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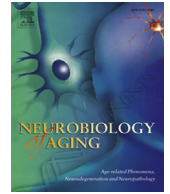
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# Death by color: differential cone loss in the aging mouse retina<sup>☆</sup>

Alexander Cunea, Michael B. Powner, Glen Jeffery\*

Institute of Ophthalmology, University College London, London, UK



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## ABSTRACT

Differential cell death is a common feature of aging and age-related disease. In the retina, 30% of rod photoreceptors are lost over life in humans and rodents. However, studies have failed to show age-related cell death in mouse cone photoreceptors, which is surprising because cone physiological function declines with age. Moreover in human, differential loss of short wavelength cone function is an aspect of age-related retinal disease. Here, cones are examined in young (3-month-old) and aged (12-month-old) C57 mice and also in complement factor H knock out mice (CFH<sup>-/-</sup>) that have been proposed as a murine model of age-related macular degeneration. In vivo imaging showed significant age-related reductions in outer retinal thickness in both groups over this period. Immunostaining for opsins revealed a specific significant decline of >20% for the medium/long (M/L)-wavelength cones but only in the periphery. S cones numbers were not significantly affected by age. This differential cell loss was backed up with quantitative real-time polymerase chain reaction for the 2 opsins, again showing S opsin was unaffected, but that M/L opsin was reduced particularly in CFH<sup>-/-</sup> mice. These results demonstrate aged cone loss, but surprisingly, in both genotypes, it is only significant in the peripheral ventral retina and focused on the M/L population and not S cones. We speculate that there may be fundamental differences in differential cone loss between human and mouse that may question the validity of mouse models of human outer retinal aging and pathology.

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## 1. Introduction

Cell loss is a key feature of aging. However, vulnerability in aging is not spread evenly across cellular populations, as in nearly all cases there are subpopulations who display selective susceptibility to loss of function and death with age. Cell death is a significant feature of the aging retina that has been well documented in the rod population in man and rodents, where approximately 30% of these cells are lost over life, whereas those that remain have significantly shorter outer segments. In rodents, this loss is present at 2 years of age whereas in man it is established around 70 years of age (Cunea and Jeffery, 2007; Curcio et al., 1993). The high magnitude of cell loss here is probably linked to the high metabolic demand of the outer retina (Barot et al., 2011; Liang and Godley, 2003; Winkler, 1981; Winkler et al., 1999). Surprisingly, data on cone loss with aging is less clear. Although there is agreement on the decline in the cone driven photopic

electroretinogram (ERG) with age in mice, data on changes in the underlying cone photoreceptor population do not reflect this. Three studies have combined electrophysiological data and cone counts in mice. Williams and Jacobs (2007), argue that cone densities in the mouse do not change with age even when aged 2 years. Gresh et al. (2003) similarly argue that in normally pigmented mice, there is no significant change in cone numbers over the age of 17 months. These studies are supported by Li et al. (2001) in 1-year-old animals. Again, these authors argue that there is no underlying cone loss with age. The miss match between structure and function is puzzling, particularly in light of human data showing specific age-related deficits in the photopic ERG (Neveu et al., 2011) and reduced central cone numbers (Song et al., 2011), although the latter was based on limited in vivo imaging. Further, with aging and disease, human S cone function may be particularly impaired, implying that this cone subtype is relatively vulnerable (Cho et al., 2000; Greenstein et al., 1989; Haegerstrom-Portnoy, 1988; Heron et al., 1988; Johnson et al., 1988).

Mice have unusual patterns of cone distribution. Like most mammals they only have 2 cone opsins, a short-wavelength opsin (S) and a combined medium/long wavelength opsin (M/L). Further, some cells can express both opsins (Bowmaker, 2008; Rohlich et al., 1994). However, these have differential distributions with M/L cones widely distributed or more common dorsally and S cones predominantly present in the ventral retina (Rohlich et al., 1994),

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\* Corresponding author at: Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V 9EL, UK. Tel.: +44 2076086837; fax: +44 2076086909.

E-mail address: [g.jeffery@ucl.ac.uk](mailto:g.jeffery@ucl.ac.uk) (G. Jeffery).

although patterns of distribution may be more complex (Applebury et al., 2000; Neitz and Neitz, 2001). Hence, if age-related cone cell death is differential, then examining its impact may critically depend on the geographic regions selected for analysis. Here, we address the issue of age-related cone loss using normal C57 mice, and those that have features of advanced aging and/or pathology in the form of complement factor H knockouts (CFH<sup>-/-</sup>), which is regarded by some as a model of age-related macular degeneration and experiences elevated outer retinal inflammation with aging (Coffey et al., 2007; Hoh Kam et al., 2013). We separate cones into different subtypes based on their opsin expression and into those from different regions. We reveal significant cone loss in the first year of life that is focused specifically on the M/L population in the ventral retina.

## 2. Methods

### 2.1. Mice

C57BL/6J mice (n = 20) and CFH<sup>-/-</sup> mice on C57 background (n = 20) were used. They were of 2 ages: young, 3-month-old and old, 12-month-old. These ages have been selected because at the age of 3 months the mouse eye has reached normal adult size, and 12 months because both Li et al. (2001) and Gresh et al. (2003) show clear cone mediated reductions in ERG function at this stage. Direct comparisons between man and mouse in terms of age are difficult to assess, as the wild mice only live for 3–4 months and man only for around 35 years. However, a 12-month-old mouse may be regarded as aged as they are no longer fertile. Few mice will live in the laboratory for >24 months. All animals were used with University College London ethics committee approval and under a UK Home Office animal project license. All animal procedures conformed to the United Kingdom Animals Scientific Procedures Act 1986.

### 2.2. Optical coherence tomography

Optical coherence tomography (OCT) imaging was used to determine the overall structure of the retina between the 2 genotypes. One study has suggested that even young CFH<sup>-/-</sup> have retinæ 25% thicker than wild-type animals (Williams et al., 2013), implying a potential developmental difference which would undermine the question we address. To provide high-resolution images of retinæ, *in vivo* mice (n = 5 per group, 4 groups) were anesthetized with an intraperitoneal injection of 6% Ketamine, (Fort Dodge, UK), 10% Dormitor (Pfizer, UK), and 84% sterile water. Pupillary dilatation was not necessary. Methylcellulose lubricant was placed on the corneas, and the noncontact OCT probe (BiopTigen, Morrisville, NC, USA) was positioned in front of the cornea placing the optic nerve (ON) at the center of the image. Retinal OCT was performed using a rectangular volumetric scan with a B-scan length of 1.50 mm and a width of 3.00 mm at a rate of 1300 scans. Beginning at a distance of 0.4 mm from the ON head, 3 measurements were made along an orthogonal axis of the retina. Total retinal thickness and that of the outer nuclear layer (ONL) were made on either side of the ON using Adobe Photoshop CS5 extended. Six measurements were made adjacent to each other at each location. These 6 values were averaged as one representative of the location. Subsequently, 1 eye from each mouse was used for immunostaining and the other for quantitative real-time polymerase chain reaction (qPCR).

