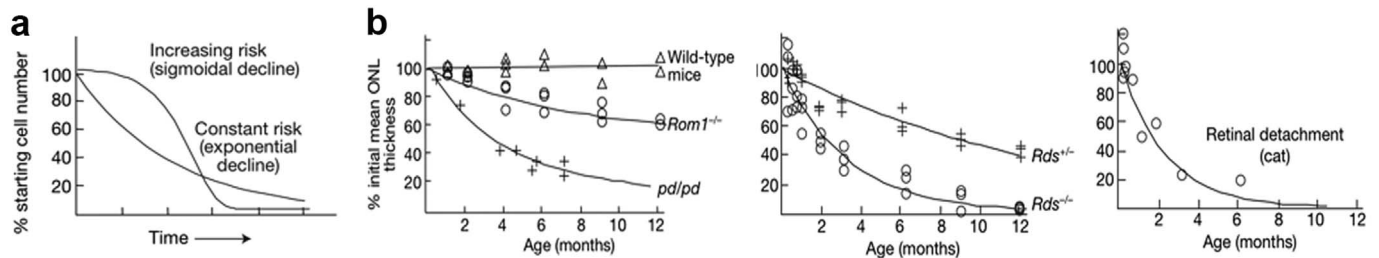
Vascular remodeling is intimately coupled to retinal energy metabolism. Surges in neuronal energy demands are met with vascular proliferation. Conversely, neuronal atrophy and lower retinal metabolic requirements result in vascular pruning. Vascular remodeling is, therefore, an early sign of neuronal metabolic changes. Classic evidence describes the importance of glucose as a primary retinal fuel, sustaining energy production and the biosynthesis of building blocks for growth through aerobic glycolysis. The prevailing assumption has been that glucose is the only fuel used by photoreceptors. However, a substantial proportion of retinal fuel for oxidation (OXPHOS) was shown not to originate from glucose, in work conducted half a century ago. We

recently showed that lipids, are used by photoreceptors as fuel for OXPHOS through fatty acid  $\beta$ -oxidation, and are an important energy source for the retina.

Nutrient availability and downstream Krebs cycle metabolites help govern the retinal vascular supply, ensuring adequate neuronal energy homeostasis. Mitochondrial disorders, inherited or acquired, tilt this precisely regulated energy balance often resulting in retinal vascular changes and neuronal loss. Photoreceptors and retinal ganglion cells are uniquely susceptible to bioenergetic dysfunction because of their considerable metabolic requirements. Many important questions arise from the variable penetrance of mitochondrial disorders in the eye, such as their regional susceptibility, their delayed onset, and the gender-specific and environmental triggers that initiate disease. More importantly, the complex compensatory mitochondrial adaptation that almost universally delays neuronal demise with disease progression is ill



**Fig. 8. Experimental models of pathological angiogenesis.** (a) Oxygen-induced retinopathy: the mouse model of proliferative retinopathy. Neonatal mice are exposed to 75% oxygen from P7 to P12, which induces loss of immature retinal vessels, leading to a central zone of vaso-obliteration (VO). Mice are returned to room air at P12, and the central avascular retina becomes hypoxic, triggering both vascular regrowth and the formation of pathologic neovascular (NV) tufts culminating at P17. (b) Laser-induced choroidal neovascularization (CNV) model. Detailed flowchart of the procedure to obtain consistent laser-induced CNV lesions. Flat-mounted choroid vessels are stained with IB4 at day 7 after laser photocoagulation. 10 × Scale bar: 200 μm, 40 × Scale bar: 50 μm. ON, optic nerve. (c) Mice model of retinal angiomatous proliferation (RAP): *Vldlr*<sup>-/-</sup> mice. Mice deficient for *Vldlr* spontaneously develop pathological RAP-like vascular lesions invading the photoreceptor layer at P12 and reaching the RPE at P16. Figure modified, with permission, from (Connor et al., 2009; Gong et al., 2015; Joyal et al., 2016).



**Fig. 9. Constant risk of photoreceptor death and survival.** (a) Irrespective of the underlying cause, the risk of neuronal death appears constant, manifesting as an exponential decline in cell number. If the risk of neuronal death were increasing, as presumed with mitochondrial diseases and the accumulation of free radicals over time, one would observe a sigmoidal decline in cell number, which is rarely reported. Compensatory pathways must, therefore, exist to palliate for neuronal death. (b) Examples of the exponential decline of photoreceptor degeneration. Wild-type and *Rom1*<sup>-/-</sup> mice, and photoreceptor dysplasia (*pd/pd*) in miniature schnauzers (left). Retinal degeneration of heterozygous (*Rds*<sup>+/-</sup>) and homozygous mice (*Rds*<sup>-/-</sup>) carrying a null mutation in the gene encoding peripherin/*rds* (middle). Experimental retinal detachment in the cat (right). Figure modified, with permission, from (Clarke et al., 2000).

understood. Therapeutic strategies that harness these inherent protective mechanisms may help prevent neuronal death and pathological angiogenesis, delaying vision loss.

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