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*JCI Insight.* 2019. [https://doi.org/10.1172/jci.insight.131928](https://doi.org/10.1172/jci.insight.131928).

Effective treatments and animal models for the most prevalent neurodegenerative form of blindness in the elderly, called age-related macular degeneration (AMD), are lacking. Genome-wide association studies have identified lipid metabolism and inflammation as AMD-associated pathogenic pathways. Given liver x receptors, encoded by NR1H3 and NR1H2, are master regulators of these pathways, herein we investigated the role of LXR in human and mouse eyes as a function of age and disease, and tested the therapeutic potential of targeting LXR. We identified immunopositive LXR fragments in human extracellular early dry AMD lesions and a decrease in LXR expression within the retinal pigment epithelium (RPE) as a function of age. Aged mice, lacking LXR, presented with isoform dependent ocular pathologies. Specifically, loss of the Nr1h3 isoform results in pathobiologies aligned with AMD, supported by compromised visual function, accumulation of native and oxidized lipids in the outer retina, and upregulation of ocular inflammatory cytokines, while absence of Nr1h2 is associated with ocular lipoidal degeneration. Therapeutically, LXR activation, ameliorated lipid accumulation and oxidant-induced injury in RPE cells in vitro, and decreased ocular inflammatory markers and lipid deposition in a mouse model, in vivo, providing translational support for pursuing LXR-active pharmaceuticals as potential therapies for dry AMD.

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LXRs regulate features of age-related macular degeneration and may be a potential therapeutic target

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Conflict of interest statement: The authors have declared that no conflicts of interest exists.
Abstract

Effective treatments and animal models for the most prevalent neurodegenerative form of blindness in the elderly, called age-related macular degeneration (AMD), are lacking. Genome-wide association studies have identified lipid metabolism and inflammation as AMD-associated pathogenic pathways. Given liver x receptors, encoded by \textit{NR1H3} and \textit{NR1H2}, are master regulators of these pathways, herein we investigated the role of LXR in human and mouse eyes as a function of age and disease, and tested the therapeutic potential of targeting LXR. We identified immunopositive LXR fragments in human extracellular early dry AMD lesions and a decrease in LXR expression within the retinal pigment epithelium (RPE) as a function of age. Aged mice, lacking \textit{LXR} presented with isoform dependent ocular pathologies. Specifically, loss of the \textit{Nr1h3} isoform results in pathobiologies aligned with AMD, supported by compromised visual function, accumulation of native and oxidized lipids in the outer retina, and upregulation of ocular inflammatory cytokines, while absence of \textit{Nr1h2} is associated with ocular lipoidal degeneration. Therapeutically, LXR activation, ameliorated lipid accumulation and oxidant-induced injury in RPE cells in vitro, and decreased ocular inflammatory markers and lipid deposition in a mouse model, in vivo, providing translational support for pursuing LXR-active pharmaceuticals as potential therapies for dry AMD.
**Introduction**

Dysregulated lipid metabolism and inflammation are key contributors to the development of multiple diseases of aging including the ocular neurodegenerative disease, age-related macular degeneration (AMD), a leading cause of blindness in the elderly in the Western and developing Worlds, that affects over 130 million people in toto (1, 2). A hallmark of the “early” dry clinical sub-type of AMD is choriocapillary dropout (3) as well as accumulation of lipid- and protein-rich deposits, known as drusen, below the retinal pigment epithelial (RPE) layer, support cells to the retina (4, 5). While the pathogenesis of early dry AMD remains to be fully understood, an array of risk factors have been identified, cueing disease-associated signaling pathways, most notably, age, along with environmental (e.g. smoking), and genetic factors (e.g. genes associated with inflammatory, complement, and lipid regulating pathways) (6-9). In spite of the wealth of accepted knowledge regarding potential risk factors, treatment options remain tenuous for patients suffering from the “early” dry form of AMD. Therefore, there is a considerable need to not only identify critical signaling pathways that drive the initiation and progression of AMD, but also to develop appropriate animal models that could then be used as a platform to test potential therapies and further discover disease regulating pathways.

Nuclear receptors are the largest family of transcription factors in the human genome that facilitate the expression of a variety of genes important in maintaining cellular homeostasis during development and aging (10, 11). Importantly, they play a regulatory role in diseases of aging including Alzheimer’s disease (12, 13), cardiovascular disease (14), and cancer (15). Given the commonality between pathogenic pathways regulated by nuclear receptors in age-related diseases and AMD, in an attempt to identify candidate receptors that may be important in disease initiation and progression, we previously developed a nuclear receptor atlas of human RPE cells, cells
vulnerable in AMD (16). The liver X receptor (LXR; NR1H), emerged as a promising target, in light of its role as a regulator of cholesterol homeostasis (17, 18) and inflammation (18), two pathways modulated in AMD (19). Further justification for investigating the role of LXR in the aging eye comes from genome-wide association studies, which have found a correlation between single nucleotide polymorphisms in genes involved in cholesterol metabolism/transport and AMD, including member 1 of human transporter sub-family ATP binding cassette transporter (ABCA1), cholesteryl ester transfer protein (CETP), hepatic triglyceride lipase C (LIPC), and lipoprotein lipase (LPL) (9, 20, 21), known LXR target genes (22).

To test the hypothesis that the LXR signaling pathway regulates AMD pathology and may serve as a potential target for therapy, we interrogated the role of the LXR pathway in ocular cells vulnerable in AMD. We evaluated the expression of LXR in human RPE cells derived from donor eyes as a function of age, examined the distribution of LXR in human donor eyes as a function of disease, and compared LXR expression throughout compartments of the ocular posterior pole. Activation of the receptor was further evaluated in vitro, under AMD relevant stressed conditions with pharmacologic LXR ligands. The ocular function and phenotype of aged mice lacking the LXR isoforms (Nr1h3−/−, Nr1h2−/− and Nr1h3−/−/Nr1h2−/−) were catalogued and the therapeutic potential of activating the LXR signaling pathway on the ‘AMD’ phenotype, including extracellular lipid accumulation and inflammation in the back of the mouse eye, was tested. We report a decrease in NR1H3 expression in human RPE cells as a function of age, and accumulation of LXR immunopositive fragments within drusen of dry AMD eyes, reinforcing vulnerability to AMD development through this pathway. We found that LXR activating ligands are able to improve mitochondrial function, and decrease lipid load in RPE cells following injury. Furthermore, we established that the absence of LXR effects the visual function and ocular
phenotype of mice differentially, with the Nr1h3 isoform resulting in the accumulation of extracellular lipid- and protein-rich deposits, phenotypic features of dry AMD, while the Nr1h2 isoform also resulted in non-AMD phenotypes, namely stellate cataract formation and RPE associated lipoidal degeneration. Detailed analysis of mouse eyes lacking Nr1h3 revealed a pro-inflammatory microenvironment, as evidenced by the accumulation of immune cells in the outer retina, and confirmed through analysis of the cytokine profile of the mouse RPE-choroid. Finally, LXR activation was able to reduce AMD relevant pathogenic phenotypes including posterior pole ocular inflammation and lipid deposition in mice expressing apoB100, a model for aging and early AMD-associated phenotypes (23, 24).