### 2.3. Retinal wholemounts

Mice were killed by cervical dislocation. Eyes were immediately enucleated and placed in 4% paraformaldehyde in phosphate

buffered saline (PBS, pH 7.2) for 1 hour, then washed in PBS and the retinæ dissected free from the eye cup. Four to 6 radial cuts were made to produce wholemounts. They were then placed in a blocking solution of 5% normal donkey serum, 3% Triton X-100, and PBS for 2 hours (NDS, Jackson Labs USA). Wholemounts were labeled with primary antibodies, rabbit polyclonal antibody against Red/Green M/L opsin: (1:5000, AB5405, Temecula California 92590) and goat polyclonal antibody against S opsin protein OPN1SW (1:1000, sc-14636 L1906, Santa Cruz Biotechnology) and left overnight at room temperature. Tissues were then washed and incubated for 2 hours in fluorescent secondary antibodies at a dilution of 1:2000 made up of 2% NDS, 3% Triton X-100, and PBS. In the negative control tissue, primary antibodies were omitted. All wholemounts were cover slipped with Vectashield (Vector Labs, Burlingame, USA).

### 2.4. Quantitative real-time polymerase chain reaction

As an independent measure for comparison with immunostaining, qPCR was additionally used to identify and quantify photoreceptor subtype specific RNA expression. After performing OCT, mice were kept overnight in standardized dim lighting condition with only indirect illumination at approximately 5 lux. They were killed as mentioned previously, their eyes immediately enucleated, and the retinæ dissected free and immediately snap frozen.

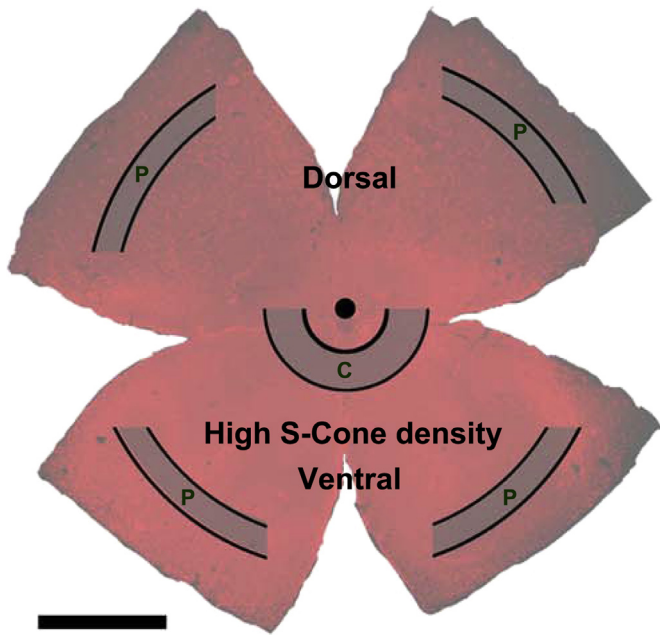
Total RNA was extracted using the Tri Reagent (Molecular Research Center, Inc Cincinnati, OH, USA). Complementary DNA was prepared (QuantiTect Reverse Transcription Kit, QIAGEN) and polymerase chain reaction (PCR) performed in an ABI 7900HT FAST Real Time PCR system using Promega qPCR Master Mix, S Opsin forward: TGTACATGGTCAACAATCGGA, reverse: ACACCATCTCCA-GAATGCAAG and M/L Opsin forward: CTCTGCTACCTCCAAGTGTGG, reverse: AAGTATAGGGTCCCCAGCAGA. Raw data were analyzed using the DART-PCR software, version 1.0 (Peirson et al., 2003). Relative expression was calculated and normalized to beta actin. Mann-Whitney *U* test was performed for statistical analysis.

### 2.5. Analysis of immune staining patterns

Whole-mounted retinæ were divided into 2 different regions, central and peripheral. The peripheral region was defined as an annulus with a width of approximately 180 µm starting approximately 260 µm from the borders of the retina. The central region was defined as an annulus starting approximately 250 µm from the center of the ON, having a width of approximately 300 µm. A schematic illustration of the areas in which cells were counted is given in Fig. 1. The rationale was to quantify S, and M/L opsin expressing cone numbers in regions where their density was both high and low and also to quantify cells positive for both opsins. Cells were not analyzed quantitatively in the dorsal aspect of the central region because within this area there was a significant gradient in S cone density, and the region was affected by the deep cut made dorsally for the purposes of orientation.

### 2.6. Measurement of the S- and M-cone density in defined regions

Multiple images were captured at ×200 within regions of interest at slightly different planes of focus, so as not to miss any labeled cells. These were exported in a JPEG format at a resolution of 3840 × 3072 pixels. Analysis was undertaken in Adobe Photoshop CS5 extended. Individual cones were clear in all the images and were commonly spaced without overlapping with one another. Further, many had a cone like morphology. Counts undertaken at defined regions in the different young and old mice of both



**Fig. 1.** Outline drawing of the retina showing central (C) and peripheral (P) ventral and dorsal regions used for cell counts. The image was taken from a retina that was stained with primary antibodies for S opsin. A ventral to dorsal gradient of S opsin cells could be seen. Scale bar = 1 mm.

genotypes were made with 6 retinæ per group and a minimum of 3000 cells counted in defined regions. Hence, counts for the separate cone types in old CFH<sup>-/-</sup> mice at each location were drawn from 6 retinæ. Statistical comparisons throughout were undertaken using a 1-tailed Mann-Whitney *U* test.

