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REVIEW ARTICLE

Oxysterols and retinal degeneration

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Retinal degeneration, characterised by the progressive death of retinal neurons, is the most common cause of visual impairment. Oxysterols are the cholesterol derivatives produced via enzymatic and/or free radical oxidation that regulate cholesterol homeostasis in the retina. Preclinical and clinical studies have suggested a connection between oxysterols and retinal degeneration. Here, we summarise early and recent work related to retina oxysterol-producing enzymes and the distribution of oxysterols in the retina. We examine the impact of loss of oxysterol-producing enzymes on retinal pathology and explore the molecular mechanisms associated with the toxic or protective roles of individual oxysterols in different types of retinal degeneration. We conclude that increased efforts to better understand the oxysterol-associated pathophysiology will help in the development of effective retinal degeneration therapies.

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KEYWORDS
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1 | INTRODUCTION

Oxysterols are a group of oxygenated products from cholesterol or its precursors (Guillemot-Legris et al., 2016). A single oxysterol can be produced by an enzyme-mediated reaction, a nonenzymatic route or from a combination of both processes (Figure 1). A sterol ring of cholesterol is oxidised by nonenzymatic routes including free radical and nonradical modification (Murphy & Johnson, 2008), and endogenous oxysterols such as 7-ketocholesterol (7-KC, abbreviations and nomenclature listed in Table S1) and 7β-hydroxycholesterol (7β-HC), which are also predominantly produced via nonenzymatic routes. A side chain of cholesterol is oxidised through enzymatic routes, typically catalysed by cytochrome P450 (CYP) enzymes. The CYP family members, including **CYP3A4**, **CYP3A5**, **CYP7A1**, **CYP7B1**, **CYP11A1**, **CYP27A1**, **CYP39A1** and **CYP46A1**, are well-characterised monooxygenases in oxysterol metabolism. CYP11A1 and CYP27A1

Abbreviations: 20R,22R-diHC, 20R,22R-dihydroxycholesterol; 22R-HC, 22(R)-hydroxycholesterol; 24S,25-EC, 24(S),25-epoxycholesterol; 24S-HC, 24(S)-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; 27-COOH, 3β-hydroxy-5-cholestenoic acid; 27-HC, 27-hydroxycholesterol; 4β-HC, 4β-hydroxycholesterol; 7-DHC, 7-dehydrocholesterol; 7-KC, 7-ketocholesterol; 7α,24S-diHC, 7α,24(S)-dihydroxycholesterol; 7α,25-diHC, 7α,25-dihydroxycholesterol; 7α,27-diHC, 7α,27-dihydroxycholesterol; 7α/β-HC, 7α/β-hydroxycholesterol; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; ABCA1, ATP-binding cassette subfamily A member 1; ABCG1, ATP-binding cassette subfamily G member 1; ABCG4, ATP-binding cassette subfamily G member 4; CYP, cytochrome P450; DHCR7, 7-dehydrocholesterol reductase; LDLR, low-density lipoprotein receptor; oxLDL, oxidised LDL; SCAP, sterol regulatory element binding proteins cleavage-activating protein; SREBP, sterol regulatory element-binding protein.

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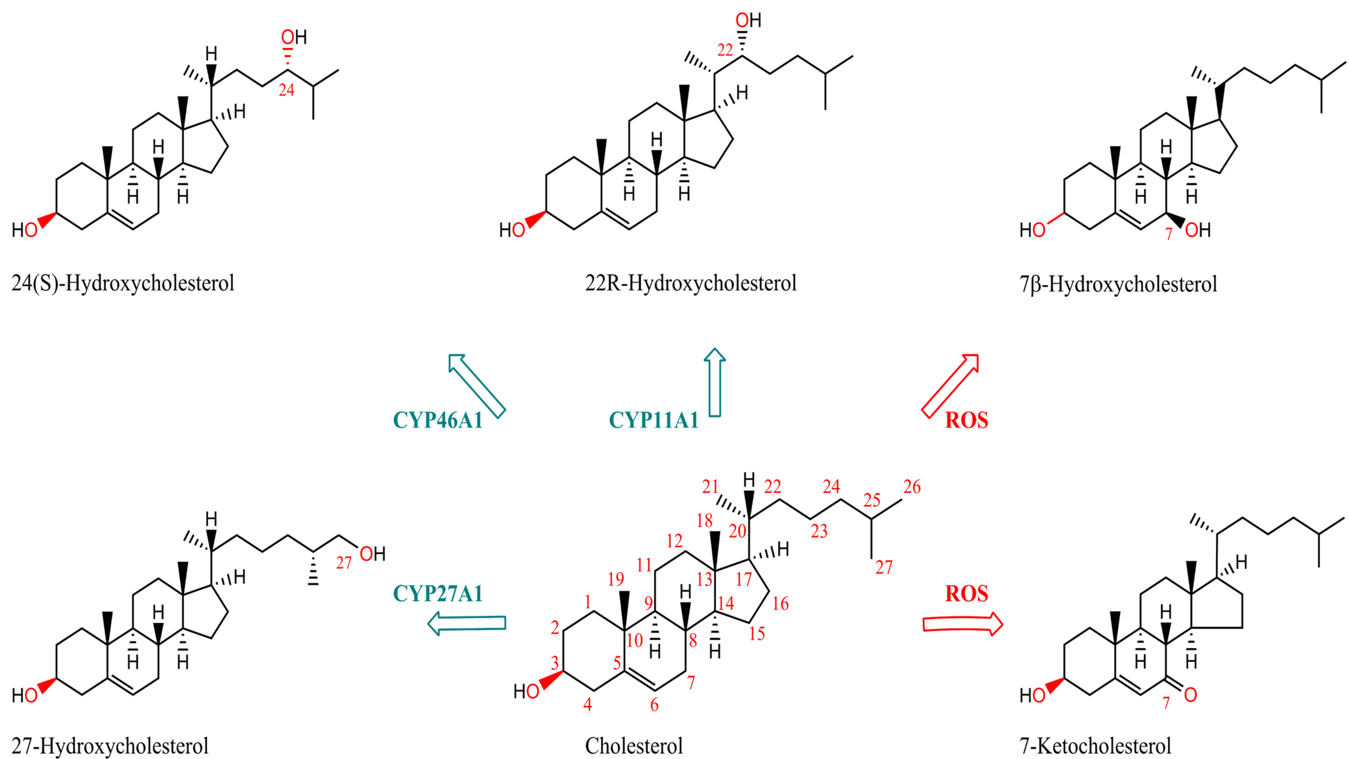

FIGURE 1 Structure of oxysterols generated in retina and retinal pigment epithelial (RPE) cells. The main oxysterols that are oxidised from cholesterol or its precursors in either enzymatic or non-enzymatic way. The enzymatic oxygenation reactions are mainly mediated by cytochrome P-450 family (green arrows). The nonenzymatic oxysterol-generating reactions are catalysed by reactive oxygen species (ROS) (red arrow)

are localised to mitochondria, while the other CYP members are localised to endoplasmic reticulum. CYP3A4 coverts cholesterol to 4β-hydroxycholesterol (4β-HC) and 25-hydroxycholesterol (25-HC), while CYP3A5 converts cholesterol only to 4β-HC (Bodin et al., 2002); CYP7A1 catabolises cholesterol to 7α-hydroxycholesterol (7α-HC) and catabolises 7-dehydrocholesterol (7-DHC) to 7-KC, while CYP7B1 oxidises 25-HC and **27-hydroxycholesterol (27-HC)** to, respectively, 7α,25-dihydroxycholesterol (7α,25-diHC) and **7α,27-dihydroxycholesterol** (7α,27-diHC); CYP11A1 metabolises cholesterol to 22(R)-hydroxycholesterol (22R-HC) and from there to 20R,22R-dihydroxycholesterol (20R,22R-diHC) then to **pregnenolone**; CYP27A1 converts cholesterol to 25-HC or to 27-HC (predominant); CYP46A1 oxidises cholesterol to 24(S)-hydroxycholesterol (24S-HC), which can be metabolised to 7α,24(S)-dihydroxycholesterol (7α,24S-diHC) by CYP39A1. Some CYP enzymes can also oxidise cholesterol precursors to form oxysterols, such as CYP46A1 that metabolises cholesterol precursor, **desmosterol**, to 24(S),25-epoxycholesterol (24S,25-EC). Enzymatically and nonenzymatically formed oxysterols can be transported to the liver where they are further converted to bile acids, which facilitates the elimination of excess cholesterol from extrahepatic tissues. Oxysterols play an important role in a diverse range of cellular functions and are involved in cancer, metabolic disorders and neurodegenerative diseases (Griffiths & Wang, 2019; Guillemot-Legris et al., 2016).

