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Topical cyclodextrin reduces amyloid beta and inflammation improving retinal function in ageing mice

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ABSTRACT

Retinal ageing results in chronic inflammation, extracellular deposition, including that of amyloid beta (Aβ) and declining visual function. In humans this can progress into age-related macular degeneration (AMD), which is without cure. Therapeutic approaches have focused on systemic immunotherapies without clinical resolution. Here, we show using aged mice that 2-Hydroxypropyl-β-cyclodextrin, a sugar molecule given as eye drops over 3 months results in significant reductions in Aβ by 65% and inflammation by 75% in the aged mouse retina. It also elevates retinal pigment epithelium specific protein 65 (RPE65), a key molecule in the visual cycle, in aged retina. These changes are accompanied by a significant improvement in retinal function measured physiologically. 2-Hydroxypropyl-β-cyclodextrin is as effective in reducing Aβ and inflammation in the complement factor H knockout ($Cfh^{-/-}$) mouse that shows advanced ageing and has been proposed as an AMD model. β-cyclodextrin is economic, safe and may provide an efficient route to reducing the impact of retinal ageing.

1. Introduction

Ageing is associated with cellular decline which is partly linked to metabolic rate. The outer retina has the highest metabolic demand in the body required to maintain the oxygen demanding photoreceptor population (Linsenmeier and Padnick-Silver, 2000). Here with age there is progressive accumulation of extracellular material including neurotoxic amyloid beta (Aβ) (Isas et al., 2010), lipids (Curcio et al., 2005a,Curcio et al., 2005b,Wang et al., 2010) and proteins that are inflammatory such as complement (Hageman et al., 2001). These accumulate on Bruch's membrane (BM) restricting the exchange of metabolic nutrients between the outer retina and its blood supply. In mice these deposits are relatively linear along BM. In humans, deposits are focal and are called drusen. These are a key risk factor for age-related macular degeneration (AMD) when they accumulate in the central retina. With progressive deposition and inflammation 30% of the photoreceptor population is lost in both humans and rodents in normal ageing (Cunea and Jeffery, 2007,Curcio et al., 1993).

In humans, retinal ageing can develop into AMD where progressive inflammation and deposition result in central retina atrophy. Mice lack this area of specialisation and do not develop retinal atrophy but do suffer from similar deposition, inflammation and cell loss across the retina. AMD is the leading cause of blindness in those over 65 years in the West and is growing rapidly as populations age (Klein et al., 1997,Klein et al., 2004). In 50% of cases it is linked to immune vulnerability being associated with polymorphisms of complement genes (Edwards et al., 2005,Haines et al., 2005). Currently, there is no cure for this neurodegenerative disease, although systemic immunotherapeutic approaches have tried to reduce retinal Aβ load (Catchpole et al., 2013,DeMattos et al., 2001,Ding et al., 2011; Salloway et al., 2014). However, topical drug administration has largely been ignored as it was seen as unlikely to be effective due to drug dilution before it reached the retina. This assumes that drugs would have to pass through the anterior eye and vitreous before entering the retina. However, penetration of the drug may be obtained via the conjunctiva and sclera into the retina (Sigurdsson et al., 2007).

Cyclodextrins (CDs) are a family of cyclic polysaccharide compounds with a hydrophilic shell enclosing a hydrophobic cavity. This structure allows them to form water–soluble complexes with otherwise insoluble hydrophobic compounds. This has led to their utilisation as carriers to increase the aqueous solubility and stability of hydrophobic drugs (Loftsson and Duchene, 2007; Stella and He, 2008). They have undergone extensive safety studies and are

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approved by the Food and Drug Administration (FDA) (Stella and He, 2008) for pharmaceutical use and dietary supplements. Topical administration results in their rapid retinal accumulation, presumably entering the eye via the conjunctiva (Loftsson et al., 2008; Sigurdsson et al., 2007).

Recently, CDs systemic delivery has shown efficacy in an Alzheimer's mouse model (Yao et al., 2012) reducing the size of Aβ plaques in the brain and upregulating genes associated with cholesterol transport and Aβ clearance. Further, systemic delivery significantly reduces lipofuscin deposits in the retina, which are an age related lipid rich pigmented deposit that accumulates in the retinal pigmented epithelium (Nociari et al., 2014). CDs are known to bind to cholesterol (Irie et al., 1992,Ohtani et al., 1989) and at high concentrations, they serve as a cholesterol sink. At low concentrations, CDs act as a cholesterol shuttle, transporting it between membranes (Atger et al., 1997; McCauliff et al., 2011). Hence, they can clear cholesterol which is known to be deposited on BM and whose presence has been linked to AMD (Pikuleva and Curcio, 2014).

Here, we ask whether topical CDs delivery has the ability to erode Aβ and reduce inflammation in the aged mouse retina and what impact this has on retinal function. This was based on our prior observation that CDs had the ability to enter the retina via the conjunctiva. We explore this in normal aged mice but also ask if it has similar abilities in terms of Aβ and inflammation alone in aged complement factor H mice ($Cfh^{-/-}$) that have been proposed as a murine model of AMD as it shares a genotype with 50% of AMD patients (Coffey et al., 2007). While there remain significant questions regarding mouse AMD models due to the absence of a macular, the aged $Cfh^{-/-}$ mouse does experience elevated deposition and inflammation and has reduced photoreceptor numbers and compromised visual function (Hoh Kam et al., 2013).