Our studies collectively, highlight the importance of the NR1H3 signaling pathway in the health of aged RPE cells, and present a novel mouse model for the dry AMD phenotype featuring lipid rich sub-RPE deposits along with accumulation of sub-retinal immune cells. This model may serve as a platform to test new therapies and identify additional therapeutic targets. Importantly, our results provide translational support for approaches including direct activation of the LXR pathway, which is capable of reducing AMD associated pathologies and may be a therapeutic target for not only the early dry form of AMD, but also other ocular and non-ocular diseases characterized by either abnormal lipid deposition or tissue immune cell infiltration.
Results

LXR expression in hRPE cells decreases as a function of age

Given the critical role of advanced age in the development of AMD, we measured the relative expression of NR1H3 (LXRα) and NR1H2 (LXRβ) in human RPE cells isolated from donor tissue as a function of age (Figure 1, Figure S1 and Table S1). A significant negative correlation was found between relative NR1H3 and NR1H2 expression levels and age (Figure 1A and Figure S1A). This negative correlation remained significant for NR1H3 expression, not NR1H2 in the ‘at-risk for AMD’ age range of 60-94 years, suggesting a steady decline in NR1H3, but a plateau in NR1H2 expression in human RPE cells (Figure 1B and Figure S1B). In a smaller cohort of RPE tissue from middle to advanced aged donors, expression of LXR target genes ABCA1 (n=12) and APOE (n=15) were found to increase with age (Figure S2A and S2B). The purity of freshly isolated RPE cells from human donors was assessed by measuring the expression of bestrophin-1 (BEST1) and retinal pigment epithelium specific protein 65/retinoid isomerohydrolase RPE65 (RPE65) freshly isolated RPE, retina and choroid from corresponding donors. RPE samples (n = 23) were found to have a 2.13 X 10^2 – 4.52 X 10^5 fold higher expression of BEST1 and 4.98 X 10^2 - 2.15 X 10^4 fold higher expression of RPE65, relative to ARPE19 cells. Additionally, the expression levels of BEST1 and RPE65 in retina and choroid were negligible (0.01 - 0.3 fold change for BEST1 and 0.009 - 0.2 fold change for RPE65) as compared to the RPE samples, reflecting the purity of the samples. Importantly, all the components of the LXR pathway were expressed in freshly isolated tissue and cultured cells (Figure 1C). We next evaluated the localization of NR1H3 and NR1H2 protein in retinal cross-sections from ‘AMD and age-matched non-effected ‘normal’ donor eyes via immunohistochemistry. In sections from non-effected donors, NR1H3 immunoreactivity was observed in RPE cells and select cones (Figure 1D, 1E, 1F), while NR1H2 immunoreactivity was
also present throughout the inner retina (Figure 1J, 1K and 1L). In AMD donor sections, NR1H3 immunoreactivity was less evident in the RPE cell layers (Figure 1G, 1H, and 1I) but still present, though at lower levels, and NR1H3 immunopositive fragments were seen in large drusen in 6 out of 9 donor tissues (Figure 1 and S3), in addition to cone cells as shown by R/G opsins staining (Figure S4). This staining pattern was confirmed using a different primary antibody to NR1H3 (Figure S4). NR1H2 immunoreactivity in drusen was also abundant in addition to diffuse staining within the neural retina of AMD eyes, reflecting the ubiquitous nature of this isoform (Figure 1M, 1N and 1O).

**The LXR pathway is biologically active in AMD vulnerable cells**

NR1H3 and NR1H2 activities were examined in human primary RPE cells (hRPE), and the macaque derived RF/6A choroidal endothelial cell (CEC) lines. Receptor transcriptional activity was assessed by measuring the binding of the receptor-ligand complex to the gene response element using a luciferase reporter assay. Ligand activation by GW3965 and TO901317, two non-steroidal LXR active pharmaceuticals (Table S3), significantly increased LXR promoter activity in hRPE (GW3965: 4-fold, TO901317: 2-fold; Figure 2A) and CEC (GW3965: 2.5-fold, TO901317: 5-fold; Figure 2D) cells. This induction was reversed following siRNA induced knockdown of NR1H3 and NR1H2 expression. Conversely, LXR antagonist (GSK2033) treatment did not result in promoter binding. Ligand activation by GW3965 and TO901317, significantly induced expression of LXR target genes, fatty acid synthase (FASN) and sterol regulatory element-binding protein 1 (SREBF1) in both hRPE (from a 93 year old, female; Figure 2B and 2C) and CEC (Figure 2E and 2F) cells. Similar induction patterns of LXR target genes in response to agonists were observed in two additional primary RPE cell lines (from a 15 and 60 year old).

Collectively, these results demonstrate that components of the LXR pathway are expressed, and
LXR activity and target gene expression can be modulated through the use of pharmacological drugs and siRNA, respectively, in ocular cells vulnerable in AMD. Furthermore, variation in the magnitude of LXR induction and expression, illustrates the differential nature and cell-specific effects of these ligands.

**LXR activation differentially ameliorates oxidant injury and lipid overload in a ligand dependent manner in the eye**

The retina is susceptible to oxidant injury culminating from inadequate neutralization of generated reactive oxygen species (ROS). This high ROS load is a consequence of the retina’s daily exposure to visible light, its high oxygen consumption, the daily phagocytosis of photoreceptor outer segments, as well as its polyunsaturated fatty acid-rich environment (25). Human RPE cells were exposed to agents simulating oxidant injury [i.e. NaIO₃, H₂O₂, and cigarette smoke extract (CSE)], and lipid overload [i.e. palmitic acid (PA) and alpha linolenic acid (αLA); Table S3]. The effects of LXR modulation in response to oxidant- and lipid-induced damage to the RPE cells were determined by evaluating the activity assay, mitochondrial membrane potential using the JC-1 assay, lipid buildup in the cells by adipored assay and cell viability by CellTiter-Blue® assay. We found that the treatment with oxidant injury and lipids did not directly induce LXR activity in RPE cells (Figure S5). Therapeutically, a 24-hour pre-treatment of RPE cells with the LXR ligand, TO901317 was able to prevent the compromise to the mitochondrial membrane potential in NaIO₃, H₂O₂, and CSE treated cells, whereas GW3965 pretreatment was able to block mitochondrial injury in lipid treated cells (Figure 3A and S6). LXR antagonist GSK2033 pretreatment had no appreciable effect on the mitochondrial membrane potential. Additionally, we measured the neutral lipid content in RPE cells in response to oxidant injury and lipid overload and examined if LXR pathway modulation could prevent the accumulation of neutral lipids in RPE cells. Lipid
treatments (PA and αLA) induced a build-up of neutral lipids within cells, which was ameliorated by pretreatment with GW3965 (Figure 3B). The agonist TO901317, and antagonist GSK2033, did not impact lipid accumulation in RPE cells in vitro. Finally, cell viability assessments confirmed that the drug pretreatments and injury treatments were sub-lethal for the course of the assay (Figure 3C). These results revealed the therapeutic potential of activating the LXR pathway to avert oxidant injury and lipid accumulation in RPE cells. Importantly, the two LXR agonists tested appear to work via distinct pathways, as evident by their selective action in response to diverse injury agents.