Retinal degeneration occurs in a group of retinopathies that are characterised by the progressive death of retinal neurons. There are different types of retinal degeneration, mainly classified either as inherited or complex retinal disorders. Inherited retinal degeneration, such as retinitis pigmentosa, is caused by genetic defects, primarily affecting photoreceptor cells, whereas complex retinal degeneration involves a combination of genetic and environmental risk factors and includes conditions such as diabetic retinopathy, age-related macular degeneration (AMD) and glaucoma. Cholesterol is enriched in the neural retina and in retinal pigment epithelial (RPE) cells and plays a critical role in maintaining retinal function (Pikuleva & Curcio, 2014). Major oxysterol-producing enzymes have been reported to be expressed in the retina and various oxysterols have been shown to be distributed in the retina (Fliesler & Bretillon, 2010; Pikuleva & Curcio, 2014). In this review, we will discuss oxysterol metabolism in the retina and its function in retinal degeneration.

2 | RETINAL STRUCTURE AND FUNCTION

The human retina is a 0.5-mm-thick circular disc with a diameter of about 30–40 mm that is positioned at the back of the eye. The retina is a highly organised light-sensitive structure which consists of a complex network of sensory neurons and associated cells which are tightly packed together (Figure 2a). The photoreceptor cells are the initial light detecting cells and their capability to detect light is dependent on their interaction with the retinal pigment epithelial cells.

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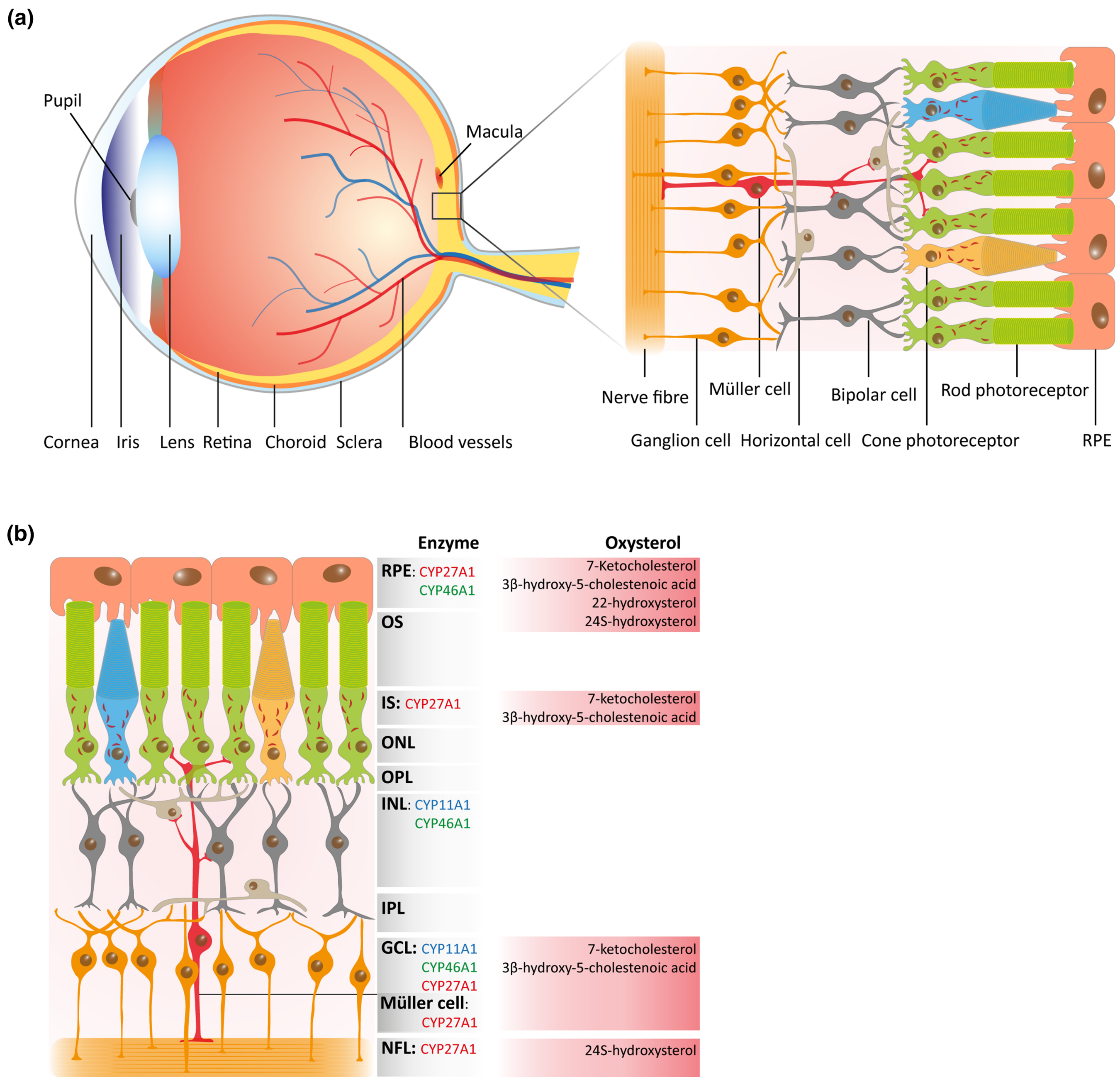

FIGURE 2 Retinal structure and localisation of oxysterols and major oxysterol-metabolism enzymes. (a) Structure of human eye and retina. Left panel: human retina (yellow) is located on the rear hemisphere of the eyeball. From outermost to innermost, the layers are the sclera, the choroid, and the retina. The macula is an oval pigmented area located at the central retina where the highest concentration of cones is found. Retinal blood vessels enter the retina in conjunction with the optic nerve, with branches distributing in the choroid layer. The cornea is a transparent layer at the front of the eye, lying anterior to the iris and pupil. Right panel: human retina consists of different types of cells: retinal pigment epithelium (RPE) cells, rod photoreceptors, cone photoreceptors, bipolar cells, horizontal cells, amacrine cells, ganglion cells, and Müller cells. They form several layers of the retina (from outermost to innermost): outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fibre layer (NFL). Müller cells span the entire retina. (b) Distribution of oxysterol-producing enzymes and presentation of main oxysterols in the retina. IS, inner segments; OS, outer segments

