INVESTIGATING OUTCOMES OF GENE REPLACEMENT THERAPY IN THE RPE65-DEFICIENT DOG

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ABSTRACT

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Treatment of Leber Congenital Amaurosis (LCA) caused by *RPE65* gene mutations with recombinant adeno-associated virus (rAAV) mediated subretinal gene replacement therapy has been shown to be safe and effective through phase I/II clinical trials. The purpose of the four studies that comprise this thesis were to further evaluate gene therapy outcomes using the RPE65-deficient canine model. To study the success of treatment in the presence of retinal degeneration treatment of older RPE65-deficient dogs (2-6 years of age) was performed, demonstrating improved retinal function, as assessed by electroretinography (ERG), and improved vision testing outcomes. The effect of immune responses on treatment the second eye after prior treatment of the first eye of RPE65-deficient dogs was also studied, finding evidence of safety and efficacy equal to that seen in the first treated eye. These findings supported inclusion of the second eye in current human clinical trials, and this has since commenced. For these and subsequent studies a description of the phenotype of older RPE65-deficient dogs, and changes with age were evaluated, this included description of a previously unreported region of photoreceptor loss at the *area centralis*. Additionally, these studies required evidence that improvements in vision testing outcomes of an objective canine vision testing apparatus were due to the given therapy alone, and this was provided.

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CHAPTER 1 Introduction

1.1 Inherited retinal dystrophies

The inherited retinal dystrophies are a large group of disorders that result in impaired visual function. The estimated worldwide prevalence is approximately 1 in 1,500.[1] This group of diseases is caused by mutations in genes that code for proteins involved in the development, function and survival of retinal neurons, photoreceptors, and the retinal pigment epithelium (RPE). While most commonly onsetting in adulthood, inherited retinal dystrophies are also the leading cause of childhood blindness in both developed and developing countries.[2] The inherited retinal dystrophies can be classified as stationary (non-progressive) disorders or progressive disorders. The progressive inherited retinal dystrophies can be further classified by whether they affect the central retina, or are generalized as is the case for Retinitis Pigmentosa (RP), the largest and perhaps best known family of these diseases. Many of the inherited retinal dystrophies primarily affect photoreceptors, and these can be classified according to the type of photoreceptor that is initially affected.[3] Disorders that primarily affect the rod photoreceptors but with secondary cone photoreceptor dysfunction are common types of RP and are referred to as rod-cone dystrophies. Cone-rod dystrophies are less common and are characterized by initial development of cone disease, or concomitant loss of both cones and rods.[4] Many genomic loci and specific gene mutations associated with inherited retinal disorders have been identified, and the most up to date list can be found online, http://www.retnet.org.

1.1.1 Retinitis pigmentosa

Retinitis Pigmentosa is the largest family of inherited retinal dystrophies, caused by abnormalities of the photoreceptors or RPE of the retina. Disorders in the RP family have common pathologic and phenotypic features, but are caused by numerous and distinct genetic mutations. The worldwide prevalence approximates 1 in 4000, with dominant, recessive, X-linked, mitochondrial and digenic modes of inheritance described.[5-12] Pathologically, the family of RP disorders have the common features of a progressive degeneration of photoreceptors typically by apoptotis.[13, 14] This is appreciated histologically or in the living patient by optical coherence tomography, as thinning of the outer nuclear later (ONL) of the retina that contains the nuclei of photoreceptors. In retinitis pigmentosa ONL thinning may later be followed by changes of the inner retinal layers and the RPE, which degenerate later in the disease process.[15] Retinitis pigmentosa results in symptoms of progressive bilateral nyctalopia and loss of peripheral visual fields, typically starting in adolescence or early adulthood.[16-19] These changes are also accompanied by an abnormal or diminished electroretinogram and funduscopic abnormalities.[20-23] The vision loss progresses to involve cone-mediated vision and usually results in legal blindness in middle age, although the rate of progression varies considerably between patients.[19, 24]

The variability in progression and severity of RP largely reflects the numerous and diverse mutations known to cause the condition, although there can be variability between patients with the same gene mutation.[25] Some gene mutations can cause syndromic RP where

abnormalities of one or more organ systems occurs in addition to retinal disease, though such disorders are less common.[26] Currently 51 genes associated with nonsyndromic autosomal dominant, autosomal recessive and x-linked RP have been mapped or cloned (http://www.retnet.org). The majority of these gene defects affect the photoreceptors themselves or the underlying RPE, with photoreceptor defects generally resulting in more severe disease then RPE defects.[27]

1.1.2 Leber Congenital Amaurosis

Leber Congenital Amaurosis (LCA) is an inherited retinal dystrophy and the most common cause of blindness in children with an estimated global prevalence of 1 in 81,000.[28-30] LCA is very similar to RP, being a progressive disease affecting the photoreceptors and RPE. Like RP there is progressive thinning of the retinal ONL and the clinical features of progressive bilateral nyctalopia and visual field deficits.[28] However unlike RP, Leber Congenital Amaurosis is a congenital retinal dystrophy characterized by visual impairment from birth.[31] Other clinical features typical of LCA are nystagmus, sluggish pupillary light reflexes and a normal fundus appearance in young individuals.[2] With respect to retinal function as assessed by electroretinography, both light-adapted (photopic) and dark-adapted (scotopic) ERG recordings are typically markedly attenuated.[32, 33] Visual function deteriorates slowly but over a variable period, with most patients being blind by the third to fifth decade of life, concordant with the slow progressive retinal degeneration.[34-36]

Like Retinitis Pigmentosa, LCA is caused by many different gene mutations, of which the majority of the known mutations are autosomal recessive although autosomal dominant LCA is

also described.[25] Of the 19 described mutations that cause LCA two are known to affect genes encoding proteins involved in the visual cycle, *RPE65* and *RDH12.*[37] Table 1.1 shows the gene mutations identified to date that result in LCA. The different gene mutations contribute to some heterogeneity of the phenotype of LCA patients. Visual acuities have been reported to range from 20/40 to no light perception, with patients having *CRB1* or *RPE65* mutations demonstrating the widest variation in residual vision, and patients with *AIPL1* or *RPGRIP1* mutations consistently having more severe visual impairment.[38] With respect to funduscopic appearance, regions of peripheral chorioretinal atrophy are reported in patients with *AIPL1* and *RPE65* mutations, and yellow punctate foci are more often observed in patients with *AIPL1, CRB1, RPE65* and *RPGRIP1* gene mutations.[32, 38] Generally LCA patients have some preservation of retinal architecture early in the course of the disease, however there is individual variability, with patients with *AIPL1* mutations having significant loss of photoreceptors at an early age.[39] In contrast, in-vivo assessments of outer nuclear layer (ONL) thickness of patients with *RPE65* mutations by optical coherence tomography have shown that despite the significant visual impairment from a young age, the retinas of these patients retain an appreciable photoreceptor layer into adulthood.[40]

Table 1.1 Gene mutations that result in a phenotype of LCA (http://www.retnet.org)

Symbol	Gene	Inheritance	Locus	% of total LCA cases
LCA1	*GUCY2D*	Autosomal recessive	17p13.1	11.7%
LCA2	*RPE65*	Autosomal recessive	1p31.2	6%
LCA3	*SPATA7*	Autosomal recessive	14q31.3	Unknown
LCA4	*AIPL1*	Autosomal recessive	17p13.2	5.3%
LCA5	*Lebercilin*	Autosomal recessive	6q14.1	1.8%
LCA6	*RPGRIP1*	Autosomal recessive	14q11.2	4.2%
LCA7	*CRX*	Autosomal recessive & autosomal dominant	19q13.32	1%
LCA8	*CRB1*	Autosomal recessive	1q31.3	9.9%
LCA9	*NMNAT1*	Autosomal recessive	1p36	Unknown
LCA10	*CEP290*	Autosomal recessive	12q21.32	25%-43%
LCA11	*IMPDH1*	Autosomal dominant	7q32.1	8.3%
LCA12	*RD3*	Autosomal recessive	1q32.3	0.1%
LCA13	*RDH12*	Autosomal recessive	14q24.1	2.7%
LCA14	*LRAT*	Autosomal recessive	4q32.1	0.5%
LCA15	*TULP1*	Autosomal recessive	6p21.31	<1%
LCA16	*KCNJ13*	Autosomal recessive	2q37.1	Unknown
Not numbered	*IQCB1*	Autosomal recessive	3q13.33	Unknown
Not numbered	*CABP4*	Autosomal recessive	11q13.1	Unknown
Not numbered	*OTX2*	Autosomal dominant	14q22.3	Unknown

1.1.3 *RPE65* gene mutations and LCA

To date more than 60 mutations in the *RPE65* gene have been identified and associated with LCA and RP.[41-44] LCA caused by *RPE65* mutations is designated LCA Type 2. Missense mutations may alter the stability and functional capacity of the resultant mutant protein.[45-48] This variability in the residual function of the mutant protein contributes to the phenotypic heterogeneity of LCA Type 2.[46-49] Probable functional null mutations are also described.[50-52] The *RPE65* gene encodes for RPE65 protein which is a key enzyme in the visual cycle and is

essential for recycling of the visual pigment required for photoreceptors to respond to light. RPE65 functions as an isomerohydolase in the visual cycle.[53] Recent studies have suggested that there is an alternative as yet undiscovered isomerohydrolase which is part of an alternative visual cycle utilized by cone photoreceptors. This may contribute to the residual vision of patients with *RPE65* null mutations.[54]

1.1.4 The RPE65 protein

The RPE65 protein is expressed in the retinal pigment epithelium. The RPE is a monolayer of cells lying adjacent to the photoreceptors, essential for the health and normal function of the neural retina. RPE65 protein was first identified in 1993 in efforts to evaluate the RPE in health and disease; monoclonal antibodies raised against human RPE cells identified a novel protein associated with RPE cell membranes, 65-kDa in size and therefore named RPE65.[55] The RPE65 protein was subsequently shown to be 61-kDa in size and comprised of 533 amino acids.[56, 57] The crystal structure of RPE65 was determined and published in 2009.[58] The *RPE65* gene that encodes RPE65 contains 14 coding exons, spanning 20 kb and was mapped to human chromosome 1, p31 by fluorescence in situ hybridization.[56, 57] RPE65 protein is expressed in the RPE of rats from embryonic days 4-5, coinciding with the appearance of photoreceptor outer segment membranes and is subsequently expressed continuously throughout life.[59] The role of RPE65 is as the isomerohydrolase responsible for the conversion of all-*trans* to 11-*cis* retinoids as a key part of the visual cycle (described in detail below).[53, 60, 61] It has also been suggested that RPE65 protein is expressed in cone photoreceptors.[62] Though a more recent study did not find any evidence for a direct role of RPE65 in cone photoreceptor

function, this would appear to be depend on the strain of mouse studied.[63, 64] While the presence and role of RPE65 in cones remains unclear at this time, if present it would lend support to the hypothesis that cones utilize a different retinoid processing cycle in addition to the canonical visual cycle. This does not mean however that retinoid regeneration in cones is RPE65 independent, with RPE65 shown to be essential for both rod and cone function.[65, 66]

1.1.5 RPE65, the isomerohydrolase of the visual cycle

RPE65 is an evolutionarily conserved 61-kDa membrane associated protein present in the smooth endoplasmic reticulum of RPE cells.[55, 67, 68] As the isomerohydrolase it plays an essential role in the visual cycle by which 11-*cis* retinal is regenerated after light exposure. 11-*cis* retinal plays a crucial role in vision, in photoreceptors it is bound to an opsin signaling protein, forming a visual pigment molecule (e.g. rhodopsin in rods). Opsins are membrane-bound proteins with seven trans-membrane helices that enclose a binding pocket for 11-*cis* retinal.[69] Alone, opsins are not photosensitive, and will only absorb visible light once coupled with 11-*cis* retinal. Additionally, the sensitivity of individual visual pigments to different wavelengths of light is determined by interactions between 11-*cis* retinal and opsin specific for that particular photoreceptor.[70]

The first stage of the visual cycle involves activation of a visual pigment molecule such as rhodopsin by a photon of light, initiating a series of reactions that result in photoreceptor hyperpolarization, a process known as phototransduction. Light induced activation of rhodopsin

isomerizes 11-*cis* retinal to all-*trans* retinal, inducing conformational changes to opsin which subsequently activates the G protein transducin.[71] The guanosine triphosphate (GTP) bound subunit of transducin then activates a phosphodiesterase (PDE) that hydrolyzes cyclic guanosine monophosphate (cGMP) into guanosine monophosphate (GMP). This reduces the concentration of cGMP in the photoreceptor outer segment, leading to the closure of cGMP gated ion channels in the outer segment membrane and photoreceptor hyperpolarization.[72] In the dark there is on-going glutamate release at the photoreceptor:bipolar cell synapse, and when the photoreceptor is hyperpolarized in response to light the amount of glutamate release decreases. This leads to depolarization or hyperpolarization of different classes of bipolar cells. Further processing by other neurons in the inner retina occurs before this signal is transmitted to the visual cortex.

Following light activation, a rapid recovery of the photoreceptor is necessary for it to respond to subsequently absorbed photons of light. This requires the inactivation of each of the previously activated components. The activated rhodopsin must be phosphorylated, and the transducin inactivated by hydrolysis of the bound GTP to GDP.[73] Though rhodopsin phosphorylation is a multistep process, transducin inactivation is slower and dictates the recovery kinetics of a rod photoreceptor.[73] After hydrolysis the GDP dissociates from PDE, deactivating it.[74] Restoration of basal cGMP concentration is stimulated by the lowered calcium concentration that occurs with hyperpolarization of the photoreceptor; the dissociation of calcium allows guanylate-cyclase-activating proteins (GCAPs) to activate guanylate-cyclase (GC), thereby restoring the basal cGMP concentration.[75] Additionally the 11-*cis* retinal must

be regenerated. The regeneration of 11-*cis* retinal takes place in the RPE.

All-*trans* retinal is released from the activated opsin into the inner leaflet of the photoreceptor disc bi-layer where it complexes with phosphatidylethanolamine. The resulting N-retinylidine-phosphatidylethanolamine is then transported to the photoreceptor disc surface by retina specific ATP binding cassette transporter (ABCR).[76] It is released into the cytoplasm as all-*trans* retinal and reduced to all-*trans* retinol (Vitamin A) by all-*trans* retinol dehydrogenase.[77] The all-*trans* retinol exits the photoreceptor and once bound to interphotoreceptor retinoid binding protein (IRBP) it crosses the sub-retinal space, to enter the RPE.[78] Once in the RPE, cellular retinoid binding protein (CRBP) transfers the all-*trans* retinol to the first visual cycle enzyme, lecithin retinol acyl transferase (LRAT), which generates all-*trans* retinyl esters.[79] All-*trans* retinol from the systemic circulation also enters the visual cycle through the RPE basal surface for esterification by LRAT. Coupled hydrolysis and isomerization of all-*trans* retinyl esters by the isomerohydrolase RPE65 then yields 11-*cis* retinol.[61] Cellular retinaldyhyde binding protein (CRALBP) then binds and delivers the 11-*cis* retinol to 11-*cis* retinol dehydrogenase for the final enzymatic step, oxidization to 11-*cis* retinal.[80, 81] Finally IRBP is proposed to facilitate the transport of 11-*cis* retinal from the RPE across the subretinal space to the photoreceptors, protecting it from isomerization en route.[78, 82, 83] The 11-*cis* retinal then binds with opsin to complete the cycle. So without RPE65, 11-*cis*- retinal levels are significantly reduced or absent and retinyl esters accumulate in the RPE.

It is important to note that studies of the visual cycle were performed on rod photoreceptors.

Cones likely rely on the same system, but it is suggested that there is also an alternate cone specific visual cycle pathway thought to involve Müller glial cells.[84, 85]

1.2 Murine models of RPE65 deficiency

To date, three murine models with RPE65 deficiency and resulting retinal disease have been reported and extensively studied.[47, 67, 86] These murine models have contributed much to our understanding of RPE65 deficiency, however they have some limitations, for instance unlike the human eye mice lack a fovea or region of higher photoreceptor density. Adapted for their night vision, anatomically the retina of this nocturnal species is rod dominated, with rods comprising approximately 97% of photoreceptor population.[87, 88] The smaller eye of the mouse also means procedures such as subretinal injection require modification of the microsurgical techniques that would be used in human patients. Cone photoreceptor subtypes are also different, across the murine retina 95% of cone photoreceptors co-express both S-opsin and M-opsin with a peak spectral sensitivity of 508 nm.[89] The remaining 5% of cones are genuine S cones that express only S-opsin and are most efficiently stimulated by U.V. light.[89] In humans in addition to rods that have a spectral sensitivity of 491nm, there are three cone photoreceptor subtypes S (blue), M (green), and L (red) cones with spectral sensitivities of 430, 530, and 561 nm respectively.[90]

1.2.1 RPE65 knockout (*Rpe65 -/-*) mouse

In 1998 Redmond *et al*, described the generation of RPE65 knockout (*Rpe65* -/-) mice, a model of RPE65 deficiency that has since been widely studied.[67] The model was created by replacing

the first three exons and intervening introns of the *Rpe65* gene by homologous recombination using a neomycin resistance cassette.[67] Like RPE65-LCA patients these mice are visually impaired in dim-light and have diminished rod and cone driven ERG responses.[67, 91] A progressive retinal degeneration occurs so that by 6-7 months of age, the photoreceptor outer nuclear layer is approximately 70% of normal thickness and approximately half normal thickness by 12-18 months of age.[67, 92] This largely reflects slow progressive rod photoreceptor apoptosis, the rate of which may vary with environmental and genetic variables such as coat color and hence ocular melanin, and ambient light with cyclic-light rearing resulting in a more rapid rate of degeneration than dark-rearing.[93, 94] Woodruff *et al* showed that the rod photoreceptors of RPE65 knockout mice act as if strongly adapted by a background light even in its absence.[95] They have a low sensitivity to light, containing a high concentration of largely unliganded opsin unbound to arrestin which activates the visual cycle at a low level, leading to closure of cGMP-gated ion channels.[95, 96] It is this continuous, pathologic light-independent opsin signaling that induces the Bcl-2 related pathway and Bax-dependent apoptosis of the rods.[14, 36, 97] Recently an early transient wave of apoptosis of rods was also identified, appearing at post-natal day 13, peaking between P16 and P19, and quickly decreasing from P19 to P25.[98] This early apoptotic response did not lead to a detectable decrease in the retinal outer nuclear layer thickness and was dependent on Bax-induced apoptotic pathway.[98]

In contrast, the cone photoreceptors of RPE65 knockout mice show a fast rate of degeneration with an early loss of a large proportion of cones by 1 month of age, most dramatically affecting

the S cones of the inferior retina.[99] Examination of double knockout *Nrl-/-Rpe65-/-* mice lacking rods and RPE65 confirmed this rapid loss of cone photoreceptors.[65] More Recently it has been shown that 11-*cis* retinal is variably required for trafficking of cone opsins to cone photoreceptors, unlike rod opsin (rhodopsin) which traffics to the rod photoreceptors without this reliance on 11-*cis* retinal.[100] This manifests as mislocalization of cone opsins within the synaptic pedicle, cell body and inner segments of cone photoreceptors.[100] The mislocalized cone opsins alter photoreceptor physiology and induce apoptosis thus contributing to the greater rate of cone photoreceptor apoptosis relative to rods.[100, 101] Additionally Hamann *et al* showed that rod photoreceptor apoptosis is Bax-induced, while the early degeneration of cones is not mediated by pro-apoptotic Bax.[36] This suggested that two independent apoptotic pathways are activated in rods and cones in RPE65 knockout mice. Palmitoylation state may also play a role, since palmitoylation has been found to stabilize rod opsin and cone opsins are unpalmitoylated.[102, 103] Hence when there is a paucity of chromophore as in RPE65 disease, destabilization of unpalmitoylated cone opsins may contribute to the more rapid cone degeneration.

The RPE65 knockout mouse also accumulates all-*trans* retinyl esters in lipid droplets within the RPE.[67, 104] These RPE inclusions have been shown to be lysosomal storage bodies for retinyl esters, retinosomes, that contain substantial amounts of retinoids which accumulate from 3 weeks of age due to the block in the visual cycle.[104, 105] The effect of these lipid droplets on RPE function is not presently known, similarly it is not known if they contribute to degeneration of RPE cells or photoreceptors.

1.2.2 *Rpe65*^{rd12} mouse

A naturally occurring murine model of RPE65 deficiency also exists, the *rd12* mouse. These mice have a nonsense mutation in exon 3 of *Rpe65* resulting in a premature stop codon and absence of RPE65 expression.[86] Phenotypically the *Rpe65*^{rd12} mouse is very similar to the RPE65 knockout mouse. Like the RPE65 knockout mouse, there is a slowly progressive loss of rod photoreceptors and more rapid and marked loss of cones. Mice less then 2 months of age display voids in the rod outer segments proposed to represent reduced photoreceptor disc density.[86] Using peanut agglutinin lectin (PNA) staining Li *et al* showed a dramatic decrease in cones by 21 days of age.[106] By 3 months-of-age, all cones in the central retina are degenerate, with some PNA-positive cones remaining in the dorso-temporal quadrant of the retina.[86] This is similar to the pattern seen in the RPE65 knockout mouse.[99] The *Rpe65*^{rd12} mouse has a progressive thinning of the outer nuclear layer of the retina, being near-normal thickness until around 8 weeks of age but reduced to approximately 60% thickness by 7 months of age.[86, 107] This rate of retinal degeneration is quite similar to that reported in the RPE65 knockout mouse. RPE cells in *Rpe65*^{rd12} mice are also described to have occasional small lipid-like droplets at 3 weeks of age, increasing in number and size with age. Of note, by 27 months of age the RPE cells are reportedly atrophied and hypopigmented.[86]

1.2.3 *Rpe65*^{R91W} knockin mouse

A third mouse model was reported by Samardzija *et al*, generated by targeting exon 4 of the *Rpe65* gene and modifying codon 91 from CGA to TGG.[47] This is a model of RPE65 disease caused by missense mutations that are more common then null mutations in human patients.[51, 108] Like the other murine models there is a slow progressive decline in rod photoreceptor number with increasing age. Retinal morphology is also very similar and shows a similar decline in thickness as the RPE65 knockout mouse.[47] However this model has a slower cone degeneration than RPE65 knockout and $Rpe65^{rd12}$ mice.[47, 109] Using this model Samardzija *et al* provided evidence that competition between rod and cone opsins for available 11-*cis* retinal occurs, and suggest that the rods trap more of the available chromophore which may contribute to the more rapid cone photoreceptor apoptosis.[109]

1.3 A canine model of Leber Congenital Amaurosis

Canine models of human retinal dystrophies have become increasingly important for evaluation of therapies to restore vision.[110-113]. One such model is the RPE65-deficient dog, used for pioneering proof of concept gene therapy trials, and continued evaluation of alternate therapies and modifications to existing techniques.[110, 111, 114, 115]. The canine globe size is very similar to that of humans meaning that similar surgical approaches can be used for treating retinal disease. Also unlike mice, dogs have an *area centralis* thathas a higher rod and cone density than other regions of the retina.[116] Additionally the cone-rich *area centralis* of the dog has an S-cone population approximating 10% of cones in this region, similar to the human parafoveal area, which has 8%–10% S cones.[116-118] However the canine retina is not directly comparable to that of the human retina that contains a fovea which is S-cone and rod free,

approximating 100–150 µm in diameter.[117, 118] Unlike humans that have three cone subtypes (described in section 1.2), dogs are dichromats, having 2 cone subtypes, L/M (red/green) and S (blue) cones, with peak sensitivity at approximately 555 nm and 429 nm respectively.[119-122] Canine rod photoreceptors also have a slightly higher spectral sensitivity then that of human rods (491nm), with a peak at about 508 nm.[90, 119]

1.3.1 The RPE65-deficient (*RPE65 -/-*) dog

In 1989 a canine model of autosomal recessive LCA was described by Narfström *et al* in a colony of Swedish Briard dogs.[123] First described as a model for congenital stationary night blindness as they were blind at lower light levels, the causal gene mutation was subsequently identified as a null mutation in *RPE65*, a four base-pair deletion resulting in a frame-shift and premature stop codon.[124, 125] Immunohistochemical studies showed that affected dogs lack RPE65 protein in the RPE confirming the validity as a model for human RPE65-LCA.[110, 114, 126] The lack of 11-*cis* retinal production means that normal visual pigment formation cannot occur, resulting in a phenotype of severely reduced rod and cone photoreceptor sensitivity and visual impairment.[30]

