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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; Cunea et al., 2008). The high magnitude of cell loss here is probably due to the high metabolic demand of the outer retina (Barot et al., 1993). However, these have differential distributions with M/L cones widely distributed or more common dorsally and S cones mostly located towards 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.

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−/−) 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−/− 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.
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−/−), 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−/− 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−/− have retinae 25% thicker than wild-type animals (Williams et al., 2013), implying a potential developmental difference which would underlie the question we address. To provide high-resolution images of retinae, in vivo mice (n = 5 per group, 4 groups) were anesthetized with an IP 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 and the OCT image at the center of the retina. 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 for 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 retinae 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 (1:1000, 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 retinae 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: TGTACATGTGCAAAATCGGA, reverse: ACACCATCTCCAGAATGCAAG and M/L Opsin forward: CTCTGTACTCTCAAGGTTG, reverse: AAGTAGGGTCCACCAGCA. 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 retinae 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 Figure 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
3. Results

3.1. In vivo analysis

Retinae 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−/−: 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−/−: 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−/− 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−/− 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−/−, 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−/− mice (p = 0.021). The reduction in C57 animals was 27% and in CFH−/− it was 18%. Statistically significant differences were also present for the CFH−/− 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−/− mice.

S opsin 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−/− 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 opsin with age reflected the data shown with immune labeling for opsin. 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 opsin).

![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.](image-url)
ratio change: C57: 34.4%, CFH−/−: 34.7%), with that change in the CFH−/− being statistically significant (C57: $p = 0.075$, CFH−/−: $p = 0.027$, 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.
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+/− 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 retinae 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 retinae, 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 retinae at 1 and 17 months of age. They examined frozen sections and wholemounts, but the wholemounts 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 retinae 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 retinae. 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+/− model

There are other key discrepancies between our results and those undertaken previously. The CFH+/− 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+/− 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.,
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/C0/C0 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., 2010). The relative changes in cone subtype with age, genotype, and retinal region. All counts are from 6 retinae 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, 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/C0/C0: 17.6% reduction, p = 0.0210). Fewer S opsin cells were also found with age (C57: 11.7% reduction, CFH/C0/C0: 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/C0/C0 mice but just failed to reach statistical significance in C57 mice (C57: 22.7% reduction, p = 0.051, CFH/C0/C0: 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/C0/C0 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.
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 differ 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 opsins (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.

Fig. 6. qPCR data. Ratio bars for each genotype showing the relative proportion of M/L 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: −7.8%− 34.7%). Although in young animals the population of M opsin constitutes approximately to 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.

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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