As shown in Figure 2a, the retinal pigment epithelial in the vertebrate eye lies between the outer segments of the photoreceptors and the choroidal blood supply. It is composed of a single layer of tightly packed columnar hexagonal epithelial cells containing pigment granules (melanin) and other organelles involved in the digestion of photoreceptor outer segment membranes into phagosomes (Hu et al., 2008; Strauss, 2005). The melanosomes containing the pigment melanin are involved in the absorption of the light energy that strikes the retina. The retinal pigment epithelial is aligned along Bruch's membrane through its basolateral membrane. Bruch's membrane provides a matrix essential for the interaction of the retinal pigment epithelial with blood flow in the capillaries of the choroid. The retinal pigment epithelial

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therefore forms a tight-junction epithelium between the blood flow of the choroid and the outer photoreceptor segments, forming the outer blood–retinal barrier that enables selective permeability between the choroid and neurosensory retina (Steinberg, 1985; Strauss, 2005). This barrier is vital for the highly selective bidirectional processes involving the transport of nutrients from the blood to the photoreceptors (choroid to retina) and for the movement of water and metabolites from the retinal side to the blood (retina to choroid) (Strauss, 2005). The pigments in the retinal pigment epithelial absorb light and reduce its scattering, thereby improving image resolution and helping to protect the retina from any large influx of light. The retinal pigment epithelial cells act as storage reservoirs of metabolites and vitamins and are critical for maintenance of retinal adhesion (Katz & Gao, 1995).

The retina is composed of at least six types of neural cells (rods, cones, bipolar, horizontal, amacrine and ganglion cells) and one type of glial cell (Müller glia) derived from the neural ectoderm. These cells are compactly arranged in several layers including the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fibre layer (NFL) (Figure 2a,b) (Purves et al., 2001). The human retina comprises over 110 million rods and over 5 million cones (Remington, 2012) and these two types of photoreceptors are arranged in parallel fashion in the photoreceptor layer of the retina. Cones are responsible for photopic (bright illumination) central vision while the rods are responsible for scotopic (dim illumination) peripheral vision. Although rods outnumber cones by ~20-fold, cones are crucial for good visual acuity and colour discrimination. Human rods are highly sensitive, possessing the capacity to detect single photons, whereas each cone requires receipt of more than 100 photons to generate a comparable response (Mustafi et al., 2009). Phototransduction, in which a photon of light is changed to an electrical signal, takes place in the outer segments of the rod and cone photoreceptors. The electrical signals created by these retinal cells are then transferred via bipolar cells to the ganglion cells that convey the information to the brain through the optic nerve, resulting in the experience of vision (Remington, 2012). There is a striking difference in the circuitry associated with rod and cone photoreceptors, specifically in the mode of transmission of signals from rods and cones to ganglion cells. Typically, a single cone cell communicates with a single bipolar cell that in turn transmits information to a single ganglion cell, whereas several rod cells can communicate with one bipolar cell, with several of the latter then feeding into a single ganglion cell. This convergence of signals by rods helps them to pool small signals in dim light, while the one-to-one relationship between cones–bipolar–ganglion cells maximises visual acuity (Mustafi et al., 2009; Solomon & Lennie, 2007).

3 | CHOLESTEROL HOMEOSTASIS IN THE RETINA

The retina has a well-regulated cholesterol input and output that maintains cholesterol homeostasis (Fliesler & Bretillon, 2010). The retina can directly take up cholesterol from the systemic circulation predominantly through a low-density lipoprotein receptor (LDLR)-mediated process in the retinal pigment epithelial, which has LDLR localised to the basolateral side, facing the choroid (Tserentsoodol et al., 2006). Local cholesterol synthesis also contributes to cholesterol input in the retina. Lin et al. (2016) demonstrated that *in situ* biosynthesis of cholesterol accounted for 72% of the cholesterol input in mouse retina. Excess cholesterol in the retina is either removed via the reverse cholesterol transport and/or metabolised to oxysterols by CYP enzymes (Fliesler & Bretillon, 2010); however, quantitative contributions of the reverse cholesterol transport and oxysterol formation to the removal of excess cholesterol in the retina remain elusive. Cholesterol efflux is the first step of the reverse cholesterol transport pathway, which is mediated by acceptors (HDL and apolipoproteins) and **ABC transporters** (ATP-binding cassette subfamily A member 1 (**ABCA1**), ATP-binding cassette subfamily G member 1 (**ABCG1**) and **ABCG4**. Recent work from our laboratory and elsewhere has demonstrated effective cholesterol efflux in retinal pigment epithelial and choroidal endothelial cells (Biswas et al., 2017; Lyssenko et al., 2018; Storti et al., 2017).

Proteins associated with regulation of cholesterol homeostasis (sterol regulatory element-binding protein (SREBP); sterol regulatory element binding proteins cleavage-activating protein (SCAP); insulin-induced genes (INSIGs); liver X receptor α and β, **LXR-α**/NR1H3 and **LXR-β**/NR1H)) are expressed in the retina (Zheng et al., 2012). SREBP-2 is the major regulator of cholesterol homeostasis. When intracellular cholesterol is at a low level, SCAP escorts SREBP-2 from endoplasmic reticulum to the Golgi apparatus, where SREBP-2 is cleaved by two proteases, resulting in the release of the amino-terminal of transcription factor functional domain and upregulation of cholesterol synthesis and uptake associated genes. When cholesterol level is high, cholesterol binds to SCAP and triggers SCAP physically interacting with INSIGs, leading to retaining SCAP in the endoplasmic reticulum and blocking SREBP-2 associated transcriptional activation (DeBose-Boyd et al., 1999; Hua et al., 1993; Yang et al., 2002). Furthermore, oxysterols inhibit cholesterol synthesis by binding to INSIGs and preventing SREBP-2 processing (Adams et al., 2004; Radhakrishnan et al., 2007). Additionally, oxysterols can regulate cholesterol homeostasis by activating the liver X receptor signalling pathway and enhancing cholesterol efflux (Fu et al., 2001; Janowski et al., 1999; Lehmann et al., 1997).

4 | OXYTEROLS IN THE RETINA

4.1 | Expression of major oxysterol-producing enzymes in the retina

Some oxysterol-producing enzymes, such as the CYP family members, have been shown to express in the retina (Figure 2b). Guarneri et al. (1994) first reported that CYP11A1 was localised to ganglion cells and cells of the inner nuclear layer of rat retina. CYP11A was also shown to be similarly localised in hamster retina, although here its

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expression in the cells of the inner nuclear layer was higher than that in rat retina (Jaliffa et al., 2005). Lee et al. (2006) examined expression of CYP27A1 in monkey retina and found that CYP27A1 was strongly expressed in photoreceptor inner segments and was also expressed at low levels in the retinal pigment epithelial, choroid, ganglion cells, nerve fibres and Müller cells. CYP46A1 had higher mRNA levels in the neural retina and low levels in the retinal pigment epithelial in bovine samples (Bretillon et al., 2007). CYP46A1 was strongly localised to ganglion cells and was also present in the inner nuclear layer of rat retina (Bretillon et al., 2007; Ishikawa et al., 2016); CYP46A1 also had similar localisation in mouse retina (Ramirez et al., 2008). While CYP27A1 and CYP46A1 were also expressed in the human retinal pigment epithelial cell line, ARPE-19 (Biswas et al., 2017) and monkey endothelial cell line, RF/6A (Biswas et al., 2018). In the human retina, mRNA of *CYP11A*, *CYP27A1* and *CYP46A1* has been detected in the neural retina and retinal pigment epithelial. CYP11A was localised to ganglion cells and the cells of the inner nuclear layer in both human and rodent retinas. CYP11A1 was also expressed in Bruch's membrane, the retinal pigment epithelial and photoreceptor inner segments in human retina. CYP27A1 was predominantly localised to Bruch's membrane, retinal pigment epithelial, photoreceptor inner segments, ganglion cells and nerve fibre layer. CYP46A1 had a localisation in human retina similar to that of CYP27A1, although was expressed at a lower level (Zheng et al., 2012). Early work in the same lab demonstrated that CYP27A1 was expressed at a higher level in human neural retina (403–510 fmol·mg^{-1} protein) than was CYP46A1 (58–63 fmol·mg^{-1} protein), while CYP27A1 was also expressed in human retinal pigment epithelial at 1110–2060 fmol·mg^{-1} protein, about 2.7- to 4.0-fold higher than in the neural retina (Heo et al., 2011; Liao et al., 2011).