Affected dogs have disorientation of the rod outer segment discs from 5 weeks of age, shortening of rod inner segments from 4 months of age, and degeneration of peripheral rod photoreceptors from 7 months of age.[127, 128] Other authors describe a later-onset photoreceptor degeneration after 17 months of age.[110, 125, 129] Hence it has been suggested that some variation between the different colonies studied may exist.[54] Consistent

with the murine models, lipid droplets accumulate in the RPE, first noted at 3.5 months of age, then increasing in size and number.[127-129] Most descriptions of the histopathologic changes are limited to dogs less then 1.5 years of age with a notable exception of a report by Wrigstad *et al* describing an eye from a 7-year-old dog which showed a reduction of photoreceptors centrally and almost complete photoreceptor degeneration peripherally with severe changes of the inner retina in the peripheral fundus.[128]

An immunohistochemical study by Hernandez *et al* in younger RPE65-deficient dogs showed no mislocalization of cone opsins, findings that contrast with those reported in murine models where M/L and S opsins are mislocalized and fail to traffic properly.[99, 101, 129] The dogs studied by Hernandez *et al* also differed from the murine models with regard to rod opsin, showing mislocalization from the photoreceptor outer segment to the ONL, a feature not described in the murine models.[100, 129] The rod bipolar cells of the RPE65-deficient dogs also showed changes. Specifically there was enlargement and retraction of the terminals of rod bipolar cells, and increased dendritic aborizations extending into the ONL of the retina from rod bipolar cells and ON bipolar cells (receiving input from both rods and cones).[129] A subtype of horizontal and amacrine cells (GABAergic cells) were also reduced in number, in the retina gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter that mediates lateral surround inhibition, the need for which could be anticipated to be reduced in RPE65 deficiency.[129, 130]

Where the visual function of RPE65-deficient dogs has been described, there are also some

discrepancies in the literature. Young *RPE65* -/- dogs are consistently reported to have poor dim light vision, however descriptions of their vision in bright light varies, described as normal by some authors, and as being severely impaired by others.[125, 131, 132] Here different techniques have been used by different authors, with some relying on subjective descriptions, and efforts have been made to reduce the subjectivity of vision assessment in more recent studies.[114, 133, 134] Additionally functional MRI studies have been preformed as part of therapy studies and in the untreated dog there were minimal cortical responses in the primary visual areas of the lateral gyrus.[135] The affected dogs also exhibit nystagmus although the degree is variable.[126, 136, 137]

Electroretinograms performed on young affected dogs show a marked reduction in rod and cone photoreceptor sensitivity, specifically the rod-driven dark-adapted threshold of response is elevated and response amplitudes are reduced.[110, 123, 125, 131, 132] Light-adapted cone flicker responses are of very low amplitude to unrecordable.[110, 123, 125, 131, 132] Surprisingly, given the low amplitude cone flicker responses, the normally cone dominated light-adapted flash ERG response shows a- and b-wave amplitudes very similar to those of normal dogs.[110] This is proposed to be due to a greater rod photoreceptor contribution, where background light that normally suppresses rods does not fully suppress the markedly desensitized rods in these *RPE65* -/- dogs.[110]

The appearance of the fundus of young RPE65-deficient dogs is reportedly normal.[123, 125] However changes in the funduscopic appearance have been described in two older dogs. A five

and a half year-old dog was reported to have multifocal yellow-white spots in the tapetal fundus, and a 6 year old dog with changes consistent with retinal thinning and retinal vasculature thinning.[138]

1.4 Gene therapy to restore RPE65

Retinal dystrophies such as RPE65-LCA have been considered untreatable for many years. Gene therapy to replace a mutant or absent protein has been recently evaluated as a technique to improve the visual function of affected individuals. RPE65-LCA was considered a good candidate for evaluation of this treatment as the retinal architecture is relatively well preserved in young individuals where the absence or low level of RPE65 is the main factor precluding vision due to the visual cycle blockade. Due to possibility of an immune response to the novel protein in the presence of a mutant protein, individuals with a null mutation and no RPE65 were also considered better candidates.

1.4.1 Gene therapy

Gene therapy is the introduction of genetic material into target cells with the goal of curing disease or slowing disease progression.[139] The approach used depends on whether the underlying disease mutation leads to a gain or loss of function, whether the gene product function affects cell survival or development, and the genes location at a cellular and tissue level.[140] Most efforts have been directed towards treating loss-of-function mutations by the addition of a normal copy of the mutated gene, as is the case for RPE65-deficiency.[140] Other

approaches include the modification of messenger RNA to avoid the consequences of a mutation, inhibiting the expression of a mutated gene, or direct repair of the genetic mutation.[140] Introduction of a normal copy of a mutated gene requires a vector to carry genetic material into a cell. Viruses are widely used as they can gain entry to cells by binding to cell surface receptors.[141] Alternate strategies to deliver genes of interest without viral packaging are being explored but require overcoming the hurdles of achieving nuclear localization and achieving stable long-term transgene expression.[141] Irrespective of the technique used, the goal is to achieve expression of the therapeutic gene in the target cells under the control of regulatory elements, while ensuring no pathogenic or adverse effects occur, such as marked immune responses to either the expressed protein or the vector.[139]

1.4.2 Gene therapy for retinal disease

The eye has unique features that make it well suited for gene replacement therapy for inherited retinal dystrophies. First the anatomic structure of the eye including optical transparency allows accurate delivery of a vector to targeted retinal cells under direct visualization using standard microsurgical techniques with reduced risk of off-target effects.[142] Second the feature of ocular immune privilege, this phenomenon attenuates immune responses in the eye that might otherwise cause undesirable inflammation or limit transgene expression. Ocular immune privilege prevents or modifies the innate and adaptive immune responses through the presence of a blood-retinal barrier, peripheral tolerance of eye-derived antigens (anterior chamber-derived immune deviation (ACAID)), and soluble and cell-surface immunomodulatory factors that suppress the cells and molecules that mediate innate and adaptive immune

responses.[143] Despite its name ACAID does not just apply to the anterior chamber, it is an altered systemic immune response that excludes CD4+ T helper cells, and B cells that secrete complement-fixing antibodies when antigens are present in the vitreous and subretinal space.[143] This is achieved because intraocular antigen presenting cells migrate directly into the bloodstream and traffic to the marginal zone of the spleen.[143] Together these features mean many of the concerns that have arisen from previous gene therapy trials in other organ systems are mitigated.

1.4.3 Vectors for retinal gene replacement therapy

Delivery of a therapeutic gene to retinal tissue can be achieved by both viral- and non-viral-based methods. At present despite techniques such as electroporation, the transfection rate using non-viral vectors is low and transgene expression is short-lived.[141, 144] Viral vectors have therefore been the method of choice for gene delivery to the eye to date. Viral vectors commonly used for ocular gene transfer are adenoviral, lentiviral and adeno-associated viral (AAV) vectors [145]. Of these, recombinant adeno-associated virus (rAAV) vectors are increasingly utilized, being able to transduce the RPE, photoreceptor cells, ganglion cells and Müller cells of the retina.[146, 147] Recombinant AAVs are derived from wild-type AAV, a parvovirus that has a 4.7-kb genome made up of *rep* and *cap* genes that code for 4 replication and 3 capsid proteins, flanked by two 145-bp inverted terminal repeats (ITRs).[147] The recombinant AAV (rAAV) vectors retain only the ITRs, leaving almost 4.7 kb for packaging of therapeutic DNA and appropriate cell-specific promoters.[147] A further advantage of rAAV vectors is lack of integration into the genome, rather allowing stable, long-term transgene

expression by existing as extragenomic circular episomes (unlike wild-type AAV), decreasing the risk of insertional oncogenesis.[147, 148] Early rAAV vectors used genomic and protein capsid components that were both of serotype 2 (AAV2/2).[149] It is the capsid serotype that determines the tropism of the vector.[149] So to alter tissue tropism hybrid 'pseudotyped' rAAV vectors have been developed, where an AAV plasmid is packaged in a capsid from an AAV of a different serotype. For example unlike rAAV2/2, rAAV2/5 (AAV2 genome packaged in AAV5 capsid) has improved photoreceptor transduction and faster onset of transgene expression compared to AAV2/2 vectors.[142]

One of the main limitations of rAAV vectors is limited packaging capacity.[150] However other viral vector types have disadvantages of potentially greater concern, adenoviral vectors for example induce a strong cytotoxic T lymphocyte mediated immune response that limits the duration of transgene expression.[151] Lentiviruses insert into the genome raising concerns of insertional mutagenesis, although strategies to circumvent this are being developed.[152] Irrespective of the type of viral vector used, the route of administration is largely dependent on the targeted cell type. For instance delivery by subretinal injection places the therapeutic gene adjacent to the photoreceptors and RPE, whereas intravitreal injections can target retinal ganglion cells of the inner retina but do not result in efficient transduction of the outer retina (photoreceptors and RPE).[142]

1.4.4 Gene therapy for RPE65 deficiency

RPE65 deficiency was considered a good candidate for gene replacement therapy, as the

primary defect is the visual cycle blockade and studies of young RPE65-deficient mice and dogs showed good preservation of photoreceptors. The canine model was used to show initial proof of principle for gene therapy. In 2001 Acland *et al* described use of an AAV2 vector carrying wildtype canine *RPE65* cDNA with a CMV/CBA promoter to target the retinal pigment epithelium following subretinal injection in the RPE65-deficient dog, with resultant improvements in retinal function and vision.[114] These results were repeated and expanded upon in several studies, demonstrating safety and efficacy in the canine model.[110, 126, 135, 137, 153-157] These studies showed improvement of both rod and cone photoreceptor-driven function after gene therapy as assessed by ERG. The threshold of rod driven flash ERG responses approximated that of normal dogs after treatment, being elevated by approximately 4 log units in untreated RPE65-deficient dogs. Most studies evaluating longevity of effect have seen ERG improvements remain stable for 8 months to 4 years, with the exception of one study by Benicelli *et al* that saw a significant decline in responses by 3 months.[110, 126, 154, 157] Improved vision, as assessed by behavioral vision testing after treatment, was reflected by functional MRI which showed weak responses at the lateral gyrus prior to treatment but significant cortical activation at this area by one month after treatment.[135] Immunohistochemical studies also showed expression of RPE65 protein in the RPE over the treated area.[114, 137] Analysis of retinoids in treated eyes of RPE65-deficient dogs showed detectable levels of 11-*cis* retinal, restricted to the region of the therapeutic injection.[110] Retinal morphology in the treated area was reportedly unchanged after treatment, having a comparable ONL thickness with affected untreated RPE65-deficient dogs of the same age.[110, 153] One study reported thinning of the ONL in the region of the subretinal bleb in eyes treated

with high doses of $1.5x10^{12}$ vector genomes (vg) and above.[158] With respect to the lipid inclusions noted in the RPE, Narfström *et al* reported disappearance of this feature in the treated area of a single RPE65-deficient dog, however other reports have not corroborated this finding.[137]

Subretinal delivery of human *RPE65* cDNA using rAAV vectors has also been performed in RPE65 knockout mice and *Rpe65*^{rd12} mice. These studies in murine models showed improved rod and cone photoreceptor function, improved visually guided behavior, and reduced accumulation of all-*trans* retinyl esters.[159-161] Evaluation of visually evoked potential (VEP) to test the integrity of the visual pathway after gene replacement therapy in the *Rpe65*^{rd12} mouse showed a relatively intact visual pathway but a systematic loss of function of pathways carrying high temporal frequency luminance information.[162] Notably, treatment of 17-26 month-old RPE65 knockout mice with advanced retinal degeneration showed that subretinal gene replacement therapy was effective but in a smaller percentage of eyes.[40] While the focus has remained on the use of AAV vectors, gene therapy using an integration-deficient lentiviral vector in *Rpe65*^{rd12} mice has been shown to effectively transduce the RPE with resultant expression of RPE65 and improvements in rod photoreceptor driven ERG responses, stable for at least 2 months after treatment.[152]

1.4.5 Human clinical trials of gene therapy for RPE65-LCA

The first human clinical trial of gene therapy for RPE65-LCA was initiated in February 2007 in London (NCT00643747). Two further trials started later the same year in Philadelphia (NCT00516477) and (NCT00481546), and preliminary results of a fourth clinical trial initiated in Israel was recently reported (NCT00422721) (www.clinicaltrials.gov). Each used replication deficient rAAV2 vectors carrying human *RPE65* cDNA delivered by subretinal injection.[163-166] These initial phase I/II studies sought to demonstrate first safety and second efficacy in RPE65-LCA patients. With respect to safety early results were positive, finding no significant adverse events, toxicity, or untoward immune responses.[163-166] The treatment also effectively improved vision albeit to a variable degree, however the dramatic improvements in the ERG that occurred in the canine and murine models were not seen.[163-166] These initial results of the phase I/II clinical trials confirmed the validity of gene replacement therapy for inherited retinal disease in human patients and led to continued evaluations. Now with 40 human patients treated to date, with regard to rescue and duration of rescue the clinical trials have shown persistence of improved dim light vision out to 3 years post-treatment, improvements in pupillary light responses, lessened nystagmus and changes in dark-adaptation kinetics, but variable improvements in visual acuity.[167-171] With regard to safety there have been no reports of systemic adverse events to date, ocular adverse events have however been reported and include retinal detachment, choroidal effusion, ocular hypotension and ocular hypertension. In all cases these adverse events have been attributed to the surgical procedure itself.[167-171] With respect to immune responses, humoral responses monitored by measuring the titer of circulating antibody to AAV2 capsid have persisted for variable durations, with some patients having elevated titers out to 3 years post-treatment.[163-167, 169-171]

However most patients had titers below the normal population mean.[163-167, 169-171] The studies have also shown that a single subretinal AAV2 treatment does not induce a consistent or pronounced AAV2 capsid antigen-specific immune response.[171] Treatment has also not induced a T-cell immune response to the AAV2 capsid or RPE65 protein in any treated subject to date, even in the presence of pre-existing peripheral AAV2-specfic memory T cells.[163-167, 169-171] Monitoring of the distribution of the vector in the peripheral blood by quantitative polymerase chain reaction have identified no vector genome copies, consistent with a lack of escape of subretinally administered AAV2 vector into the circulation.[163-167, 169-171]

1.5 Questions addressed in this thesis

While it is apparent that treatment of RPE65-LCA patients by gene replacement therapy is safe and successful, improving various measures of vision, the dramatic improvements seen in the canine and murine models have not been emulated.[163-171] This raises the question; why the difference, a question which may help identify methods to improve treatment efficacy. One important difference was that canine and murine pre-clinical studies almost exclusively report treatment of young individuals, in dogs this was typically less then 1 year of age where photoreceptor preservation was good to excellent and the RPE showed few changes.[110, 114, 127, 128, 137, 157] RPE65-LCA clinical trials have therefore begun treatment of younger patients in efforts to improve outcomes. However there is wide phenotypic variation in degree of retinal architecture preservation between individual RPE65-LCA patients, which may confound outcomes.[40, 54, 108] We looked at the question from the opposite standpoint, arguing that if our description of the phenotype of these dogs confirms little phenotypic

variation between individual colony dogs of the same age, as anticipated, an effect of age on the success of gene replacement therapy could be evaluated here. The question we sought to address was if gene therapy in older RPE65-deficient dogs with more advanced retinal degeneration would result in poorer outcomes. This would support treatment of younger patients, or treatment of individuals (or retinal areas) with better preserved retinal structure.

A second question is whether gene therapy treatment of the second eye of human patients will have an attenuated response or adverse inflammation due to immune priming by prior treatment of the first eye. A study in which the second eye of *Rpe65*^{rd12} mice was treated after prior treatment of the first eye supports the safety and efficacy of treating the second eye if low vector doses are administered.[172] Certainly the immune privileged nature of the subretinal space supports this concept, a study evaluating bilateral intravitreal injections 1 month apart in C57BL/6J mice showed poor reporter gene expression in the second treated eye.[173] Immune responses in one species may not be a predictor of the response in other species. Therefore sought to evaluate whether subsequent treatment of the second eye of RPE65-deficient dogs would give similar rescue to that achieved in the first eye. This would be important to test before starting to treat the second eye of human patients.

Before addressing these questions we noted that there is limited description of the phenotype of older RPE65-deficient dogs. There are detailed descriptions of the phenotype of young

RPE65-deficient dogs less then 2 years of age, but only 7 dogs older then 2 years have had some phenotypic description.[128, 135, 137, 157] Information on changes in vision or ERG measures of retinal function with age are lacking. Additionally, descriptions of the progression of retinal architecture changes with age are incomplete and descriptions of the degree of photoreceptor preservation in young dogs vary.[127-129] Published information on the residual vision of young RPE65-deficient dogs assessed using variably objective techniques is also inconsistent, with some authors reporting reduced dim-light vision, others complete blindness.[125, 131, 132, 174] Clarification of the phenotypic features of the RPE65-deficient dogs used in our studies is therefore of importance before addressing the above questions, and will also provide information useful for future studies using this model of RPE65-LCA.

Additionally the repeatability of current methods of vision testing have not been demonstrated for dogs. Given the importance of the canine models of RPE65-LCA with regard to gene therapy trials and the testing of other therapies to restore vision, accurate and reproducible quantitative assessment of canine vision is essential.[110, 111] As the use of canine models to the study inherited retinal dystrophies grows such information will also prove valuable if the studied device is used to evaluate vision in other models. The importance of evaluating vision in those studies being that electroretinography is a measure of retinal function only, while vision requires cortical integration of retinal signals and is the end goal of treating inherited retinal diseases. Additionally, as reported in the phase I/II clinical trials for RPE65-LCA, treatment can rescue enough photoreceptors to improve vision, without a measurable change in the ERG, as this records a summed photoreceptor driven response.[163-165, 167, 169] To date obstacle

course based tests to assess canine vision have been widely reported. To reduce subjectivity and produce results suitable for statistical analysis, methods such as counting the number of mistakes the dog makes, using blinded observers to score performance, and recording obstacle course transit time have been used.[114, 133, 134] An objective vision testing device that allows measurement of device transit time and ability to identify an open exit under different lighting levels has been recently described by Gearhart *et al* [174]. This device has previously been shown to be accurate for discriminating affected from unaffected dogs with inherited retinal dystrophies, and also for distinguishing between two retinal dystrophies.[174] What is not known at this time is the validity of repeated measurements using this or other devices before and after a given therapy.

1.5.1 Study hypotheses

These questions led to four separate studies that together comprise this Master's thesis. These studies are listed here with the specific hypothesis tested.

1) Repeatability of an objective canine vision testing apparatus.

Hypothesis;

a) Time to exit an objective vision testing device will improve significantly with repeated testing of untreated RPE65-deficient dogs of the same age.

b) Correct choice of exit tunnel using an objective vision testing device will improve significantly with repeated testing of untreated RPE65-deficient dogs of the same age.

2) Changes in phenotype of the RPE65-deficient dog with age.

Hypotheses;

Visual function

a) Young RPE65-deficient dogs will rapidly and correctly choose the open exit using an objective four choice vision testing device in bright light conditions.

b) Using an objective vision testing device both outcomes (time to exit and correct choice of exit) will decline significantly with age.

Electroretinography

c) Amplitude of rod and cone driven ERG responses will decline significantly with age.

d) Threshold of scotopic and photopic flash ERG responses will decline significantly with age.

Histopathology

e) There will be significant decline in thickness of all retinal layers with increasing age.

f) Lipid inclusions in the RPE will increase significantly in size and number with increasing age.

Fundus examination

g) Funduscopic evidence of retinal degeneration will be appreciated in RPE65-deficient dogs older then 5 years.

3) Gene therapy in older RPE65-deficient dogs (2-6 years of age) with subretinal injection of rAAV2/2.h*RPE65*.h*RPE65*

Hypotheses;

a) Significant improvement in amplitude of rod and cone driven ERG responses will be seen after treatment.

b) Significantly improved vision testing outcomes (time to exit and correct choice of exit) will be seen after treatment of these same dogs.

c) There will be a significant decline in ERG and vision testing outcomes with increasing age at time of treatment.

4) Gene therapy in the second eye of RPE65-deficient dogs after prior treatment of the first eye.

Hypotheses;

a) Amplitude of rod and cone driven ERG responses will not be significantly different between first and second treated eyes.

b) Vision testing outcomes (time to exit and correct choice of exit) will not be significantly different between first and second treated eyes.

c) Immune responses will not correlate with ERG or vision testing outcomes of first or second treated eyes.

CHAPTER 2 Materials and methods

2.1 Status of work underway at program commencement

An RPE65-deficient dog colony was established at Michigan State University using semen from an RPE65-deficient dog to inseminate a laboratory beagle. The F1 generation was bred to produce homozygous affected dogs. Until homozygous affected dogs were used for breeding the colony dogs were genotyped using a PCR assay amplifying the region and resolving the amplicons by electrophoresis on a high percentage agarose gel. By 2008 the colony was large and several studies had been completed including safety and efficacy of subretinal injection of different AAV2/2 vector titers in support of regulatory application for a human phase I/II gene therapy trial (NCT00643747, www.clinicaltrials.gov), and assessment of intravitreal retinoid therapy.[111, 164]

2.2 Work completed as part of Master's thesis

A large part of my work was directed towards collation and analysis of ERG results, vision testing data, fundus images and histopathology that resulted from the above efforts prior to and during my 4 years at Michigan State University. I played a smaller role performing electroretinograms, taking RetCam images and assessing vision using Gearhart *et al*'s vision testing device. I also performed globe harvesting and fixation, and subsequent evaluation of histologic sections. To help categorize a retinal lesion that had not previously been described I contributed to design and the subsequent implementation of a protocol for evaluating the *area*

centralis with RetCam imaging, semi-thin sections and electron microscopy. In terms of study design my main contribution was to design and implement of a method for evaluating the repeatability of vision testing results (Study 2 - Chapter 4).

Most of the methods used in this thesis are included in the methods section of each individual study. The four studies that make up this dissertation follow as separate chapters as each has or will be submitted as an individual publication. The subretinal injection technique is not described in detail in the studies, with references to early work being cited. Therefore a detailed description of this technique is included in this Materials and methods chapter.

2.3 Subretinal injection

Subretinal injections for rAAV mediated gene therapy were performed under inhalant anesthesia. Operated eyes and adnexa were prepared for surgery by application of a 1:50 dilution of povidone-iodine solution and placement of a sterile fenestrated drape and eyelid speculum. Two stay sutures of 4–0 silk (Ethicon, Inc.) were placed in the perilimbal conjunctiva at the 3 and 9 o'clock positions to manipulate the globe into primary gaze. The pupil was dilated with 1% tropicamide (Mydriacyl, Alcon Inc.) and 10% phenylephrine hydrochloride (AK-Dilate, Akorn Inc.), and under direct visualization through an operating microscope (Opmi6, Zeiss Inc.) subretinal injections were performed via a transvitreal approach using a commercial subretinal injector (RetinaJect, Surmodics Inc.). This involved introducing the subretinal injector

through the pars plana and advancing it across the vitreal cavity toward the retinal surface. The 39 gauge cannula of the injector was then extended to press on the retinal surface and as injection commenced the fluid pressure created a retinotomy. The injected fluid (250 to 500 µL) containing the rAAV vector entered the subretinal space to induce a retinal detachment. In later experiments a 3-port vitrectomy was performed prior to subretinal injection because this allowed for an easier subretinal injection with more control over bleb formation, and also more closely mimicked the technique used in the human clinical trials. This surgical technique involved incising the conjunctiva to the level of the sclera, 5mm posterior to the limbus in the inferotemporal quadrant. The sclera was then cauterized and a microvitreoretinal blade was then advanced through the pars plana. A suture of 7-0 Vicryl suture was placed across the sclerotomy before inserting a 2.5-mm, 23-gauge infusion cannula into the eye and tying it in place. Intraocular pressure was maintained at 20 mm Hg, Two additional sclerotomies were made in the superonasal and superotemporal quadrants, one for a 23-gauge endoilluminator and one for an Accurus Innovit virector (Alcon, Inc). Vitreous was removed with machine settings of 1200 to 1800 cuts per minute and 150 to 200 mm Hg suction. Subretinal injection then proceeded as described above.

CHAPTER 3

Reliability of an objective canine vision testing apparatus for repeated assessment of vision

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3.1 Abstract

Objective: To evaluate for any difference in vision testing outcomes with repeated evaluations of the same dogs.

Animals studied: Four 11-month old RPE65-deficient dogs.