2. Materials and methods

2.1. Animals

8-9 months old C57BL/6 and 6–7 months old $Cfh^{-/-}$ mice which were backcrossed onto C57BL/6 genetic background for more than 10 generations were used. Animals were housed under a 12/12 light dark cycle with access to food and water ad libitum. All animals were used with University College London ethics committee approval that conformed to the United Kingdom Animal License (Scientific Procedures) Act 1986 (UK). UK Home Office project license (PPL 70/6571).

2.2. Treatment regime

C57BL/6 mice (n = 10) were treated with 3 μl of 10% 2-Hydroxypropyl-β-cyclodextrin (β-CD) (Sigma Aldrich, UK) in phosphate buffered saline (pH 7.4) as eye drops bilaterally 3 times daily for 3 months. Controls (n = 5) were untreated.

The $Cfh^{-/-}$ mice were divided into 3 groups. The first (β-CD LT) was treated with 3 μl of 10% 2-Hydroxypropyl-β-cyclodextrin (β-CD) (Sigma Aldrich, UK) as above as eye drops 3 times a day for 3 months (n = 10). The second (control) of $Cfh^{-/-}$ mice (n = 5) was left untreated. The third group (β-CD ST) was treated with 3 μl of 10% β-CD (Sigma Aldrich, UK) as above as eye drops 3 times a day for 3 days per month for 3 months (n = 5).

2.3. Electroretinogram (ERG)

After treatment C57BL/6 animals were given full field flash ERG to assess retinal function in response under scotopic and photopic conditions similar to Hoh Kam et al. (2013) using the ColorDome Ganzfeld ERG (Diagnosys LLC, Cambridge, UK). Mice were dark-adapted overnight for scotopic measurements and anaesthetised with 6% Ketamine, (National Veterinary Services Ltd, UK) 10% Dormitor, (National Veterinary Services Ltd, UK) and 84% sterile water at 5ul/g intraperitoneal injection. Pupils were dilated (1% Tropicamide, MINIMS, Bausch & Lomb, France) prior to recordings. Ground and reference subdermal electrodes were placed subcutaneously near the hindquarter and between the eyes respectively and the mouse placed on a heated pad (37 °C). Recording gold electrodes were placed on the cornea. ERG was carried out under scotopic conditions for both eyes simultaneously, with increasing stimulus strengths using a 6500 K white light at; 3.5×10^{-6}, 3.5×10^{-5}, 3.5×10^{-4}, 0.03, 0.3, 2.8 and 28.1 cd s/m^2. After the scotopic series mice were adapted to a 20 cd/m^2 background for 20 min. Then photopic responses to white light flash stimuli of 0.3, 2.8, 28.1 and 84.2 cd s/m^2 were recorded with a background light of 20 cd/m^2. An average of 20–25 readings were taken for each intensity. Statistical differences between groups were evaluated by using random ANOVA.

2.4. Immunohistochemistry

After ERGs, C57BL/6 mice were culled by cervical dislocation as were the $Cfh^{-/-}$ mice from which recordings were not undertaken. Eyes were collected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 1 h, cryopreserved in 30% sucrose in PBS and embedded in optimum cutting temperature (OCT) compound (Agar Scientific Ltd). 10 μm cryosections were thaw-mounted on a slide and incubated for 1 h at room temperature in a 5% Normal Donkey serum in 0.3% (v/v) Triton X-100 in PBS, pH 7.4. This was followed by an overnight incubation with either a mouse monoclonal antibody to Aβ 4G8 (1:100, Covance), a mouse monoclonal antibody to RPE65 (1:500, Merck Millipore, UK), both were conjugated with an Alexa Fluor 568 (Invitrogen, UK), or a rat monoclonal antibody to complement C3b (C3b) (1:50, Hycult biotechnology) diluted in 1% Normal Donkey Serum in 0.3% Triton X-100 in PBS. For the $Cfh^{-/-}$ mice, we used a goat polyclonal to complement C3 (1:500, Cappel MP Biomedicals, Cambridge, UK). After primary antibody incubation, sections were washed several times in 0.1 M PBS then slides stained for active C3b were incubated in a secondary antibody, donkey anti-rat conjugated with Alexa Fluor 488 (Invitrogen, UK) and for a donkey anti-goat conjugated with Alexa Fluor 488 (Invitrogen, UK) for C3, made up in 2% Normal Donkey Serum in 0.3% Triton X-100 in PBS at a dilution of 1:2000 for 1 h at room temperature. Negative controls were undertaken by omitting the primary antibody. After secondary antibody incubation, sections were washed and nuclei stained with DAPI. Slides were then washed in 0.1 M PBS followed by washes in Tris buffered Saline (pH 7.5). Slides were mounted in Vectashield (VECTOR Laboratories) and coverslipped.

For lipid detection retinal sections were stained with a saturated solution (3%) of Sudan Black B in 70% ethanol for 1 h at room temperature and then washed in several changes of distilled water. Slides were mounted in glycerol and then coverslipped.

2.5. Western blots

Eyes were dissected on ice and the retina and RPE-choroidal tissues were snap frozen in liquid nitrogen. Protein was then extracted by homogenising the samples in 2% SDS with protease inhibitor cocktail (Roche diagnostics), and centrifuged at 13,000 ×g. The supernatant was transferred to a new microcentrifuge tube and will be used for Western blots of C3 and RPE65. Aβ was extracted from the resultant pellet with 70% formic acid and the mixture was then centrifuged at 13,000 ×g. The supernatant was then