4.2 | Distribution of oxysterol in the retina

Based on the expression of oxysterol-producing genes (discussed above) and high level of oxidative stress (Wright et al., 2010), oxysterols in the retina are generated via enzymatic and radical reactions in a way similar to that seen in other tissues. A number of studies have examined oxysterols in the retinas of different species and found that oxysterols are present in the retinal pigment epithelial, photoreceptor inner segments, ganglion cells and nerve fibre layer (Moreira et al., 2009; Rodriguez et al., 2014; Rodriguez & Fliesler, 2009) (Figure 2b). Moreira et al. (2009) examined oxysterols in primate retinas using high-pressure liquid chromatography–mass spectrometry and immunohistochemistry. 7-KC, which is predominantly generated by non-enzymatical oxidisation (Figure 1 and Anderson et al., 2020), was present in the neural retina at 1–1.5 pmol·nmol^{-1} free cholesterol and in retinal pigment epithelial/choroid at 5–8 pmol·nmol^{-1} free cholesterol. Immunostaining showed that 7-KC was localised to monkey choriocapillaris, retinal pigment epithelial and Bruch's membrane (Moreira et al., 2009). Rodriguez and Fliesler (2009) investigated the effect of photodamage on generation of the oxysterols 7-KC and 7α/β-hydroxycholesterol (7α/β-HC) in rat neural retina. They reported that light-treated rats had an approximately six fold increase in 7-KC (control rats: 1.6 pmol·nmol^{-1}; treated rats: 12.7 pmol·nmol^{-1} cholesterol) and a 50-fold increase in 7α/β-HC (control rats: trace only; treated rats: 25.0 pmol·nmol^{-1} cholesterol), indicating increased production; further immunohistochemistry showed that the increased 7-KC was localised to the retinal pigment epithelial, photoreceptor inner segments and ganglion cells of photodamaged retinas (Rodriguez & Fliesler, 2009). In aged mice, a significantly high level of 7-KC was accumulated beneath the retinal pigment epithelial (Indaram et al., 2015). 7-KC was also examined in bovine retina and was found to be lower in the neural retina (25 pmol·mg^{-1} protein) than in the retinal pigment epithelial (41 pmol·mg^{-1} protein); a further two 7-KC-derived oxysterols, 3β,27-dihydroxy-5-cholesten-7-one (7-KCh-27OH) and 3-hydroxy-5-cholesten-7-one-26-oic acid (7-KCh-27COOH), were detected at low levels in bovine retinal pigment epithelial (7-KCh-27OH: 1 pmol·mg^{-1} protein; 7-KCh-27COOH: not quantified) but not in the neural retina (Heo et al., 2011). Measurement of 7-KC in monkey neural retina and retinal pigment epithelial/choroid demonstrated that 7-KC level was increased during ageing and was three to four times higher in the retinal pigment epithelial/choroid (1–68 pmol·nmol^{-1} cholesterol) than in the neural retina (1–18 pmol·nmol^{-1} cholesterol). Similarly, 7-KC in aged human retinal pigment epithelial (2483.8 pmol·nmol^{-1} cholesterol) was around 150 times higher than that in the neural retina (16.5 pmol·nmol^{-1} cholesterol) (Rodriguez et al., 2014). Drusen, cholesterol- and lipid-rich extracellular deposits underneath the retinal pigment epithelial, also contain an extremely high level of 7-KC (734.9 pmol·nmol^{-1} cholesterol) (Curcio et al., 2005; Rodriguez et al., 2014). However, 7-KC in human plasma is unaffected by ageing and is not associated with age-related macular degeneration, the leading cause of blindness in aged population (Lin et al., 2018), suggesting increased in situ synthesis of 7-KC in neural retina and retinal pigment epithelial with ageing.

Using gas chromatography–mass spectrometry, Mast et al. (2011) examined the CYP enzymes-mediated oxysterol production, 24(S)-hydroxycholesterol (24S-HC), produced by CYP46A1); 27-hydroxycholesterol (27-HC) and 3β-hydroxy-5-cholestenoic acid (27-COOH) (produced by CYP27A1); 22(R)-hydroxycholesterol (22R-HC) and pregnenolone (produced by CYP11A1) in bovine and human retinas; they found abundant cholesterol (total) in the bovine neural retina (158 nmol·mg^{-1} protein) and retinal pigment epithelial (136 nmol·mg^{-1} protein) but detected very low levels of oxysterols, including 24S-HC (total) at 0.036 nmol·mg^{-1} protein in retina and at 0.021 nmol·mg^{-1} protein in the retinal pigment epithelial, 22R-HC (total) at 0.007 nmol·mg^{-1} protein in neural retina and at 0.010 nmol·mg^{-1} protein in retinal pigment epithelial, and pregnenolone (total) at 0.019 nmol·mg^{-1} protein in the neural retina and at 0.029 nmol·mg^{-1} protein in the retinal pigment epithelial. However, 27-HC was undetectable in both the bovine neural retina and retinal pigment epithelial; 27-HC oxidised 27-COOH (free) was present in both neural retina (0.025 nmol·mg^{-1} protein) and retinal pigment epithelial (0.09 nmol·mg^{-1} protein), suggesting that when 27-HC is generated by CYP27A1, it might be immediately oxidised to

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27-COOH by the same enzyme. In the bovine choroid, only 24S-HC (total) and 22R-HC (total) were detected at, respectively, 0.018 nmol·mg^{-1} protein and 0.001 nmol·mg^{-1} protein. Furthermore, significantly higher amounts of 27-COOH (free) was found in human neural retina (0.037–0.125 nmol·mg^{-1} protein) than in the retinal pigment epithelial (0.002–0.01 nmol·mg^{-1} protein), while free 24S-HC and pregnenolone were detected at lower levels (0.001–0.004 nmol·mg^{-1} protein for both) in the neural retina but was not detected at all in the retinal pigment epithelial; free 22R-HC and 27-HC also were undetectable in both the neural retina and retinal pigment epithelial (Liao et al., 2011; Mast et al., 2011).

5 | LOSS OF OXYSTEROL-PRODUCING ENZYMES IS ASSOCIATED RETINAL PATHOLOGY

5.1 | CYP27A1 and CYP46A1

Mutations in the *CYP27A1* gene cause cerebrotendinous xanthomatosis (CTX), which is a rare autosomal recessive metabolic disease that has systemic and neurological symptoms (Cruysberg et al., 1995; Dotti et al., 2001) and involves a variety of ocular abnormalities, including cataract, cholesterol-like crystals in the vitreous humour, premature retinal senescence, retinal vessel sclerosis, optic disk paleness, drusen and retinal pigment epithelial defects (Cruysberg et al., 1995; Dotti et al., 2001; Morgan et al., 1989). *Cyp27a1*$^{-/-}$ mice have been extensively characterised. Such mice do not exhibit the classic CTX symptoms such as cataract, xanthomas in tendon and brain, and atherosclerosis (Honda et al., 2001; Repa et al., 2000; Rosen et al., 1998). However, retinal pathology in *Cyp27a1*$^{-/-}$ mice was first characterised in Dr. Pikuleva's laboratory (Omarova et al., 2012). *Cyp27a1*$^{-/-}$ mice demonstrated defects in retinal cholesterol homeostasis and developed cholesterol deposits beneath the retinal pigment epithelial, neovascularisation and Müller cell activation (Omarova et al., 2012).