Procedures: Vision was evaluated using a previously described four-choice vision testing device. Four evaluations were performed at 2-week intervals. Each evaluation comprised seven tests in the device at each of six different white light intensities (bright through dim) and each eye was evaluated separately. Two outcomes were recorded, correct choice of exit (selection of the one open exit from four tunnel choices) and time to exit. Both outcomes were analyzed for significance using ANOVA. We hypothesized that performance would improve with repeated testing (more correct choices and more rapid time to exit).

Results: Choice of exit did not vary significantly between each evaluation ($P=0.12$), in contrast time to exit lengthened significantly ($p = 0.012$), and showed greater variability in dim light conditions.

Conclusions: We found no evidence to support the hypothesis that either measure of outcome improved with repeated testing; time to exit increased with repeated testing. The data also support the importance of including a choice based assessment of vision in addition to measurement of device transit time.

3.2 Introduction

Canine models of human retinal dystrophies have become increasingly important for the testing of therapies to restore vision, as seen in some key recent studies.[110-113] Consequently, methods for accurate, reproducible and quantitative assessment of canine vision are required. Obstacle course tests have been commonly used to assess vision but can be subjective in nature. To reduce the subjectivity and produce results suitable for statistical analysis, the number of mistakes the dog makes can be counted, blinded observers can use a grading system to score performance, and the time taken to negotiate a standardized obstacle course recorded.[114, 133, 134] Here the availability of an objective assessment of canine vision is considered critically important with the increasing emphasis that is placed on the use of dog retinal dystrophy models in assessment of therapies.[110-113, 175] Such a vision testing device has been described by Gearhart *et al*, and has previously been shown to be accurate for discriminating affected from unaffected dogs with inherited retinal dystrophies, and also for distinguishing between two retinal dystrophies.[174]

The device first described by Gearhart *et al* is a four-choice vision testing device.[174] This device consists of a central starting box with 4 exit tunnels that can be closed at the far end. One tunnel is randomly selected to be open for each test and two measures of outcome are recorded, whether the open tunnel is chosen on the first attempt by the dog to exit the device on each occasion (correct choice of exit) and the time taken to exit the device (time to exit)). Testing is performed at different lighting-levels to allow assessment of both rod and cone mediated vision.[174] We have subsequently utilized this method to evaluate the outcome of

therapeutic trials in RPE65-deficient dogs.[111] Dogs affected with this mutation see well in bright light but lack dim light vision from an early age, this is due largely to a block in the visual cycle with these dogs showing normal retinal development and a very slowly progressive retinal degeneration.[114, 123-125, 127, 128]

The vision testing device developed by Gearhart *et al* relies on a natural behavior of dogs; a desire to exit an enclosed box.[174] With repeated testing this behavior might be affected by factors such as the dog's familiarity or comfort with the device. The possibility of detection of non-visual cues could also conceivably affect testing outcomes. We therefore sought to investigate whether we could detect any variation in vision testing outcomes with repeated trials of the same dogs. To investigate these questions we tested RPE65-deficient dogs from the same litter with repeated evaluations in the device to determine if vision testing improved over repeated evaluations.

3.3 Materials and methods

3.3.1 Animals

Four 11-month-old RPE65-deficient dogs (2 males and 2 females) from the same litter produced in a colony maintained at Michigan State University were used in this study. The dogs had no ophthalmic abnormalities apart from those attributable to their RPE65 disease status. Housing was under standard 12-hour light and dark cycles. The procedures performed were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and approved by Michigan State University's

Institutional Animal Care and Use Committee.

3.3.2 Vision testing device

Visual performance was assessed using the vision testing device previously described with slight modifications.[174] A solid topped, 1.2 meter square junction box, 90cm high, was constructed of wood with a wire-reinforced canvas tunnel extending from each of the four sides. These circular canvas tunnels had openings 69cm in diameter and were 80cm long. All dogs were placed in the junction box through a door located in one corner of the device. Snug-fitting foam and plastic end caps were used to cover three ends of the four tunnels prior to placement of the dog in the device. The device was located in a room free of windows, lit by 4 lights placed between the exit tunnels of the device with dimmer switches used to control light intensity (Figure 3.1).

Figure 3.1 Modification of Gearhart *et al's* vision testing device. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

3.3.3 Study design

Four evaluations were performed at 2-week intervals for all dogs and two outcomes assessed, ability to identify the open exit on the first attempt 'correct choice of exit', and device transit time 'time to exit'. This was performed at each of six different white light intensities (35-45, 15-17, 8-9, 1-1.5, 0.2-0.4, and 0.02-0.04 cd/m^2). The eyes of the four dogs were tested separately 7 times at each light intensity and correct exit choice (yes or no) and time to exit the device recorded to generate mean 'correct choice of exit' (n/7) and mean 'time to exit' (seconds). Results were obtained for each eye at the 6 light intensities for the four dogs for each of the four evaluation weeks (Table 3.1).

Table 3.1 Study design and sample data

Study design

Dog	Week	Eye	Light intensity cd/m^2
09-076	1	**OD**	**35 to 45**
09-077	2	OS	15 to 17
09-079	**3**		8 to 9
09-080	4		1 to 1.5
			0.2 to 0.4
			0.02 to 0.04

					Results	
Dog	Week	Eye	Light intensity cd/m^2	Test	Correct exit (y/n)	Exit time (seconds)
09-076	3	OD	35-45	1	yes	3
09-076	3	OD	35-45	2	yes	11
09-076	3	OD	35-45	3	yes	5
09-076	3	OD	35-45	4	yes	9
09-076	3	OD	35-45	5	yes	2
09-076	3	OD	35-45	6	yes	2
09-076	3	OD	35-45	7	no	3
				Mean	**6 of 7**	**5 seconds**

Note: results for the right eye of dog 09-076 on week 3 at the brightest light intensity

3.3.4 Study parameters

Light levels were set prior to each test using a photometer (IL 1700 Research Radiometer with SED033 silicon light detector; International Light, Inc, Peabody, MA) to ensure the lighting levels were equal at the entrances of all tunnels. Before testing at each light intensity a standard period of time was allowed for the dogs to acclimate to the altered lighting levels in the testing room. The time period allowed ranged from 1 minute for the brightest light intensity through to 5 minutes for the dimmest light intensity. Once a light intensity was set, each eye of all four dogs was tested 7 times in the device before moving onto the next light intensity. Evaluations were performed with one eye covered with an eye mask to allow vision in each eye to be assessed separately, thus simulating the technique used when assessing therapeutic outcomes. For each of the 7 tests in the device the open exit tunnel was randomly selected and changed between tests by removing the styrofoam covering before the dog entered the device. No auditory or other stimuli were given to the dogs to encourage them to exit the device and food rewards were not given, however the dogs were praised when they exited the device. The dogs were not acclimated to the investigators prior to the start of the study and were not familiarized with the room or the testing device prior to initiation of the study. The light intensity, open exit tunnel, order the dogs were run through the device, and first eye tested were selected randomly using a random-number generator (Excel; Microsoft Corp., Redmond, WA).

3.3.5 Data analysis

The response variables were time to exit (seconds) and correct choice of exit (n/7). The factors that could affect each response variable were week, light intensity, eye and dog (Table 3.1). Data were analyzed using a four factor ANOVA with the fixed factors of week, light intensity, eye and the random factor of dog. The errors were assessed for normality by examination of the histogram and normal probability plot. P values were calculated with significance set at $p < 0.05$. Post-hoc comparisons were by means of Bonferroni t test (m=15 for light intensity and m=6 for week) to identify the specific time-points that were significantly different. Data were analyzed using SAS Proc Mixed v 9.1.3.

3.4 Results

There was a significant effect of light intensity on time to exit and correct choice of exit. As lighting levels decreased, time to exit the device increased ($p < 0.0001$) and the number of correct choices of exit declined ($p < 0.0001$). There was no significant variation in either time to exit or correct choice of exit, between right and left eyes at any light intensity (p values ranged from 0.34 to 0.9, and 0.21 to 0.87 respectively).

3.4.1 Effect of trial

Assessment of correct choice of exit found no significant difference between the four trials (P=0.12) (Figure 3.2). In contrast there was a significant increase in time to exit the device with an increasing number of tests ($p = 0.01$); this difference became greater at the lower light intensities (interaction P=0.01) (Figure 3.2). On post-hoc analysis there was a significant increase in time to exit at the lowest light intensity (0.02-0.04 cd/m^2) between weeks 1-3 and

1-4, at the second dimmest light intensity (0.2-0.4 cd/m^2) between weeks 1-4, and at intensity 1.0-1.5 cd/m^2 between weeks 1-3, 1-4, 2-3, and 2-4 (all P<0.006).

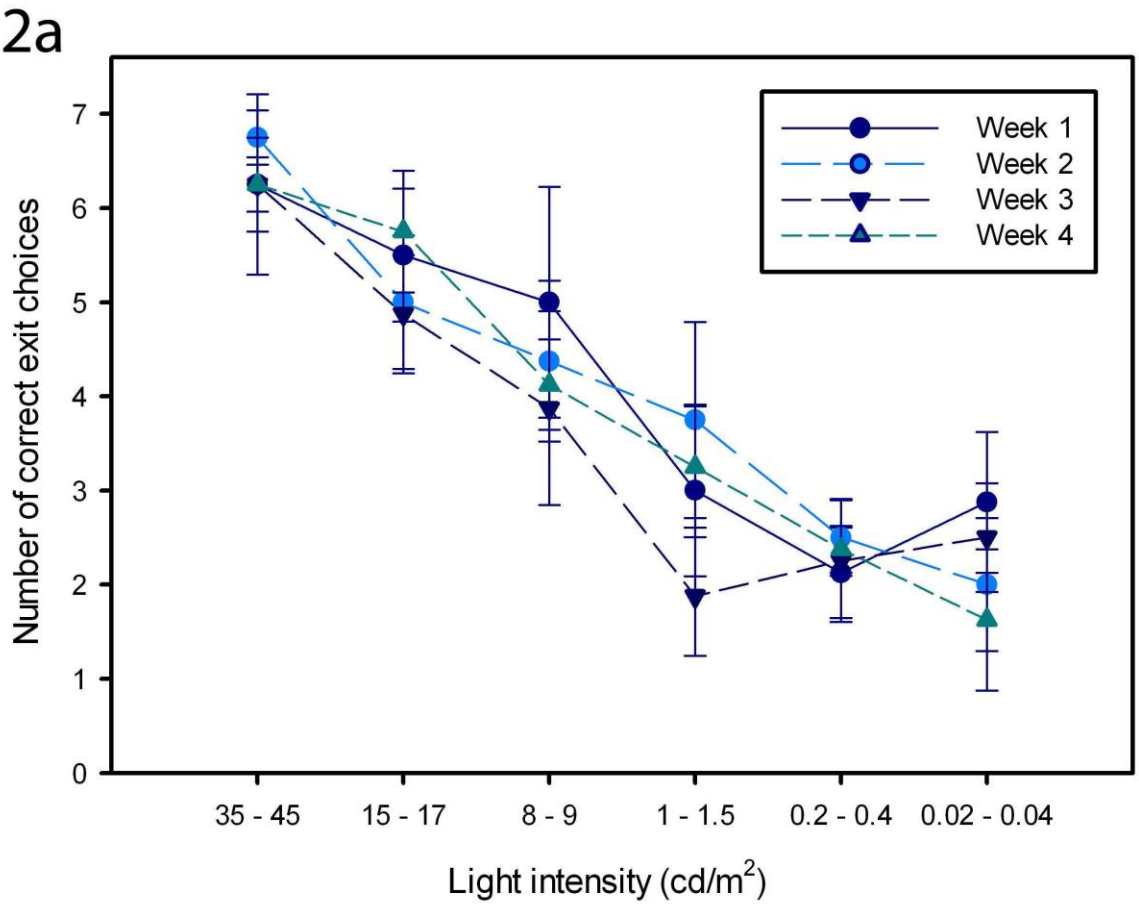

Figure 3.2 Test-retest variability. Mean correct choice of exit is displayed with standard deviation (2a); Consistent with their RPE65 disease status performance declines as light intensity decreases, but importantly there was no significant effect of study week on choice of

exit. Mean time to exit the device is displayed with standard deviation (2b); As light intensity declines the dogs take longer to exit the device. Here there was a significant effect of study week on exit time. Post-hoc analysis identified three significant data points identified here by the asterisks.

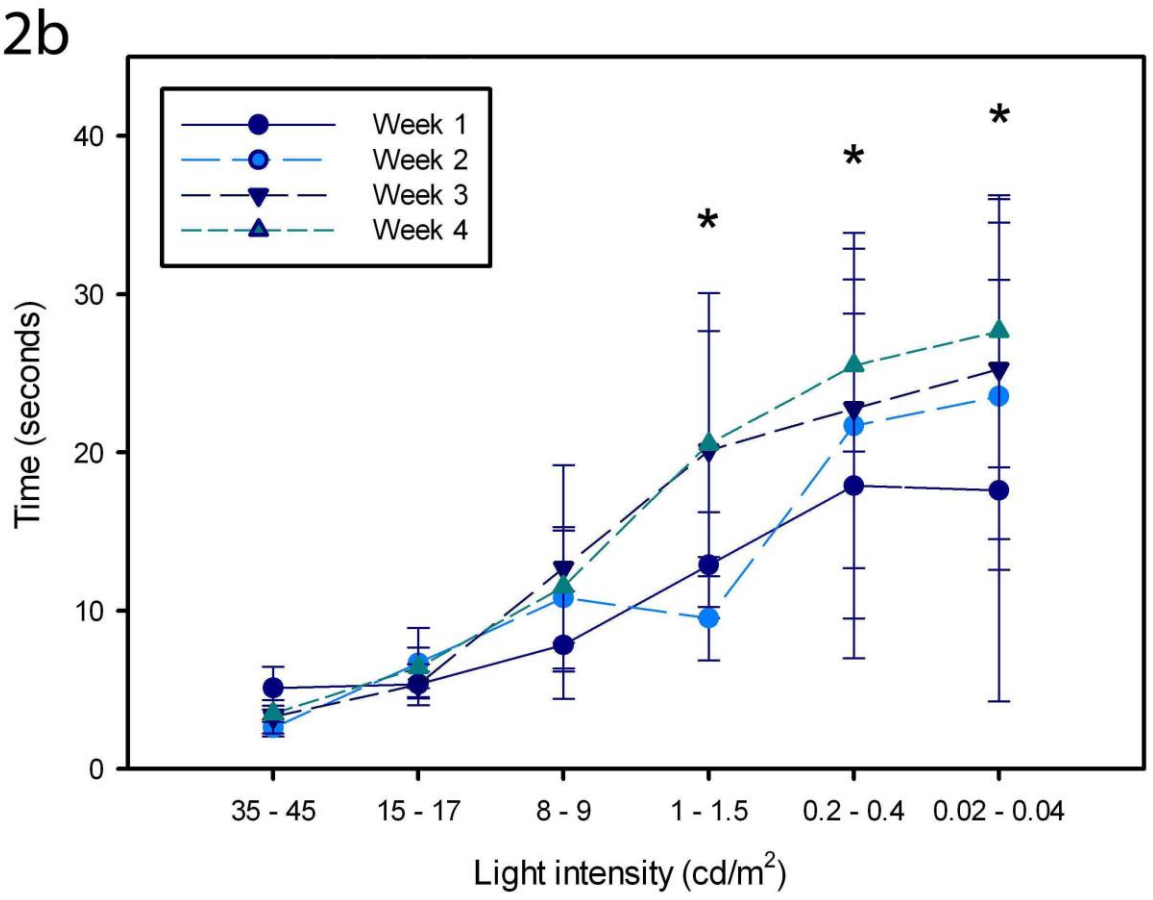

Figure 3.2 continued

3.5 Discussion

The vision testing device originally described by Gearhart *et al*, was shown to be accurate and sensitive for distinguishing affected from unaffected dogs in two different canine models of inherited retinal disease (rcd3 corgis and RPE65-deficient dogs), as well as for distinguishing the vision differences between the two dog models.[174] The results of the current study provide evidence that there is no improvement in either measure of outcome (correct choice of exit or time to exit) with repeated evaluations. This provides evidence to that valid comparison of outcomes can be performed for dogs evaluated on more than one occasion with confidence that any improvement is due to a given therapy.

For correct choice of exit we saw no significant effect of repeated evaluations of the same dogs. However time to exit the device did show significant variability with lengthened exit times observed at dim light intensities with repeated testing. We believe it is unlikely that this lengthening of exit time was due to deterioration of vision with disease progression, because RPE65-deficient dogs have a very slowly progressive disease that would not be expected to have progressed to a detectable degree over the time course of the study.[123, 124, 127, 128] It seems more likely that this finding was consistent with increased familiarity with the device, reducing the dogs urge to escape from the enclosed box.

From a practical standpoint, if the apparent lengthening of exit time from the device is due to familiarity with the device rather than progression of vision loss in the RPE65-deficient dogs used in the study, this would tend to mask subtle improvements in visual function that could

result from therapy. However if the vision testing device was being used to monitor vision changes over time the inter-test intervals would most likely be much greater then the 2-week intervals in this study. Hence familiarity with the device could be anticipated to play a much smaller role, and disease progression may have a bearing on vision testing outcomes. For this study very close intervals of 2 weeks between the 4 evaluation weeks were chosen to increase the chance of detecting some non-visual cue that might improve outcomes.

The findings of this study support the validity of vision testing results using the Gearhart device for monitoring response to therapy by repeated assessments of vision. Use of this objective vision testing device offers advantages over the more subjective techniques such as obstacle course based evaluations, and can be used to test vision under scotopic, mesopic or phototopic conditions. The availability of an objective assessment of canine vision such as this is considered critically important with the increasing emphasis that is placed on the use of dog retinal dystrophy models in assessment of therapies.[110-113, 175] The assessment of vision is important because, as shown by phase I and II clinical trials in RPE65-LCA patients, it is possible to rescue function in sufficient photoreceptors to improve vision, while there are not sufficient numbers rescued to result in a measurable change in the electroretinogram (which records a summed response originating from photoreceptors and resulting in inner retinal neuron responses).[163-165, 167, 169] The limitation of the device, as pointed out by Gearhart *et al*, is that it is not assessing visual acuity.[174] More sophisticated tests would be required if the goal were to assess visual acuity. Our findings in this study, while supporting the validity of using this device for repeated tests of vision should be considered in light of the fact that only 8 eyes of

four dogs were studied. We sought to reduce the effect of variability between dogs with RPE65-deficiency by including dogs from a single litter.

The vision testing device originally described by Gearhart *et al*, has previously been shown to be accurate for discriminating affected from unaffected dogs with inherited retinal dystrophies, and also for distinguishing between two retinal dystrophies.[174] Here we provide evidence that there is no improvement in outcomes with repeated testing using this device. This supports the suitability of using this device for assessing for improvements in vision in response to therapeutic intervention, with confidence that the change seen is due to the given therapy.

CHAPTER 4

Changes in phenotype of the RPE65-deficient dog with age

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4.1 Abstract

Background: Gene augmentation therapy in the *RPE65* -/- dog provided proof-of-concept data that led to clinical trials for the gene therapy treatment of human patients with Leber Congenital Amaurosis caused by *RPE65* gene mutations. Despite common use of the canine model, only limited information exists on phenotype progression with age. In the present work we investigated electroretinographic, histopathologic, vision testing outcomes and funduscopic appearance of the *RPE65* -/- dog over a wide age range.

Methodology/Principal Findings: Data was collected from 47 *RPE65* -/- dog ranging from 2 months to 8 years of age. Dark and light adapted electroretinography showed raised response thresholds and an inconsistently recordable, low amplitude cone flicker response. With increasing age there was a decline in both light and dark-adapted a- and b-wave amplitudes. On plastic embedded histopathologic sections there was progressive thinning of the outer nuclear layer and corresponding reduction in photoreceptor nuclei numbers with age. Vision was assessed with a vision testing device and showed that dogs of all ages had comparable results to *RPE65* +/+ dogs in bright light, while at lower light levels the dogs were functionally blind. Funduscopic evaluation of all dogs showed an ophthalmoscopically detectable region of tapetal hyper-reflectivity located at the region of highest rod and cone photoreceptor density. This correlated with focal outer nuclear layer thinning at this location.

Conclusions/Significance: The data presented here provides descriptive information on the natural course of disease in the *RPE65* -/- dog. Information that is relevant for current and future studies utilizing this canine model.

4.2 Introduction

Leber Congenital Amaurosis (LCA) type 2 is an early onset childhood retinal dystrophy that results in devastating loss of vision.[2] It is caused by heritable mutations in the *RPE65* gene that is expressed in the retinal pigment epithelium (RPE), where it encodes RPE65 protein. RPE65 is a retinoid isomerase essential for the RPE visual cycle, its deficiency results in disrupted synthesis of 11-*cis* retinal, the component of rhodopsin that is isomerized by light exposure to initiate phototransduction.[58] This block in the visual cycle is accompanied by a slow progressive retinal degeneration due in part to light independent signaling by free opsin in rods, which would normally be bound to 11-*cis* retinal.[95, 176] Three murine models of RPE65-deficiency have been reported. A RPE65 knockout mouse described by Redmond *et al* in 1998.[67] A naturally occurring retinopathy in *rd12* mice shown by Pang *et al* in 2005 to be due to a *Rpe65* gene mutation resulting in a premature stop codon, and most recently a model generated by modification the *Rpe65* gene generating a mutant protein, described by Samardzija *et al* in 2008.[47, 86] However it is the naturally occurring *RPE65 -/-* dog, identified by Narfström *et al* in the 1980's that has received the most attention with respect to gene augmentation trials.[123] Perhaps surprisingly for such a widely used model there is little published information about progression of the *RPE65* -/- dog phenotype with age (Table 4.1). Here, the cost to maintain colonies and the time taken to allow the animals to age to determine the long-term effects of disease progression has been a limitation.

Table 4.1 Published data on untreated RPE65-deficient dogs and (eyes).[110, 114, 123, 125, 127, 128, 131, 133, 135-137, 155-157]

Age	Histology	ERG	Vision Assessment	Optical Coherence Tomography	Electron Microscopy	Fundus appearance
1 - 3 mths	1(1)					
3 - 12 mths	12(12)	43(73)	44(64)		5(5)	17(32)
1 - 2 yrs		2(2)	10(10)	9(9)		
2 - 3 yrs	1(1)	1(2)	1(2)			1(2)
3 - 4 yrs		1(1)				
4 - 5 yrs						
5 - 6 yrs						
6 - 7 yrs	1(1)				1(1)	
7 - 8 yrs						
TOTAL	15(15)	47(78)	55(76)	9(9)	6(6)	18(34)

The canine model, was first described in a colony Swedish Briard dogs, displaying visual deficits at lower light levels.[123] The causal gene mutation in *RPE65* was identified by Veske and colleagues in the late 1990's, a four base-pair deletion with a subsequent frame-shift and premature stop codon.[124] Later, immunohistochemical analysis of retinal sections showed a lack of RPE65 in the affected retinal pigment epithelium.[110, 114, 126] It was trials of gene augmentation therapy in the *RPE65* -/- dog first reported by Acland *et al*, that provided critical proof-of-concept studies culminating in three separate clinical trials of gene therapy treatment in patients with *RPE65* gene mutations.[114, 163-165] Here, the canine model allowed the use of standard surgical techniques, with a comparable globe size to the human. Additionally the canine eye has regions of higher density packing of photoreceptors, the *area centralis* and visual streak.[116] As the trials progress the canine model continues to be used to assess

refinements of gene augmentation therapy, and also to evaluate alternative therapeutic strategies.[111]

Published information on the residual vision of young *RPE65* -/- dogs varies, with some authors reporting reduced dim-light vision, and others reporting complete blindness.[125, 131, 132] More recently, detailed objective assessment showed that brighter light vision was minimally impaired in young affected dogs, with accurate and rapidly exit a four-choice vision testing device at full room lighting.[174] Changes in visual function with age have not been reported. There is a similar paucity of information on retinal function in older *RPE65* -/- dogs. Descriptions of electroretinographic (ERG) assessment of retinal function in younger dogs are more consistent, reporting a marked reduction in photoreceptor sensitivity with an elevated dark-adapted threshold of response and reduced response amplitudes, along with absent light-adapted cone flicker responses.[110, 123, 125, 131, 132] Interestingly, the light-adapted response of some dogs may have larger amplitude light-adapted a- and b-waves than those of normal dogs, perhaps because the background light normally used to suppress rod photoreceptor function is not sufficient to fully suppress the markedly desensitized rods in the *RPE65* -/- dog.[110] Again information on changes in the electroretinogram response of these dogs with age is absent from the literature.