**Loss of Nr1h3 in vivo compromises visual function**

Given the role of LXR in regulating lipid metabolism and inflammation, two pathways associated with development of AMD, and our observation that LXR expression in human RPE cells decreases with age, we examined the visual function of aged LXR knockout mice, after confirming that the absence of LXR globally also effects LXR gene expression locally in the RPE/choroid (Figure S7). Visual function was assessed by recording ERGs from 10-14 month old Nr1h3−/− (n = 11), Nr1h2−/− (n = 11), Nr1h3−/−/Nr1h2−/− (n = 2), and Nr1h3+/−/Nr1h2+/+ wildtype (WT, n = 15) controls. Evaluation of the ERG recordings revealed a decline in scotopic a-wave (approximately 41%), scotopic b-wave (approximately 26%), and photopic b-wave amplitudes (approximately 28%) of Nr1h3−/− mice compared to WT controls (Figure 4A, 4B, and 4C), indicating that in the absence of Nr1h3, visual function may be compromised at several levels, including the photoreceptors, and retinal cells post-synaptic to photoreceptors. We also evaluated the visual function of Nr1h3−/− mice as a function of age and observed an age-related decline in scotopic a-wave and b-wave amplitudes (4-5 month olds compared to 10-12 month olds; Figure S8), supporting a role for Nr1h3 in the maintenance of retinal cell health. Upon further examination,
gene-specific differences in the localization of retinal cell markers for photoreceptors (Figure S9A and S9B), Müller and bipolar cells (Figure S9C and S9D) were not seen, and no differences in retinal layer thicknesses were observed (Figure S10). However, a significant decline (24.9%) in the c-wave amplitude in Nr1h3−/− mice compared to WT controls (Figure 4D) was observed, reflecting the contribution of the RPE to the visual function decline observed in these mice (26).

While Nr1h2−/− mice did not display any gene-specific differences in visual function, the Nr1h3−/−/Nr1h2−/− mice followed a similar trend to the Nr1h3−/− mice (Figure 4). Finally, no significant isoform specific differences were seen in dark adaptation and a-wave flash sensitivity. Some LXR dependent changes in the recovery kinetics for a-wave amplitude were seen, but only at early time points (Figure 4E and 4F).

Isoform specific ocular pathologies indicate absence of LXRα is associated with an early dry AMD phenotype.

In vivo imaging allowed examination of the morphology of the fundus and OCT images from aged Nr1h3−/−, Nr1h2−/−, and Nr1h3−/−/Nr1h2−/− and WT controls. Whereas the fundus and OCT images from the WT mice were not remarkable (Figure 5A and 5B), the Nr1h3−/− mice exhibited hypopigmented regions in the fundus (Figure 5C, 5D, S11A, S11B, S11C, S11D, S11E, S11F, S11G, and S11H). Similarly, Nr1h3−/−/Nr1h2−/− mouse fundus images, presented with pigmentary changes in the fundus (Figure 5G and 5H). Nr1h2−/− mice, in addition to retinal changes, developed stellate cataracts (Figure 5E, 5F, and S11I, S11J, S11K and S11L), resulting in cloudy fundus images. The overall architecture of the inner retinas of LXR null mice were evaluated ex vivo in cross-sections and no gene specific differences in the overall architecture and thickness of the inner retinal layers were found [Nr1h3−/− (n = 5), Nr1h2−/− (n = 3), Nr1h3−/−/Nr1h2−/− (n = 4) and WT (n = 4); Figure 5I, 5J, 5K, 5L, S10]. High magnification evaluation of the outer retina of LXR mice using cTEM
allowed for detailed analysis of the ultrastructure of the RPE-choroid complex. While eyes from
WT mice exhibited normal RPE-choroid morphology with organized basal infoldings and apical
processes of the RPE (Figure 5M and 5N), in Nr1h3−/− mice, continuous sub-RPE deposits were
observed below disrupted basal infoldings, seen as amorphous electron dense material,
interspersed with ‘streamers’, a phenotype typical of early dry AMD (over 40% of the length of
BrM; Figure 5O, 5P and S12). The RPE phenotype of Nr1h2−/− mice had distinct pathologies
including accumulation of large lipid droplets within the RPE, reminiscent of lipoidal
degeneration, which is not a characteristic of AMD (Figure 5Q and 5R) (27, 28). Predictably,
Nr1h3−/−/Nr1h2−/− mice, displayed both phenotypes, large lipid droplets within the RPE in
combination with accumulation of extracellular electron-dense sub-RPE deposits (Figure 5S and
5T). Based on these morphological findings we conclude that in the eye the alpha isoform of LXR
regulates extracellular lipid deposition, an early phenotypic feature of dry AMD, while the beta
isoform is responsible for intracellular lipid accumulation as well as stellate cataract, an ocular
pathology of the anterior segment.

Nr1h3 regulates lipid deposition in the outer retina

Accumulation of neutral lipids with age in Bruch’s membrane and extracellular to the RPE has
been correlated with drusen formation (29, 30). Given the reported role of LXR in regulating lipid
metabolism, we stained cryosections from Nr1h3−/− (n = 4), Nr1h2−/− (n = 4), Nr1h3−/−/Nr1h2−/− (n =
3) and WT (n = 3) mice with the histochemical stain oil red o (ORO) in order to evaluate the
presence of neutral lipids within the retina. While the RPE and BrM of WT mice was absent of
neutral lipids (Figure 6A), Nr1h3−/− mice displayed robust staining in sub-RPE deposits and within
BrM (Figure 6B). Nr1h2−/− mice displayed a strong globular staining pattern within and below the
RPE cell layer (Figure 6C). Nr1h3−/−/Nr1h2−/− mice also displayed strong ORO positive staining not
only within the RPE cells but also in sub-RPE deposits (Figure 6D). ORO positive staining in the tips of the photoreceptor outer segments, as seen in the WT panel served as a positive internal control (Figure 6A). Another characteristic of sub-RPE deposits in AMD is the accumulation of apolipoprotein E (APOE), a regulator of cholesterol transport, and oxidized lipoproteins (31, 32). Probing Nr1h3−/− (n = 4), and WT (n = 3) eyes with antibodies targeting APOE and oxidized phospholipids present in oxidized LDL (E06), revealed higher levels of APOE and E06 immunoreactivity below the RPE in Nr1h3−/− (Figure 6F) compared to WT sections (Figure 6E), suggesting the buildup of oxidized lipids along the length of BrM. QFDE imaging is a technique that allows for further interrogation of lipoprotein particles in human aged BrM (33-36). En face visualization with QFDE displayed severely disrupted basal infoldings of the RPE in Nr1h3−/− (Figure 6I, 6J, 6K and S13), compared to uniform and organized basal infoldings in WT mice (Figure 6G and 6H). A different angle and depth of fracture plane allowed us to examine the structure of the RPE basement membrane (BM), inner collagenous layer (ICL), and elastic layer (EL) of BrM. While the WT mice exhibited normal morphology, across BrM (Figure 6L), select regions within BrM of Nr1h3−/− mice presented with an accumulation of fused round particles (Figure 6M and S13D). A region of the Nr1h3−/− BrM, which lacked the accumulation of these spherical particles is also shown (Figure 6N). Given that the choriocapillaris is also compromised in dry AMD, we also visualized the ultrastructure of the choriocapillary (CC) surface and found that in wild type mice, fenestrations in the CC appear as circular pores or “wagon wheels” (Figure S14A, S14B, S14E and S14F), but in Nr1h3−/− mice, fewer fenestrations were observed, and the “spokes” of the fenestrations were fewer in the “wagon wheels” (Figure S14C, S14D, S14G and S14H). These results provide a unique representation of lipid deposition in regions below the RPE and details of changes in RPE morphology associated with the loss of Nr1h3. Finally, an evaluation
of RPE autofluorescence revealed a similar isoform-dependent pattern of severity, with significant upregulation seen in \( \text{Nr1h3}^{-/-} \) (16.7%) and \( \text{Nr1h3}^{-/-}/\text{Nr1h2}^{-/-} \) (20.0%) compared to the WT (Figure 6O, 6P, 6Q, 6R, and 6S) mice and no significant changes seen in \( \text{Nr1h2}^{-/-} \) RPE autofluorescence levels. These findings suggest that lipofuscin accumulation in the RPE cells, which may be a result of dysregulation of lipid metabolism and clearance, as well as accumulation of extracellular neutral lipid, and apolipoprotein are at least in part attributable to \( \text{Nr1h3} \).