transferred to a new microcentrifuge tube and the pellet discarded. The formic acid in the supernatant was evaporated using a speed-Vac concentrator (The Eppendorf Vacuum Concentrator Model 5301, Brinkmann) and the protein pellet was reconstituted in 10% dimethyl sulfoxide in 2 mol/L Tris–HCl. Protein concentration was measured with an absorbance of 595 nm and Bovine Serum Albumin was used as a standard protein concentration. Equal amounts of proteins (50 μg/ml) were separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The nitrocellulose membranes were pre-treated with 5% non-fat dried milk in 1 M PBS (pH7.4) for 1 h and incubated overnight at 4 °C with either a goat polyclonal antibody to C3 (1:500, Cappel), a rabbit monoclonal to RPE65 (1:1000, Abcam), a mouse monoclonal antibody to Aβ 4G8 (1:1000, Covance) or a mouse monoclonal to α-tubulin (1:1000, Millipore) followed by several washes in 0.05% Tween-20 in 1 M PBS. The membranes were then incubated with the respective secondary antibodies; rabbit anti-goat HRP conjugated (1:2000, Dako), goat anti-rabbit HRP conjugated (1: 3000, Dako) and goat anti-mouse HRP conjugated (1:10,000, Thermo Scientific) for 1 h. Immunoreactivities were visualised by exposing x-ray films to blots incubated with ECL reagent (SuperSignal West Dura, Thermo Scientific). Total protein profile was determined by staining blots with Ponceau S solution to check the transfer efficiency and quantification. Protein bands were then photographed and scanned. The absolute intensity of each band was then measured using Adobe Photoshop CS5 extended.

2.6. Analysis

2.6.1. Measurement of the expression of Aβ, C3b, RPE65 and C3 along the RPE and Bruch's membrane interface by immunostaining

Fluorescence images were taken in JPEG format at ×400 using an Epi-fluorescence bright-field microscope. Images were montaged and the integrated density, which is the product of the area chosen (in pixels) and the mean grey value (measurement of the brightness), were recorded using Adobe Photoshop CS5 extended. The lasso tool was used to draw a line all the way around the RPE and Bruch's membrane interface to measure the amyloid beta, RPE65, C3 and the C3b expression in this area.

2.6.2. Measurement of the amount of Aβ, C3 and RPE65 in Western blots

Scanned images of the immunoblots were inverted to grayscale format and the mean gray value was measured for each protein band by using the lasso tool to draw a line all the way around the edges of the band using Adobe Photoshop CS5 extended. The absolute intensity was calculated by multiplying the mean gray value and the pixel value. The protein bands were quantified and their ratios to alpha tubulin were calculated and plotted into graphs.

2.6.3. Statistical analysis

A Mann–Whitney U test was used to compare groups and a one way ANOVA with post hoc analysis with Dunn's multiple comparison test was used for the three groups. Data was analysed using Graph pad Prism, version 5.0 (Graphpad, San Diego, CA).

3. Results

3.1. β-CD significantly reduces Aβ and inflammation and elevates RPE65 in aged C57BL/6 mice

We administered β-CD as single eye drops for 3 months in old C57BL/6 mice in which Aβ deposition and inflammation were established (Catchpole et al., 2013). All β-CD-treated mice had a significant reduction of around 65% in Aβ deposition on BM when immunostained tissue was examined ($P < 0.05$, Fig. 1A and B) compared with controls. Further, while Aβ deposition was relatively focal on BM in the untreated group, in β-CD-treated mice its distribution was clearly diffuse, which may be attributable to the process of clearance (Fig. 1A, arrowheads). Western blot was also used to quantify Aβ deposition in the retina and RPE of both the β-CD-treated and untreated mice (Fig. 1C). The results showed that β-CD decreased retinal Aβ levels markedly but this was not significant ($P > 0.05$).

To determine if reductions in pro-inflammatory Aβ (Johnson et al., 2002) were associated with a decreased inflammation, we immunostained adjacent section for active complement C3 (C3b) (Fig. 1C). There was a significant decline of around 75% in C3b on BM in β-CD-treated mice compared with controls ($P < 0.05$, Fig. 1D and E). Western blot results showed a decrease in the level of C3 in the β-CD-treated mice compare to controls, but this did not reach statistical significance ($P > 0.05$, Fig. 1F). Hence, topical administration of β-CD reduced Aβ deposition and inflammation in the aged outer retina.

There is evidence from amphibians that β-CD improves the visual cycle (Johnson et al., 2010), which is an enzyme pathway where opsin is recycled. This involves the removal of all-trans retinol, a potentially toxic element whose accumulation results in vulnerability to light-induced photoreceptor damage. Elevated all-trans retinol is also linked to Stargardt disease, a rare early onset form of AMD (K. Haddley, 2011; Moiseyev et al., 2010). In frogs, β-CD clears all-trans retinol in a dose-dependent manner (Johnson et al., 2010).

To determine if CDs impact on the visual cycle in aged mice we immunostained sections for retinal pigment epithelium specific protein 65 (RPE65), which is a protein expressed in the retinal pigment epithelium (RPE) that plays a critical role in recycling visual pigments. There was an approximate 30% increase in RPE65 expression in β-CD-treated mice compared to controls ($P < 0.01$, Fig. 2A and B). Differences were not simply related to intensity of labelled RPE65, but also to its distribution. In treated animals expression was continuous along the RPE, but in controls label was patchy with gaps that implied its expression was low in some RPE cells.

To further quantify RPE65 in the RPE and retina, Western blot analysis was undertaken. The results revealed that there is a significant increase in the level of RPE65 in the β-CD-treated mice compare to the untreated ($P < 0.05$, Fig. 2C). This confirms the result obtained with immunostaining and shows that β-CD improves the visual cycle.