The described funduscopic appearance of young affected *RPE65 -/-* dogs have noted no significant changes.[123, 125] One study reports the appearance of the fundus in two older dogs, a 5.5 year old dog showing multifocal yellow-white spots in the tapetal fundus and a 6

year old dog with ophthalmoscopic changes indicative of retinal thinning and superficial retinal vasculature thinning.[177] Despite the relatively normal appearance of the fundus of young *RPE65* -/- dogs, detailed histopathologic studies in younger dogs describe disorientation of the rod outer segment disc membranes from 5 weeks of age, shortening of rod inner segments from 4 months of age, and peripheral rod photoreceptor degeneration from 7 months of age.[110, 125, 127, 128] These studies also describe large lipid-like inclusions in the RPE, shown to contain retinoids that accumulate due to the block in the visual cycle.[104] There are limited descriptions in older animals. Wrigstad *et al* reported on an eye from a 7-year-old dog that showed a reduction of photoreceptors centrally and almost complete photoreceptor degeneration peripherally with severe changes of the inner retina in the peripheral fundus.[128]

In the current study we present a detailed description of the phenotype of 52 untreated *RPE65* -/- dogs 1 month to 8 years of age. This provides an extensive description of the natural course of disease in the *RPE65* -/- dog, information that is relevant for current and future studies evaluating the outcome of therapeutics for LCA type II utilizing this canine model. Here we show that the low amplitude dark-adapted ERG responses decline with age, while the extremely low amplitude 33Hz cone flicker responses remain inconsistently recordable despite the amplitude of the light-adapted a- and b-waves of young *RPE65* -/- dogs being similar to that of normal dogs. These ERG changes parallel the block in the visual cycle accompanied by a slow progressive thinning of all retinal layers with the exception of the retinal pigment epithelium (RPE), which increases in thickness with the early appearance of lipid inclusions, and the nerve

fiber layer is stable in thickness with age. While the *RPE65* -/- dogs in this study showed diminished vision in dim light, they maintained adequate retinal function to negotiate a vision testing device in bright light at comparable levels to normal dogs, out to 8 years of age. We also report a previously undescribed finding of focal thinning of the outer nuclear layer (ONL) at the region of highest rod and cone photoreceptor density in the dog.

4.3 Materials and methods

4.3.1 Animals and ethics statement

Fifty two *RPE65* -/- dogs from a colony maintained at Michigan State University were used in this study (Table 4.2). Six *RPE65* +/+ dogs from the same colony were used as controls. All dogs were cared for in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures involving animals adhered to the guidelines of this same committee, and were approved by the Institutional Animal Care and Use Committee of Michigan State University.

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Table 4.2 Studied untreated RPE65-deficient dogs and (eyes)

Age	Histology	ERG	Vision Assessment	Optical Coherence Tomography	Electron Microscopy	Fundus appearance
1 - 3 mths		4(8)		4(8)		4(8)
3 - 12 mths	5(7)	17(34)	12(24)	2(4)		17(34)
1 - 2 yrs	3(5)	6(12)	5(10)		4 (4)	6(12)
2 - 3 yrs	4(5)	6(10)	4(8)	2(4)		6(10)
3 - 4 yrs		2(4)	2(4)	1(2)		2(4)
4 - 5 yrs		1(2)	1(2)			1(2)
5 - 6 yrs		2(4)	2(4)	1(2)		2(4)
6 - 7 yrs						
7 - 8 yrs		2(4)	2(4)	1(2)		2(4)
TOTAL	12(17)	40(78)	28(56)	11(22)	4(4)	40(80)

4.3.2 Vision testing

Vision testing was performed on 56 eyes of 28 *RPE65* -/- dogs aged 4 months to 8 years. A four choice vision testing device was used as previously described [174]. Evaluation was performed for each eye individually by placement of an eye mask over the contralateral eye. No auditory or other stimuli were given to the dogs to encourage them to exit the device during a trial and food rewards were not given, the dogs were praised when they exited the device. A photometer (International Light, Peabody, MA) was used to ensure the lighting levels were equal at the entrances of all tunnels prior to testing at each light intensity. Two measures were recorded, average time to exit the device and the number of first correct choices of exit tunnel. Each eye was tested by 7 repeated trials at 3 different light intensities (0.02 – 0.04, 1.0 – 1.5 and 35 – 45 cd/m^2), and results for each eye at each light intensity averaged.

4.3.3 Electroretinography

Electroretinography (ERG) recordings were performed on 78 eyes of 40 *RPE65* -/- dogs aged 2 months to 8 years as previously described.[178] The dogs were anesthetized under inhalant isoflurane and the globes were positioned in primary gaze using stay sutures of 4–0 silk (Ethicon, Piscataway, NJ). The pupils were dilated with 1% tropicamide (Alcon Laboratories, Honolulu, HI) and 10% phenylephrine hydrochloride (Akorn, Buffalo Grove, IL). Full-field flash ERGs were recorded using ERGJet lenses (Microponent, Le Cret-du-Locie, Switzerland) and the UTAS-E 3000 electrophysiology unit with a Ganzfeld (LKC Technologies, Gaithersburg, MD). Band pass filter cutoff was set at 0.5–500Hz. Dark-adapted ERG responses were recorded following 1 h of dark adaptation, from a series of 16 white flash stimuli (-3.6, -3.18, -2.79, -2.41, -2.0, -1.6, -1.19, -0.79, -0.39, 0.00, 0.39, 0.85, 1.36, 1.9, 2.38 and 2.82 log cdS/m^2). Interstimulus intervals increased from 1s at low intensities to 360s at the highest intensity to avoid light adapting the rods. Following exposure to a background light of 30 cd/m^2 for 10min, cone-mediated flicker responses were recorded at 33Hz. Dark- and light-adapted ERGs were assessed for threshold and amplitude of response and intensity:response curves were plotted.

4.3.4 Histopathology

Retinal morphology was evaluated on plastic embedded retinal sections. Seventeen eyes were collected from 12 *RPE65* -/- dogs aged 3-36 months (Table 1.1), and 11 eyes of 6 *RPE65* +/+ dogs aged 3-36 months. The dogs were euthanized with intravenous injection of pentobarbital sodium and eyes were collected immediately after euthanasia. Incisions were made through

pars plana into the vitreous and globes were fixed in 3% glutaraldehyde, 2% paraformaldehyde, and 0.1 M sodium-cacodylate buffer (pH 7.2). After 2 hours at 4°C the anterior segment was removed, and the eyecup returned to the same fixative for 20 hours at 4°C. The eye cups were dehydrated in a graded series of ethanol solutions and infiltrated with semisoluble polymer medium (Electron Microscopy Sciences, Fort Washington, PA). After polymerization sections of the whole eyecup were taken in a superior-inferior orientation, and 3µm sections were cut through the optic nerve head and stained with H&E for light microscopic analysis.

Retinal morphology was assessed over the entire length of the retinal sections as previously described.[178] The rows of rod and cone nuclei in the outer nuclear layer were counted at 2 locations in the superior retina and 2 locations in the inferior retina. These sites were 1/3 and 2/3rds the distance from the optic nerve to the *ora serrata*. At each location cell counts were performed on 2 adjacent 200µm sections and the numbers averaged. Additionally, the thickness of each cell layer was measured at these same sites. For evaluation of the region of tapetal hyper-reflectivity observed at the area centralis of the *RPE65* -/- dogs. Four eyes of 4 *RPE65* -/- dogs euthanased at 11 months of age were processed as detailed above. Ten serial sections of the whole eyecup were taken in a superior-inferior orientation, these sections were 3µm and 300µm apart, with the first section 1 optic nerve head diameter from the temporal margin of the ONH. Sections were stained with H&E for light microscopic analysis.

4.3.5 Digital fundus images and optical coherence tomography

Complete ophthalmic examination was performed on 80 eyes, this included indirect ophthalmoscopy (Welch Allyn, Skaneateles Falls, NY, USA) and fundus photography (RetCam II,

Clarity Medical Systems, Pleasanton, CA, USA). Additionally, 20 eyes of 10 *RPE65* -/- dogs of representative ages (1 month through 8 years of age) had retinal morphology assessed in the region of the area centralis by optical coherence tomography (OCT) (Spectralis, Heidelberg, Germany). 10 eyes of 5 *RPE65* +/+ dogs were used as controls. The dogs were anesthetized under inhalant isoflurane, the globes were positioned in primary gaze using stay sutures with pupils were dilated as described above for electroretinography. The location of the area centralis was determined based on the findings of Mowat *et al*.[116]

4.3.6 Transmission electron microscopy

Four eyes of 4 *RPE65* -/- dogs were processed for scanning electron microscopy; after euthanasia the eyes were fixed as described above for histopathology, using 2.5% glutaraldehyde and 2.5% paraformaldehyde. After fixation a 0.5cm by 0.5cm square of retina that contained the *area centralis* was collected. The square of tissue was post-fixed in 2% osmium tetroxide, dehydrated in acetone series and infiltrated and embedded in spur resin. Serial sections were taken at 2µm intervals and stained with Toluidine blue until the region of retinal thinning was identified. Thin (70nm) sections were examined with transmission electron microscopy (TEM) JEOL 100CX (Japan Electron Optic Laboratory) and photographs we taken with MegaView III.

4.3.7 Statistical analysis

Vision testing outcomes were analyzed using independent samples t-tests to evaluate for differences between RPE65 -/- dogs and RPE65 +/+ dogs. To assess for correlation between age

and ERG, vision testing and histopathology outcomes, two-tailed Pearson correlation analysis was performed. Analysis was performed on histopathology data at 4 standardized sample site, maximum recorded a- and b-wave amplitudes and 33Hz cone flickers for ERG responses, and mean number of correct exit choices and mean time to exit the device for vision testing outcomes. The ERG, vision testing and histopathology results were further compared using a one-way ANOVA between different age groups. Post-hoc comparisons were by ANOVA with least significant difference (LSD) to identify the specific time-points that were significantly different. Data were analyzed using SAS Proc Mixed v 9.1.2 and data were considered significant at $p < 0.05$.

4.4 Results

4.4.1 Poor vision in dim light but maintenance of bright light vision

Twenty nine *RPE65* -/- dogs aged between 4 months and 8 years and 6 *RPE65* +/+ dogs underwent vision testing using a 4 choice of exit vision testing device. In bright light (room light levels, 35-45 cd/m^2) the *RPE65* -/- dogs performance for both 'time to exit the device' and 'correct choice of exit tunnel' was almost identical to and not statistically different from that of the *RPE65* +/+ dogs (p= 0.14 and 0.68 respectively) (Figure 4.1). As anticipated, at the two lower lighting levels (0.02 – 0.04 and 1.0 – 1.5 cd/m^2) the performance of the *RPE65* -/- dogs declined, approximating random guessing of the open exit tunnel at the dimmest light intensity. Vision testing results remained stable with age, with no significant variation in either measure of

outcome as age increased (p value range = 0.10 to 0.75), with dogs aged 3-12 months (n=12) and those aged 60-96 months (n=7) performing almost identically.

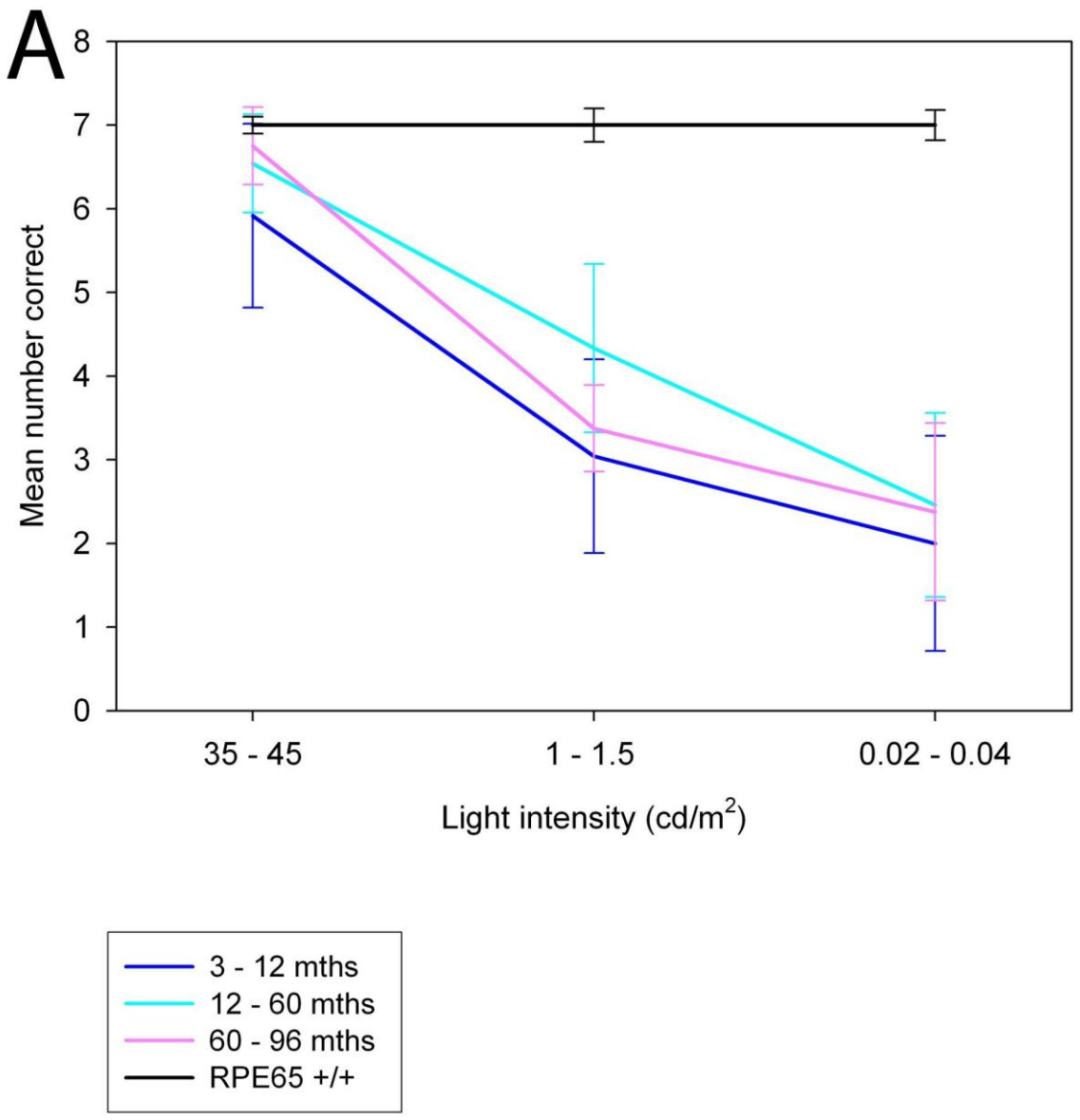


Figure 4.1 **Stable visual responses and maintenance of bright light vision.** Vision testing results are displayed; dogs are grouped as follows; *RPE65* +/+ dogs 6-14 months, n=10 eyes; *RPE65* -/- dogs 3-12 months, n=24 eyes; 12-60 months, n=24 eyes, and 60-96 months, n=8 eyes. Shown is the mean number of correct choices of exit tunnel (A), and mean time to exit the vision testing apparatus (B).

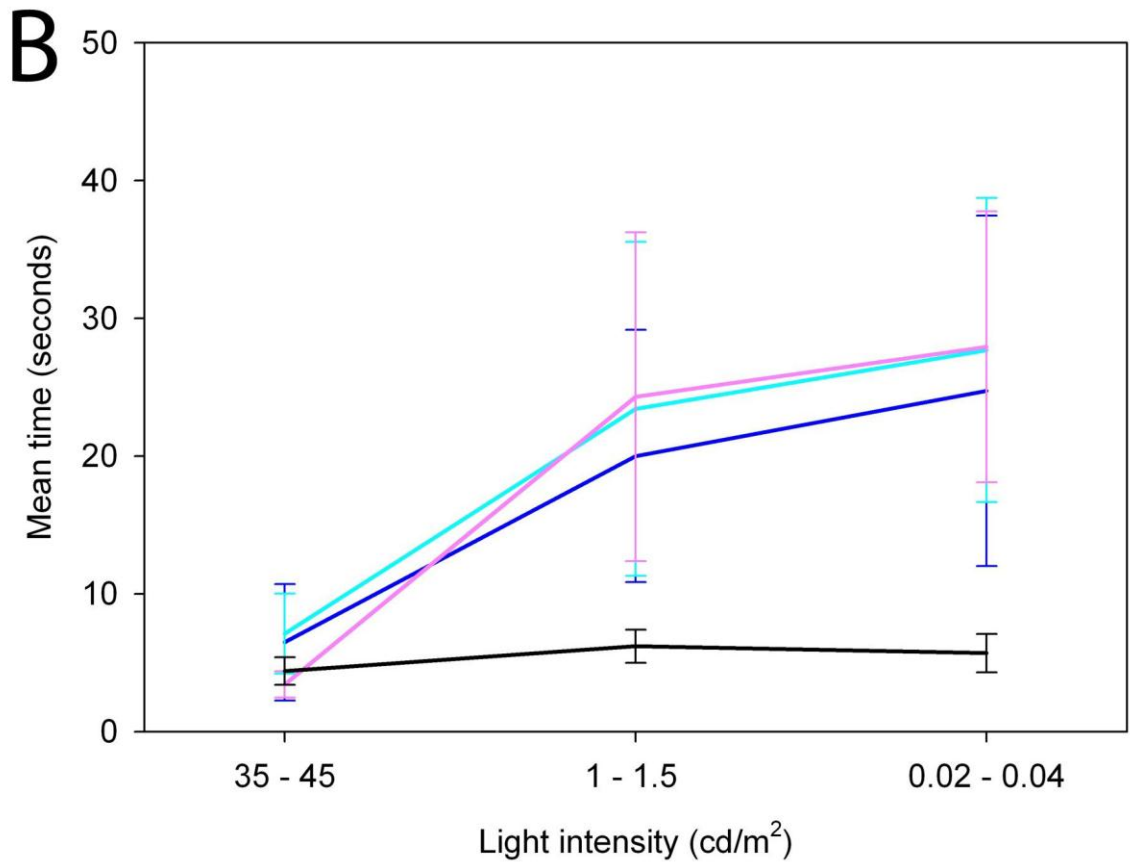


Figure 4.1 continued

4.4.2 Low amplitude dark-adapted ERG responses decline with age

Dark-adapted ERG responses were assessed for 41 *RPE65* -/- dogs, 2 months to 8 years of age. For dogs of all ages the amplitude of response of the a- and b-waves was dramatically and significantly lower than that recorded from 6 *RPE65* +/+ dogs 9-12 months of age for all flash intensities ($p= 0.001$ and <0.001 respectively). When evaluated for an effect of age on the low amplitude responses recorded from the *RPE65* -/- dogs, there was a significant decline in dark-adapted a- and b-wave amplitudes recorded at the brightest 3 light intensities (1.9, 2.38 and 2.82 log cdS/m^2) with increasing age (p value range = 0.004 to <0.000) (Figure 4.2). While the amplitude of response declined with age, there was no detectable change in the threshold of response for either a- or b-wave with age. The response threshold of the *RPE65* -/- dogs remained stable at approximately 1 and 3 log units for a- and b-waves respectively brighter than that of the *RPE65* +/+ dogs.

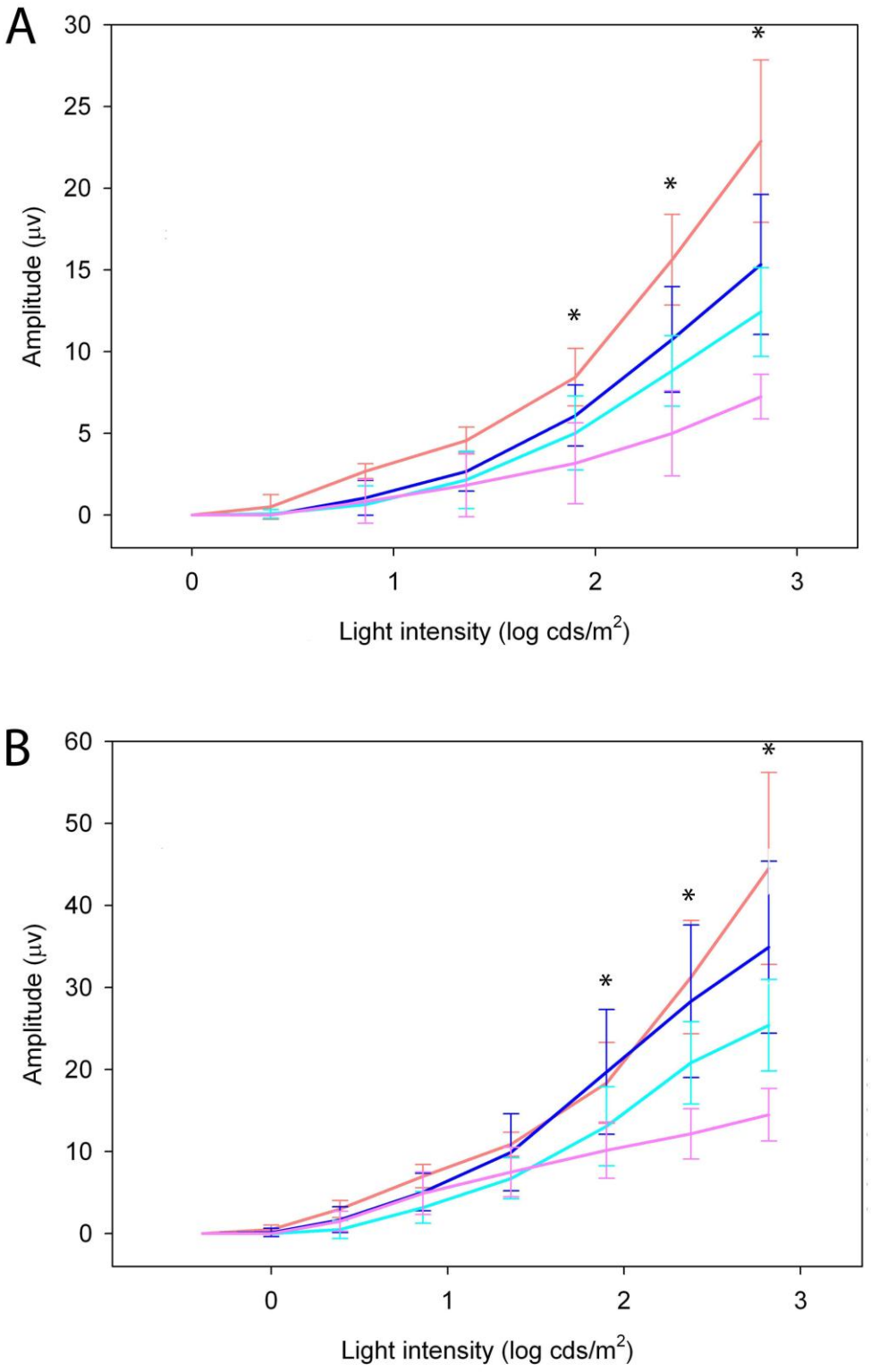


Figure 4.2 Low amplitude dark-adapted flash ERG responses decline with age.

Intensity:response series for *RPE65* -/- dogs of 4 age groups; <3 months, n=8 eyes; 3-12 months,

n=34 eyes; 12-60 months, n=28 eyes; 60-96 months, n=8 eyes (A and B). Intensity:response series of the <3 month age group and *RPE65* +/+ dogs; 9-12mths, n=10 eyes (C and D). Displayed is the mean dark-adapted a-wave (A and C), dark-adapted b-wave (B and D). Asterisks indicated significant data points. Error bars display standard deviation.