Cyp46a1$^{-/-}$ mice demonstrated a greater than 40% reduction in cholesterol turnover in the brain but had a similar level of hepatic cholesterol and bile acid synthesis (Lund et al., 2003). Saadane et al. (2019) examined the systemic and retinal cholesterol metabolism and retinal phenotypes. The systemic cholesterol metabolism of the *Cyp46a1*$^{-/-}$ mice did not change significantly but did exhibit dysregulation of retinal cholesterol homeostasis. Vision function testing found no differences in either scotopic (rod function) or photopic (cone function) electroretinogram between wildtype and *Cyp46a1* deleted mice; however, *Cyp46a1*$^{-/-}$ mice demonstrated venous beading, tortuosity, increased vascular permeability and microglial activation in the retina (Saadane et al., 2019). Since CYP27A1 and CYP46A1 are the main enzymes for cholesterol metabolism in the retina, the same group also examined retinal pathology of *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$ mice and found that these animals had significantly higher levels of retinal cholesterol compared to *Cyp27a1*$^{-/-}$ or *Cyp46a1*$^{-/-}$ alone; the mutant animals also developed abnormal retinal vasculature and had macrophage/microglial activation and increased oxidative stress in the retina. Electroretinogram tests showed that both scotopic and photopic electroretinograms were significantly lower in male double knockout mice when compared to the wildtype male mice (Saadane et al., 2014). Sterol-O-acyltransferase 1 (SOAT1) is responsible for cholesterol esterification in the retina (Saadane et al., 2016), leading to production of cholesterol esters, which are a significant component of drusen (Curcio et al., 2005). Apolipoprotein E (APOE), a major apolipoprotein in the retina, regulates cholesterol homeostasis as an acceptor for cholesterol efflux or a ligand for LDL receptor (Mazzone & Reardon, 1994; Zhao & Mazzone, 1999). To further investigate the associated function of CYP27A1 and CYP46A1 with SOAT1 or APOE in cholesterol metabolism, Petrov, Lam, et al. (2019) generated *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$/*Soat1*$^{-/-}$ and *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$/*Apoe*$^{-/-}$ mice, whose retinal structure and vasculature were normal. *Cyp27a1*$^{-/-}$*Cyp46a1*$^{-/-}$/*Soat1*$^{-/-}$ mice were found to have a normal retinal cholesterol level similar to that of *Soat1*$^{-/-}$ mice, but the *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$ mice had a high level of retinal cholesterol, suggesting a possible compensatory response in cholesterol synthesis. However, *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$/*Apoe*$^{-/-}$ mice had a level of retinal cholesterol similar to that of *Apoe*$^{-/-}$ mice. All *Cyp27a1*$^{-/-}$*Cyp46a1*$^{-/-}$/*Soat1*$^{-/-}$ mice exhibited markedly decreased scotopic electroretinograms, with decreased photopic electroretinograms seen only in the male mice. *Soat1*$^{-/-}$ mice had similar trends of decreased electroretinograms to that of *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$/*Soat1*$^{-/-}$ mice, indicating that cholesterol esterification is required for retinal function. Only male *Cyp27a1*$^{-/-}$/*Cyp46a1*$^{-/-}$/*Soat1*$^{-/-}$ mice had a significant decrease in both scotopic and photopic electroretinograms (Petrov, Astafev, et al., 2019).

5.2 | 7-Dehydrocholesterol reductase (DHCR7)

7-Dehydrocholesterol reductase (DHCR7) is the final enzyme of the Kandustch–Russel pathway in the cholesterol biosynthesis pathway and converts 7-dehydrocholesterol (7-DHC) to cholesterol. Mutations of the *DHCR7* gene cause Smith–Lemli–Opitz syndrome (SLOS), which is an autosomal recessive metabolic disorder that is characterised by increased 7-DHC and reduced cholesterol in all tissues, malformation in multiple organs and mental retardation (Nowaczyk & Irons, 2012). Smith–Lemli–Opitz syndrome patients also present with a wide range of ocular abnormalities, such as sclerocornea, cataract, paleness of optic discs and optic atrophy (Elias et al., 2003; Garry et al., 2010; Kretzer et al., 1981). Pathological analysis of a 1-month-old Smith–Lemli–Opitz syndrome male patient's eye samples showed generally normal retinal structure, suggesting that loss of DHCR7 may not affect early retinal development; nevertheless, there was extensive death of ganglion cells, disintegration of mitochondria in the retinal pigment epithelial and accumulation of cytoplasmic material underneath photoreceptors (Kretzer et al., 1981). Smith–Lemli–Opitz syndrome children have been shown to have defects in rod and rod-derived functions, showing slow kinetics of phototransduction

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activation and deactivation, possibly due to dysfunction of membrane-associated phototransduction proteins (Elias et al., 2003). A follow-up study examined visual function by electroretinogram testing of the same patient cohort with long-term supplementation with dietary cholesterol. Compared to the controls, patients exhibited a significant decrease in the rod response but no difference in the cone response, suggesting that additional dietary cholesterol may protect cone function in Smith–Lemli–Opitz syndrome patients (Garry et al., 2010).

Retinal function of DHCR7 has been studied in rodent models. *Dhcr7* null mice have significantly increased 7DHC and markedly decreased cholesterol in serum and tissues. These mice also recapitulate some Smith–Lemli–Opitz syndrome phenotypes, such as retardation of intrauterine growth and craniofacial abnormality (Fitzky et al., 2001; Wassif et al., 2001). However, Dhcr7 mutant mice only survive 18–24 h after birth, limiting the application of the model for further phenotypic characterisation. An alternative Smith–Lemli–Opitz syndrome rat model has been developed by treating normal rats with trans-*N*,*N*-bis[2-chlorophenylmethyl]-1,4-cyclohexanedimethanamine dihydrochloride (AY9944), a selective inhibitor for DHCR7. AY9944-treated rats recapitulate Smith–Lemli–Opitz syndrome phenotypes (Kolf-Clauw et al., 1996). When pregnant rats were fed with AY9944 from gestational day 6 till postnatal day (P) 28 and their offspring also received AY9944 treatment from birth to P28, the pups at P28 exhibited abnormal accumulation of 7-DHC and markedly reduced cholesterol in serum, liver, retina and brain when compared to control animals, However there was no defect in retinal development or change in electroretinogram function. indicating that loss of DHCR7 may not affect retinal development (Fliesler et al., 1999). When AY9944 treatment of the progeny was extended from 4 to 10 weeks, the offspring demonstrated retinal degeneration with shorter outer segments, reduced thickness of outer nuclear layer and decreased electroretinogram amplitudes, along with abnormal ultrastructure of retinal pigment epithelial cells, when compared to the control animals (Fliesler et al., 2004).