\( \text{Nr1h3}^{-/-} \) mice exhibit a pro-inflammatory microenvironment in the outer retina

In addition to regulating genes involved in cholesterol transport, LXR is involved in regulation of an array of pro-inflammatory genes resulting as a consequence of insults such as lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-\( \alpha \)), interleukin-1 beta (IL-1\( \beta \)), or bacterial stimulation (18), and it has been reported that LXR agonists may act as negative regulators of macrophage inflammatory gene expression (37). Of relevance, recruitment of immune cells to the outer retina are a purported feature of the AMD etiology (38). We examined the outer retina of \( \text{Nr1h3}^{-/-} \) mice for inflammatory mediators. RPE-choroid flat mounts were prepared from aged WT (\( n = 5 \)) and \( \text{Nr1h3}^{-/-} \) (\( n = 8 \)) mice, probed with a macrophage marker (ADGRE1, also known as F4/80) and stained with phalloidin to delineate the RPE cells. We observed a 3.8-fold accumulation of ADGRE1\(^+\) cells within the sub-retinal space of \( \text{Nr1h3}^{-/-} \) compared to WT mice (Figure 7A, 7B, 7C and 7D), suggesting an augmented pro-inflammatory microenvironment in the sub-retinal space. However, we did not detect striking differences in the morphology of phalloidin stained RPE cell borders (Figure S15A, S15B and S15C). To further evaluate the cytokine milieu of the outer retina, we examined the cytokine profile of the RPE-choroid tissue complex isolated from WT and \( \text{Nr1h3}^{-/-} \) mice. Out of a panel of 62 proteins, \( \text{Nr1h3}^{-/-} \) samples exhibited a significant upregulation of nine cytokines, with a primarily pro-
inflammatory profile (Figure 7E and 7F). Interestingly, at a systemic level, 10 cytokines were found to be downregulated in the plasma of \textit{Nr1h3}\(^{-/-}\) mice (Figure S16A and S16B). These results corroborate that \textit{Nr1h3} is involved in maintaining a homeostatic microenvironment in the outer retina.

**Therapeutic targeting of LXR diminishes pathogenic phenotypes associated with AMD**

There is marked accumulation of neutral lipids in the pentalaminar BrM in a normal eye with aging (39, 40). This lipid buildup in the posterior eye has been theorized to set the stage for the formation of extracellular lipid rich deposits and specifically the development of drusen and basal deposits, phenotypic characteristics of early dry AMD (39, 41, 42). Several studies have shown that this extracellular lipid deposition is at least in part, due to the accumulation of esterified cholesterol-rich apolipoprotein B (APOB) - containing lipoprotein particles (43, 44). Since mice predominantly produce the apoB48 protein, we employed a mouse that produces the full-length mouse \textit{apoB100} (23), as a proof of concept, to evaluate the therapeutic effect of modulating LXR activity. It is well established that the RPE-choroid of young \textit{apoB100} mice, produce the apoB100 isoform and there is accumulation of neutral lipids in their BrM (24). Herein, three month old \textit{apoB100} mice were fed a low fat diet (LFD) or the LXR agonist GW3965, supplemented in a low fat diet (LFD + GW), for a period of 5 months. Mice in both groups displayed a similar rate of increase in weights with age, suggesting absence of any overt systemic toxicities (Figure S17A). We measured the expression of LXR target genes, \textit{Abca1} and \textit{Srebp1c} in RPE-choroid isolated from LFD and LFD + GW treated mice and found that their expression was upregulated in the LFD + GW cohort (\textit{Abca1}: 54%, \textit{Srebp1c}: 35%, n=2), indicating that the drug was able to reach the posterior retina and activate LXR locally. Additionally, no differences were observed in the systemic levels of HDL and LDL/VLDL (Figure S17B and S17C). Fundus and OCT imaging
exhibited an improvement in the morphology of retinal layers as evident by a decrease in hypo-
pigmented regions in fundus images of LFD + GW group (Figure 8C and 8D) as compared to LFD
mice (Figure 8A and 8B). We also observed a significant decrease in GFAP staining within the
inner retina following activation of LXR in comparison to mice fed the LFD, supporting rescue of
retinal injury associated with the apoB100 mice and a decline in inflammation (Figure 8E and 8F).
Quantification of the lipoprotein deposition in BrM of apoB100 mice revealed a significant
decrease (77%) in the fraction of APOE+ length of BrM, following LXR agonist treatment (Figure
8G, 8H, and 8I). GW3965 treatment also led to a reduction in the number of ADGRE+ cells within
the sub-retinal space (81%, p=0.0598; Figure 8J, 8K, and 8L). Furthermore, examination of the
ultrastructure of the retina/RPE/choroid complex from vehicle (LFD; Figure 8M) and drug-treated
mice (LFD + GW) revealed disrupted RPE basal infoldings (Figure 8N) and intermittent thin sub-
RPE deposits (Figure 8O) in the LFD cohort. These morphological features displayed a marked
improvement in the LFD + GW group (Figure 8P, 8Q, and 8R). Furthermore, the LFD + GW group
displayed distinct mitochondria (Figure S18C and S18D) relative to the vehicle group (Figure
S18A and S18B). These results signify that LXR activation is able to therapeutically influence
inflammation and apolipoprotein E deposition in the outer retina and may be a viable path for
targeting these pathogenic pathways in AMD.
Discussion

The need to identify animal models that recapitulate early phenotypic features of complex neurodegenerative diseases such as dry AMD is a necessity to serve as a platform to not only further understand the pathobiology of disease, but also facilitate testing of potential therapies in pre-clinical studies. We hypothesized that the LXR signaling pathway is likely an important mediator of AMD pathobiology given its role as a regulator of lipid metabolism, lipid transport, and inflammation, critical pathogenic pathways associated with disease development. To this end, we interrogated human ocular samples from AMD donors for evidence of LXR involvement and found that the expression of LXR in human RPE cells, nurse cells to the retina and a primary vulnerable site in AMD, decreases as a function of age. Furthermore, we found that drusen, the characteristic lesion of early dry AMD contain fragments that are LXR immunopositive. We confirmed that the LXR signaling pathway can be activated in human RPE cells in vitro and characterized the ocular phenotype and function of Nr1h3, Nr1h2 and Nr1h3/Nr1h2 knockout mice. Interestingly, we found an isoform dependent ocular phenotype, with Nr1h3−/− mice demonstrating accumulation of sub-RPE extracellular lipids, a distinguishing characteristic of early, dry AMD, while the pathology of Nr1h2−/− mice mimic RPE lipoidal degeneration. Pre-clinical studies allowed testing the efficacy of an LXR agonist, selected based on its ability to re-establish RPE cholesterol homeostasis and mitochondrial function, in an in vivo model with phenotypic features of dry AMD. Collectively, our results have identified a novel mouse model with characteristic features of dry AMD, identified mechanisms of LXR-based therapies, demonstrated efficacy of targeting the LXR pathway, supporting developing LXR directed therapies for the treatment of neurodegenerative diseases of aging such as AMD, in which altered cellular metabolism and inflammation may play a role.
This study is the first comprehensive characterization of the posterior ocular function and phenotype of LXR knockout mice. Though redundancies have been shown in the function of the two LXR isoforms in other organs including the liver, isoform specific functions have also been discovered. Systemically, aged LXR deficient mice have been shown to display isoform specific differences in plasma cholesterol levels, with absence of \( Nr1h2 \), associated with significant elevation of total cholesterol and HDL levels and absence of either \( Nr1h3 \) or \( Nr1h2 \) associated with increased LDL levels (45). Additionally, \( Nr1h3^{-/-} \) mice have been shown to display rapid accumulation of large amounts of cholesterol, leading to impaired hepatic function (46). Conversely, \( Nr1h2^{-/-} \) mice maintain their resistance to dietary cholesterol, despite differences in the expression of genes involved in lipid metabolism (47). Similarly, differences in expression of target genes have been reported in the epididymis of 12 month old mice, with the absence of the beta isoform but not alpha associated with a significant loss of expression of the cholesterol transporters, \( Abca1 \) and \( Abcg1 \) (45). In our investigations, we found that the LXR pathway must also be critical for the development of normal visual function, as visual function deficits, mostly corresponding to the posterior region of the eye and specifically the RPE, were detected. Importantly, decrease in the function of RPE cells was primarily observed in mice deficient in the \( Nr1h3 \). Furthermore, we found that both isoforms, along with their obligate binding partners, the RXRs, are expressed in several ocular tissues of the posterior pole including the retina, RPE, and choroid. Isoform specific variations were also found in physiological and pathological processes under the control of each of the isoforms in the eye. Absence of \( Nr1h2 \) effected the anterior segment resulting in stellate cataract formation as well as accumulation of intracellular and extracellular lipid deposits, while \( Nr1h3 \) null mice primarily developed RPE extracellular lipid accumulation. When both isoforms were absent, not surprisingly, not only were all phenotypes
evident but also life expectancy was diminished with many mice perishing by 10-11 months of age.