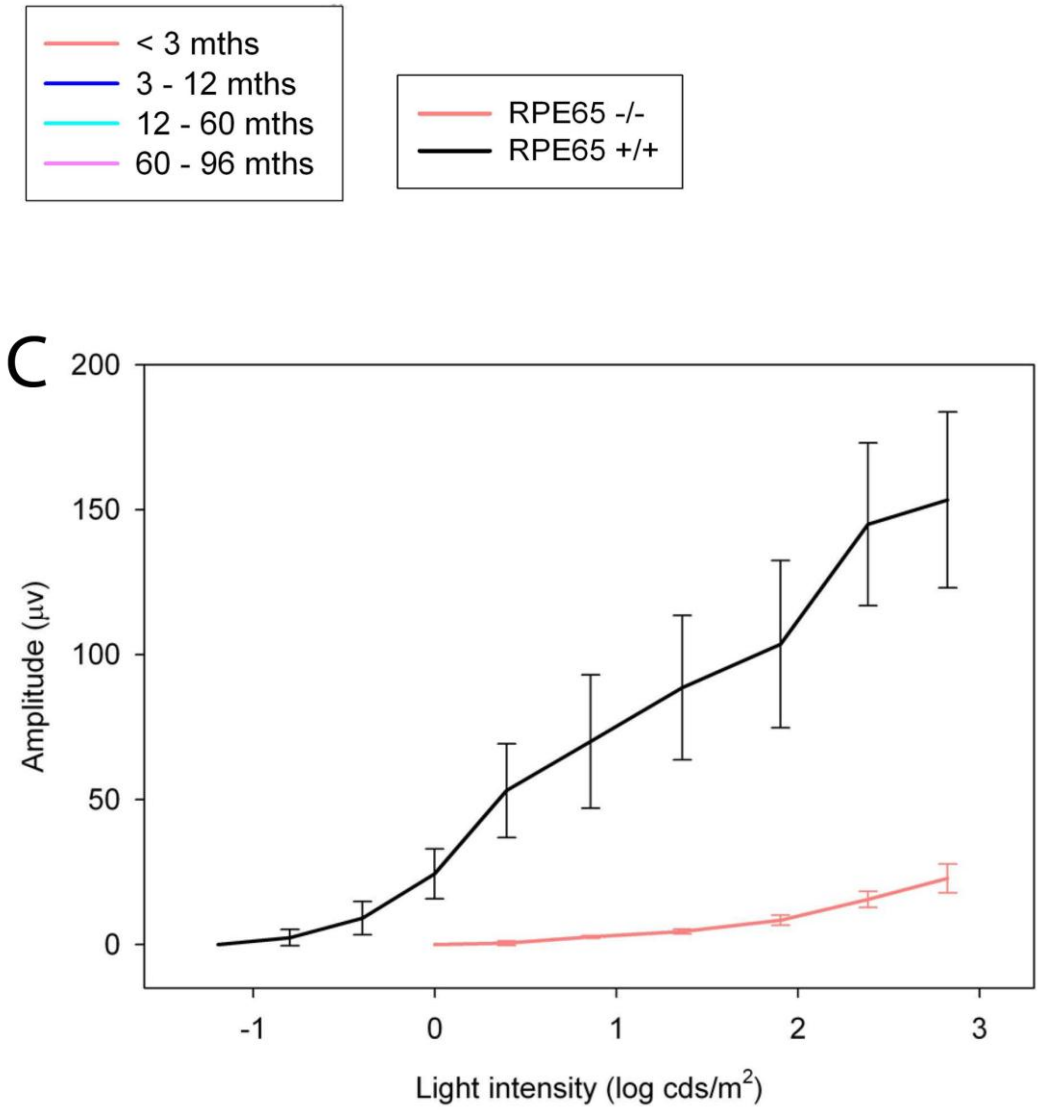


Figure 4.2 continued

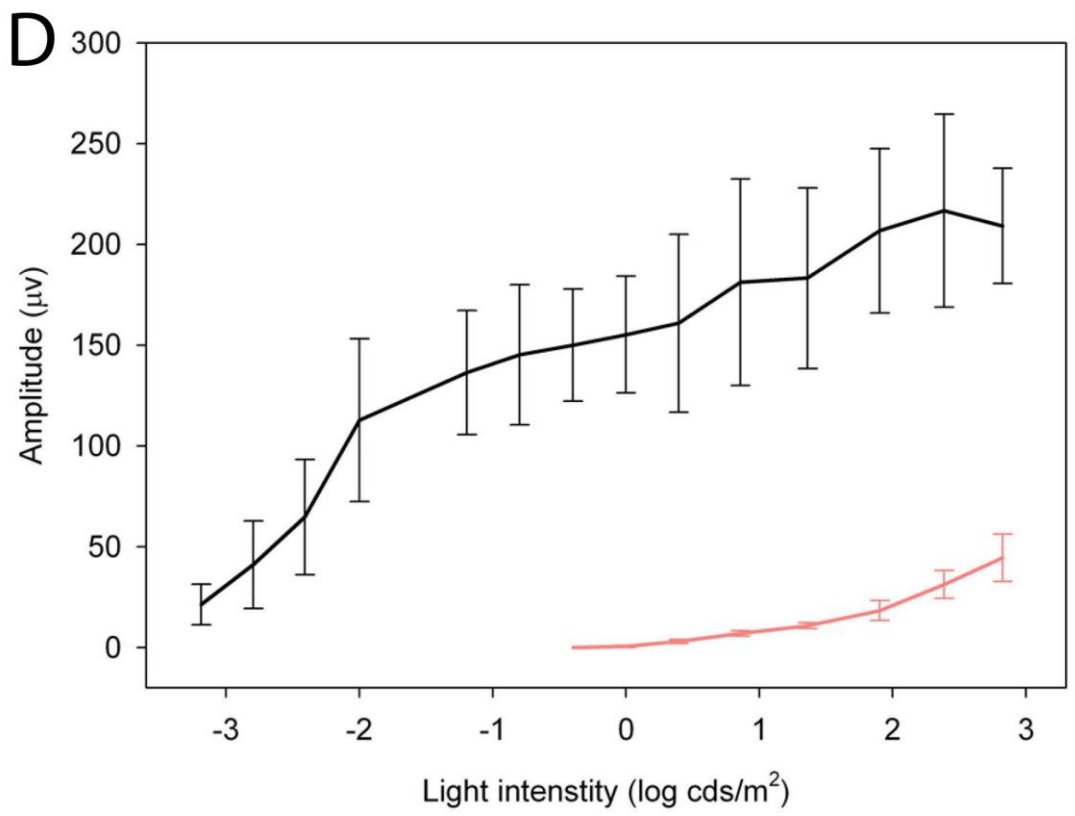


Figure 4.2 continued

4.4.3 Light-adapted flash ERG responses comparable to normal dogs

The amplitude of response of the light adapted a- and b-waves at the brightest three light intensities (1.36, 1.9 and 2.38 log cdS/m^2) in the young *RPE65* -/- dogs (<3 mths and 3-12 mths) were not significantly different to those recorded from the 6 *RPE65* +/+ dogs (p value range 0.94 to 0.12) at these same light intensities. While the bright light amplitude of response was comparable to that of the *RPE65* +/+ dogs, the threshold of response of the light adapted a- and b-waves were 0.8 and 1.6 log units respectively brighter in the *RPE65* -/- dogs. For both light adapted a- and b-waves the amplitude of response of the *RPE65* -/- dogs declined significantly with age at the brightest 3 light intensities (p value range = 0.002 to <0.000), while the threshold of response was stable with age (Figure 4.3).

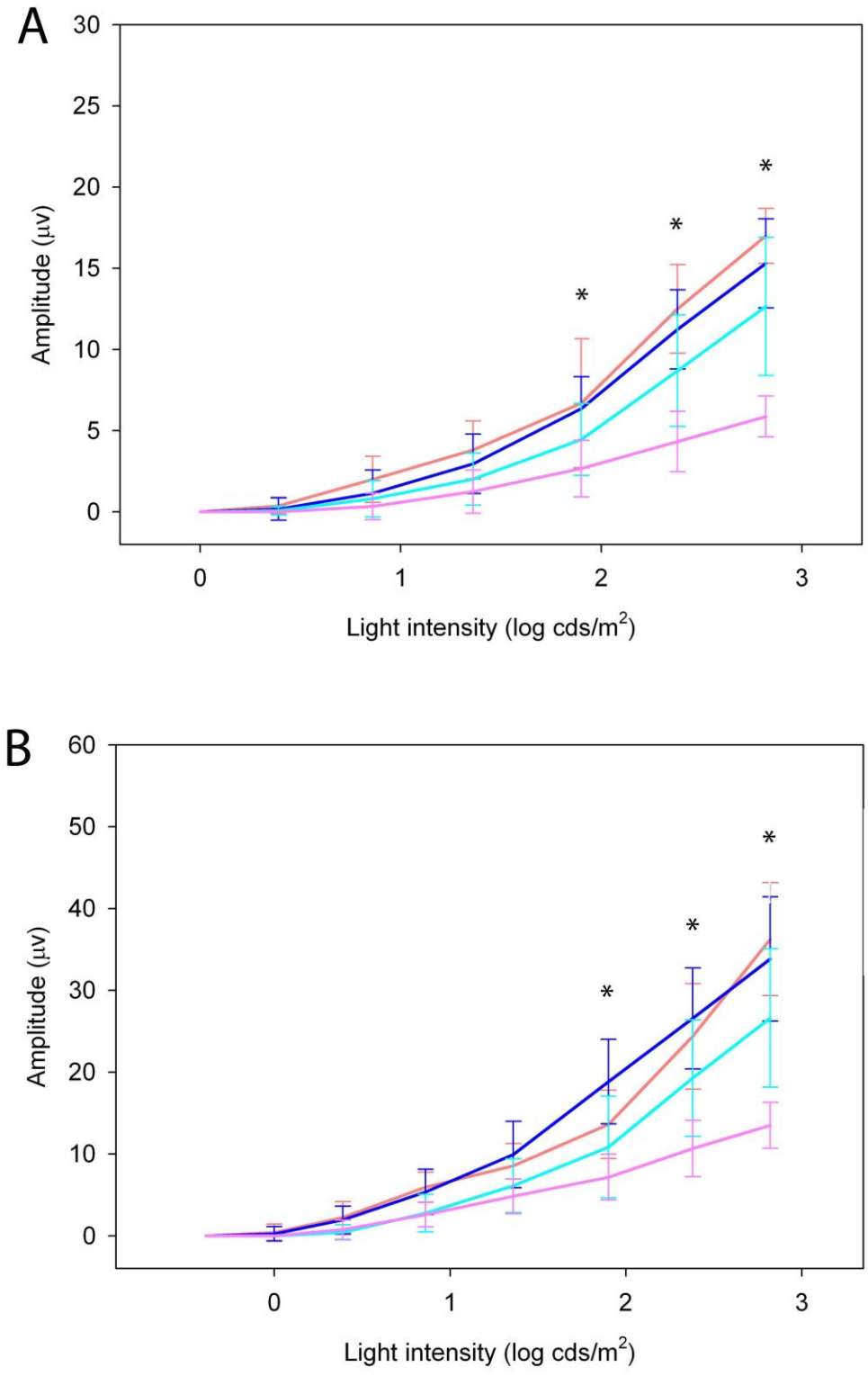


Figure 4.3 Light-adapted flash ERG amplitude comparable to normal dogs.

Intensity:response series for *RPE65* -/- dogs of 4 age groups; <3 months, n=8 eyes; 3-12 months,

n=34 eyes; 12-60 months, n=28 eyes; 60-96 months, n=8 eyes (A and B). Intensity:response series of the <3 month age group and *RPE65* +/+ dogs; 9-12mths, n=10 eyes (C and D). Shown is the mean light-adapted a-wave (A and C), light-adapted b-wave (B and D). Asterisks indicated significant data points. Error bars show standard deviation.

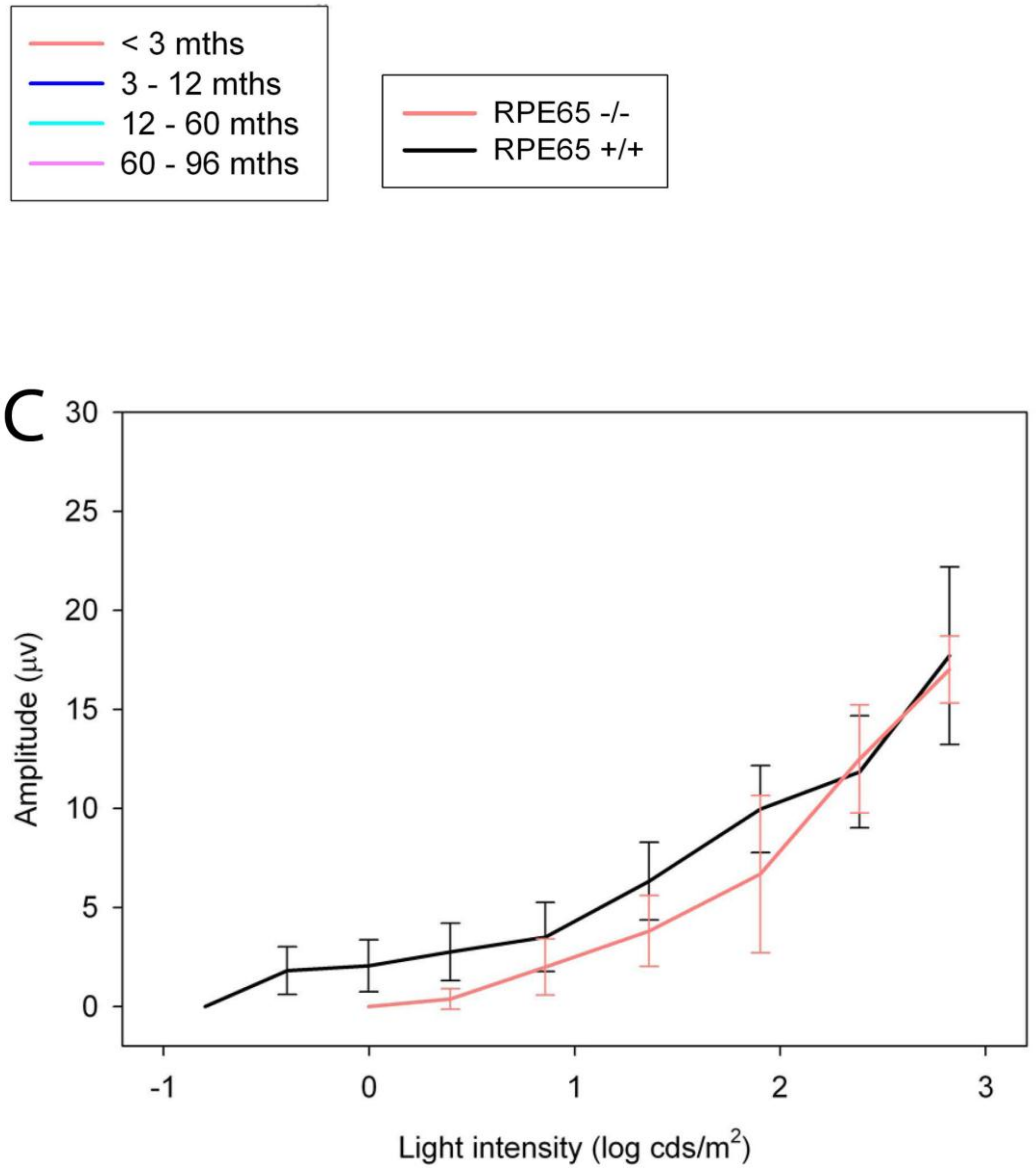


Figure 4.3 continued

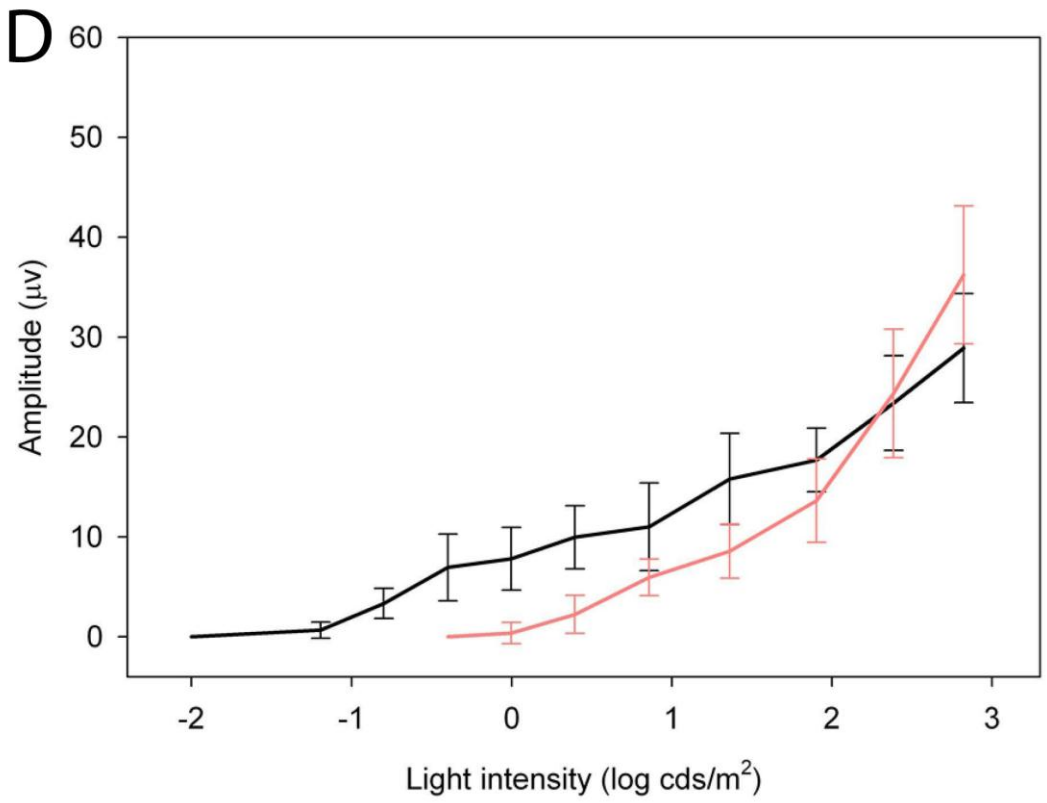


Figure 4.3 continued

4.4.4 Inconsistent very low amplitude cone flicker responses

Light adapted 33Hz cone flicker ERGs recorded immediately after light adapted flash responses showed inconsistently recordable low amplitude responses. In contrast to the *RPE65* +/+ dogs, the 33Hz light adapted cone flicker responses of the *RPE65* -/- dogs were generally of such low amplitude as to be indistinguishable from background interference. However a low amplitude, 2-4μv, recording was appreciated in 5 eyes of *RPE65* -/- dogs across all ages (Figure 4.4).

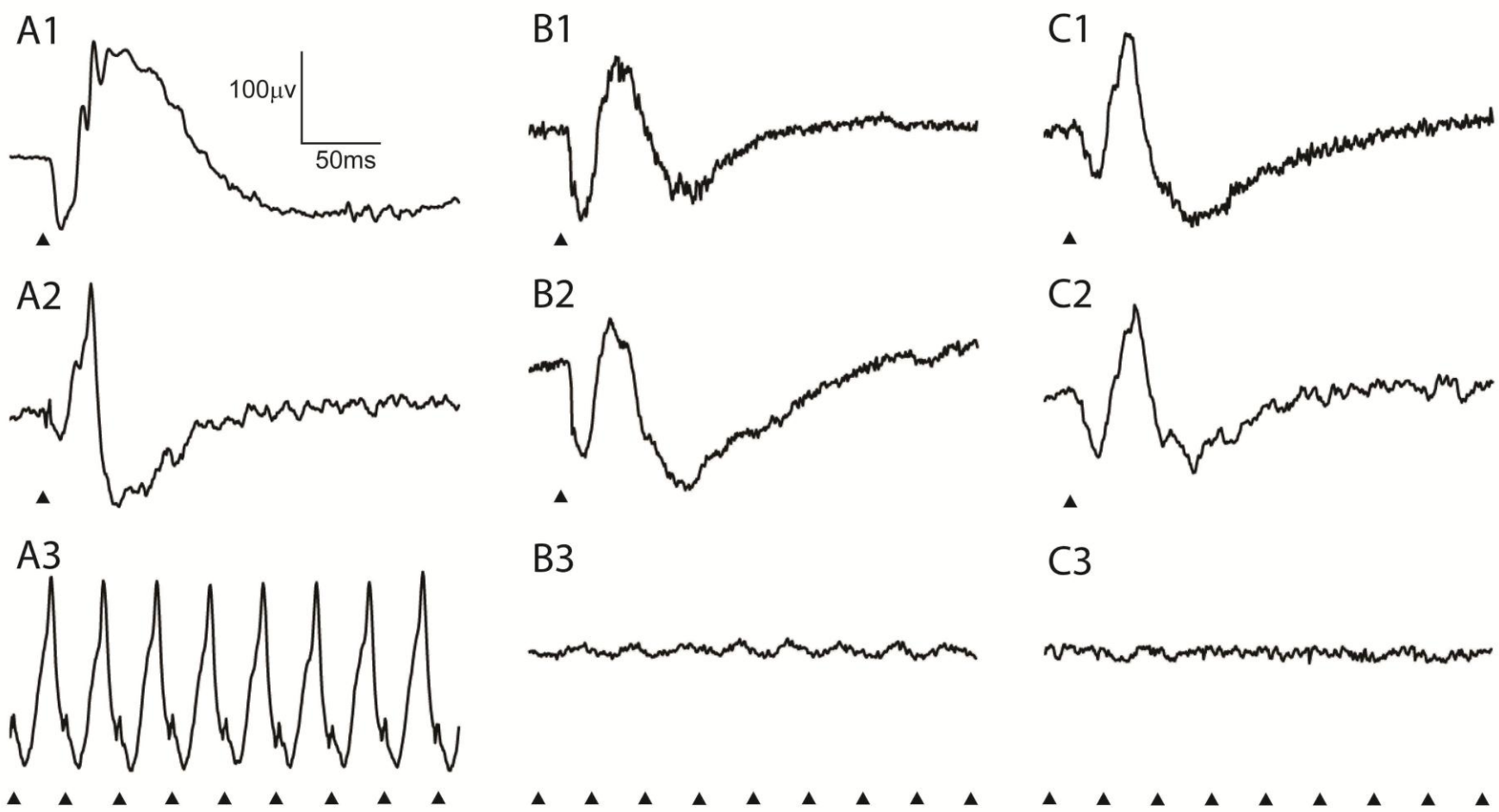


Figure 4.4 Representative ERG responses show inconsistent 33Hz cone flicker. Representative electroretinographic (ERG) responses from an *RPE65* +/+ dog (A), and *RPE65* -/- dogs from 4 age groups; 0-3 months (B); 3-12 months (C); 12-60 months (D);

and 60-96 months (E). Displayed are dark-adapted ERGs recoded at 2.82 log cdS/m^2 (A1-E1), light-adapted ERGs at 2.38 log cdS/m^2 (A2-E2), and light-adapted 33Hz flicker response (A3-E3). Scale is 20µv by 50ms for all images except A1 (100µv by 50ms).