### 3. Results

#### 3.1. *In vivo* analysis

Retinæ were imaged *in vivo*, to determine if there were any overall significant differences between the genotypes at different ages and confirm that both groups of young animals were similar. *In vivo* imaging resulted in clear retinal images in which cellular layers could be identified (Fig. 2). Here, measurements were made of the total retinal thickness and of the thickness of the ONL. Total retinal thickness was reduced in aged animals of both genotypes (C57: 5.4%, CFH<sup>-/-</sup>: 1.8%), but in neither was this statistically significant. However, measurements of the thickness of the ONL displayed a significant reduction in both groups of mice with age (C57: 14.5%, CFH<sup>-/-</sup>: 10.3%) (Fig. 3). Although this analysis reveals clear age-related changes in the outer retina, cones account for only approximately 3% of the total photoreceptor population (Carter-Dawson and LaVail, 1979). Hence, the magnitude of this difference is likely to be caused by rod loss.

#### 3.2. *In vitro* analysis

The combined cone population was counted in central and peripheral regions as defined in Fig. 1 in young and old C57 and CFH<sup>-/-</sup> mice from tissue immune-stained for both S and M/L opsin. No statistically significant differences were found for age-related changes in combined cone numbers except for in the periphery of C57 mice ( $p = 0.014$ ). However, there was also a notable but nonsignificant decline in the same region in CFH<sup>-/-</sup> mice

( $p = 0.069$ ). Differences in central regions were minimal and not significant (Fig. 4).

#### 3.3. Distribution of different cone types

These results imply there may be a regional specific decline in cone numbers. However, these data do not distinguish between the different cone subtypes. Cone counts were undertaken for cells with specific opsin label and cells with combined opsin expression at defined locations (Fig. 1). The distribution of the different cone types were as described previously, with S opsin cells displaying a marked differential distribution across the retina with their density being much greater in the ventral than the dorsal retina (Fig. 5). In the mice used here, M/L opsin positive cells were more evenly distributed, but with a bias toward the dorsal regions.

In peripheral regions, cells were counted between young and old animals within the ventral half of the central region (Fig. 1). Here, there were no statistically significant differences between young and old mice in terms of the number of cells expressing S opsin, M/L opsin, or S and M/L opsin. Rather numbers hardly differed between the 2 age groups for each opsin type (Fig. 5).

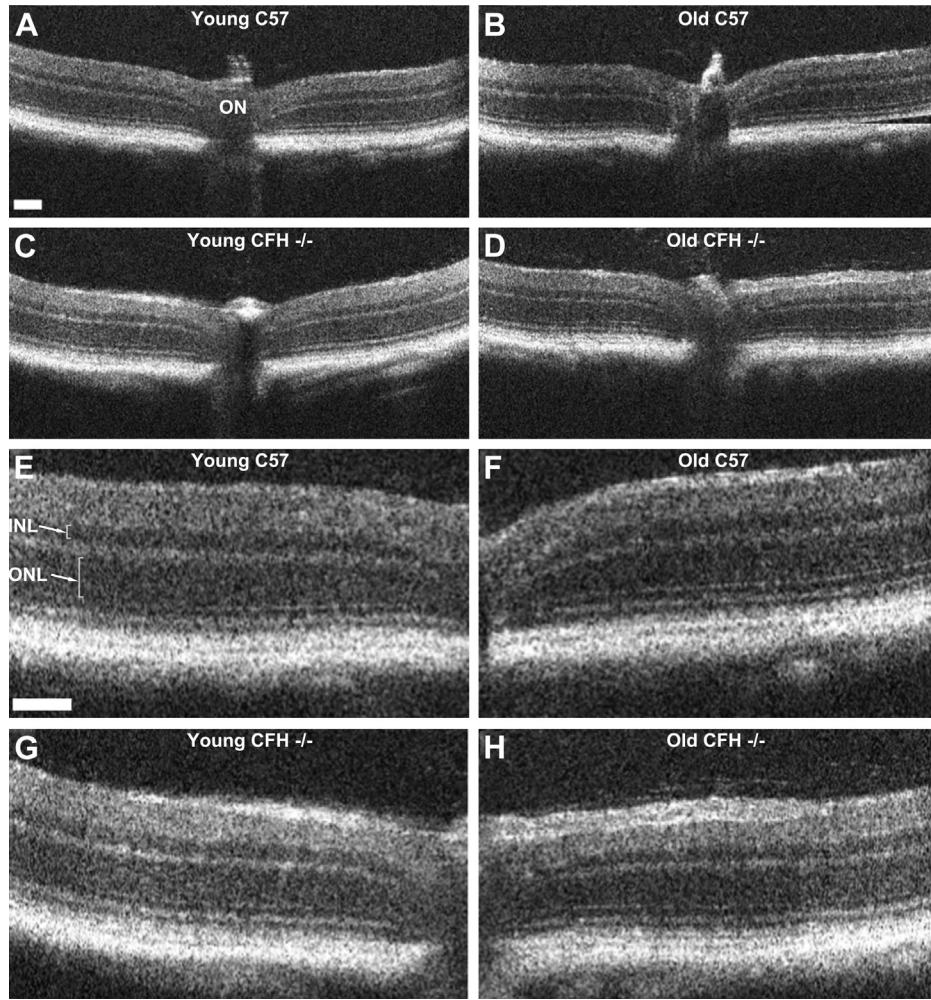
There were no statistically significant differences between young and old mice for S opsin in either C57 or CFH<sup>-/-</sup>, although in both there was a decline with age of approximately 10%–20%. However, in the periphery, there were statistically significant differences between young and old mice in these ventral regions for M/L opsin in C57 mice ( $p = 0.044$ ) and likewise for M/L opsin between young and old CFH<sup>-/-</sup> mice ( $p = 0.021$ ). The reduction in C57 animals was 27% and in CFH<sup>-/-</sup> it was 18%. Statistically significant differences were also present for the CFH<sup>-/-</sup> mice in peripheral regions for cells with combined S and M/L opsin where the decline was >30% ( $p = 0.004$ ). However, the difference in C57 mice for these cells fell just below significance with a decline of 23% ( $p = 0.051$ , Fig. 5). Hence, the identification of significant age-related cell loss depends upon the region examined, occurring only in the periphery, and is only present in cells expressing M/L opsin. In cells expressing both opsin types significant differences were confined to CFH<sup>-/-</sup> mice.

S opsins labeled cells were also counted in the dorsal half of the retina in regions where their density was low. As with these cells in the ventral retina, there was no significant decline in their number between young and old animals in either C57 or CFH<sup>-/-</sup> mice. M/L opsin cells were also counted in these regions and again there were no significant differences between young and old mice of either genotype (Fig. 5).