Ageing is also associated with an accumulation of retinal lipids and it has been argued that this may contribute to AMD (Suzuki et al., 2007; Curcio et al., 2009; Wiegand et al., 1983). Phospholipids are produced in photoreceptors and subsequently deposited in the RPE as lipofuscin in the daily shedding of outer segments and their phagocytosis (Bok, 1985) potentially leading to RPE dysfunction and photoreceptor death (Suzuki et al., 2007). To reveal phospholipids and lipids, sections from both treated and controls were stained with Sudan Black B, which is a histochemical lipids stain (Fig. 2D). It was not possible to assess staining on BM because melanin obscured the staining patterns. However, staining on outer segments was clear and there were marked qualitative differences between β-CD-treated and control mice with reduced staining in the former group (Fig. 2D).

3.2. β-CD significantly improves retinal function in aged C57BL/6 mice

Significant reductions in Aβ and inflammation along with

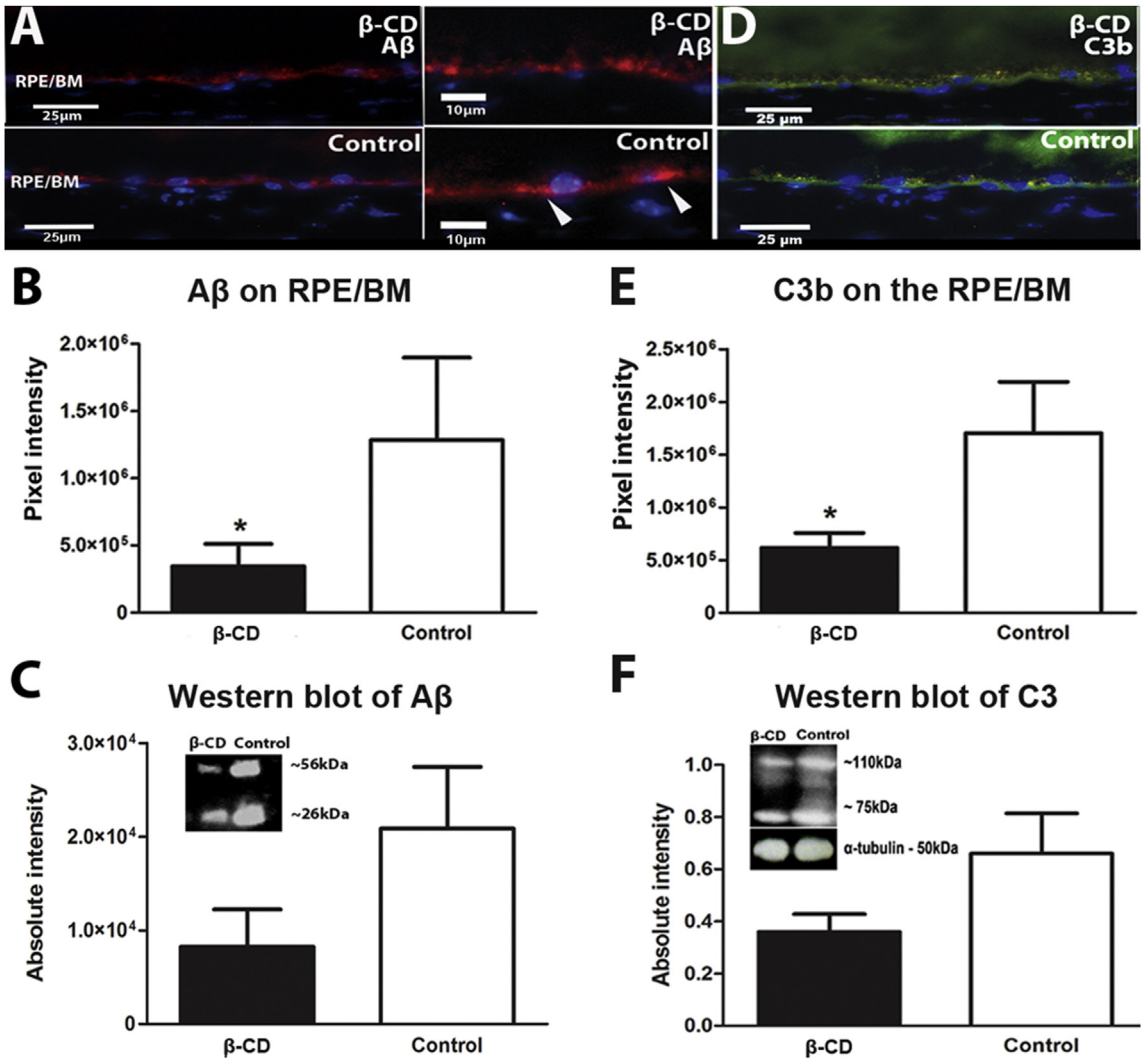

Fig. 1. β-CD treatment efficiently removes Aβ and complement C3b along the RPE/BM interface in the C57BL/6 mice. A. Retinal sections from mice of both β-CD-treated and untreated groups immunostained with a mouse monoclonal Aβ 4G8 (red) and the nuclei counterstained with DAPI (blue). Centre panels are close-ups of the RPE/BM interface showing that the Aβ expression in the control group is more focal and punctate (arrowheads). B. β-CD-treated mice have significantly reduced Aβ deposition along the RPE/BM interface compared to controls ($P < 0.05$). C. Graph showing the quantification of Aβ 4G8 in the retina and RPE. The Western blot results showed that β-CD decrease retinal Aβ levels. D. Retinal sections immunostained with a rat monoclonal antibody to active C3b (green) and counterstained with DAPI (blue). E. Graph showing β-CD-treated mice have significantly lower C3b expression along the RPE/BM interface ($P < 0.05$) than controls. F. Graph showing the quantitative measurement of C3 in the retina and RPE. β-CD-treated mice have less level of inflammation compare to controls but this did not reach significance ($P > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased RPE65 expression may be associated with improved retinal function. This was measured with scotopic and photopic ERG recordings. There are marked reductions in the amplitude of the individual ERG waves with age and pathology (Arden, 2006). Clear significant improvements in amplitudes of the aged ERG waves of both rod and cone function were found in treated mice (Fig. 3A and B) but no difference was seen in the latencies (data not shown). Clear significant improvements were marked at higher luminance where an increase of approximately 28% was shown in the scotopic a-wave amplitude ($P < 0.05$, Fig. 3C) at 28.1 cd s/m^2 and 25% in the scotopic b-wave amplitude ($P < 0.01$ at 2.8 and $P < 0.05$ at 28.1 cd s/m^2, Fig. 3D). A significant 20% improvement in the photopic b-wave was also seen in treated mice at the highest luminance, 84.2 cd s/m^2 ($P < 0.05$, Fig. 3E). Hence, both the a- and b-waves under both scotopic and photopic conditions were significantly improved in β-CD-treated mice, suggesting that β-CD treatment improved both rod and cone function and hence improved visual function over a large dynamic range.

3.3. β-CD significantly reduces Aβ and inflammation in the BM of a murine model of AMD

Having established that β-CD is effective in reducing features of outer retina ageing we ask two further questions. First, is β-CD also effective in treating aged *Cfh*$^{-/-}$ mice in terms of Aβ and inflammation as it is in normal ageing when given the same dosage patterns? *Cfh*$^{-/-}$ mice suffer from premature retinal Aβ and inflammation accumulation (Catchpole et al., 2013) and share a genotype with 50% of AMD patients (Edwards et al., 2005; Haines et al., 2005). Second, if so, can this be achieved with a much lower dosing pattern? Hence, we only dosed at 10% of the level for the long term treatment in C57BL/6 mice with a total of 27 eye drops per eye over 3 months.

Fig. 4 shows data from both long term treatment (β-CD LT) in *Cfh*$^{-/-}$ mice and also short term treatment (β-CD ST) where dosing was reduced by 90%. In both groups, Aβ was significantly reduced on BM compared to controls (Fig. 4A and C). As with the C57BL/6