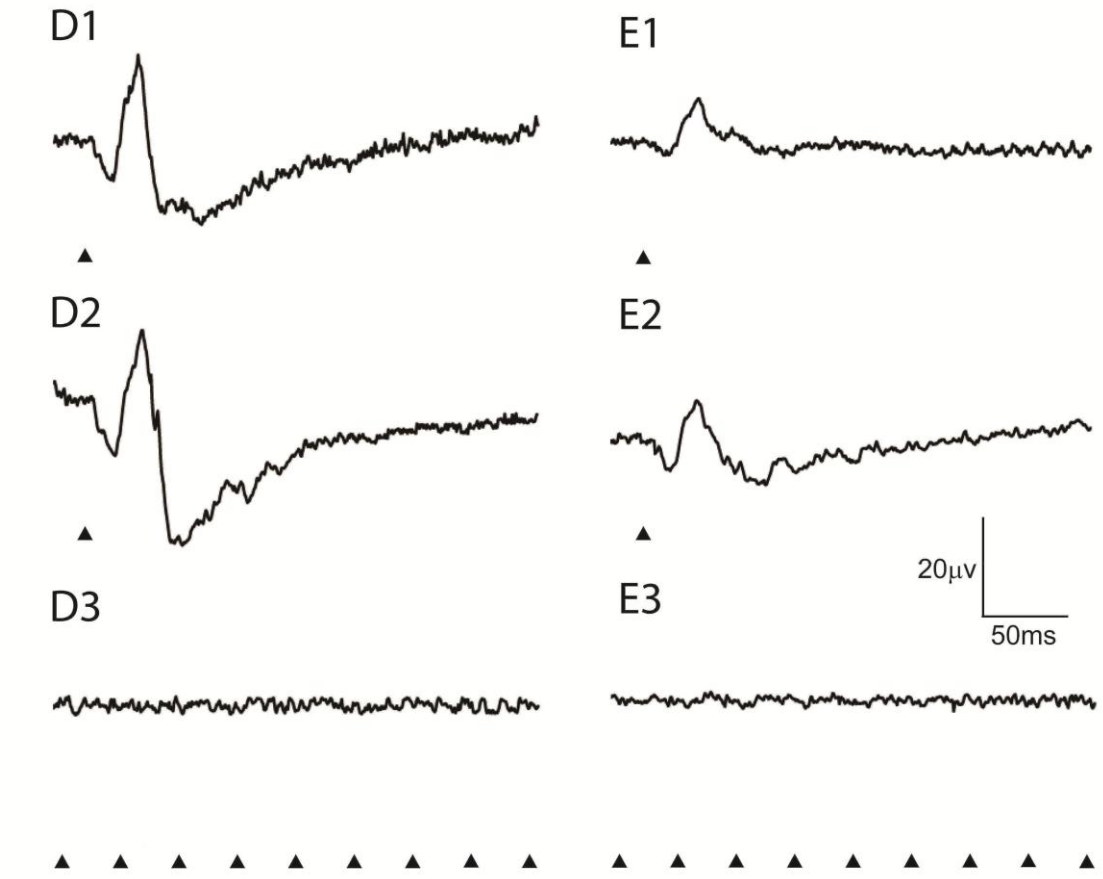


Figure 4.4 continued

4.4.5 Slow and progressive retinal thinning and development of RPE inclusions

Retinal layer thickness and outer nuclear layer cell counts were measured on plastic embedded retinal sections. Measurements were compared for 14 *RPE65* -/- dogs aged 3 months to 3 years, and 6 *RPE65* +/+ dogs, also aged 3 months to 3 years. In the youngest *RPE65* -/- dogs outer nuclear layer (ONL) cell count and thickness of all retinal layers closely approximated that of the *RPE65* +/+ dogs. However as age increased the ONL cell count declined significantly in the *RPE65* -/- dogs (p value = 0.004). Changes to the retinal architecture in the *RPE65* -/- dogs were not limited to the outer nuclear layer, there was appreciable thinning of the neural retina over the ages assessed (Figure 4.5A). All retinal layers decreased significantly in thickness over the ages assessed (p values ranged from 0.02 to 0.001), exceptions were the RPE that increased in thickness (p value = 0.01) and the nerve fiber layer that remained relatively static in thickness over the ages studied (Figure 4.5B). The relative change in thickness of the retinal layers did not vary between the regions assessed over the ages studied.

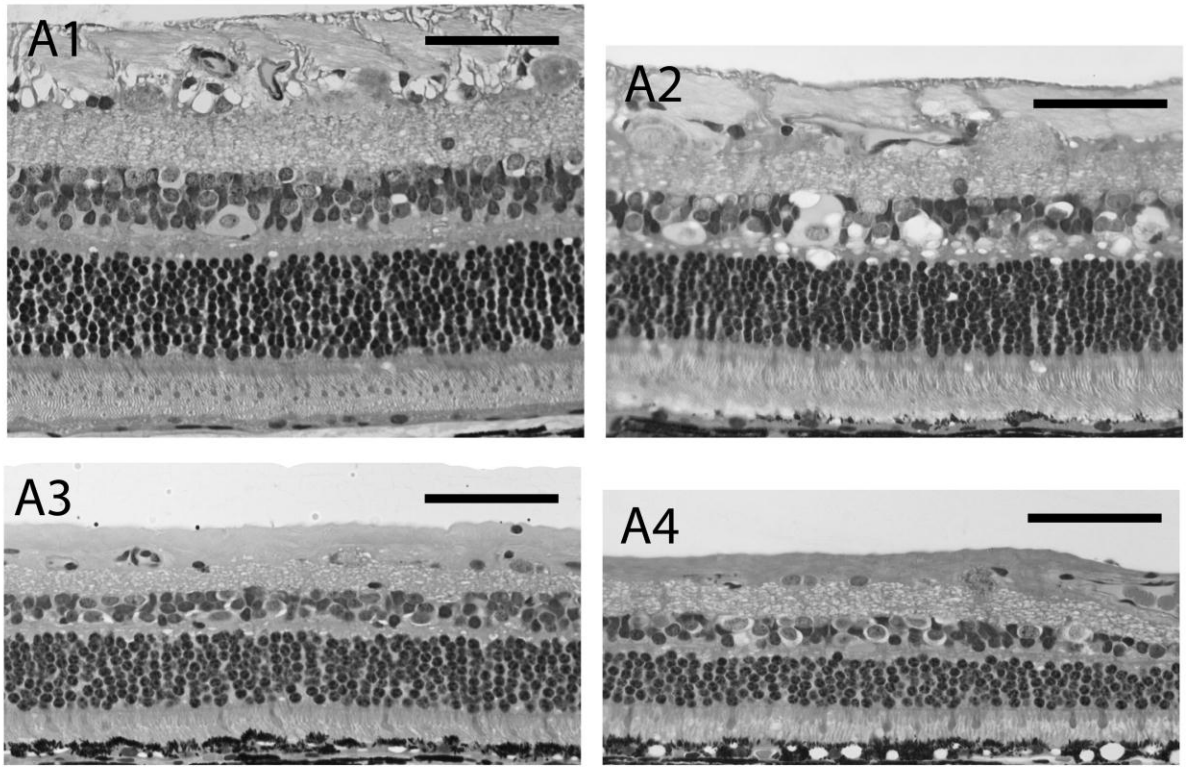


Figure 4.5A Slow, progressive decline in retinal thickness. Representative images are shown in A1-A4; an *RPE65* +/+ dog 12 months of age (A1); and *RPE65* -/- dogs, 3 months of age (A2), 16 months of age (A3), and 33 months of age (A4). (Scale bars = 50µm).

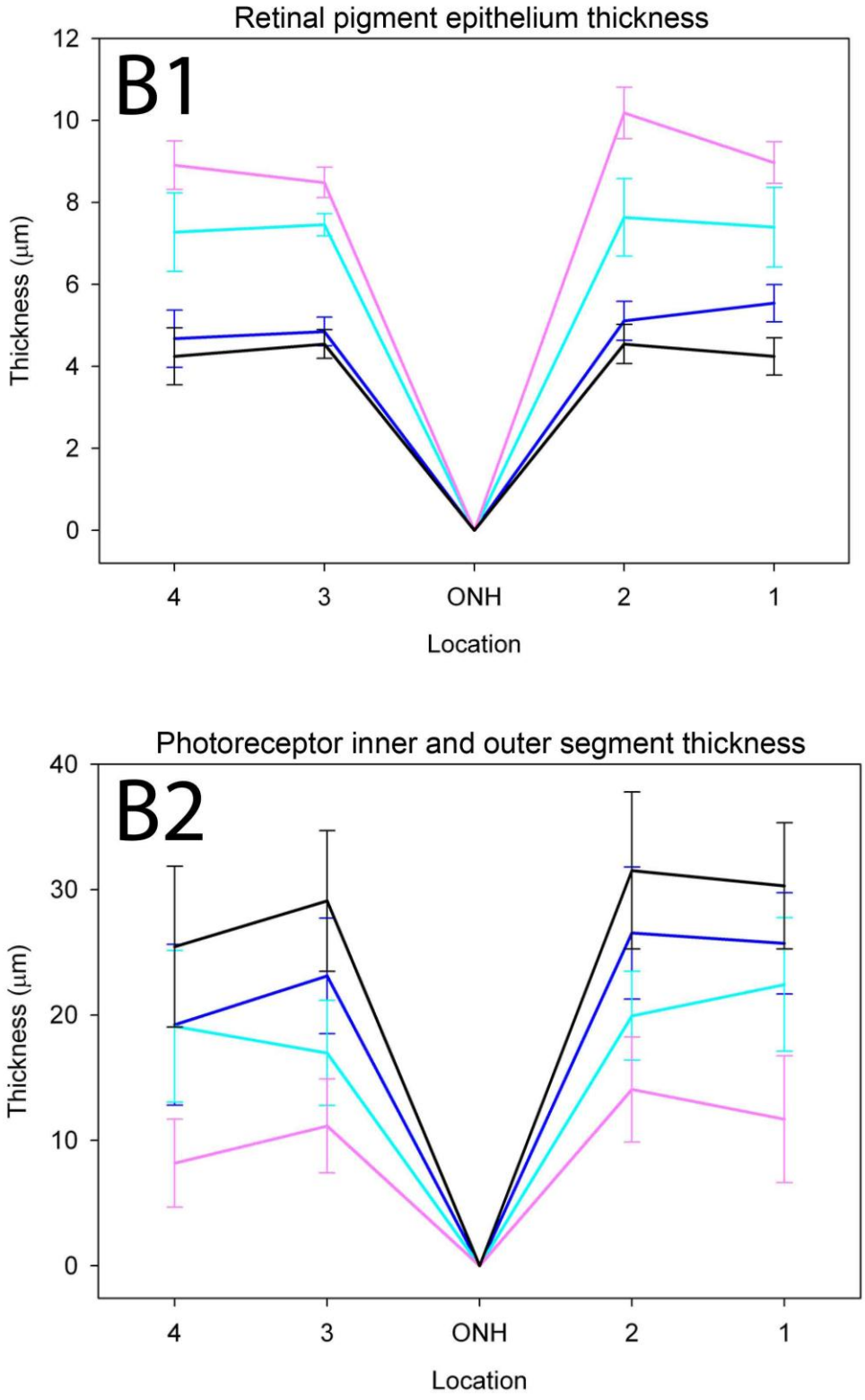
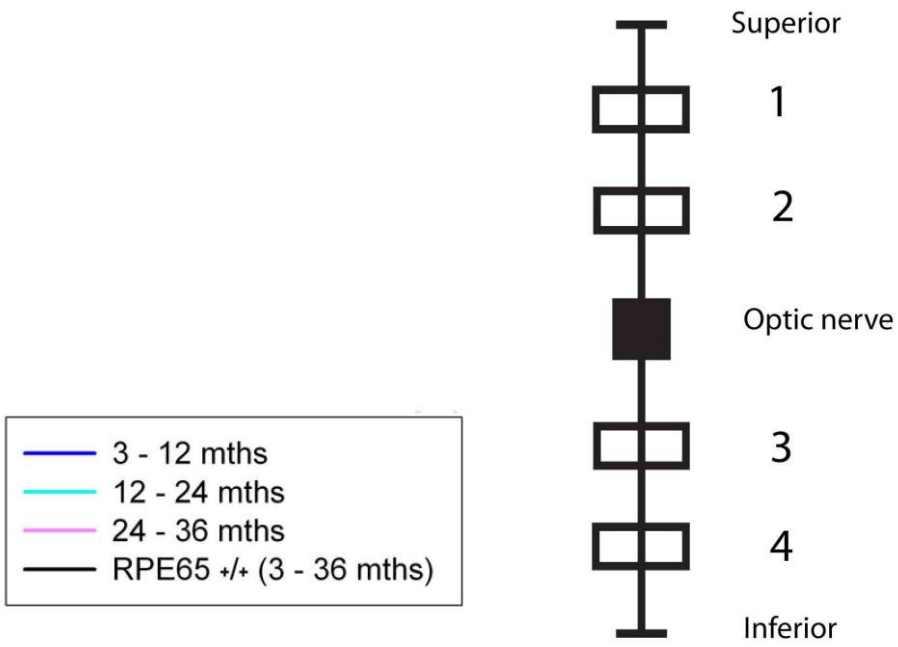


Figure 4.5B **Slow and progressive decline in photoreceptor number.** Retinal layer thickness

and outer nuclear layer cell counts are displayed in B1-B8. Measurements were performed at 4 sites, 1/3 and 2/3rds between the optic nerve and ora ciliaris retinae both superior and inferior to the optic nerve (see inset schematic). Dogs are grouped as follows; *RPE65* +/+ dogs 3-36 months, n=11 eyes; *RPE65* -/- dogs 3-12 months, n=7 eyes; *RPE65* -/- dogs 12-24 months, n=5 eyes; and *RPE65* -/- dogs 24-36 months, n=5 eyes. Retinal layers are shown as follows; Retinal pigment epithelium (B1), photoreceptor layer (B2), outer nuclear layer (B3), outer plexiform layer (B4), inner nuclear layer (B5), inner plexiform layer (B6), nerve fiber layer and ganglion cell layer (B7). Outer nuclear layer cell count is displayed (C).



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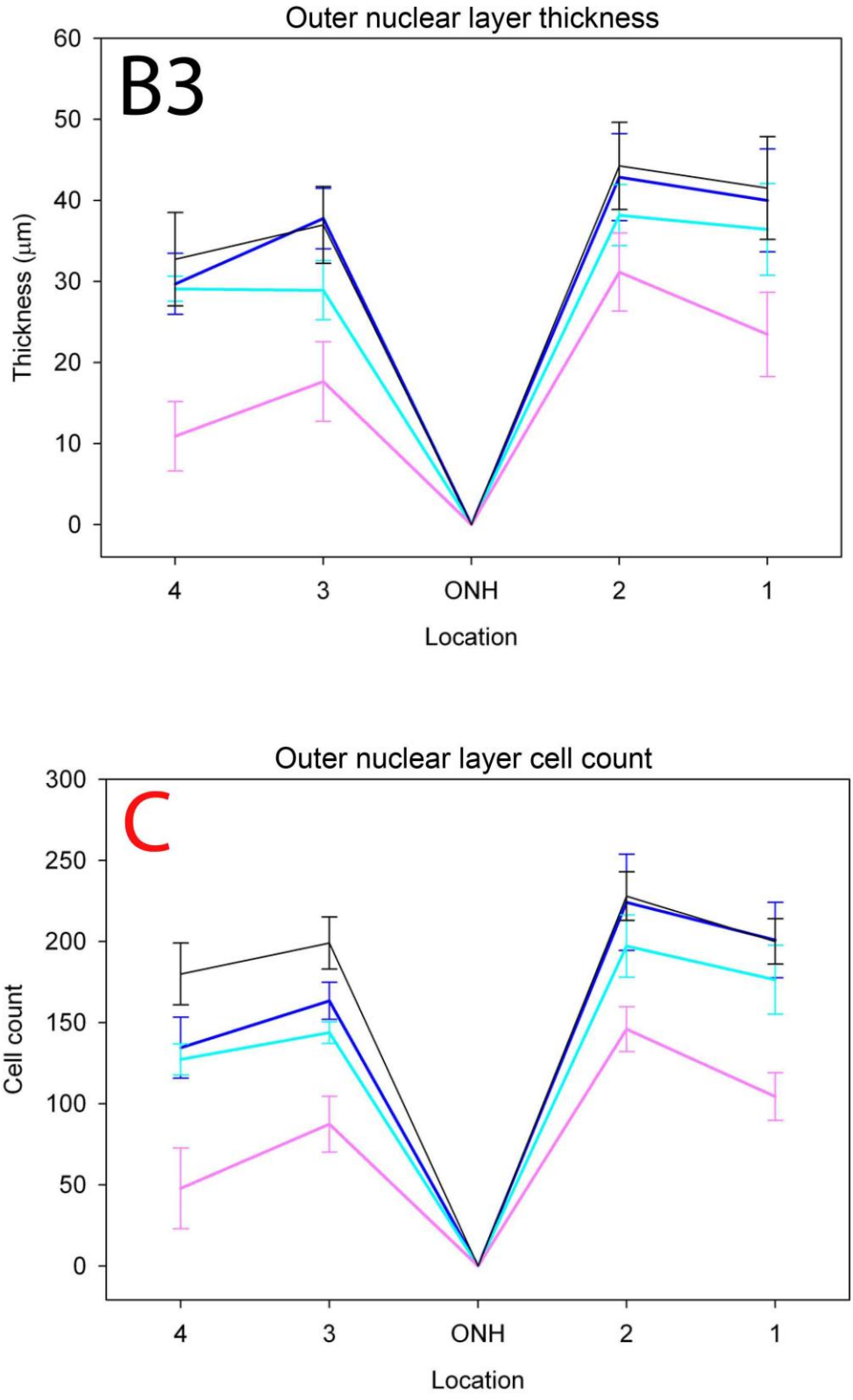

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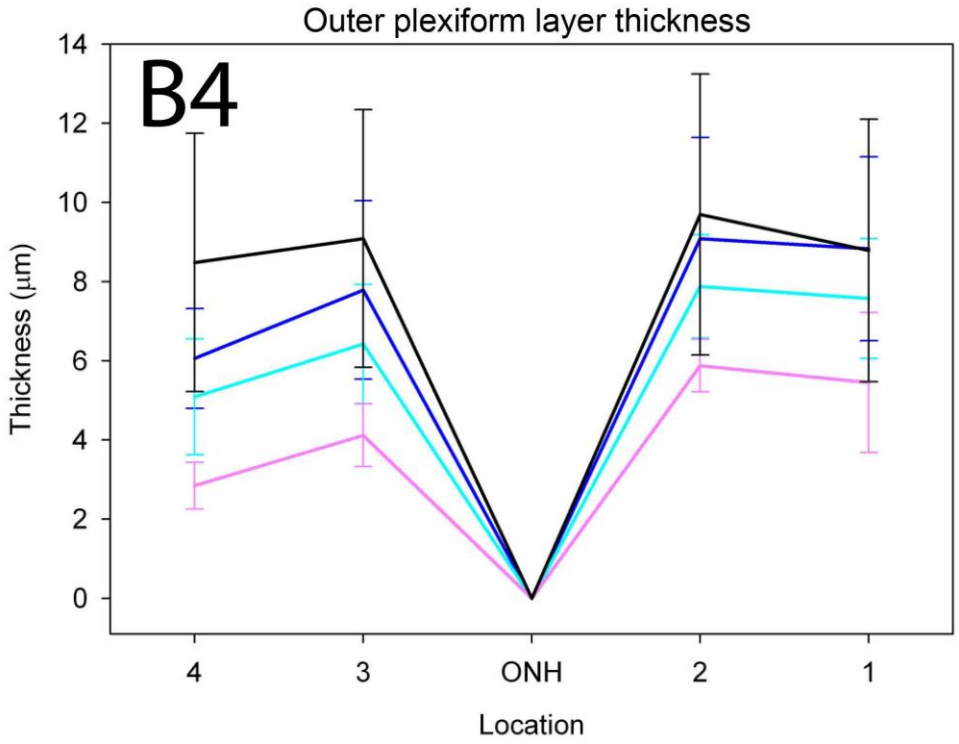

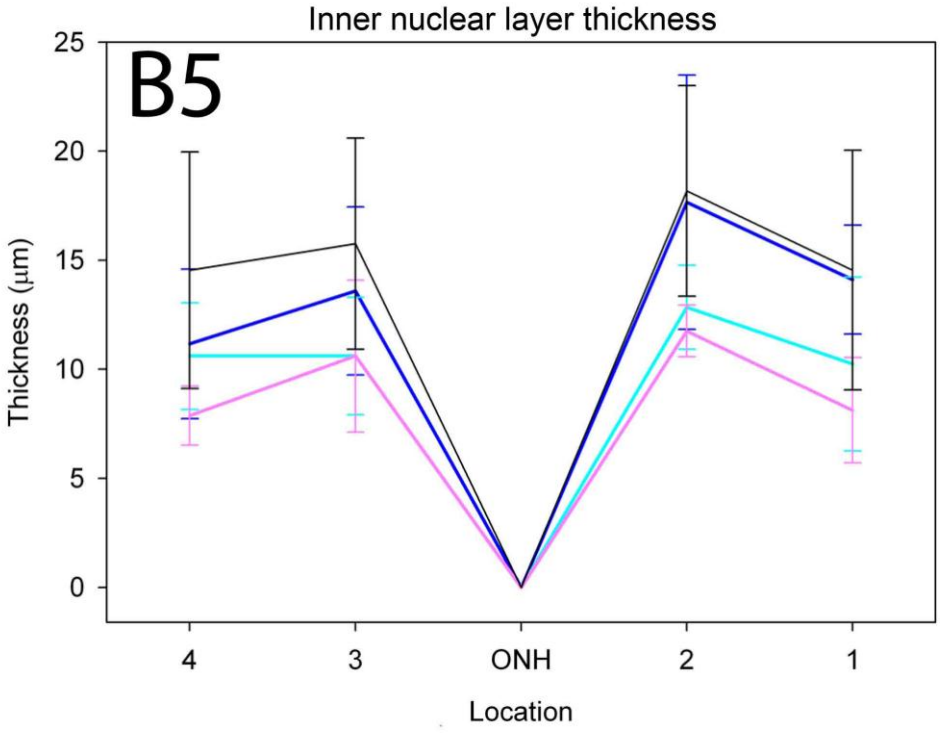

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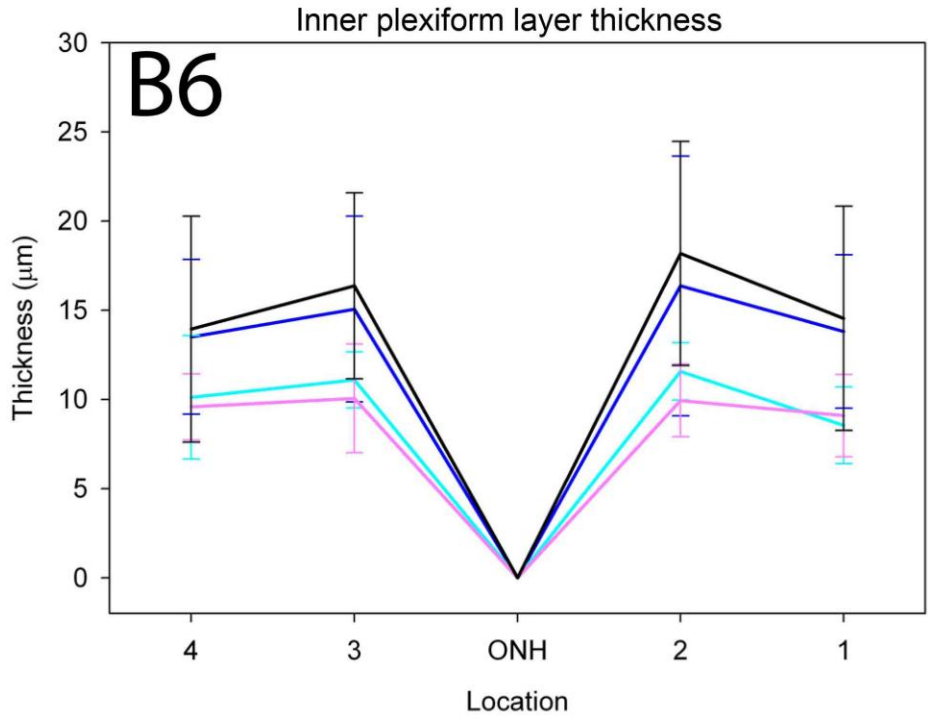


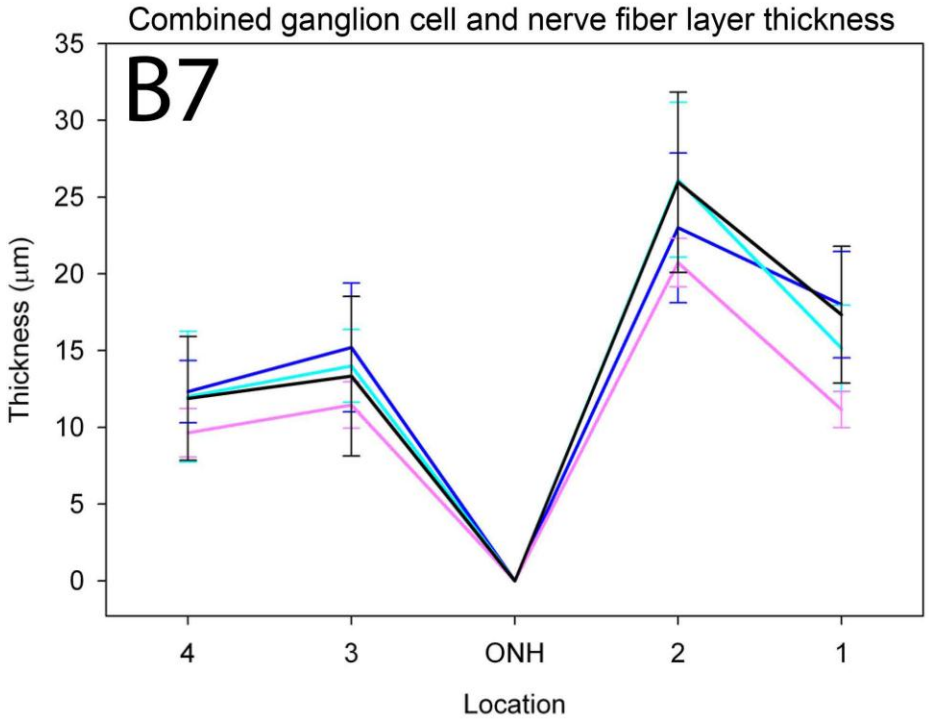


Figure 4.5B continued

The increased thickness of the RPE observed as the *RPE65* -/- dogs aged appeared to be largely due to the development of RPE inclusions, absent in the *RPE65* +/+ dog. These RPE inclusions were evaluated further in terms of number and size and found to be greatest in number in the central retina. The RPE inclusions were sparsely present at 3-6 months (1.0 +/- 1.0 inclusions per 200 microns), increasing in number to 13.8 +/- 2.6 inclusions per 200 microns at 24-36mths (Figure 4.6). A similar increase in inclusion size was appreciated as the animals aged, from a mean of 1.0 +/- 0.5 microns at 3-6 months to 27 +/- 7 microns at 24-36 months of age.