#### 3.4. qPCR analysis

The qPCR data for changes of expression of opsins with age reflected the data shown with immune labeling for opsins. However, the qPCR findings are somewhat undermined due to the fact that they do not take into account regional differences. It is not possible to compare directly old and young animals when base lining using mouse housekeeping genes because of the differential cell loss with age throughout the retina. Differential cell loss with age will mean that the proportion of the retinal messenger RNA extract that is derived from cones would be different between young and old animals. However, a ratio between the messenger RNA level expression of S and M/L opsins can be used as a proxy marker to show a preferential change in M/L opsin with age. Whereas in young animals the population of M opsin constitutes approximately 1 in 3 of the total, in old animals M opsin is reduced to only approximately 1 in 4 (S vs. M/L



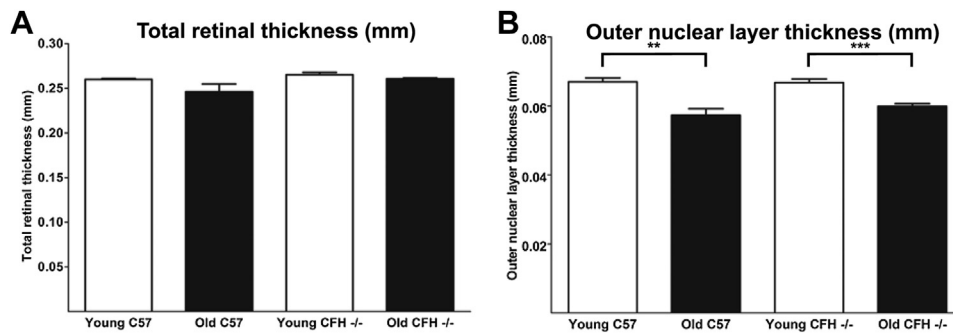


**Fig. 2.** In vivo OCT images of retinas from young and old C57 and CFH<sup>-/-</sup> mice at low (A–D) and high (E–H) magnification. Five animals from each group were imaged, 20 in total. (A–D) Images centered on the optic nerve head, whereas measurements in E–H are taken at a distance of 0.4 mm from the optic nerve head. Retinal layers can be distinguished. Scale bars = 100 μm. Abbreviations: INL, inner nuclear layer; OCT, optical coherence tomography; ONL, outer nuclear layer.

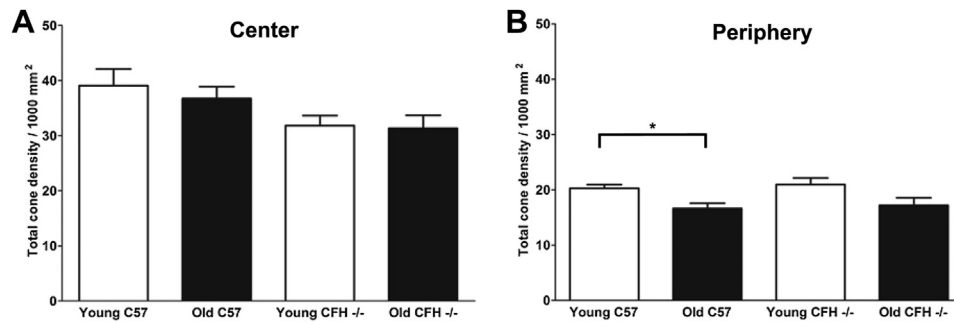
ratio change: C57: 34.4%, CFH<sup>-/-</sup>: 34.7%), with that change in the CFH<sup>-/-</sup> being statistically significant (C57:  $p = 0.075$ , CFH<sup>-/-</sup>:  $p = 0.027$ , Fig. 6). Hence, these data support the notion that M/L opsin is more vulnerable with age, consistent with the immune staining.

**4. Discussion**

Here, we show clear cone loss with aging in the mouse retina. This is focused on the M/L cone population in the peripheral retina and not on the S cones. This is in contrast to the previous studies



**Fig. 3.** Histograms of OCT data showing differences in (A) total retinal thickness and (B) outer nuclear layer thickness in young and old C57 and CFH<sup>-/-</sup> mice. Twenty animals were imaged in total, 5 per group. (A) There were no significant differences in total retinal thickness between young and old mice of either genotype. However, the retina was thinner in older than younger animals (C57: 5.4% reduction, CFH<sup>-/-</sup>: 1.8% reduction). (B) Thickness of the outer nuclear layer in young and old animals of both genotypes. Here, there were significant differences in thickness between young and old C57 (14.5%,  $p = 0.0040$ ) and young and old CFH<sup>-/-</sup> (10.3%,  $p = 0.0003$ ). Error bars = SEM. Abbreviations: OCT, optical coherence tomography; SEM, standard error of the mean. \*\* $p > 0.01$ ; \*\*\* $p > 0.001$ .



**Fig. 4.** Histograms for total cone density in the center and periphery in retinas labeled for both S and M/L opsin. Data are taken from 6 retinas per group, 4 groups. There are no significant differences between young and old in either genotype in the center. In the periphery there are differences in both genotypes with fewer cells in old than young (C57: 18.1% reduction, CFH<sup>-/-</sup>: 18.0% reduction). However, this was only statistically significant in C57 mice (C57:  $p = 0.0140$ ; CFH<sup>-/-</sup>:  $p = 0.0688$ ). Hence, cell loss is a greater feature in the periphery than in the center in both genotypes and of roughly similar magnitude although more variable in CFH<sup>-/-</sup>. \* $p > 0.05$ .

that have failed to find any cone loss with age in mice (Gresh et al., 2003; Li et al., 2001; Williams and Jacobs, 2007). Also, unexpectedly, in spite of studies showing advanced aging and/or pathology in the CFH<sup>-/-</sup> mouse (Coffey et al., 2007), we do not find overwhelming evidence for elevated cone loss in this mouse model compared with the C57 mice. Our results demonstrate significant cone loss over the first year in peripheral M/L cones, which is in contrast to data for the rod population that indicate that significant cell loss here is marked in the second year of life and may be less significant earlier (Cunea and Jeffery, 2007; Fox and Rubinstein, 1989; Kolesnikov et al., 2010).

#### 4.1. The aging mouse outer retina

Cone topography in the mouse retina is unusual (Bowmaker, 2008; Rohlich et al., 1994). Although there remain some controversy over the exact distribution of the different cone types and the proportion and location of those expressing both opsins, it does not undermine our data. Cone distributions in both genotypes were identical. Even if opsin co-expression was underestimated in our study, it does not change the nature of the results presented, that reveals that cells containing S opsin were not subject to the loss found in those containing M/L opsin in the ventral retina.