Retinal degeneration in AY994-treated rodents is supposed to be caused by 7-DHC-derived oxysterols (Fliesler & Xu, 2018). 7-DHC is a strong reactive lipid molecule, sensitive to free radical oxidation, so generation of oxysterols from 7-DHC is predominantly via non-enzymatic oxidation but also via enzymatic oxidation (Fliesler & Xu, 2018; Xu et al., 2009). Xu et al. (2010) first identified 14 novel oxysterol species from 7-DHC via free radical oxidation and found the oxysterol mixture is toxic to Neuro2a cells at ≥10 μM possibly via inhibition of proliferation and induction of differentiation (Korade et al., 2010; Xu et al., 2010). 7-DHC-derived oxysterols have been identified and are accumulated in blood, liver, brain and retina of AY9944-exposed rats. Particular 7-KC in the AY9944-treated retina is more than 30 times higher compared to that of the untreated controls (Xu et al., 2011, 2012). Intravitreal injection of 7-KC caused pan-retinal degeneration in normal rats, indicating the toxicity of 7-KC (Xu et al., 2012). Further *in vitro* experiments demonstrated that 7-DHC-derived oxysterols, including 7-KC, caused toxicity to mouse cone, monkey retinal pigment epithelial and rat Müller glial cells (Pfeffer et al., 2016). The cause of retinal degeneration by 7-DHC-derived oxysterols in AY9944-treated rats must at least be partially due to oxidative damage, given that co-treatment with cholesterol and antioxidants is more effective in protecting against retinal degeneration in AY9944-treated rats than treatment of rats with cholesterol alone (Fliesler et al., 2018).

6 | OXYSTEROLS AND AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration is a chronic disease that affects the macula, the central cone dominated feature of the retina (Mitchell et al., 2018). The disease affects an ageing demographic and inevitably causes a loss of central vision due to growing abnormalities within the macula. Age-related macular degeneration is one of the leading causes of visual loss in the developed world, particularly in those of Caucasian ethnicity (Mitchell et al., 2018). This degenerative condition is becoming increasingly prevalent due to an ageing population with predictions suggesting that age-related macular degeneration will double in frequency over the next two decades (Robman et al., 2017). Age-related macular degeneration is a complex, multifactorial disease process. Ageing is known to be the single most significant risk factor, but both genetic and environmental factors also contribute significantly to age-related macular degeneration aetiology. When age-related macular degeneration has progressed to its late stage, it is classified into two major forms, dry (geographic atrophy) and wet (choroidal neovascularisation, CNV). The wet type is the significantly less common (about 10%) of the two, but its progression is fast and severe, accounting for 90% registration of legal blindness caused by age-related macular degeneration. While there is no effective treatment for dry age-related macular degeneration, anti-vascular endothelial growth factor (VEGF) therapy is effective in wet age-related macular degeneration, causing regression of choroidal neovascularisation and preservation of visual function (Mitchell et al., 2018).

The clinical hallmark of age-related macular degeneration is abnormal accumulation beneath the retinal pigment epithelial of extracellular deposits (drusen) and basal linear deposits (BLinD) (Pikuleva & Curcio, 2014). Histopathological analyses has shown that cholesterol is enriched in these deposits, suggesting that defective reverse cholesterol transport may play an important role in the pathogenesis of age-related macular degeneration (Curcio et al., 2001, 2005; Wang et al., 2010). Retinal pigment epithelial cells progressively degenerate in age-related macular degeneration, leading to the death of photoreceptors in the macula. The retinal pigment epithelial cells are responsible for cholesterol homeostasis in the retina by supplying cholesterol to photoreceptor cells and by clearing excess cholesterol into the choriocapillaris via the reverse cholesterol transport (Biswas et al., 2017; Lyssenko et al., 2018; Storti et al., 2017). Oxysterol-producing genes are abundantly expressed in the retinal pigment epithelial (Heo et al., 2011; Liao et al., 2011; Zheng et al., 2012), which is exposed to a high level of oxidative stress (Datta et al., 2017).

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Oxidative stress is proposed to induce cholesterol oxidation, so oxysterols may be actively produced in the retinal pigment epithelial via enzymatic and free radical reactions. In fact, oxysterol species have been detected and localised to the retinal pigment epithelial (Figure 2b) and there may be a functional role for 7-KC, 24S-HC and 27-HC in age-related macular degeneration.

The extremely high levels of 7-KC in the aged retinal pigment epithelial and drusen, coupled with an observed increase of 7-KC in the retinal pigment epithelial as a result of photodamage, support the notion of a link between 7-KC and age-related macular degeneration (Moreira et al., 2009; Rodriguez et al., 2014; Rodriguez & Fliesler, 2009). Early work showed that 7-KC treatment decreased cell viability, induced cell death and increased reactive oxygen species (ROS) production in human retinal pigment epithelial cells (Dugas et al., 2010; Olivier et al., 2016; Ong et al., 2003; Rodriguez et al., 2004a, 2004b) and in porcine retinal pigment epithelial cells (Joffre et al., 2007). 7-KC is the most abundant oxysterol in oxidised LDL (ox-LDL) (Brown et al., 1996; Oh et al., 2016). Ox-LDL induces inflammation in the retinal pigment epithelial and choroidal endothelial cells (Biswas et al., 2017, 2018). 7-KC treatment also induced production of inflammatory cytokines including **IL-1β**, **Il-6**, **IL-8 (CXCL8)**, **IL-18**, and **TNF-α** in retinal pigment epithelial cells (Dugas et al., 2010; Huang et al., 2012; Joffre et al., 2007; Larrayoz et al., 2010; Moreira et al., 2009; Shi et al., 2015; Yang et al., 2019), possibly via **MEK**/**ERK**, **Akt/PKB**, **PKC** and **NF-κB** pathways (Larrayoz et al., 2010; Moreira et al., 2009; Yang et al., 2019). Huang et al. (2012) reported that 7-KC also caused endoplasmic reticulum stress in human retinal pigment epithelial cells. Given that the retinal pigment epithelial is responsible for the daily renewal of the photoreceptor outer segment via phagocytosis, impaired or decreased phagocytosis is likely to contribute to age-related macular degeneration (Kevany & Palczewski, 2010). Recently, it has been shown that intravitreal injection of 7-KC in rats has resulted in disorganised and decreased apical microvilli of retinal pigment epithelial and detachment of apical microvilli from the photoreceptor outer segments, leading to defects in outer segment phagocytosis (Yang et al., 2019).

Retinal microglia are resident macrophages that are distributed in the inner retina of young, healthy individuals. With ageing or under stress conditions, these microglia migrate to the outer retina and become activated, demonstrating proinflammatory and proangiogenic properties. Microglial activation is associated with various types of retinal degeneration, including age-related macular degeneration (Karlstetter et al., 2015). 7-KC has been reported to co-localise with subretinal microglia in aged mice. 7-KC chemoattractively influenced microglial migration to the subretinal space, and enhanced microglial activation, indicated by inflammasome activation, increased the expression of proinflammatory cytokines (IL-1β, IL-6, IL-18 and TNFα) and of proangiogenic factors (vascular endothelial grow the factor, **PDGFb** and **ICAM-1**). Conditional culture media from 7-KC-exposed microglia stimulated endothelial cell migration, while transplantation of 7-KC-exposed microglia in a Matrigel-induced choroidal neovascularisation model significantly promoted choroidal neovascularisation formation (Indaram et al., 2015).