That the LXR signaling pathway may be important in the aging eye is not surprising, given the importance of cholesterol regulation in the retinal milieu. The retina relies on both local synthesis as well as delivery from systemic circulation to replenish its cholesterol reserves (48, 49). Furthermore, it employs HDL-mediated reverse cholesterol transport and metabolism of cholesterol to more soluble oxysterols for its elimination (49, 50). The excess cholesterol may remain in the retina and undergo oxidation as a result of exposure to light and endogenous free radicals (51, 52). Given the fact that LXRs, key regulators of cholesterol homeostasis, are expressed in retina and RPE and, oxysterols act as endogenous ligands for LXRs, it is plausible that LXRs may be the principal regulators of lipid metabolism and transport in the eye. This was confirmed by our in vivo assessments, which show accumulation of neutral and oxidized lipids below the RPE and within BrM, as evident by positive ORO and E06 staining in the absence of Nr1h3. Accumulation of oxidized lipids is an important contributor to AMD pathogenesis as these oxidized lipids can directly contribute to tissue injury or recruit inflammatory mediators in the outer retina (53). QFDE TEM is an imaging technique which has previously been used to visualize lipid particles, believed to be lipoproteins in human aged donor eyes (54). In conjunction with conventional TEM we were able to study the topology and ultrastructure of lipid deposits in detail in the RPE/BrM complex. While normal and healthy RPE basal infoldings in the WT mice were seen, Nr1h3−/− mice displayed regions of severely disrupted basal infoldings amidst healthy regions. The en face images of RPE/BrM/choriocapillaris complex from Nr1h3−/− mice acquired by this technique displayed ‘deposits’ above the inner collagenous layer. Noteworthy, the Nr1h3−/− mice did not etch as well as the WT mice with QFDE, suggesting the presence of low volatiles such as
an abundance of lipids. This corroborates the histological and immunohistochemical findings in our mouse model of the presence of neutral lipids, using oil red o staining, and apolipoproteins within BrM and below the RPE, pathologies characteristic of human AMD. This is the first demonstration of the pathology of the posterior pole of Nr1h3−/− mouse eye using QFDE and specifically visualization of disrupted basal infoldings and depots of spherical/lipid-like particles. Collectively our imaging results confirm that loss of Nr1h3 leads to accumulation of lipid in the RPE/BrM complex, which may stem from dysregulation of lipid clearance pathways in the eye.

Considerable evidence has emerged to indicate that the LXR pathway regulates inflammation and immune processes. In vivo, LXR deficient mice have a sustained state of heightened pro-inflammatory mediators, with specifically Nr1h3−/− mice displaying exacerbated CCl4-induced lesions, in addition to inflammation and collagen deposition in a model of chronic liver injury (55). In a model of retinal injury by N-methyl-D-aspartate, LXR activation reduced retinal neurotoxicity of the treatment and inhibited the increase of phospho-p38 MAPK and proinflammatory cytokine TNF-α in the treated mice (56). Additionally, activation of the LXR pathway represses a set of inflammatory genes including Nos2, mt-Co2, Il6 and Il1b, after LPS, TNFα, or, IL-1β stimulation (37). In our study, we have shown the accumulation of ADGRE1+ macrophages in the outer retina of Nr1h3−/− mice, indicating a pro-inflammatory micro-environment. We also measured the cytokine profile of the RPE-choroid complex and found a proinflammatory cytokine profile in Nr1h3−/− mice compared to the WT mice. This panel of cytokines includes IGFBP3, IGFBP5, IL12 P70, IL13, CCL9, CXCL2, SELP, CCL5 and LEPR. IGF-axis gene polymorphisms have been associated with advanced AMD and specifically IGFBP3 and IGFBP5 have been shown to be expressed in RPE progressive phenotypes, namely normal, early reactive, and myofibroblasts (57, 58). IL13 levels have also been found to be upregulated in
the aqueous humor of patients diagnosed with neovascular AMD, suggesting a pathological role in AMD progression (59). CCL9 and CXCL2 have been reported to be involved in the recruitment of neutrophils to the site of injury and might act as a chemoattractant for the ADGRE1+ macrophages (60). Furthermore, CCR5 shows a trend to be elevated in a subset of monocytes isolated from treatment naive neovascular AMD patients (61). Interestingly, we observed that systemic levels of cytokines significantly decreased in Nr1h3−/− mice compared to the controls, some with reported protected properties. CXCL1 has been reported to confer a neuroprotective function in a model of experimental autoimmune encephalomyelitis (62). CSF1 has been implicated in repair following acute kidney injury suggesting that a decline in systemic CSF1 levels may have a detrimental effect on tissue homeostasis (63). The contrary trend seen in RPE-choroid and systemic levels of IGFBP3, CCL9 and CXCL2, together with the ocular phenotype observed in knockout mice implies that local cytokine milieu may have a more dominant role in maintaining homeostasis and/or contributing to disease, especially in an organ such as the eye, which enjoys immune privilege. Though it remains to be seen if the inflammation observed in these mice is a direct consequence of the absence of LXR or if it occurs in response to the elevated lipid accumulation, we construe that loss of Nr1h3 leads to a pro-inflammatory ocular environment, which may aid in the progression of the disease by altering RPE and/or choroidal endothelial cells.

Finding and developing therapies for dry AMD has been a challenge to date. This dilemma is in part, due to the complexity of this neurodegenerative disease, with multiple retinal cells affected and pathogenic pathways involved, raising the question of which cells and/or pathways should be targeted. One strategy has been to target genetic risk factors or modify environmental factors associated with disease progression. An alternative strategy would be to strengthen the
armor of AMD-vulnerable cells and reinforce pathways that are compromised as a function of age. 478

We found that expression of LXRs decreases as a function of age and, in particular, in the ‘elderly’ risk age-group, supporting the hypothesis that activating LXR may have beneficial effects on the overall health of RPE cells. Two factors must be considered in pursuing pre-clinical testing of an LXR targeted therapy.