Mice are nocturnal and largely photophobic. The M/L cone loss recorded here was in the ventral periphery, and it may be relevant that this corresponds to upper visual fields. Mice commonly navigate with their nose down to improve ground scent location and engage their whiskers with the environment. In such a position central and dorsal retinal regions will be exposed to less ambient light than the ventral retina, and this may be related to the differential patterns of cone loss. However, it remains to be demonstrated that normal light exposure influences cell loss at this location in mice. It also remains to be determined, if there are specific structural differences between M/L and S cones in mice as have been found in human and/or primate retinas as discussed in the following.

Three previous studies in the mouse have demonstrated cone functional decline with age, but failed to find evidence for cell loss. This is surprising, particularly as rod loss is a key feature of aging in mouse and man (Cunea and Jeffery, 2007; Curcio et al., 1993). The most likely explanation for this discrepancy is sampling. Williams and Jacobs (2007) sampled 21 sites in whole mounted mouse retinas, but give no further information. The mice in which they present data for cone counts were largely from 200- to 800- day-old, but no loss was found in spite of the extreme age of some. Gresh et al. (2003) examined retinas at 1 and 17 months of age. They examined frozen sections and whole mounts, but the whole mounts were stained with PNA lectin that marks all cones and were sampled at the central and peripheral locations with the average

taken from the 2 measures. But the number of retinas examined is unclear. Additionally, specific opsin staining was only undertaken in the sectioned material. Li et al. (2001) examined mice at 2, 6, and 12 months of age, which is probably comparable with 20–70 years in age span in humans, but all their histology was again in sectioned retinas. It is highly likely that the sectioned material provides a very limited window on cell loss because the number of cones identified in these preparations is small compared with numbers in whole mounts. Although these studies all examined ERGs comprehensively and even pigmentation phenotype differences (Gresh et al., 2003), none specifically focused on counts in whole mounted preparations from defined regions for specific cone subtypes. This is probably the reason for the discrepancy between our results and those of others that have gone before.