24S-HC can directly cross the blood–brain barrier and be transported to the liver by circulating lipoproteins, facilitating cholesterol elimination from the brain (Björkhem, 2006). 24S-HC also functions as an activator for nuclear transcription factors, such as liver X receptors -α and -β (Lehmann et al., 1997), to upregulate the expression of cholesterol transport genes including *ABCA1* in both glial cells and neurons (Fukumoto et al., 2002) and apolipoprotein E in astrocytes (Liang et al., 2004), leading to increased cholesterol efflux and mediation of cholesterol homeostasis. Additionally, 24S-HC can suppress **amyloid β** production by hindering amyloid precursor protein trafficking in Alzheimer's models (Urano et al., 2013). Amyloid β has been localised to the drusen of age-related macular degeneration patients and is thought to contribute to age-related macular degeneration pathogenesis (Isas et al., 2010; Luibl et al., 2006). Anti-amyloid therapy suppresses retinal damage and retards vision loss in an age-related macular degeneration mouse model (Ding et al., 2011). Although 24S-HC is reported to be higher in the plasma of age-related macular degeneration patients than that of controls (Lin et al., 2018), the level of 24S-HC in the retina and retinal pigment epithelial of age-related macular degeneration patients may be different. So, we propose that 24S-HC may play a protective role in age-related macular degeneration by enhancing cholesterol transport and reducing amyloid β formation in neural retina and the retinal pigment epithelial.

27-HC is undetectable in neural retina and retinal pigment epithelial, possibly due to being immediately oxidised to 27-COOH by CYP27A1 (Mast et al., 2011). Although there is no difference in plasma 27-HC levels of age-related macular degeneration patients and controls (Lin et al., 2018), we believe there to be a connection between 27-HC and age-related macular degeneration based on our recent work (Biswas et al., 2017, 2018). The **translocator protein (TSPO)** is a mitochondrial membrane protein that is responsible for delivering cholesterol from the mitochondrial outer membrane to the inner membrane where it is metabolised to 27-HC by CYP27A1. In turn, 27-HC activates liver X receptor signalling to upregulate the expression of ABCA1, ABCG1/4, and apolipoprotein E and promotes cholesterol transport in non-steroidogenic cells, including the retinal pigment epithelial (Figure 3) (Biswas et al., 2017; Papadopoulos et al., 2015). We found that translocator protein is expressed in the human retinal pigment epithelial and monkey choroidal endothelial cells. In mouse retina, its expression was extremely high in the retinal pigment epithelial and was seen to decline with age. Knockout of translocator protein resulted in decreased cholesterol efflux and cholesterol accumulation in the retinal pigment epithelial cells. Translocator protein ligands (FGIN-1-27, **emapunil (XBD173)** and **etifoxine**) promoted cholesterol efflux in the retinal pigment epithelial and choroidal endothelial cells and upregulated expression of oxysterol metabolism and cholesterol trafficking genes. Translocator protein ligands also suppressed oxidised LDL-induced oxidative stress and inflammation (Biswas et al., 2017, 2018). The data suggests that translocator protein-mediated cholesterol trafficking via 27-HC may play an important role in the pathogenesis of age-related macular degeneration.

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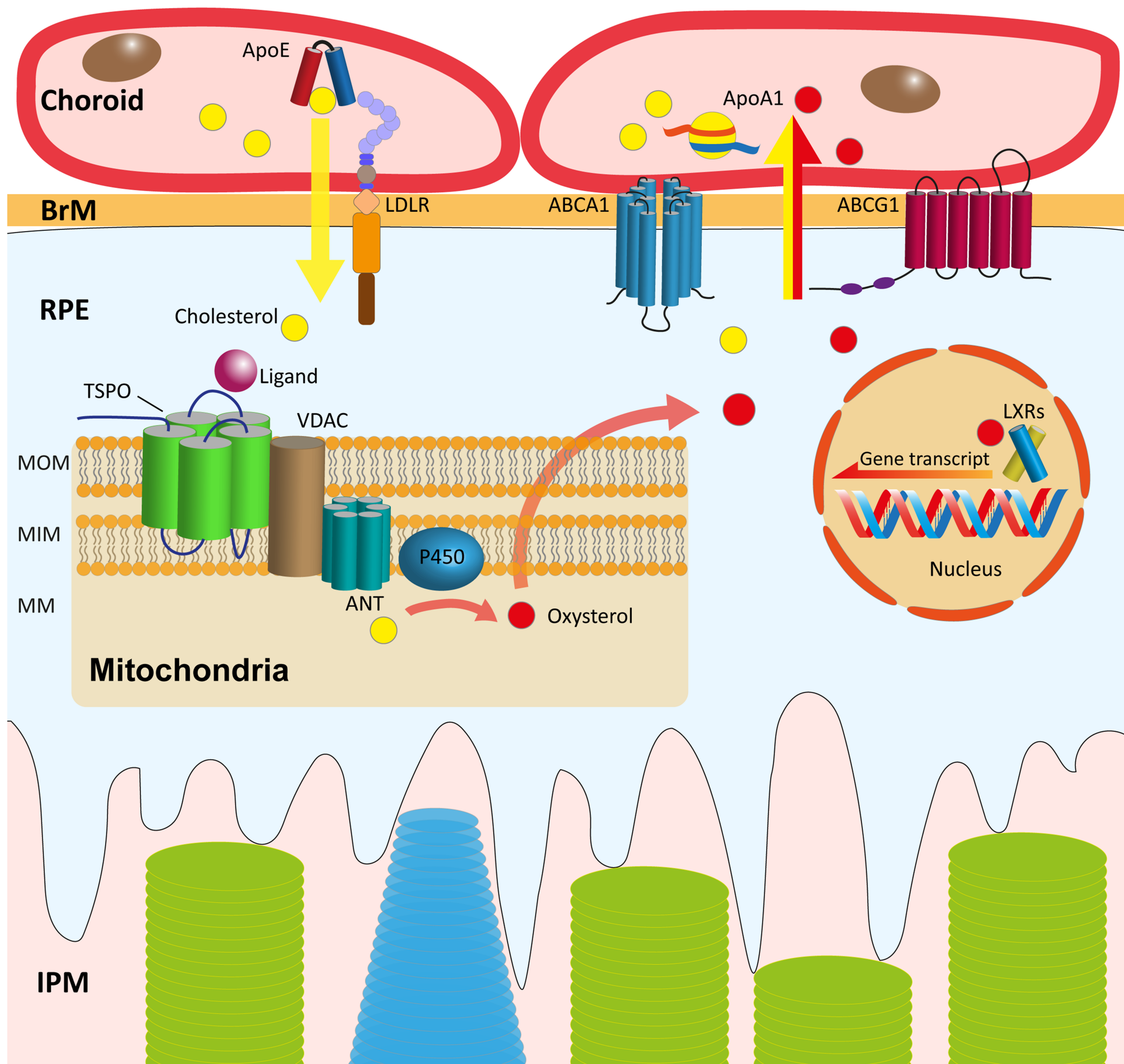

FIGURE 3 Translocator protein (TSPO)-mediated cholesterol efflux in retinal pigment epithelial (RPE) cells. The RPE uptakes low-density lipoproteins (LDL) from the choroidal circulation mainly via LDL receptor (LDLR). Cholesterol is released from LDL degradation in the RPE and delivered to the mitochondria, where cholesterol is transported from mitochondrial outer membrane (MOM) to mitochondrial inner membrane (MIM) by TSPO protein complex containing TSPO, steroidogenic acute regulatory protein (StAR, not shown), the voltage dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and other regulatory proteins (not shown). Oxysterol is generated by CYP27A1 to 27-hydroxycholesterol (27-HC), which upregulates expression of cholesterol transports (e.g. ABCA1 and ABCG1A) via activation of the liver-X receptor (LXR) signalling pathway and enhances cholesterol efflux. Ligands can bind TSPO, enhance mitochondrial cholesterol trafficking, and promote cholesterol efflux. BrM, Bruch's membrane; MOM: mitochondrial outer membrane; MIM: mitochondrial inner membrane; MM: mitochondrial matrix; IPM, interphotoreceptor matrix; ApoA1: apolipoprotein A1; ApoE: apolipoprotein E; ABCA1: ATP-binding cassette transporter member 1; ABCG1: ATP-binding cassette subfamily G member 1