The *first* is the choice of activating ligand. To date, several activating LXR ligands have been reported to modulate lipid metabolism and inflammatory pathways in different injury models, including the eye. Activating LXR with GW3965, has been shown to provide a protective effect in experimental diabetic retinopathy and an inner retinal damage mouse model (56, 64), while the LXR agonist TO901317 has been shown to ameliorate amyloid β-induced inflammatory responses in human RPE cells as well as the neural retina (65, 66). It is important to note however, that LXR ligands, are not without side-effects, most notably temporary hyper-triglyceridemia. Furthermore, the TO901317 compound, the first LXR ligand to be identified and one that has been tested frequently by researchers, has potential off-target effects through activation of other nuclear receptors including FXR and PXR (67, 68). The selectivity of LXR ligands can be attributed to several factors, including degree of induction of genes involved in lipid transport, namely *ABCA1, ABGAI, APOE*; or trans-repression of proinflammatory genes such as nitric oxide synthase (*iNOS*), cyclooxygenase-2 (*PTGS2*), or proinflammatory cytokines in microglia and astrocytes (47, 69, 70). It has further been proposed, but unproven that GW3965 and TO901317 may bind to both isoforms with varying degrees of binding affinity (71). In our studies, in vitro assays allowed comparison of the function of agonists GW3965 and TO901317, versus the antagonist GSK2033 in RPE and choroidal endothelial cells. We found these activating ligands bind to both isoforms, NR1H3 and NR1H2, as evident by the induction of promoter activity and target gene expression,
in the presence of siRNA targeting individual isoforms. Our in vitro, AMD-relevant endpoints for assessing LXR ligand efficacy, included mitochondrial membrane potential, as a measure of mitochondrial health, and lipid accumulation. The choice to assess the mitochondria was in light of the extensive evidence supporting a contributory role of mitochondrial dysfunction, changes in mitochondrial number and size, increased mitochondrial DNA damage and a decline in DNA damage repair in AMD (72-75). Furthermore, the mitochondrial enzyme cytochrome P450 27A1 (CYP27A1), regulates cholesterol homeostasis, bile acid biosynthesis, activation of vitamin D₃, and removal of the cytotoxic metabolite 7-ketocholesterol, and its absence in mice results in upregulation of cholesterol biosynthesis in the mouse retina, along with sub-RPE accumulation, (76, 77), supporting additional contributions of mitochondrial function to AMD pathogenesis. We found that the ability of LXR activation to prevent mitochondrial injury when RPE cells are exposed to multiple oxidative insults, including treatment with sodium iodate, hydrogen peroxide, cigarette smoke extract, and lipid overload was selective and based on the type of oxidant injury. TO901317, was effective in countering ‘classical’ oxidant injury such as NaI₃O₃, H₂O₂ and CSE whereas, GW3965 was effective in ameliorating mitochondrial injury induced by lipid treatment. Similarly, we found that only GW3965 was able to prevent intracellular lipid accumulation in response to treatment with PA and αLA in RPE cells.

The second factor to be considered for pre-clinical studies is the choice of animal model. We tested the therapeutic effect of LXR activation on lipid deposition and inflammation in the apoB100 mouse model, which is characterized by accumulation of neutral lipids below the RPE (24), a phenotypic feature of aging and potential precursor to development of AMD. Furthermore, in these mice, the cholesterol transporter, apoE, also accumulates below the RPE in the space where sub-RPE deposits develop (32). We chose to deliver the drug via diet, as systemic delivery
of LXR ligands have been used to target the brain and both GW3965 and TO901317 have been shown to pass the blood-brain-barrier (78, 79). We observed that apoB100 mice fed a diet rich in GW3965 for 5 months display a decline in expression of inflammatory markers (GFAP and ADGRE1) within the retina, and, apolipoprotein E deposition within Bruch’s membrane, supporting the therapeutic potential of targeting LXR to target these AMD associated pathologies.

Several studies to date have examined the phenotype of tissue specific knockdown of downstream target genes of the LXR, including ABCA1 in photoreceptor and RPE cells (80, 81). In the absence of Abca1 in mouse photoreceptors, there is accumulation of neutral lipid rich sub-retinal debris and Aif1+ sub-retinal cells, along with compromise in visual function (81) while the absence of Abca1 in the mouse RPE demonstrated retinal degenerative changes including significant RPE and retinal degeneration concomitant with RPE lipoidal degeneration, a phenotype also seen in our Nr1h2−/− mice (80). Given previous findings that Abca1 levels are impacted more so in the absence of the beta isoform than alpha, it is plausible that downstream effectors other than Abca1 may be involved in the accumulation of lipids extracellular to RPE cells (e.g. the lipid-rich drusen phenotype). Additionally, phenotypic characterization of Nr1h2−/− mice, which exhibit inflammation of the optic nerve and loss of ganglion cells with aging, suggests that the beta isoform is a major contributor to the regulation of inflammation in the inner retina (82). Ban et al targeted the lipid efflux pathway in macrophages by deleting Abca1 and Abcg1, and found that the double knockout mice present with extracellular lipid-rich deposits in the outer retina, below the retina, concomitant with photoreceptor dysfunction (83). Collectively, these reports in conjunction with our results support that both LXR isoforms are involved in the regulation of inflammation and lipid deposition in inner and outer retina, but in an isoform-specific manner and the need for future studies examining the impact of LXR using tissue specific knockout models.
Overall, our study establishes the LXR pathway as a key regulator of lipid transport and inflammation in the eye. Aged mice lacking the alpha isoform represent a new mouse model of the dry AMD phenotype and may be used as a platform to test therapies specifically targeting excess extracellular lipid accumulation and inflammation. Further, we identified mechanisms of LXR-based therapies and demonstrated in vivo efficacy of targeting the LXR pathway. Importantly, our results underscore the importance of the health of RPE cells, located at an important interface to regulate delivery of cholesterol to the retina as well as its elimination, highlighting their importance as a target for ocular pathologies governed by excessive lipid accumulation in the posterior pole (84). Broadly, this translational study supports developing LXR targeted therapies for the treatment of neurodegenerative diseases of aging, in which altered cellular metabolism and chronic inflammation may play a role.
Methods

Human tissue and cell lines: Use of human donor eyes for research was approved by the Duke University Institutional Review Board. Details of methods associated with tissue collection and cell culturing are provided in the supplementary section.

Animals. Male and female Nr1h3/Nr1h2+/+ (WT), Nr1h3−/−, Nr1h2−/−, Nr1h3−/−/Nr1h2−/− mice on the C57BL/6J background were used to examine the effect of the absence of the Nr1h2 and 3 in vivo. Mice which express apoB100 (apoB100 mice, genetic background: 50% C57BL/6 and 50% 129/Sv) (24) were used to evaluate the effect of a LXR agonist in vivo. Additional details are provided in the supplementary section.

Statistical methods and rigor. Statistical methods for data analysis included two-tailed Student’s t-test and two-way ANOVA, with Tukey’s/Donnett’s (as recommended by GraphPad Prism) multiple comparison test using GraphPad Prism. Values were considered statistically significant at p < 0.05 and are indicated in the figures. For in vitro experiments including transcriptional activity assays, JC-1, adipored and cell viability, samples were run in triplicate and experiments were performed a minimum of three times (technical and biological replicates = 3).