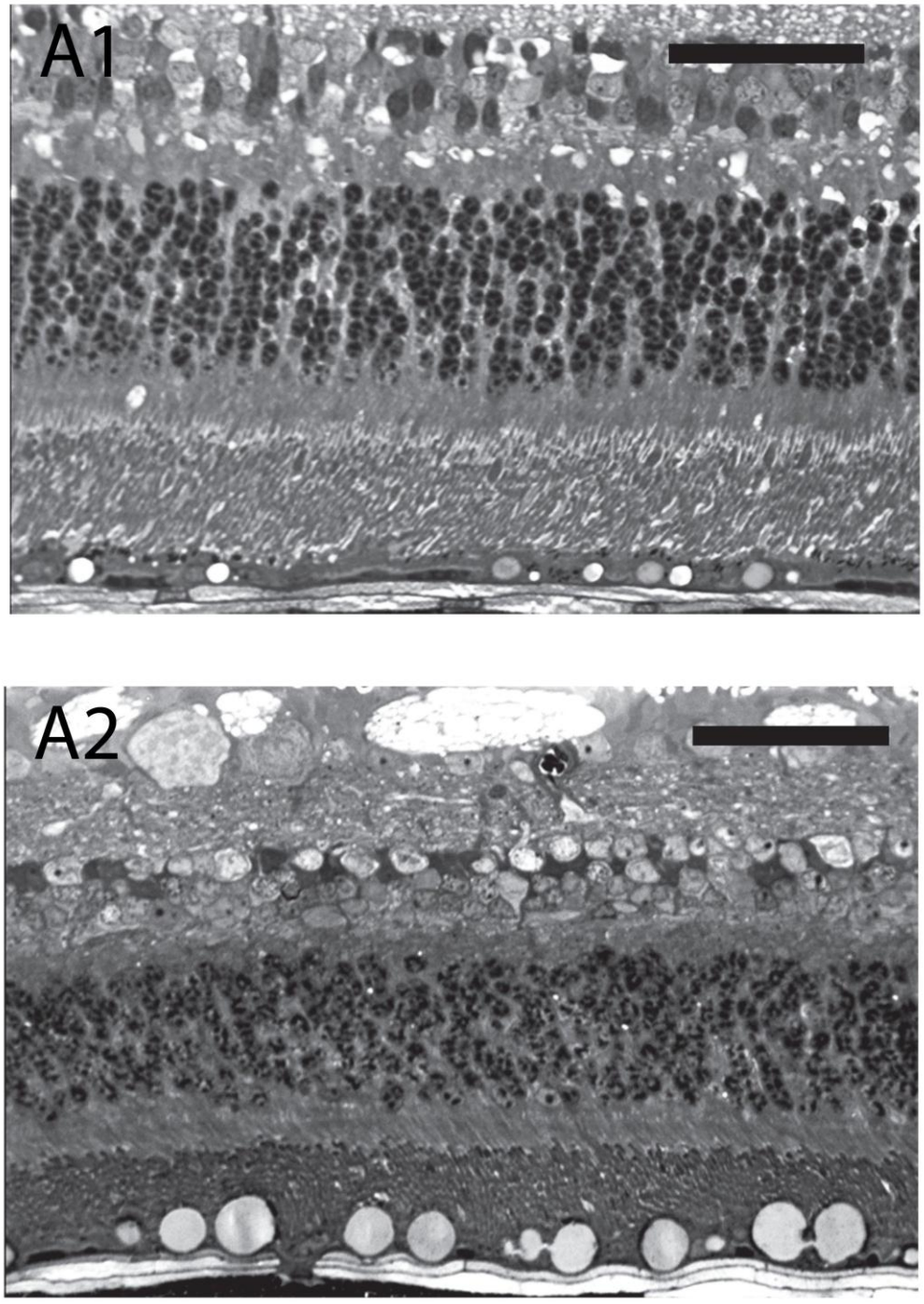
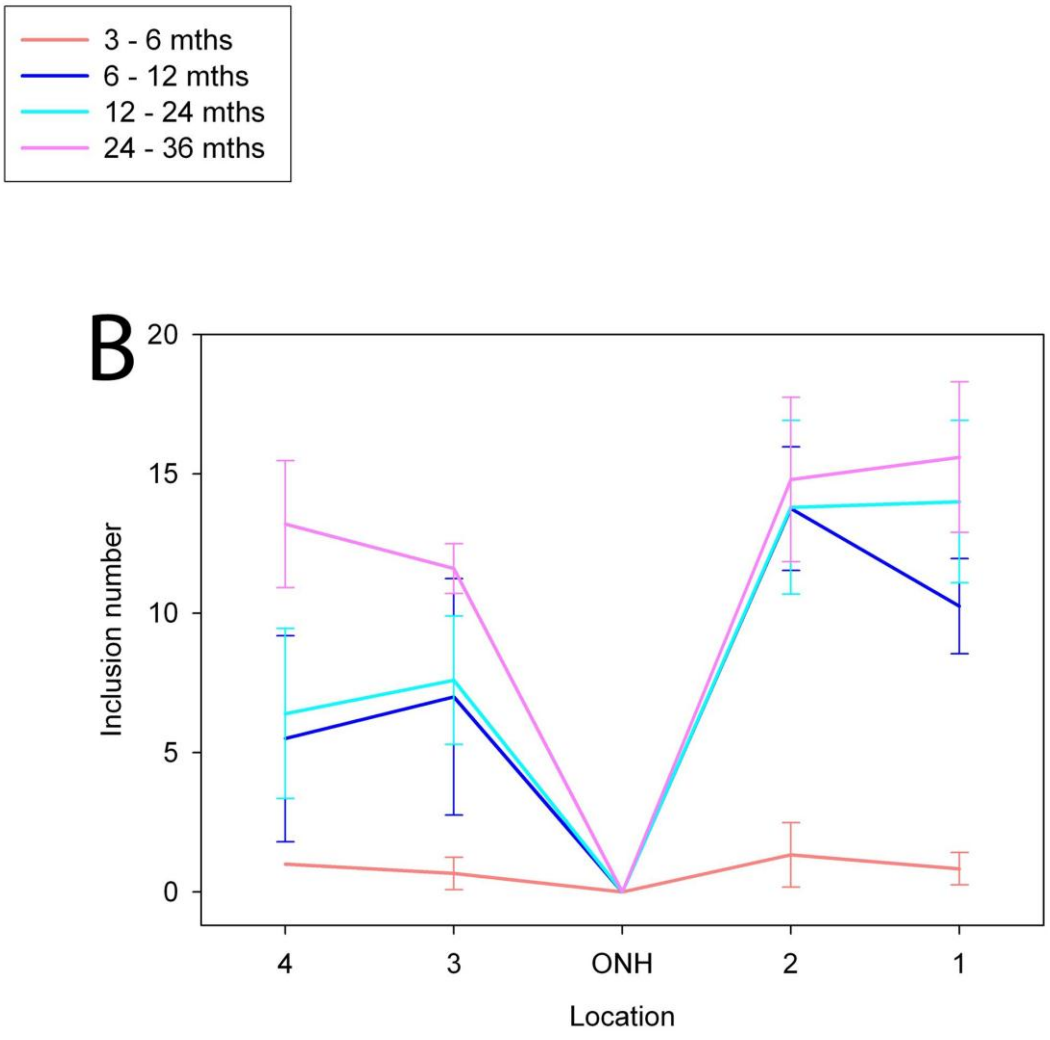


Figure 4.6 RPE inclusions increase in size and number with age. Retinal sections of *RPE65* -/- dogs are shown with inclusion size and number measurements performed at 4 sites as shown in Figure 4.5. Dogs are grouped as follows; 3-6 months, n=3 eyes; 6-12 months, n=4 eyes; 12-24

months, n=5 eyes; 24-36 months, n=5 eyes. Representative images showing RPE inclusions are displayed; an *RPE65* -/- dog 3 months of age (A1), and an *RPE65* -/- dog 36 months of age (A2). (Scale bars = 50µm). RPE Inclusion measurements are shown; inclusion number per 200 microns (B) and RPE inclusion size in microns (C).



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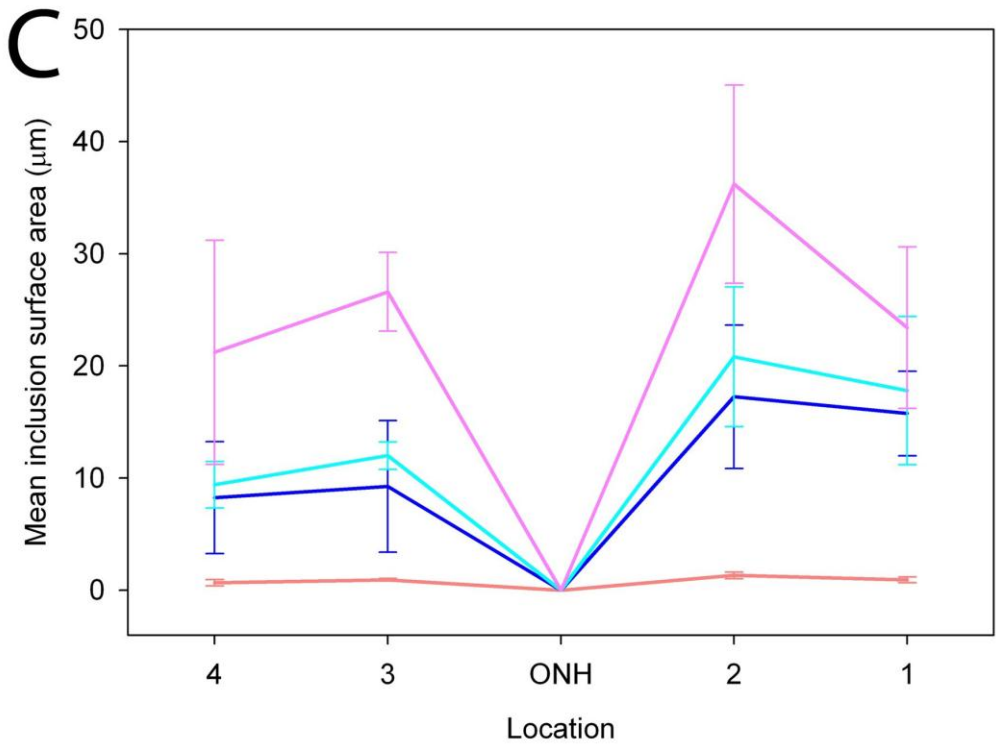


Figure 4.6 continued

4.4.6 Tapetal hyper-reflectivity and ONL thinning at area centralis

All 52 *RPE65* -/- dogs examined in this study had an ophthalmoscopically detectable region of tapetal hyper-reflectivity temporal and slightly superior to the optic nerve head. This is the location of the region of highest photoreceptor density in the dog, the canine area centralis [116]. The lesion was first apparent on fundic exam in 3 month old dogs as a small pinpoint area of tapaetal hyper-reflectivity. This finding was accompanied by a region of tapetal hypo-reflectivity that extended horizontally at and above the level of the area centralis, a region corresponding to the canine visual streak [116] The size of hyper-and hypo-reflective regions increased slowly in size with age (Figure 4.7). These findings were not appreciated in any of the *RPE65* +/+ or *RPE65* +/- dogs in the colony.

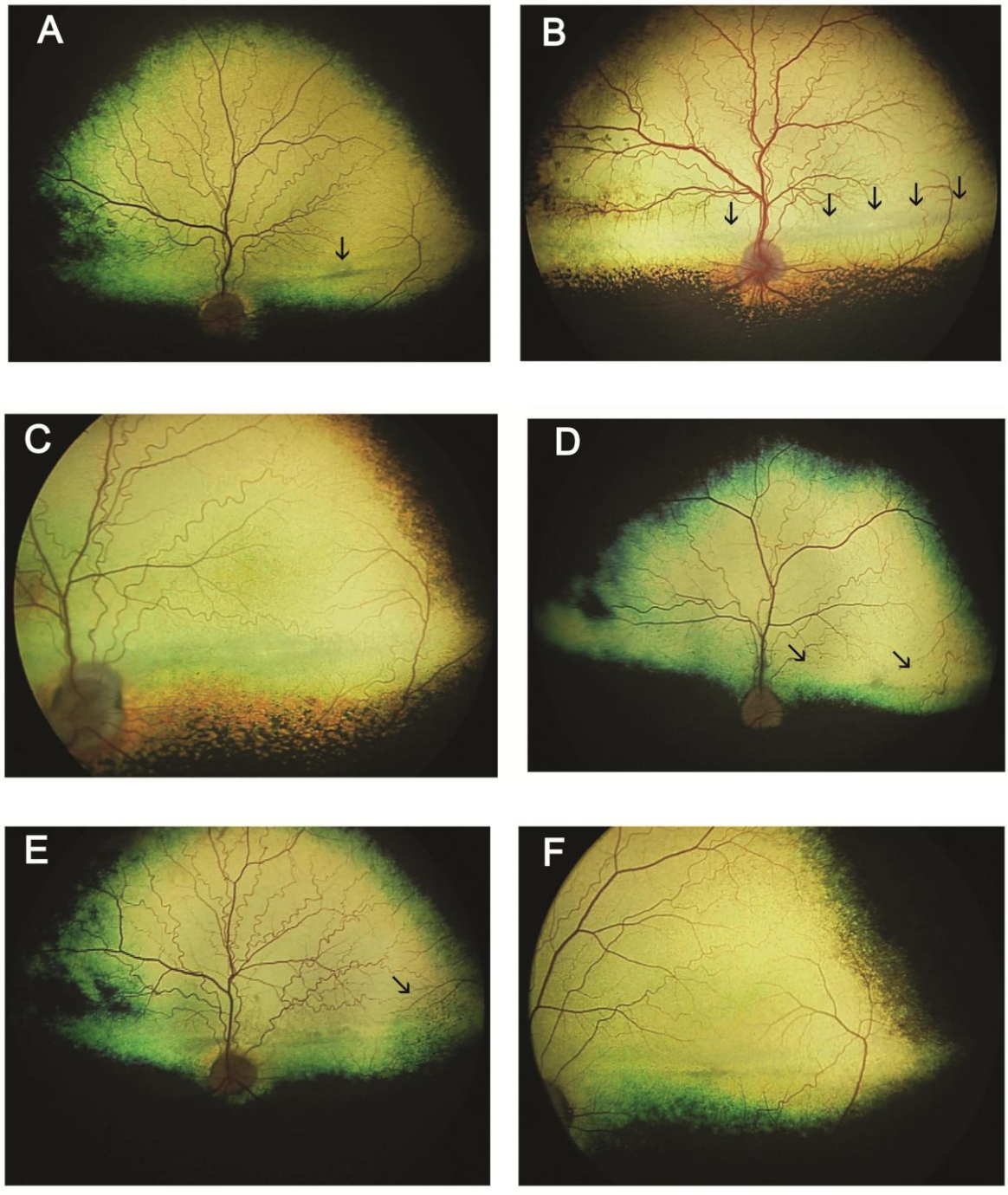


Figure 4.7 Funduscopic changes in the *RPE65* -/- dog. Representative images (Retcam®, Clarity) illustrating funduscopic changes of *RPE65* -/- dogs. Tapetal hyper-reflectivity at the area centralis (arrow) in a dog 24 months of age (A), and tapetal hypo-reflectivity along the visual

streak (arrows) in a dog 39 months (B). Higher magnification view of the changes in tapetal reflectivity in a dog 47 months of age (C). Pigment foci scattered across tapetal fundus (arrows) in a dog 42 months of age (D), and a region of pigment foci (arrows) in a dog 49 months of age (E). Higher magnification view of small pigment foci in a dog 56 months of age (F).

Figure 4.7 continued

In-vivo optical coherence tomography (OCT) (Specralis®, Heidelberg, Germany) was performed on 22 eyes of 11 *RPE65* -/- dogs ranging in age from 1 month to 8 years. The region of retinal thinning was readily apparent in all eyes, detectable at 1 month of age, before ophthalmoscopic appreciation of the lesion (Figure 4.8) OCT performed over the region of the area centralis in 10 eyes of 5 *RPE65* +/+ dogs identified no such lesion. Histopathologic assessment of these findings was performed on 4 eyes of 4 *RPE65* -/- dogs and showed a focal region of dramatic thinning of the outer nuclear layer in the area centralis (Figure 4.9). Transmission electron microscopy was performed on this region, identified by serial sections taken 2µm through the area centralis. Rod outer segments were short and outer nuclear layer in this area thinner then in surrounding regions as appreciated on routine histpathology. Rod outer segment morphology, RPE structure and lipid inclusions in this location were otherwise comparable with that observed in surrounding areas (Figure 4.10).

In addition to the region of tapetal hyper-reflectivity observed in all dogs, the appearance of pigment foci was detected in the tapetal fundus of the majority of eyes of dogs aged over 23 months (Figure 4.7). Additional funduscopic changes appreciated included pinpoint hyper-reflective foci seen in 3 eyes of dogs of variable ages, and scatted across the tapetal fundus. Both eyes of 2 of the 3 dogs older than 60 months also displayed mild retinal vasculature attenuation, this finding was not appreciated in any of the other dogs in this study.

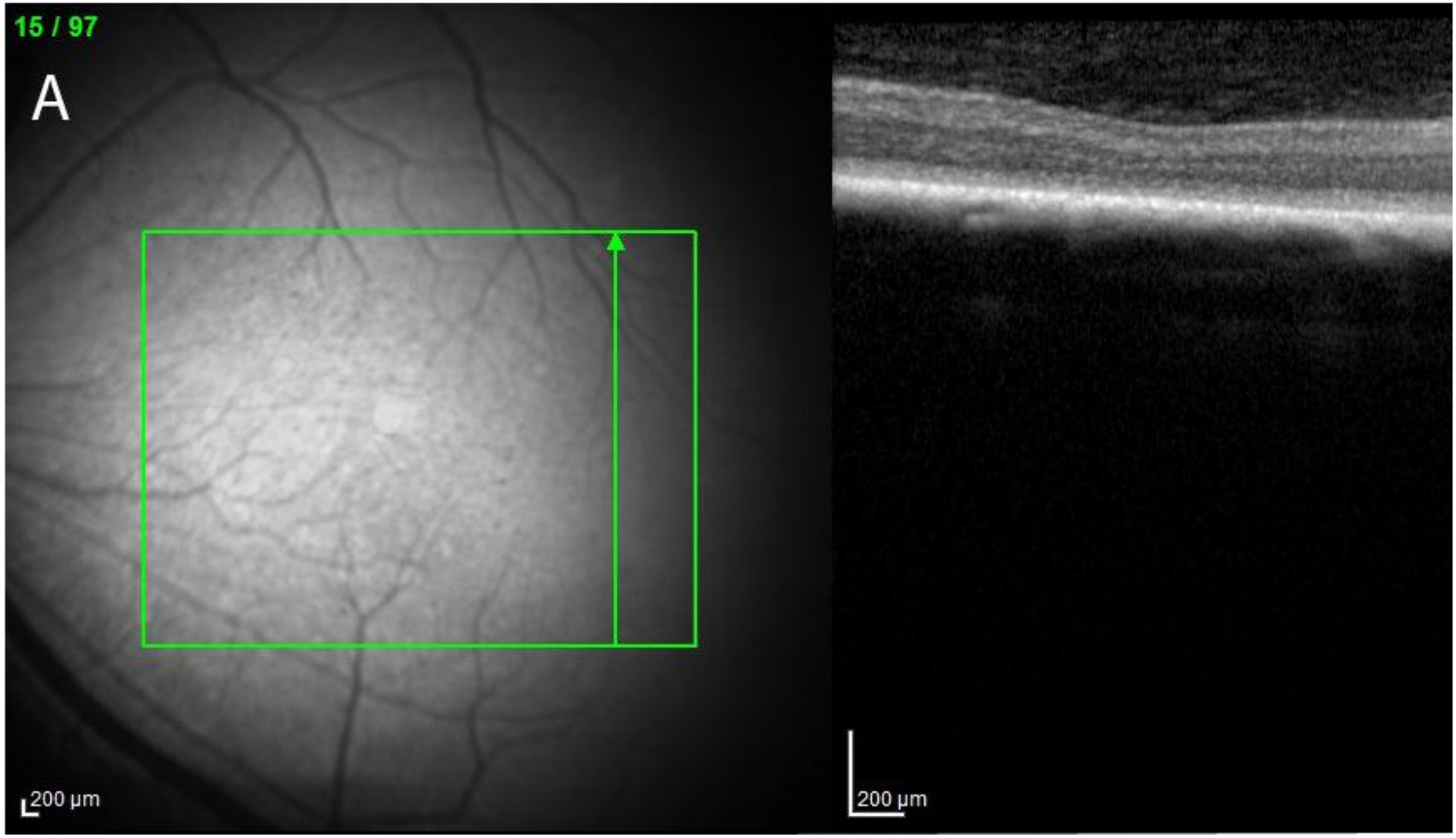
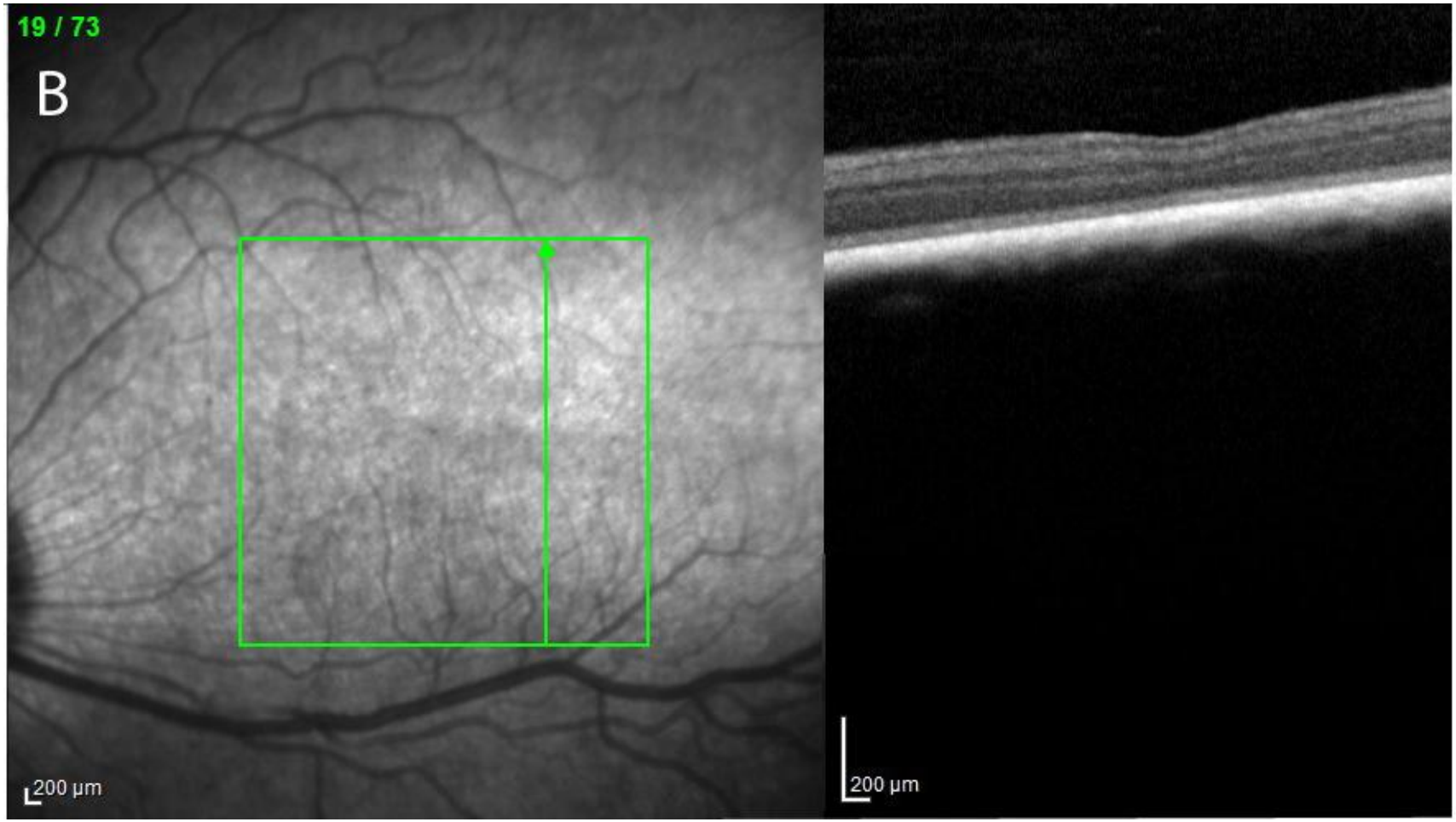