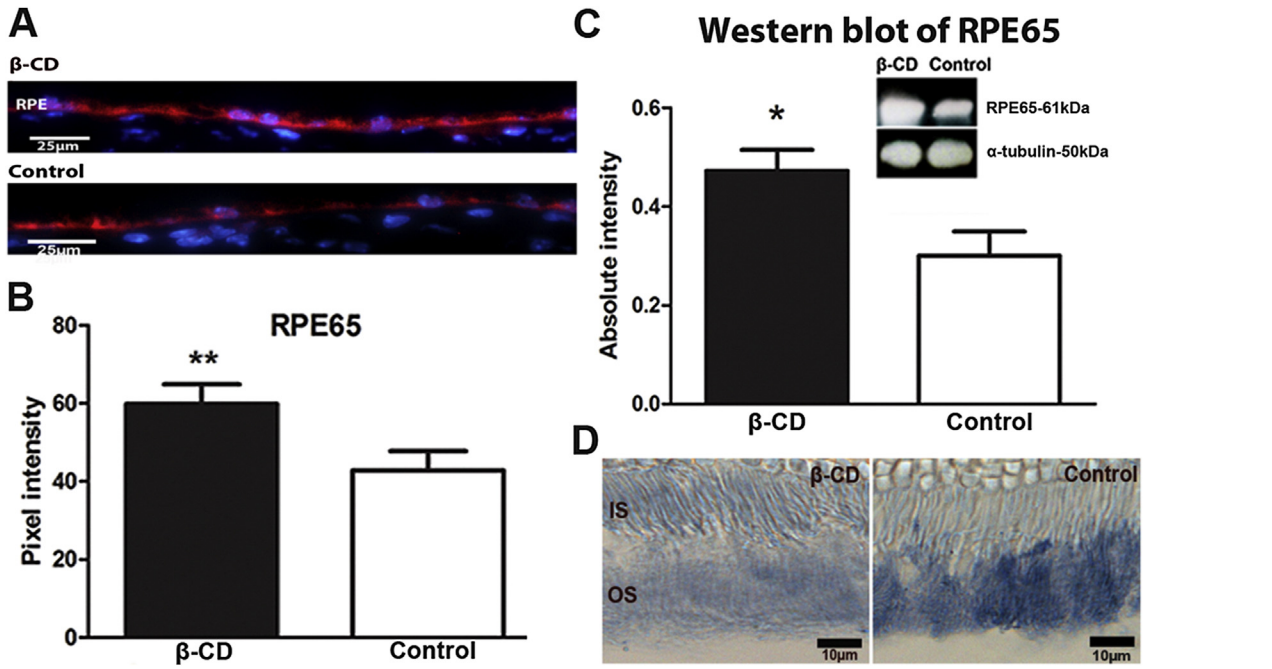

Fig. 2. β-CD treatment efficiently increase RPE65, a visual cycle protein in the aged C57BL/6 mice. A. Retinal sections immunostained with a monoclonal antibody to RPE65 (red). Nuclei were counterstained with DAPI (blue). RPE65 expression in the RPE is significantly higher in the β-CD-treated mice and is continuous compared to controls where the expression is weaker and there are areas of no staining is seen. B. Differences in RPE65 expression between β-CD-treated and untreated mice were significantly different with β-CD-treated increasing RPE65 expression ($P < 0.01$). C. Graph showing the quantitative measurement of RPE65 in the RPE and retina. It shows that the level of RPE65 is significantly increase in β-CD-treated mice ($P < 0.05$). D. Representative micrographs of photoreceptors from retinal sections of both β-CD-treated and control C57BL/6 mice stained with Sudan Black B (greyish blue) for lipids and phospholipids on photoreceptor outer segments. There were more lipids in the outer segments of control than in the β-CD-treated mice. Mean ± SEM. n = 8 for the β-CD-treated animals and n = 5 for the untreated animals. RPE/BM − Retinal pigment epithelium/Bruch's membrane, IS - inner segment, OS − outer segment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mice, Aβ removal in both $Cfh^{-/-}$ groups resulted in a patchy distribution of Aβ on BM (Fig. 3A). C3 expression was also significantly reduced in the $Cfh^{-/-}$ mice given the same high dosage pattern as the C57BL/6 animals (Fig. 4B and D). However, reductions in C3 expression along BM of the short term treatment group were not significant (Fig. 4D). Hence, reducing dosage by 90% was effective in reducing Aβ but not C3. This may be because Aβ is pro-inflammatory and reductions in inflammation may lag behind Aβ removal.

4. Discussion

This study shows that 3 months topical treatment with β-CD has a significant impact on the aged mouse outer retina, reducing Aβ and C3 expression along with lowering lipid levels. This treatment also increased RPE65 expression and improved retinal function in aged mice. These results were reflected in treatment of $Cfh^{-/-}$ mice where our aims were more limited, only targeting Aβ and C3. β-CD remained effective here even when dosing was reduced by 90%, but only in terms of Aβ deposition. β-CD therapeutic abilities are probably related to its hydrophobic internal structure and hydrophilic outer surface, which increase the interaction and solubility of materials such as lipids and Aβ. CDs have been used previously with success in Alzheimer mouse models and to clear Aβ in the brain (Yao et al., 2012) and in the retina to reduce lipofuscin (Nociari et al., 2014), but in both studies administration has been systemic. However, it is known that topical administration results in 60% delivery to the retina, compared with only 40% when given systemically (Sigurdsson et al., 2007). Hence, systemic delivery is an inefficient route that does not target selectively. In spite of this, CDs have been used widely as vehicles for hydrophobic drug delivery. They are safe, FDA approved and economic.

In mice, Aβ and C3 increase progressively with age on BM (Hoh Kam et al., 2010. Catchpole et al., 2013) and this probably reduces outer retinal perfusion and may increase hypoxia. The same is true of lipid deposition. Lipid is a constituent of outer segments and it is likely that this material accumulates with age as phagocytosis efficacy probably declines. While we show reductions in each of these elements, our study is not the first demonstration of the impact of β-CD on deposition in the aged mouse outer retina. It has been shown that β-CD delivered systemically also reduces lipofuscin that accumulates with age (Nociari et al., 2014). Each of these deposited materials eroded by β-CD have been implicated in AMD (Chen et al., 2010; Neale et al., 2010; Curcio et al., 2011; Curcio et al., 2005a, 2005b; Curcio, et al., 2001).

Our results are similar to those obtained by Yao, J. et al. (Yao et al., 2012) who used systemic β-CD delivery in an Alzheimer's mouse model to reduce brain Aβ deposition and microgliosis. The mechanism by which cyclodextrins reduce Aβ may be related to their modulation of cellular cholesterol, which has a complex relationship with amyloid precursor protein (APP) and Aβ metabolism (Grimm et al., 2007). APP and β- and γ-secretases, which are enzymes involved in Aβ metabolism, are colocalised in cholesterol rich lipid rafts. Hence, lowering cholesterol directly may affect APP processing and reduce Aβ production. Yao et al. (Yao et al., 2012) have also shown that β-CD increases expression of genes critical for lipid transportation, notably ABCA1 involved in increasing apolipoprotein E lipidation and improving Aβ clearance (Hirsch-Reinshagen et al., 2005).

β-CD treatment has a wider role than reducing deposition as it significantly improves retinal function and the visual cycle, both of which decline with ageing. β-CD removes lipofuscin bisretinoids that are by-products of the retinal pigmented epithelium resulting from phagocytosing and these are toxic to RPE cells and as such