Study Approval. The study protocols were approved by the Duke University Institutional Animal Care and Use Committees and all animal experiments were performed in accordance with the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Supplementary methods. Additional details of methods including RNA isolation, qPCR, siRNA transfection, transcriptional activity, adipored, and cell viability assays, JCI assay, blood collection, cytokine array, and post-mortem tissue analyses are provided in the supplementary section and Table S5.
Author Contributions

MC, PLY, and FT participated in the collection of samples, data, or data analysis. MEB performed autofluorescence measurements. ENI and JWR performed QFDE. PT provided LXR knockout mice. RAR, SN and RSA, assisted in visual function testing, ERG analysis, or Fundus-OCT imaging. JTH provided apoB100 mice. MC and GM wrote the manuscript. GM edited the manuscript. All authors have read and approved the manuscript for publication.
Acknowledgements

This research was supported by the National Eye Institute grants R01 EY027802, R01 EY028160 (to GM), P30 EY005722 (to the Duke Eye Center), EY000331 (to the Stein Eye Institute, Core Grant for Vision Research), R01 EY019287-06 (to RSA), P30 EY02687 (to Washington University, Core Grant for Vision Research) and R01 EY027691 (to JTH-Robert Bond Welch Professor); the Edward N. & Della L. Thome Memorial Foundation Award (to GM), the Carl and Mildred Almen Reeves Foundation (to RSA), the Starr Foundation (RSA); the Bill and Emily Kuzma Family Gift for Retinal Research (RSA); the Jeffrey Fort Innovation Fund (RSA); the Glenn Foundation for Medical Research and the Thome Foundation (RSA); and the Research to Prevent Blindness, Inc (RPB) Core grant (to the Duke Eye Center). We are grateful to the donors and donor families for their generosity. We thank Dr. Neal Peachy for assistance with the c-wave recording protocols. Sincere thanks to Drs. Donald P. McDonnell and Ching-Yi Chang for plasmids used in transcriptional activity assays and valuable discussions. We thank Dr. Abdoulaye Sene, Dr. Xiaoping Qi, Mr. Eddie Meade, and Mr. Michael Lekwuwa, for technical support.
References:


Figures and figure legends

**Figure 1.**

**NR1H3 expression in RPE declines with age.** (A) Expression of NR1H3 in RPE cells isolated post-mortem from donors (18 to 94 years old; fold change relative to ARPE19, n = 31, Pearson R squared was calculated, p < 0.0001). (B) Expression of NR1H3 in RPE cells isolated post-mortem from donors (60 to 94 years old; fold change relative to ARPE19, n = 25, Pearson R squared was calculated, p = 0.018). (C) Agarose gel image of genomic DNA amplification products of NR1H3, NR1H2 and their obligate binding partners RXRA and RXRB in retina, freshly isolated human RPE cells (fRPE), ARPE19 cells, primary human RPE cells (hRPE), human choroid and RF/6A cells.
β-Actin (*ACTB*) was used as a loading control. Representative images of NR1H3 (12HCLC) immunohistochemical (IHC) staining (dark grey) in retina/RPE/choroid complex from non-AMD (D, E; arrows point to a cone and RPE cells) and AMD (G, H) donors (arrowheads point to dark grey NR1H3 staining within drusen, Inset: IgG control; Scale bar, Panel D: 50 μm, Panel E: 25 μm). Panels (F) and (I) show NR1H3 immunofluorescence (IF) staining in non-AMD and AMD donors (arrowheads point to NR1H3 staining, red; Scale bar, Panel F: 25 μm). NR1H2 IHC staining in retina/RPE/choroid complex from non-AMD (J, K) and AMD (M, N) donors (arrowheads point to dark grey NR1H2 staining within drusen). Panels (L) and (O) show NR1H2 IF staining in non-AMD and AMD donors (arrowheads point to NR1H2 staining within drusen, green). Total number of eyes probed with antibodies (n = 2 – 10 from age-matched non-AMD and AMD donors, respectively). IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer, OS: Outer segments, RPE: Retinal pigment epithelial cells.
Figure 2.

The LXR pathway is biologically active in AMD vulnerable cells. (A) LXR activity in hRPE cells transfected with DR-4 luciferase reporter and siC (control, non-targeting siRNA), siNR1H3, siNR1H2, or siNR1H3/NR1H2, and treated with LXR agonists GW3965 and TO901317, or antagonist GSK2033. DMSO was used as a vehicle control (n = 3, Mean + SEM a: p < 0.05 relative to siC DMSO, b: p < 0.05 relative to drug-siC-treated cells, 2-way ANOVA and Tukey’s multiple comparison). Expression of LXR target genes FASN (B) and SREBF1 (C) in hRPE cells in response to treatment with LXR agonists, GW3965 and TO901317 and antagonist, GSK2033 (n = 3, Mean + SEM, a: p < 0.05 relative to siC DMSO, b: p < 0.05 relative to drug-siC-treated cells, 2-way ANOVA and Tukey’s multiple comparisons). (D) LXR activity in CEC transfected with DR-4 luciferase reporter and siC, siNR1H2, siNR1H2, or siNR1H3/NR1H2; cells and treated with LXR agonists GW3965 and TO901317, or antagonist GSK2033. DMSO was used as a vehicle control (n = 3, Mean + SEM a: p < 0.05 relative to siC DMSO, b: p < 0.05 relative to drug-siC-treated cells, 2-way ANOVA and Tukey’s multiple comparison). Expression of LXR target genes FASN (E) and SREBF1 (F) in CEC cells in response to treatment with LXR agonists, GW3965 and
TO901317 and antagonist, GSK2033 (n = 3, Mean ± SEM, a: p < 0.05 relative to siC DMSO, b: p < 0.05 relative to drug-siC-treated cells, 2-way ANOVA and Tukey’s multiple comparison).
Figure 3.

LXR activation differentially ameliorates oxidant injury and lipid overload in a ligand-dependent manner. Human primary RPE cells from a 93 year old donor were pretreated with LXR agonists or antagonists followed by oxidant injury or lipid overload and the (A) effect of LXR activation on the mitochondrial membrane potential was measured by exposure to the JC-1 dye, in which the ratio of JC-1 monomers/aggregates was used as a measure of mitochondrial injury. (B) Intercellular lipid accumulation was quantified in adipored assays. (C) Cell viability was measured using CellTiter-Blue®. Drugs used included the LXR agonists GW3965 and TO901317; and antagonist GSK2033. DMSO and ethanol were used as vehicle controls. n = 3, Mean + SEM, a: p < 0.05 relative to vehicle control, b: p < 0.05 relative to drug control, 2-way ANOVA and Tukey’s multiple comparison.
Loss of *Nr1h3* in vivo compromises visual function. Averaged ERG responses from 10-14-month-old dark-adapted *WT, Nr1h3"/", Nr1h2"/", and Nr1h3"/"/Nr1h2"/" mice. Plots of (A) scotopic a-wave amplitudes, (B) scotopic b-wave amplitudes, (C) photopic a-wave amplitudes as a function of flash intensity are shown (*WT*: n = 15, *Nr1h3"/": n = 11, *Nr1h2"/": n = 11, and *Nr1h3"/"/Nr1h2"/": n = 2; a: p < 0.05, *Nr1h3"/" relative to *WT*; b: p < 0.05, *Nr1h2"/" relative to *WT*; Mean ± SEM; Multiple t-tests). (D) c-wave amplitudes (n = 4, Mean ± SEM, *: p < 0.05, ns: not significant, 1-way ANOVA, Dunnett's multiple comparisons test). Dark adaptation ERG was performed and results were reported as (E) sensitivity as a function of time (*WT*: n = 4, *Nr1h3"/": n = 4, *Nr1h2"/": n = 4; Mean ± SEM is shown) and (F) ratio of A wave amplitude (a wave)/pre-bleach a-wave amplitude (A wave max) (*WT*: n = 4, *Nr1h3"/": n = 4, *Nr1h2"/": n = 4; Mean ± SEM is shown).
Figure 5.