7 | OXYSTEROLS AND GLAUCOMA

Glaucoma is a leading cause of irreversible blindness, currently affecting nearly 80 million individuals worldwide and predicted to increase to 111.8 million by 2040 (Tham et al., 2014). Glaucoma is characterised by the gradual degeneration of the optic nerve and retinal ganglion cell (RGC) loss (Almasieh et al., 2012; Weinreb et al., 2014). As RGCs are nondividing cells without the capacity for regeneration, the damage of the optic nerve appears to be irreparable. Loss of RGCs is a complex process, associated with deprivation of neurotrophic factors, failure of axonal transport, mitochondrial dysfunction, neurotoxicity (oxidative stress, inflammation, and excitotoxic damage) and defects in RGC–glia interaction (Almasieh et al., 2012). Glaucoma is classified in two main types:- primary open-angle

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glaucoma (POAG) and primary angle-closure glaucoma (PACG). Most cases are primary open-angle glaucoma and involve a long-term increase in intraocular pressure (IOP). Glaucoma is a complex disease associated with multiple risk factors such as age, genetic background, and hyperlipidaemia (Wang & Bao, 2019; Weinreb et al., 2014). intraocular pressure is considered to be the most common risk factor for glaucoma, and reducing intraocular pressure is a well-established clinical approach to slowing glaucomatous degeneration. Although reducing intraocular pressure is therapeutically effective in retarding glaucoma progression, there is some evidence that disease progression is still inevitable (Heijl et al., 2002; Kass et al., 2002), suggesting that neuroprotectants are urgently needed to prevent the degeneration of RGC and optic nerve.

The link between 24S-HC and glaucoma was first proposed when a polymorphism (rs754203) in the intron 2 of *CYP46A1* gene was shown to be associated with increased risk for POAG (Fourgeux et al., 2009). Further work found that elevated intraocular pressure in the rat caused a transient increase in expression of CYP46A1 but no change in the retinal 24S-HC between experimental and contralateral eyes (Fourgeux et al., 2012). Inhibition of CYP46A1 in rats by intraperitoneal injection with voriconazole, which has been shown to effectively inhibit CYP46A1 *in vitro* and *in vivo* (Shafaati et al., 2010), led to a 37% decrease in retinal 24S-HC and impaired inner retinal function (electroretinogram test); however, the effect of CYP46A1 inhibition on ganglion cell layer was not examined (Fourgeux et al., 2014). Ishikawa et al. (2016) found elevated IOP significantly enhanced CYP46A1 expression at mRNA and protein levels in an ex vivo rat glaucoma model, while increased CYP46A1 protein was predominantly localised to ganglion cells. Retinal 24S-HC was also increased under conditions of high intraocular pressure. The authors also demonstrated that high pressure (75 mMHg) caused damage to the RGCs and that 24S-HC counteracted the damaging effects in a dose-dependent manner. 24S-HC also protected the RGCs from voriconazole-induced damage (Ishikawa et al., 2016). The data suggest that 24S-HC may have a protective role in conditions of high-intraocular pressure that are associated with glaucoma.

8 | OXYSTEROL-TARGETED THERAPEUTIC STRATEGIES FOR RETINAL DEGENERATION

8.1 | Small molecule therapy

The development of oxysterol-targeted therapy will depend on the functional properties of individual oxysterols. For example, when targeting 7-KC, a strategy is needed to inhibit 7-KC-induced toxicity (e.g. oxidative damage and inflammation) and/or to convert 7-KC to less-toxic or nontoxic metabolites; for 24S-HC and 27-HC, ideally there should be more *in situ* biosynthesis. Sterculic acid has been shown to inhibit 7-KC-induced cell death and inflammation in retinal pigment epithelial cells and suppressed laser-injury-induced choroidal neovascularisation formation in rats (Huang et al., 2012). **Resveratrol**, a natural polyphenol, has also been shown to reduce 7-KC-induced retinal pigment epithelial apoptosis and vascular endothelial growth factor secretion (Dugas et al., 2010).

There are other candidates that are worth investigating further. **Efavirenz**, an anti-HIV drug, has been shown to activate CYP46A1 and improve behaviour in the 5XFAD Alzheimer's disease mouse model (Mast et al., 2017; Petrov, Lam, et al., 2019). It would be useful to assess the therapeutic potential of efavirenz via 24S-HC function in both age-related macular degeneration and glaucoma models. translocator protein ligands, such as XBD173, promote mitochondrial cholesterol trafficking and increase the metabolism of cholesterol to 27-HC, which upregulates cholesterol transport and metabolism genes via activation of the liver X receptor signal pathway, consequently enhancing intracellular cholesterol removal (Papadopoulos et al., 2015). Translocator protein ligands have been shown to suppress oxidised LDL-induced oxidative stress and inflammation in the retinal pigment epithelial and choroidal endothelial cells (Biswas et al., 2017, 2018). XBD173 can also inhibit microglial activation and attenuate light-induced retinal degeneration (Scholz et al., 2015). Evaluation of the protective effects of XBD173 against 7-KC-induced oxidative damage, inflammation and microglia activation may help to develop new treatments for age-related macular degeneration.

8.2 | Gene therapy

Gene therapy is one possible means by which to increase levels of oxysterol-producing enzymes in the retina. Currently, this appears to be a safe and feasible approach for the treatment of inherited retinal degeneration. Adeno-associated virus (AAV) is a commonly used vector for delivery, targeting genes in the retinal cells such as photoreceptors and retinal pigment epithelial cells (Lee et al., 2019). Hudry et al. (2010) overexpressed CYP46A1 via adeno-associated virus-mediated gene therapy in the cortex and hippocampus of Alzheimer's disease mice and found that the level of 24S-HC in the brain was significantly increased, while formation of amyloid β deposits and associated pathology were markedly reduced (Hudry et al., 2010). Adeno-associated virus-mediated overexpression of CYP46A1 in the striatum globally regulates cholesterol metabolism and improves behavioural and neuronal functions in Huntington's disease mouse model (Kacher et al., 2019). Similarly, overexpression of CYP46A1 in ganglion cells via gene delivery may help to prevent glaucoma. CYP27A1 is the main enzyme responsible for elimination of 7-KC in the retinal pigment epithelial (Heo et al., 2011); it also metabolises cholesterol to 27-HC, which promotes cholesterol efflux in the retinal pigment epithelial (Biswas et al., 2017). Potentially, then, overexpression of CYP27A1 in the retinal pigment epithelial using gene therapy could be a new therapeutic strategy for age-related macular degeneration.

9 | CONCLUSIONS

Enriched expression of oxysterol-producing enzymes and the distribution of oxysterols in the retina suggest that oxysterols may have

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multiple functions in the pathogenesis of degenerative retinal diseases. This review summarises our current understanding of oxysterol biology in the retina, including biosynthesis, metabolism, function and association with retinal degeneration. Current preclinical and clinical data suggest that 7-KC, 24S-HC and 27-HC are the major oxysterols associated with retinal degeneration. More effort is required to establish a deeper understanding of oxysterol-related pathophysiology in retinal degeneration and to develop new therapeutic interventions to improve visual function in oxysterol-associated retinal disorders.

9.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Cidlowski, et al., 2019; Alexander, Fabbro, et al., 2019; Alexander, Kelly, et al., 2019).

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AUTHOR CONTRIBUTIONS

X.S. conceived this project. X.Z., R.H.A, X.Z., J.R., Z.Z. and X.S. prepared the first draft. X.Z., X.S. and N.S. revised the manuscript.

CONFLICT OF INTEREST

The authors do not have conflicts of interest to declare.

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