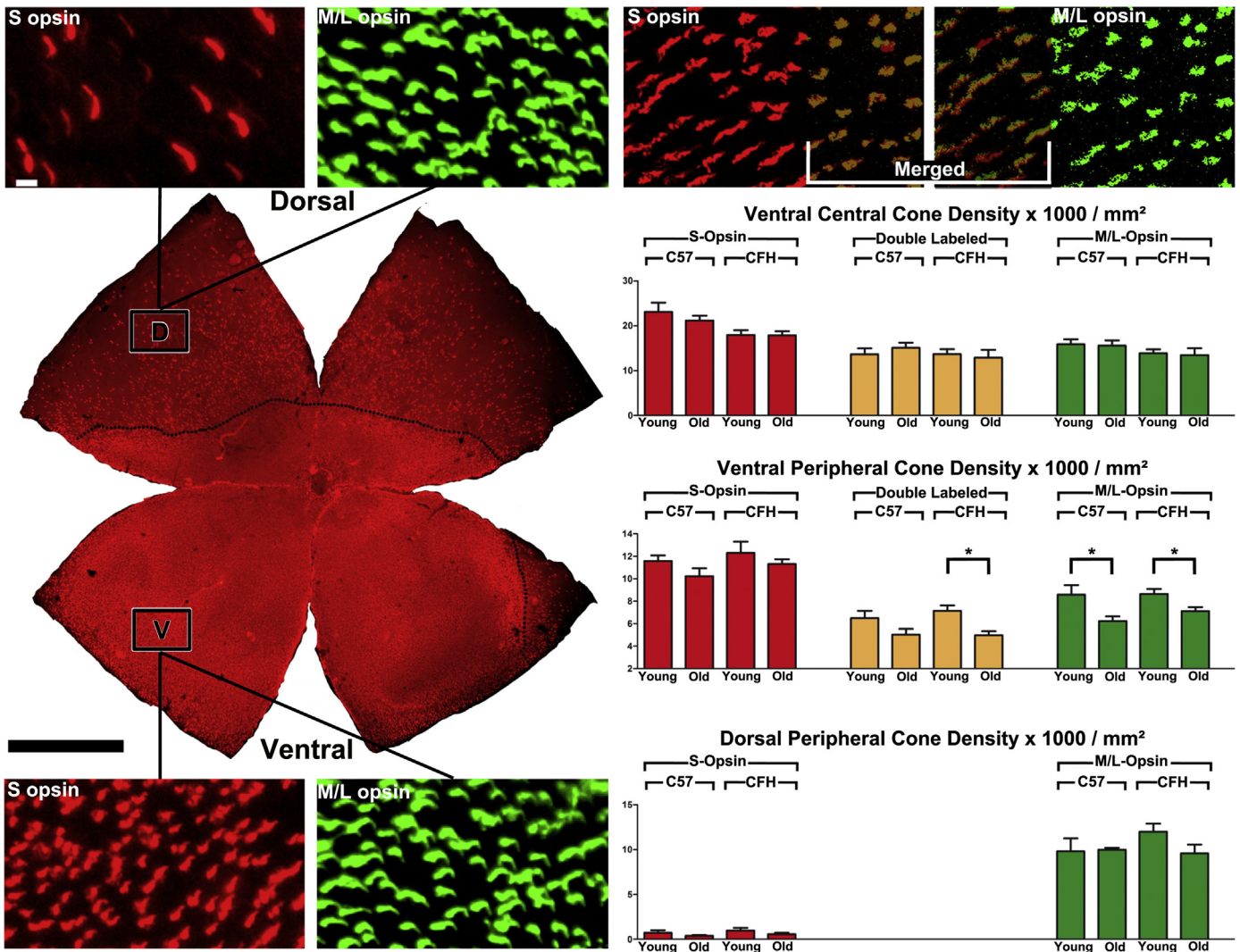
#### 4.2. CFH<sup>-/-</sup> model

There are other key discrepancies between our results and those undertaken previously. The CFH<sup>-/-</sup> mouse has been proposed as an experimental system in which to study age-related macular degeneration relevant pathways. These mice appear to suffer from higher levels of outer retinal inflammation when aging compared with normal mice (Coffey et al., 2007; Lundh von Leithner et al., 2009), but this does not seem to impact differentially on cone numbers. In fact, the CFH<sup>-/-</sup> retina is remarkably normal even in very old mice, and the deficits are far from extreme in 2-year-old animals, which are close to the end of what mouse life span can be extended to and certainly comparable with very old humans (Coffey et al., 2007). This is inconsistent with human data that shows cone photoreceptors are vulnerable to inflammatory damage. In fact, cone function in terms of their ability to follow flickering stimuli in ERG recordings is regarded as a key clinical metric of retinal inflammation in a diverse series of retinal diseases (Crawford and Igboeli, 2013; Holder et al., 2005; Noma et al., 2012). It is possible that chronic outer retinal inflammation reduces cone function without inflicting cell loss and this could explain such discrepancies. However, it is equally likely that there are fundamental differences between mice and human that are not only reflected in the highly unusual cone photoreceptor distributions found in mice and their photophobic life styles, but also in the differential vulnerability of their cone populations. If this is the case, it raises serious questions regarding the suitability of the mouse model for human retinal aging and disease.

#### 4.3. Are cones lost before rods?

It has been argued that rod loss precedes cone loss in human aging and disease (Curcio, 2001; Curcio et al., 1993; Jackson et al.,





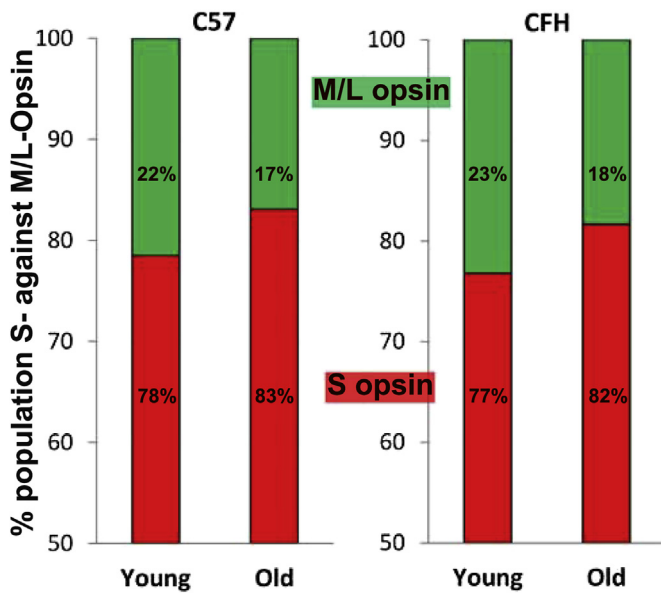
**Fig. 5.** The relative changes in cone subtype with age, genotype, and retinal region. All counts are from 6 retinas per group for each of the metrics shown. Left column: low power image of a retina stained for S opsin showing that most S opsin containing cells are located in the ventral retina. Scale bar = 1 mm. Above and below are higher magnification images reflecting relative cell densities for S and M/L cones in every region. Below are higher magnification images from the ventral high density and above of the dorsal low density regions. Right column: cone densities in different retinal regions for S, M/L, and double labeled S and M/L cones. In central and ventral regions S cones dominated, however, there were also many M/L and combined opsin cells. However, in central regions there were no differences with age in these populations. In ventral peripheral regions, there were significant differences in the number of M/L opsin cells in both genotypes in young and old animals (C57: 27.4% reduction,  $p = 0.0438$ , CFH<sup>-/-</sup>: 17.6% reduction,  $p = 0.0210$ ). Fewer S opsin cells were also found with age (C57: 11.7% reduction, CFH<sup>-/-</sup>: 7.9% reduction), but this was not significant in either genotype. There were reductions in cells expressing both opsins, with numbers reduced in old animals compared with young in both genotypes. This was statistically significant in CFH<sup>-/-</sup> mice but just failed to reach statistical significance in C57 mice (C57: 22.7% reduction,  $p = 0.051$ , CFH<sup>-/-</sup>: 30.6% reduction,  $p = 0.0046$ ). In the dorsal peripheral retina, there were more M/L cones than in other retinal regions and very few S cones, reflecting the clearly visible ventro-dorsal S cone gradient. There were no age-related differences in these cells in either genotype with age. There was a nonsignificant reduction in CFH<sup>-/-</sup> mice (20%,  $p = 0.0657$ ). Cells containing both opsins were present in the dorsal periphery, but their numbers were so low, they cannot be shown on this axis. Hence, age related cell loss is mainly confined for M/L cells in the ventral periphery.

2002; Panda-Jonas et al., 1995), but our data do not support this as a general principal. In rats, there is an approximate 25% rod loss over 24 months of age (Cunea and Jeffery, 2007). Kolesnikov et al. (2010) appear to show a similar proportional decline in the ONL thickness in mice at 30 months. Further, at 12 months Fox and Rubinstein (1989) found only an 8% age-related decline in rat rhodopsin. Our data for ONL thickness decline at 12 months is approximately 12%. However, our loss of M/L cones at 12 months in C57 mice is 27% and for S cones it is 12%. In CFH<sup>-/-</sup> M/L cone loss is 18% and for S cones it is 8% at 12 months. Taken together, it is clear that there is no evidence to argue that rod loss precedes cone loss in the mouse in the first 12 months. Rather, it appears that cone loss is actually greater over this period than the loss in rods.

4.4. Selective cone vulnerability in human aging and disease

The other issue raised by our data relates to what is commonly supposed to be the selective vulnerability of S cones in human aging and disease, which we fail to find here in mice. It has been consistently argued that human S cones are selectively lost with age (Beirne, 2013; Beirne et al., 2008; Eisner et al., 1987; Haegerstrom-Portnoy, 1988; Johnson et al., 1988; Werner et al., 2000; Zlatkova et al., 2003). Further, that they are more vulnerable in a range of diseases including diabetes (Cho et al., 2000; Greenstein et al., 1989), retinal detachment (Nork et al., 1995) and retinitis pigmentosa, and glaucoma (Greenstein et al., 1989; Heron et al., 1988). It has also been suggested that S cones may show vulnerability in age-related macular degeneration (Beirne et al., 2006; Frennesson et al.,





**Fig. 6.** qPCR data. Ratio bars for each genotype showing the relative proportion of M/L cone opsin against S cone opsin. There was a preferential decrease of M/L opsin with age (S vs. M/L ratio change: C57: 34.4%, CFH $^{-/-}$ : 34.7%). Although in young animals the population of M opsin constitutes approximately 1 in 3 of the total, in old animals M opsin is reduced to only approximately 1 in 4. This was significant in CFH $^{-/-}$  mice ( $p = 0.0278$ ), but not in C57 ( $p = 0.0754$ ). Hence, age related cell loss is preferential for M/L opsin. Abbreviation: qPCR, quantitative real-time polymerase chain reaction.