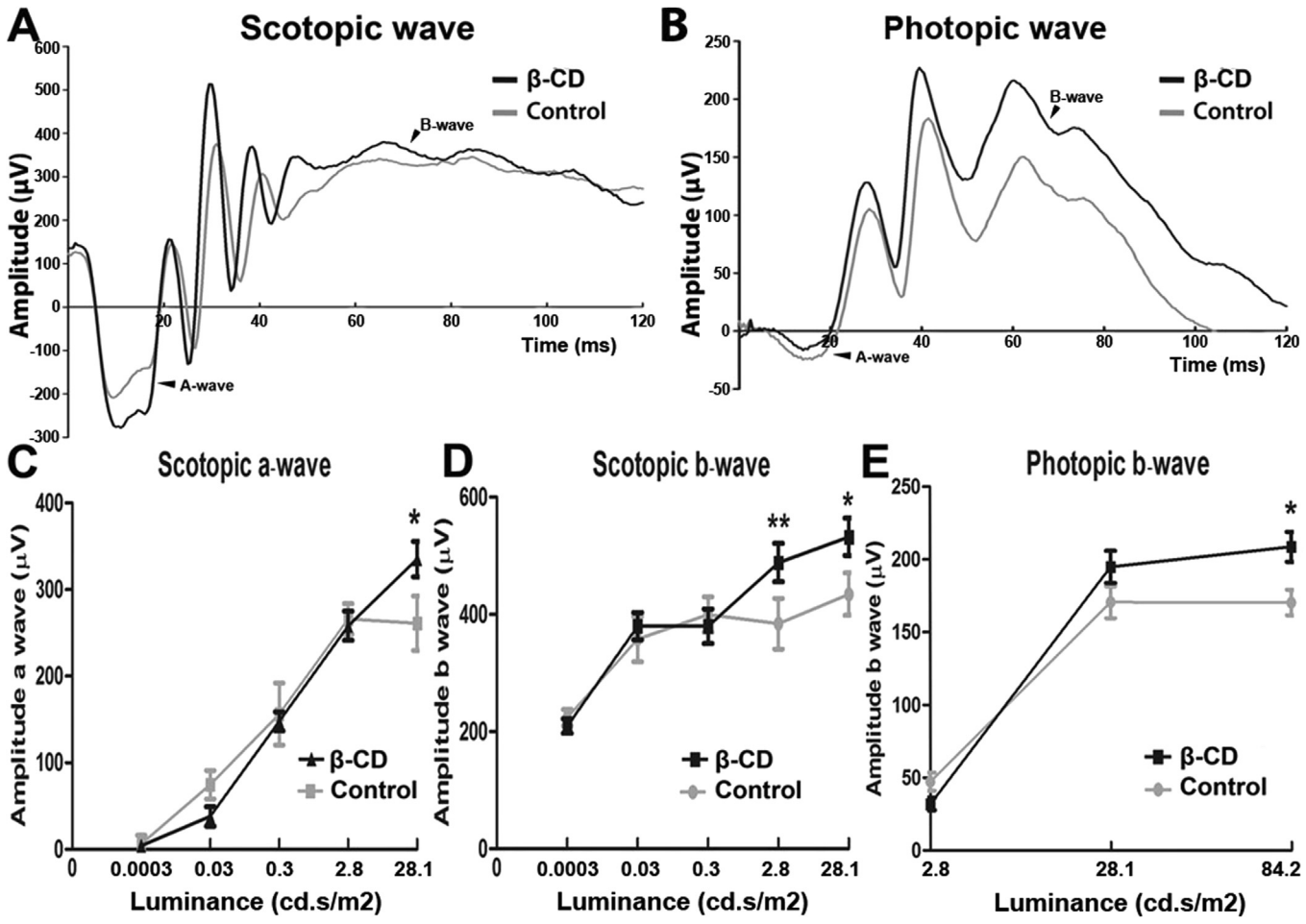

Fig. 3. ERG assessment of both scotopic and photopic responses in β-CD-treated and non-treated mice. Examples of evoked responses of a- and b-waves in A. scotopic and B. photopic conditions of both groups. C. Graph shows that at higher luminance, there is marked increase of about 28% in the scotopic a-wave amplitude of the β-CD-treated mice ($P < 0.05$) at 28.1 cd s/m^2 compared to controls. D. Graph showing scotopic b-wave response. There is a 25% increase in the scotopic b-wave amplitude ($P < 0.01$ at 2.8 cd s/m^2 and $P < 0.05$ at 28.1 cd s/m^2) of β-CD-treated mice compared to untreated group. E. Graph showing the photopic b-wave response of both groups. A significant 20% improvement in the photopic b-wave was seen in the β-CD-treated animals at the highest luminance, 84.2 ($P < 0.05$, Fig. 2C) as compared to the control group. Scotopic white light flash stimuli; 3.5×10^{-6}, 3.5×10^{-5}, 3.5×10^{-4}, 0.03, 0.3, 2.8 and 28.1 cd s/m^2. Photopic responses to white light flash stimuli were recorded with a background light of 20 cd/m^2, to flash stimuli 0.3, 2.8, 28.1 and 84.2 cd s/m^2 were recorded. Mean ± SEM. n = 10 for the β-CD-treated mice and n = 5 for the untreated group for the ERG recordings.

impact on the visual cycle (Nociari et al., 2014). Hence, their removal is likely to improve RPE cell function which is critical for the cycle. But more importantly, Johnson et al. (Johnson et al., 2010) revealed that β-CD efficiently removes all-trans retinol from frog rod photoreceptors. During the visual cycle, 11-cis retinal chromophore is photoisomerased into all-trans retinal, which is then reduced to all-trans retinol in outer segments. When this accumulates it is potentially toxic and this has been linked to Stargardt's disease (Schutt et al., 2000; Sparrow and Cai, 2001). In the frog, β-CD facilitates removal of this material and hence improves regeneration of 11-cis isomers. This may potentially explain the elevated RPE65 expression in β-CD-treated aged animals, as RPE65 is involved in the conversion of all-trans retinol to 11-cis retinal during phototransduction.

Given the improved visual cycle and reductions in Aβ and inflammation it is not surprising that the ERG improved. However, ERGs are relatively insensitive and significant changes in amplitude of the respective waves require disproportionately large changes in underlying biology before they are reliably detected. An example of this insensitivity comes from the finding that ERG thresholds are 2–3 log units less sensitive than those based on psychophysical measures (Ruseckaite et al., 2011). Hence, the true impact of β-CD on improved psychophysical visual function may be more significant than reported here.

Our improved ERGs were largely confined to higher luminance. There may be many reasons for this including differential saturation. However, recently it has been shown that age-related cone loss in mice occurs before rod loss. It is present within the first year of life while rod loss is primarily a feature of the second year (Cunea et al., 2014). Hence, as our animals were around a year of age when sacrificed, cones may have been in a more vulnerable state than rods. Further, there is a growing clinical evidence that cones may be particularly vulnerable to inflammation (see discussion in Cunea et al., 2014) and hence may have improved function follow CD treatment. However we do not have proof for this as an explanation and the specific underlying mechanism has yet to be revealed.