Pathology of *Nr1h3<sup>−/−</sup>* mice reveal isoform specific differences. Micron IV acquired in vivo images of the posterior poles of 10 to 14 month old (A, B) *WT* (*n* = 4), (C, D) *Nr1h3<sup>−/−</sup>* (*n* = 5), (E, F) *Nr1h2<sup>−/−</sup>* (*n* = 3) and (G, H) *Nr1h3<sup>−/−</sup>/Nr1h2<sup>−/−</sup>* (*n* = 4) mice. Black arrowheads point to hypo-reflective spots in the fundus. Toluidine blue stained images of plastic sections from (I) *WT* (*n* = 4), (J) *Nr1h3<sup>−/−</sup>* (*n* = 5), (K) *Nr1h2<sup>−/−</sup>* (*n* = 3), and (L) *Nr1h3<sup>−/−</sup>/Nr1h2<sup>−/−</sup>* (*n* = 4) mice showed normal retinal morphology and no gene-specific differences at the level of the inner retina (scale bar in panel I = 20 μM). Conventional electron micrographs of RPE/Bruch’s membrane/choroidal junction in (M, N) *WT* mice display normal RPE morphology, while (O, P) *Nr1h3<sup>−/−</sup>* mice illustrate continuous sub-RPE deposits (marked by red dotted line) and disorganized RPE basal infoldings. (Q, R) *Nr1h2<sup>−/−</sup>* mice develop sub-RPE deposits (marked by red dotted line) along with large lipid droplets (red arrowheads) within the RPE, while (S, T) *Nr1h3<sup>−/−</sup>/Nr1h2<sup>−/−</sup>* mice display a more
severe form of both pathologies (sub-RPE deposits: red dotted line, lipid droplets: red arrowheads) (cTEM mag bar = 1 μm). CC: Choriocapillaris, RPE: Retinal pigment epithelium, OS: Outer segments, ONL: Outer nuclear layer, OPL: Outer plexiform layer, INL: Inner nuclear layer, IPL: Inner plexiform layer.
**Figure 6.**

*NR1H3 regulates lipid deposition in the outer retina.* Representative images from retina/RPE/choroid sections of 10 to 14 month old (A) WT (n = 3), (B) *Nr1h3*<sup>−/−</sup> (n = 4), (C) *Nr1h2*<sup>−/−</sup> (n = 4), and (D) *Nr1h3*<sup>−/−</sup>/*Nr1h2*<sup>−/−</sup> (n = 3) mice stained with Oil Red O to visualize neutral lipids are shown (scale bar = 20 μm). Retina/RPE/choroid sections from 10 to 14 month old (E) WT (n = 3) (F) *Nr1h3*<sup>−/−</sup> (n = 4) were stained for APOE (red) and EO6 (green). Representative images are shown (scale bar = 20 μm). QFDE micrographs from 10 to 14 month old (G, H) WT (n = 4) and (I, J, L) *Nr1h3*<sup>−/−</sup> (n = 4) mice. In these micrographs, the fracture plan “hovers” between the RPE basal infoldings (BI) and choriocapillaris (CC). (G) In the WT mouse, the BI is located in the lower half and left side of the micrograph (BI, black border). The BI structure appears uniform and consistent. (I) In the *Nr1h3*<sup>−/−</sup> mouse, the BI region is located in the center of the micrograph and along the bottom edge (red border). Regions of the BI that have the unaffected structure (labeled BI, within black border), are predominantly adjacent to BrM, but most of the region in this micrograph shows a heavily disrupted BI (dBI). Inset (H) shows a higher magnification of a region that contains both healthy BI and BrM. Inset (J) shows the surface structure of the dBI and healthy BI. Inset (K) shows a high magnification of the dBI structure marked by the box in inset (J). Scale bar in G, I = 4 μm, inset H, J = 1 μm, inset K = 250 nm (BrM: Bruch’s membrane, OCL: Outer collagenous layer). (L) Representative image of healthy BrM from WT mouse (red dotted line; scale bar = 500 nm). (M) Representative image from *Nr1h3*<sup>−/−</sup> mouse, showing spherical particles in BrM (red dotted line and red arrowheads; scale bar = 500 nm). (N) A region of healthy BrM in the *Nr1h3*<sup>−/−</sup> mouse (red dotted line; scale bar = 500 nm). Representative images of RPE autofluorescence from 10 to 14 month old (O) WT, (P) *Nr1h3*<sup>−/−</sup>, (Q) *Nr1h2*<sup>−/−</sup>, and (R) *Nr1h3*<sup>−/−</sup>/*Nr1h2*<sup>−/−</sup> mice (RPE: Retinal pigment epithelium, OS: Outer segments). (S) Quantification of
lipofuscin autofluorescence in RPE cells (n = 2 measurements in n = 5 eyes per genotype). Mean
+ SEM, *: p < 0.05, 1-way ANOVA, Dunnett’s multiple comparisons test).
**Figure 7.**

*Nr1h3−/−* mice exhibit a pro-inflammatory microenvironment in the outer retina. RPE-choroid flatmounts from 10 to 14 month old (A) *WT* and (B, C) *Nr1h3−/−* (two examples are shown) (ON: Optic nerve) mice were stained with ADGRE1 (green) and phalloidin (red) (Scale bar = 50 μM). (D) Quantification of ADGRE1⁺ cells in RPE-choroid flatmounts from *WT* and *Nr1h3−/−* mice (*WT*: n = 5, *Nr1h3−/−*: n = 8, Mean ± SEM, *: p < 0.01. Unpaired t-test). (E) Protein isolated from RPE-choroid complexes of 10 to 14 month old *WT* and *Nr1h3−/−* mice were blotted on a Mouse Cytokine Array C3 (62 proteins). Representative images of significantly regulated cytokines (two dots/protein) are shown. (F) Quantification of staining intensity of significantly regulated cytokines (n = 4 per group, Mean ± SEM, *: p < 0.05, **: p < 0.01; Multiple t-tests)
GW3965 diminishes pathogenic phenotypes associated with inflammation and lipid deposition. Fundus and OCT images of *apoB100* expressing mice on control low fat diet (LFD) diet (A, B) and LFD + GW3965 (GW) diet (C, D) post treatment. Black arrowheads point to hypopigmented regions in the fundus. GFAP staining in retina/RPE/choroid sections from LFD (E) and...
LFD + GW (F) mice. Representative images are shown (Scale bar = 50 μm). APOE immunolocalization in retina/RPE/choroid sections from LFD (G) and LFD + GW (H) mice. Representative images are shown (Scale bar = 50μm). (I) Quantification of APOE⁺ staining (n = 3 per group, Mean ± SEM, *: p < 0.05, Unpaired t-test). ADGRE1⁺ staining in retina/RPE/choroid sections from LFD (J) and LFD + GW (K) mice (RPE: Retinal pigment epithelial cells, OS: Outer segment, Ch: choroid, dotted line depicts Bruch’s membrane). Representative images are shown (Scale bar = 50μm). (L) Quantification of sub-retinal ADGRE1⁺ cells (LFD: n = 3, Mean ± SEM, LFD + GW: n = 4, ns: not significant, Unpaired t-test). TEM images of the RPE cells from LFD treated mice, showing (M) disrupted basal infoldings (red inset), (N) compromised RPE cells, and (O) sub-RPE deposits (red dotted line). (P, Q, R) TEM images of the RPE cells from LFD + GW treated mice, showing improvement in RPE health, basal infoldings and absence of sub-RPE deposits (P: Scale bar = 2 μm).