1995; Remky and Elsner, 2005), where inflammation is likely to be an early feature (Johnson et al., 2002). Elevated inflammation is also a key feature in the CFH $^{-/-}$  mice (Coffey et al., 2007). However, it is important to moderate such finding. Studies from Werner's laboratory have emphasized the impact that age-related changes in the lens will have on S cone sensitivity, because as the lens browns with age it will absorb a greater proportion of the shorter wavelengths (Hardy et al., 2005). In spite of this, Shinomori and Werner (2012) have reported age related slowing in the S cone pathway, although other temporal properties of this pathway remain unchanged. Taken together, these results imply that changes with age in the S cone pathway may be more complex than thought when earlier studies were undertaken and may need to be re-addressed in more tightly controlled psychophysical paradigms.

If human S cones are vulnerable it may be related to structural differences as they have longer and wider inner segments than other human cone types. There is also evidence that their synaptic terminals distinguish them from other cone types. Also in human retinae they form only a small proportion of the total cone population being around 5%–10% and have a distinct pattern of distribution with none at the fovea (Calkins, 2001). Further, there may be something distinct in their membrane characteristics as it is possible to stain them selectively with fluorescent dyes (DeMonasterio et al., 1981), potentially implying that their membranes are relatively permeable.

S cones in mice are different from those in humans, in terms of their relative number, retinal distribution, and the wavelength sensitivity of their opsin (Bowmaker, 2008; Neitz and Neitz, 2001; Szel et al., 1992). Hence, it is likely that there are other fundamental differences in their structure and/or organization that may mean that they do not possess the same patterns of vulnerability as their human homologs. If this is correct, it raises a fundamental issue regarding how far mice can be used as animal models of outer retinal aging and pathology. It also raises the issue of why in mice M/L cones show selective vulnerability, the reason for which is unclear.

## Disclosure statement

The authors declare no conflicts of interest. All animals were used with University College London ethics committee approval and under a UK Home Office animal project license. All animal procedures conformed to the United Kingdom Animals Scientific Procedures Act 1986.

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## References

- Applebury, M.L., Antoch, M.P., Baxter, L.C., Chun, L.L., Falk, J.D., Farhangfar, F., Kage, K., Krzystolik, M.G., Lyass, L.A., Robbins, J.T., 2000. The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. *Neuron* 27, 513–523.
- Barot, M., Gokulandhi, M.R., Mitra, A.K., 2011. Mitochondrial dysfunction in retinal diseases. *Curr. Eye Res.* 36, 1069–1077.
- Beirne, R.O., 2013. The relationship between foveal short-wavelength-sensitive visual function and macular pigment optical density in the ageing eye. *Vis. Res.* 86, 81–86.
- Beirne, R.O., Hogg, R.E., Stevenson, M.R., Zlatkova, M.B., Chakravarthy, U., Anderson, R.S., 2006. Severity staging by early features of age-related maculopathy exhibits weak relationships with functional deficits on SWS grating acuity. *Invest. Ophthalmol. Vis. Sci.* 47, 4624–4631.
- Beirne, R.O., Zlatkova, M.B., Chang, C.K., Chakravarthy, U., Anderson, R.S., 2008. How does the short-wavelength-sensitive contrast sensitivity function for detection and resolution change with age in the periphery? *Vis. Res.* 48, 1894–1901.
- Bowmaker, J.K., 2008. Evolution of vertebrate visual pigments. *Vis. Res.* 48, 2022–2041.
- Calkins, D.J., 2001. Seeing with S cones. *Prog. Retin. Eye Res.* 20, 255–287.
- Carter-Dawson, L.D., LaVail, M.M., 1979. Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. *J. Comp. Neurol.* 188, 245–262.
- Cho, N.C., Poulsen, G.L., Ver Hoeve, J.N., Nork, T.M., 2000. Selective loss of S-cones in diabetic retinopathy. *Arch. Ophthalmol.* 118, 1393–1400.
- Coffey, P.J., Gias, C., McDermott, C.J., Lundh, P., Pickering, M.C., Sethi, C., Bird, A., Fitzke, F.W., Maass, A., Chen, L.L., Holder, G.E., Luthert, P.J., Salt, T.E., Moss, S.E., Greenwood, J., 2007. Complement factor H deficiency in aged mice causes retinal abnormalities and visual dysfunction. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16651–16656.
- Crawford, C., Igboeli, O., 2013. A Review of the Inflammatory Chorioretinopathies: The White Dot Syndromes. *ISRN Inflammation* 2013. <http://dx.doi.org/10.1155/2013/783190>. <http://www.hindawi.com/isrn/inflammation/2013/783190/cta/>
- Cunea, A., Jeffery, G., 2007. The ageing photoreceptor. *Vis. Neurosci.* 24, 151–155.
- Curcio, C.A., 2001. Photoreceptor topography in ageing and age-related maculopathy. *Eye (Lond)* 15 (Pt 3), 376–383.
- Curcio, C.A., Millican, C.L., Allen, K.A., Kalina, R.E., 1993. Aging of the human photoreceptor mosaic: evidence for selective vulnerability of rods in central retina. *Invest. Ophthalmol. Vis. Sci.* 34, 3278–3296.
- DeMonasterio, F.M., Schein, S.J., McCrane, E.P., 1981. Staining of blue-sensitive cones of the macaque retina by a fluorescent dye. *Science* 213, 1278–1281.
- Eisner, A., Fleming, S.A., Klein, M.L., Mauldin, W.M., 1987. Sensitivities in older eyes with good acuity: cross-sectional norms. *Invest. Ophthalmol. Vis. Sci.* 28, 1824–1831.
- Fox, D.A., Rubinstein, S.D., 1989. Age-related changes in retinal sensitivity, rhodopsin content and rod outer segment length in hooded rats following low-level lead exposure during development. *Exp. Eye Res.* 48, 237–249.
- Frennesson, C., Nilsson, U.L., Nilsson, S.E., 1995. Colour contrast sensitivity in patients with soft drusen, an early stage of ARM. *Doc. Ophthalmol.* 90, 377–386.
- Greenstein, V.C., Hood, D.C., Ritch, R., Steinberger, D., Carr, R.E., 1989. S (blue) cone pathway vulnerability in retinitis pigmentosa, diabetes and glaucoma. *Invest. Ophthalmol. Vis. Sci.* 30, 1732–1737.
- Gresh, J., Goletz, P.W., Crouch, R.K., Rohrer, B., 2003. Structure-function analysis of rods and cones in juvenile, adult, and aged C57bl/6 and Balb/c mice. *Vis. Neurosci.* 20, 211–220.
- Haegerstrom-Portnoy, G., 1988. Short-wavelength-sensitive-cone sensitivity loss with aging: a protective role for macular pigment? *J. Opt. Soc. Am. A* 5, 2140–2144.