There are two additional reasons to think that β-CD may have potential in dealing with problems related to retinal ageing. First, we show that β-CD is effective in aged *Cfh*$^{-/-}$ mice. These are regarded as an AMD model as they have similar genotype to 50% of AMD patients (Edwards et al., 2005; Haines et al., 2005) and suffer excess Aβ deposition and inflammation in the outer retina (Catchpole et al., 2013; Hoh Kam et al., 2013) that is associated with advanced photoreceptor loss (Hoh Kam et al., 2013). Second, we

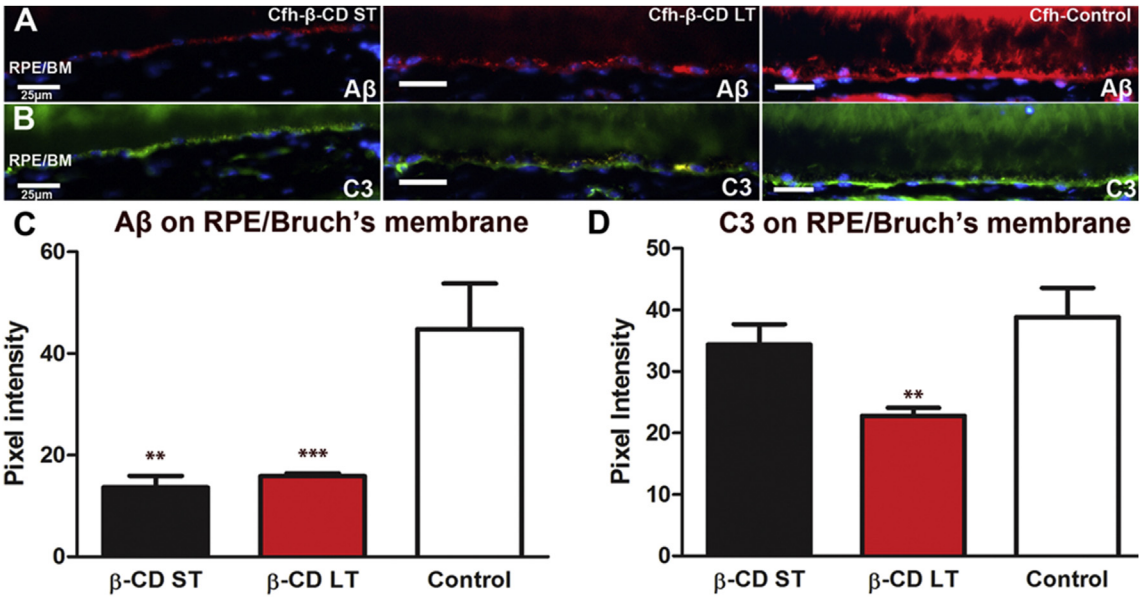

Fig. 4. β-CD treatment efficiently removes Aβ and complement C3 along the RPE/BM interface of *Cfh*$^{-/-}$ mice. A. Representative micrographs of retinal sections of *Cfh*$^{-/-}$ mice from all three groups immunostained with Aβ (red) and counterstained with DAPI (blue). Aβ is heavily expressed in controls compared to either the short treatment β-CD ST-treated mice or long treatment β-CD LT-treated mice. B. Representative micrographs of retinal sections of *Cfh*$^{-/-}$ mice from all three groups immunostained with C3 (green) and counterstained with DAPI (blue). The control group expressed more C3 than the two other β-CD-treated groups. C. Graph showing that the β-CD-treated groups have significantly lower expression of Aβ along the RPE/BM interface than the control group. ($P < 0.01$ for β-CD ST and $P < 0.001$ for the β-CD LT when compare to the control group). D. Graph showing the level of C3 expression the all three groups. The control group has a significantly higher expression of C3 compared to the β-CD LT group ($P < 0.01$) but not significantly different from the β-CD ST-treated mice. Mean ± SEM. n = 5 for the β-CD ST treated *Cfh*$^{-/-}$, n = 10 for the β-CD LT-treated *Cfh*$^{-/-}$ mice and n = 5 for the untreated animals. RPE/BM – Retinal pigment epithelium/Bruch's membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

show that significant reductions in Aβ deposition in the outer retina of these mice can be obtained with dosing at only approximately 10% of the level undertaken in the C57BL/6 mice used more extensively here. While we did reveal significant reductions in Aβ, our inability to show a significant reduction in inflammation with this low dosing may be due to time. Had the treatment been extended it is possible that inflammation would have declined in response to reduced pro-inflammatory Aβ.

A number of studies have shown reductions in retinal Aβ and inflammation with systemic immunotherapies in mice as a potential route to an AMD therapy. A key study has also shown improved ERG b-wave function in transgenic mice. However, some models are relatively complex and systemic immunotherapies are inherently problematic (Bowes Rickman et al., 2013; Ding et al., 2011). β-CD has been used extensively in humans for many years (Loftsson and Duchene, 2007). Our results imply that the effects in their use may be down to multiple factors. These include drugs that they carry and also what CD might do once they have deposited such drugs. Hence CDs may not be a reliable vehicle alone without such qualifications.

Given the data presented here using topical administration it is possible that it could be used in AMD patients. While there are very considerable retinal differences between mice and human that raise serious questions about the validity of the mouse model, this has to be balanced against other key issues. These include the lack of a realistic alternative, the pressing nature of the disease and the safe and economic route that β-CD offers.

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