- Hardy, J.L., Frederick, C.M., Kay, P., Werner, J.S., 2005. Color naming, lens aging, and grue: what the optics of the aging eye can teach us about color language. *Psychol. Sci.* 16, 321–327.
- Heron, G., Adams, A.J., Husted, R., 1988. Central visual fields for short wavelength sensitive pathways in glaucoma and ocular hypertension. *Invest. Ophthalmol. Vis. Sci.* 29, 64–72.
- Hoh Kam, J., Lenassi, E., Malik, T.H., Pickering, M.C., Jeffery, G., 2013. Complement component C3 plays a critical role in protecting the aging retina in a murine model of age-related macular degeneration. *Am. J. Pathol.* 183, 480–492.
- Holder, G.E., Robson, A.G., Pavesio, C., Graham, E.M., 2005. Electrophysiological characterisation and monitoring in the management of birdshot chorioretinopathy. *Br. J. Ophthalmol.* 89, 709–718.
- Jackson, G.R., Owsley, C., Curcio, C.A., 2002. Photoreceptor degeneration and dysfunction in aging and age-related maculopathy. *Ageing Res. Rev.* 1, 381–396.
- Johnson, C.A., Adams, A.J., Twelker, J.D., Quigg, J.M., 1988. Age-related changes in the central visual field for short-wavelength-sensitive pathways. *J. Opt. Soc. Am. A* 5, 2131–2139.
- Johnson, L.V., Leitner, W.P., Rivest, A.J., Staples, M.K., Radeke, M.J., Anderson, D.H., 2002. The Alzheimer's A beta -peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11830–11835.
- Kolesnikov, A.V., Fan, J., Crouch, R.K., Kefalov, V.J., 2010. Age-related deterioration of rod vision in mice. *J. Neurosci.* 30, 11222–11231.
- Li, C., Cheng, M., Yang, H., Peachey, N.S., Naash, M.L., 2001. Age-related changes in the mouse outer retina. *Optom. Vis. Sci.* 78, 425–430.
- Liang, F.Q., Godley, B.F., 2003. Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration. *Exp. Eye Res.* 76, 397–403.
- Lundh von Leithner, P., Kam, J.H., Bainbridge, J., Catchpole, I., Gough, G., Coffey, P., Jeffery, G., 2009. Complement factor h is critical in the maintenance of retinal perfusion. *Am. J. Pathol.* 175, 412–421.
- Neitz, M., Neitz, J., 2001. The uncommon retina of the common house mouse. *Trends Neurosci.* 24, 248–250.
- Neveu, M.M., Dangour, A., Allen, E., Robson, A.G., Bird, A.C., Uauy, R., Holder, G.E., 2011. Electroretinogram measures in a septuagenarian population. *Doc. Ophthalmol.* 123, 75–81.
- Noma, H., Funatsu, H., Mimura, T., 2012. Association of electroretinographic parameters and inflammatory factors in branch retinal vein occlusion with macular oedema. *Br. J. Ophthalmol.* 96, 1489–1493.
- Nork, T.M., Millecchia, L.L., Strickland, B.D., Linberg, J.V., Chao, G.M., 1995. Selective loss of blue cones and rods in human retinal detachment. *Arch. Ophthalmol.* 113, 1066–1073.
- Panda-Jonas, S., Jonas, J.B., Jakobczyk-Zmija, M., 1995. Retinal photoreceptor density decreases with age. *Ophthalmology* 102, 1853–1859.
- Peirson, S.N., Butler, J.N., Foster, R.G., 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res.* 31, e73.
- Remky, A., Elsner, A.E., 2005. Blue on yellow perimetry with scanning laser ophthalmoscopy in patients with age related macular disease. *Br. J. Ophthalmol.* 89, 464–469.
- Rohlich, P., van Veen, T., Szel, A., 1994. Two different visual pigments in one retinal cone cell. *Neuron* 13, 1159–1166.
- Shinomori, K., Werner, J.S., 2012. Aging of human short-wave cone pathways. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13422–13427.
- Song, H., Chui, T.Y., Zhong, Z., Elsner, A.E., Burns, S.A., 2011. Variation of cone photoreceptor packing density with retinal eccentricity and age. *Invest. Ophthalmol. Vis. Sci.* 52, 7376–7384.
- Szel, A., Rohlich, P., Caffè, A.R., Juliusson, B., Aguirre, G., Van Veen, T., 1992. Unique topographic separation of two spectral classes of cones in the mouse retina. *J. Comp. Neurol.* 325, 327–342.
- Werner, J.S., Bieber, M.L., Scheffrin, B.E., 2000. Senescence of foveal and parafoveal cone sensitivities and their relations to macular pigment density. *J. Opt. Soc. Am. A Opt. Image Sci. Vis.* 17, 1918–1932.
- Williams, G.A., Jacobs, G.H., 2007. Cone-based vision in the aging mouse. *Vis. Res.* 47, 2037–2046.
- Williams, J.A., Greenwood, J., Moss, S.E., 2013. Retinal changes precede visual dysfunction in the complement factor H knockout mouse. *PLoS One* 8, e68616.
- Winkler, B.S., 1981. Glycolytic and oxidative metabolism in relation to retinal function. *J. Gen. Physiol.* 77, 667–692.
- Winkler, B.S., Kapousta-Bruneau, N., Arnold, M.J., Green, D.G., 1999. Effects of inhibiting glutamine synthetase and blocking glutamate uptake on b-wave generation in the isolated rat retina. *Vis. Neurosci.* 16, 345–353.
- Zlatkova, M.B., Coulter, E., Anderson, R.S., 2003. Short-wavelength acuity: blue-yellow and achromatic resolution loss with age. *Vis. Res.* 43, 109–115.