Figure 4.8 **Optical coherence tomography of area centralis.** Representative images (Spectralis®, Heidelberg) taken over the

area centralis of the left eye of *RPE65* -/- dogs, illustrating focal in-vivo retinal thinning at the location of the area centralis. Dog aged 1 month (A). Dog aged 3 months (B). Dog aged 16 months (C). Dog aged 96 months (D)



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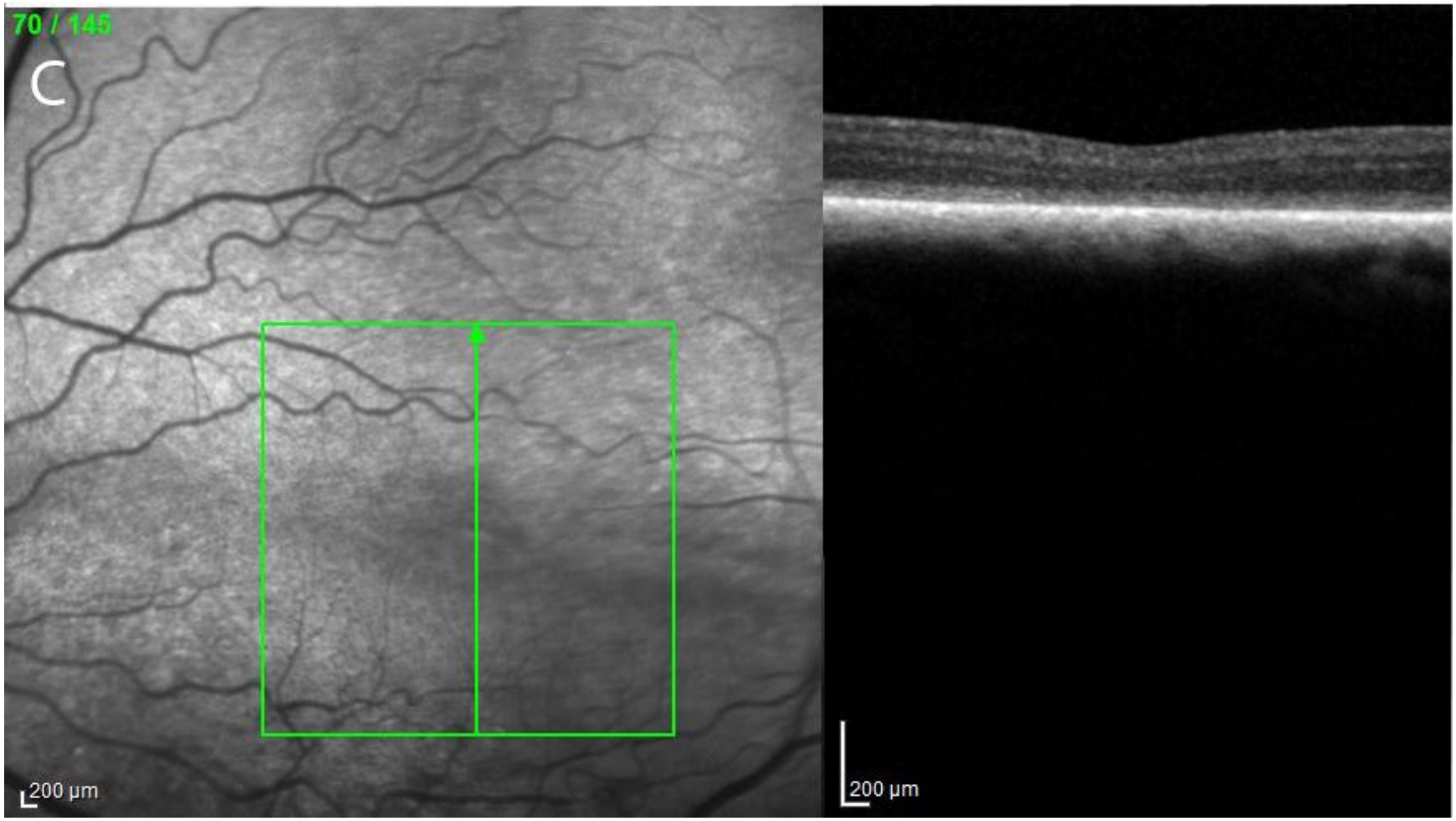

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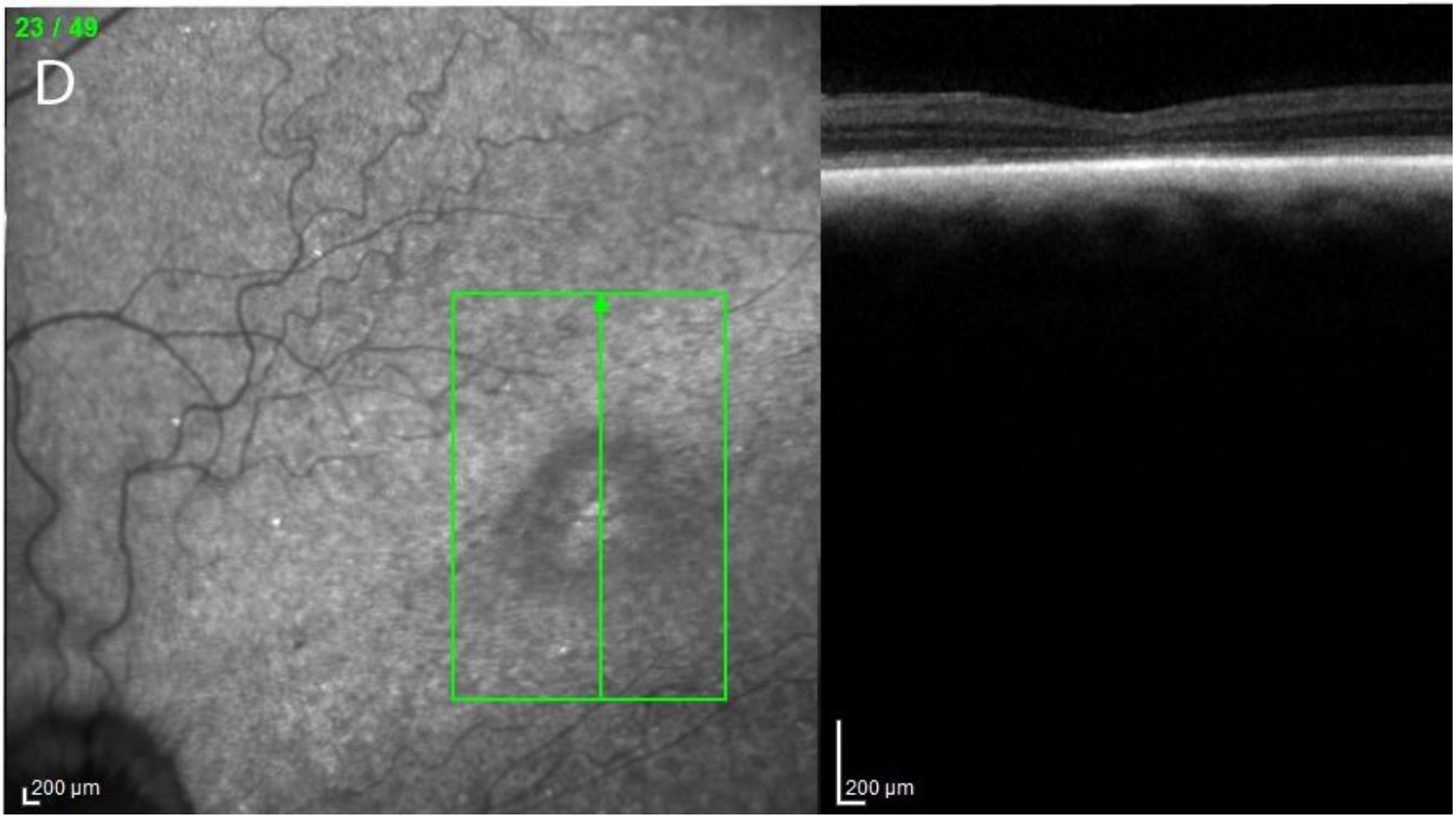

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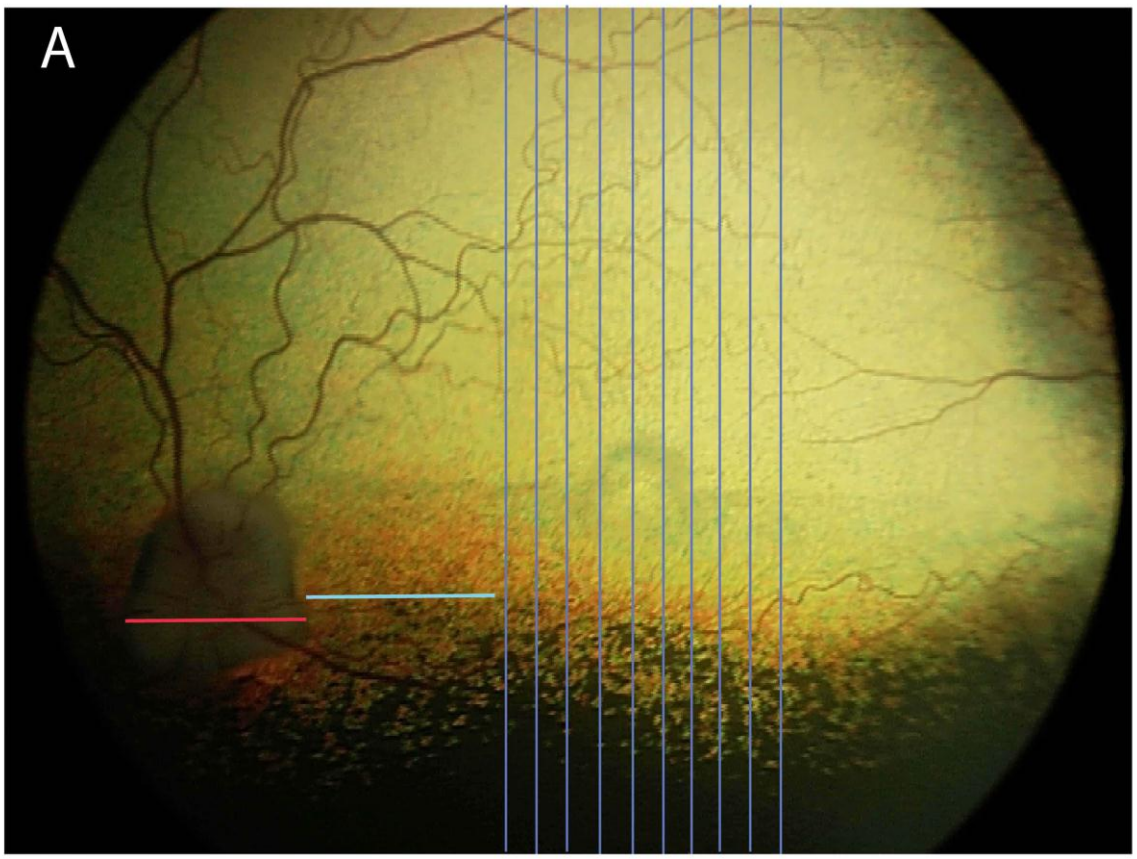
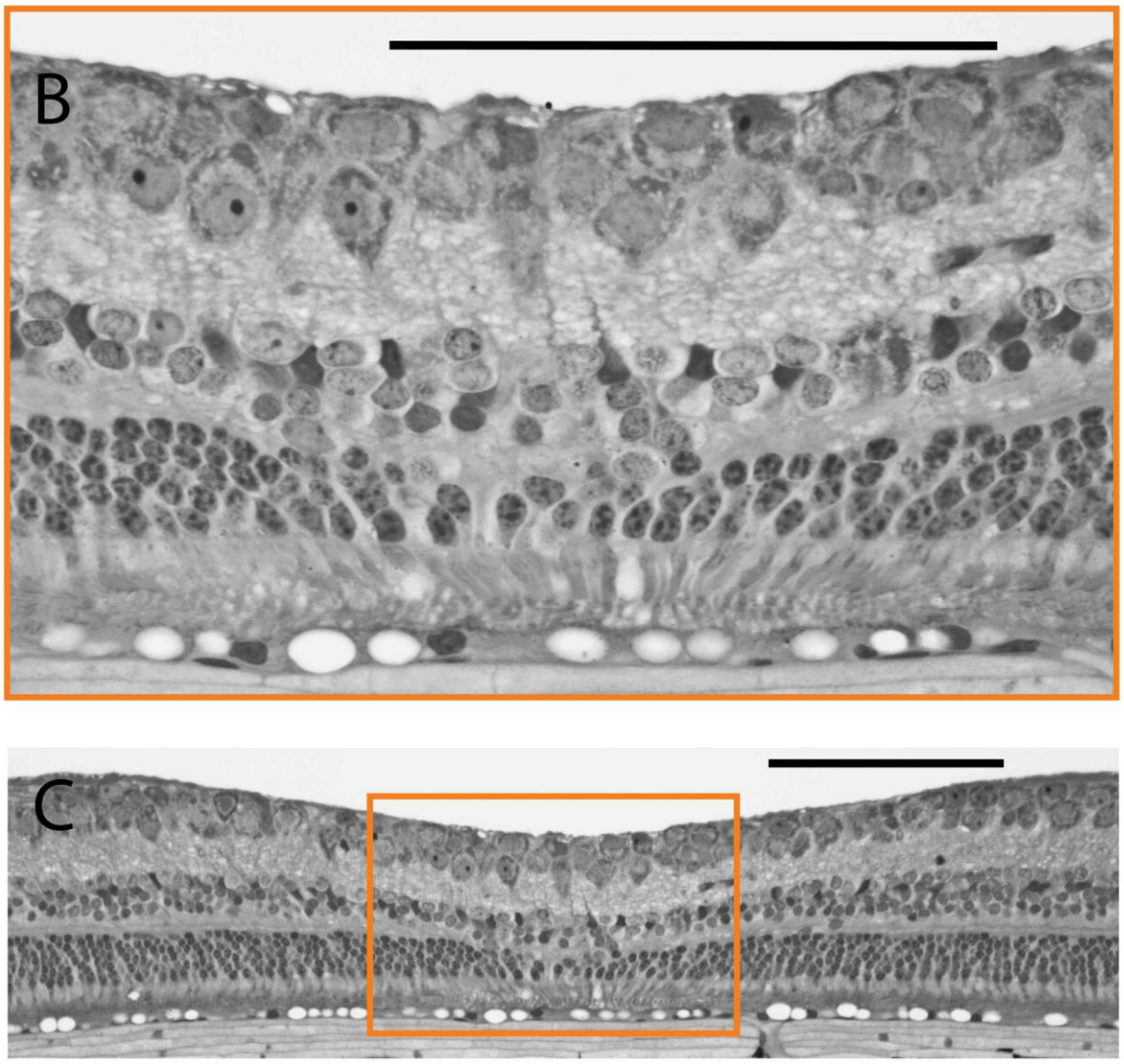


Figure 4.9 ONL thinning at the area centralis. Image of an *RPE65* -/- dog fundus showing the region of tapetal hyper-reflectivity observed in all dogs, and the location of serial sections. Sections were at 300 micron intervals (dark blue lines), commencing one optic disc diameter (red line), temporal to the optic nerve head (pale blue line) (A). Representative section (n=4 eyes of 4 *RPE65* -/- dogs) displaying focal outer nuclear layer thinning at site of area centralis lesion (B and C), (Scale bars = 35 µm).



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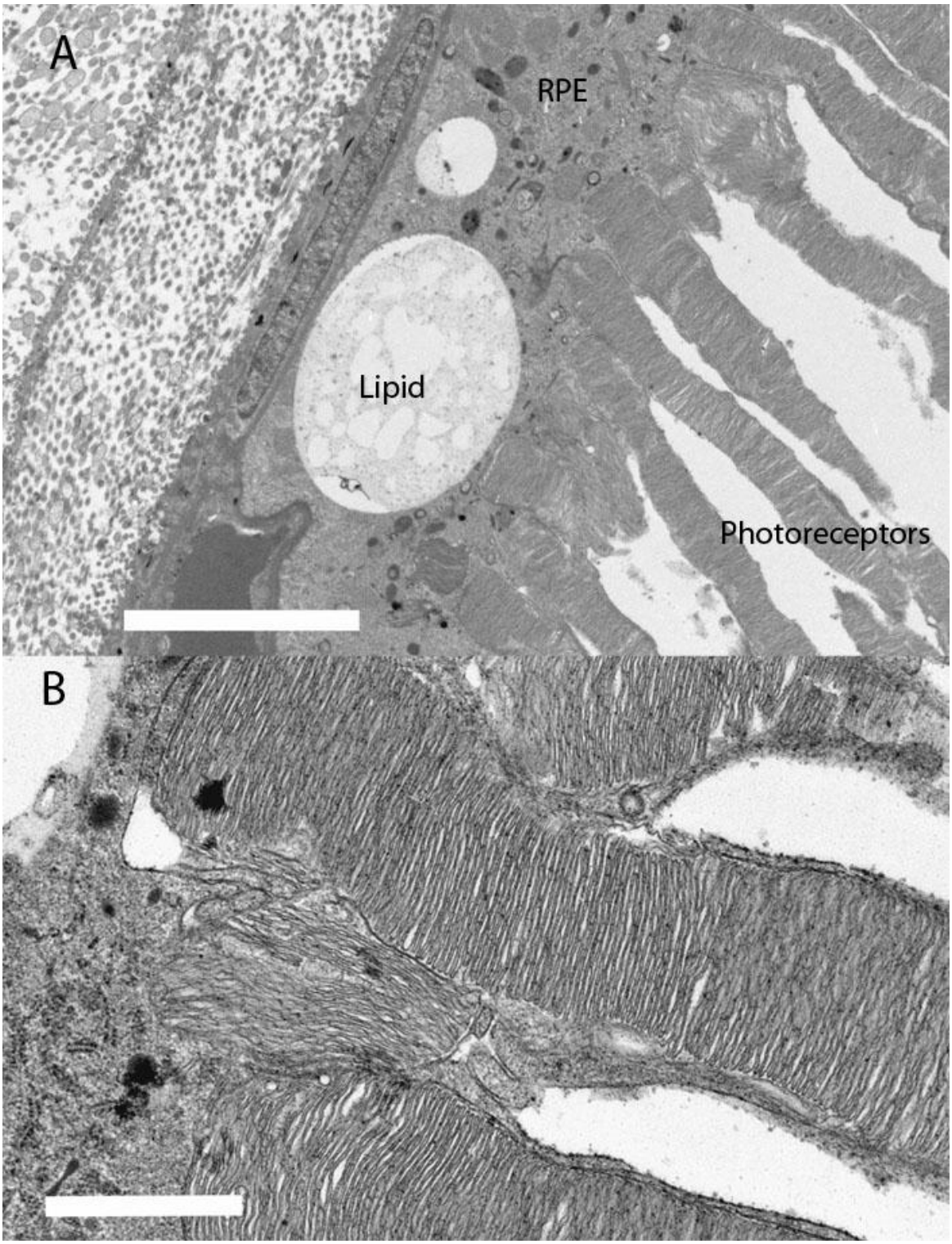

Figure 4.10 Normal photoreceptor morphology at the area centralis.

TEM images of a 1 year old *RPE65 -/-* dog. An image taken at the location of ONL thinning at the

area centralis shows photoreceptor loss and electron lucent inclusions in the RPE, scale bar = 1µm (A). Rod photoreceptors with normal disc arrangement, scale bar = 5µm (B).

Figure 4.10 continued

4.5 Discussion

The phenotype of young *RPE65 -/-* dogs has been well described, often as a prelude to gene therapy trials utilizing this model. However not only is there limited information on the progression of the phenotype with age, there are some discrepancies in the descriptions of the phenotype of young *RPE65 -/-* dogs. Most notably with respect to visual function with some authors describing diminished vision in dim light only, others reporting blindness irrespective of lighting conditions.[123-125, 137, 156] Here with the use of an objective and quantifiable vision testing device we have shown that these dogs retain bright light vision out to at least 8 years of age. We have also presented a detailed description of the electroretinographic, histopathologic and funduscopic changes that occur with age. Interestingly, with respect to vision testing outcomes we saw no change to parallel the declines in the ERG amplitude and progression of histopathologic changes observed as the affected dogs age. So despite the reduction in amplitude of response observed in the ERG assessments, and histologic evidence of retinal degeneration, these dogs retain enough functional vision to navigate out of the vision testing device at room light levels. These results are consistent with good vision at normal light levels but markedly diminished vision in dim light, vision that remains stable as the dogs age out to 8 years. It might however be anticipated that if visual acuity were able to be assessed reliably in these dogs a decline with age would be seen, consistent with descriptions of human LCA. In such individuals visual function typically deteriorates appreciably as patients reach their third to fifth decades of life.[34-36]

Consistent with previous studies we appreciated that young dogs had low amplitude dark-adapted rod driven flash ERG responses.[110, 114, 131, 137, 156] Additionally, as reported by Acland *et al*, the light-adapted flash ERG responses of the young dogs were of comparable amplitude to that of normal dogs.[114] This is suggested to be because rod suppressing background light routinely used is not adequate to suppress the responses from the desensitized rods in the *RPE65* -/- dog.[114] Under this scenario, the light-adapted ERG waveforms may represent the summation of residual cone function and that of the desensitized rods. In the present study we observed a slow progressive decline of these low amplitude light- and dark-adapted a- and b-wave recordings as the animals aged, though recordable responses were reliably measured out to 8 years of age. Additionally, despite the declining amplitudes recorded with age, the response thresholds remained stable with age. With respect to cone photoreceptor function other authors have reported no or very low amplitude 33Hz cone flicker responses in young dogs.[114, 131, 137] Our study findings were in in accord with this, but also showed that these very low amplitude responses were intermittently recorded from dogs of all but the oldest age groups. Due to the extremely low amplitude of the light adapted 33Hz flicker responses it would appear that this may not a good indicator of cone preservation in the *RPE65* -/- dog where cone function is poor. Here immunohistochemistry is be a more reliable indicator of the presence of cones, with the function of the remaining cones remaining hard to categorize at the present time.

Routine histopathology performed on dogs out to 3 years of age showed evidence of progressive thinning of all retinal layers with the exception of the RPE and ganglion cell/nerve

fiber layer. Changes in the thickness of all retinal layers was observed in the youngest dogs in this study, findings consistent with previous studies.[127, 128] Also consistent with previous reports we observed large lipid-like inclusions in the RPE, these were most numerous in the central retina and increased in number most dramatically in the 6-12 month age group.[127, 128] In these respects our histopathologic findings are concordant with those previous descriptions. Such changes might be anticipated, with older *Rpe65*^{rd12} mice reported to have an atrophied and hypopigmented RPE.[47] Of note, in the *RPE65* -/- dog Wrigstad *et al* have described complete degeneration of photoreceptors in the peripheral retina of a dog 7 years of age.[128]

At the location of the *area centralis* we observed a previously undescribed change in tapetal reflectivity in all *RPE65* -/- dogs. Histopathology and optical coherence tomography showed this to correspond to a region of dramatic thinning of the retinal outer nuclear layer. This change was appreciated in all affected dogs, visually appreciable from 3 months of age and seen as retinal thinning by OCT in dogs 1 month of age. The feature may be consistent with increased competition for available retinoid in this region of highest rod and cone photoreceptor density. The results of electron microscopy showed that the morphology of the rod photoreceptor outer segments in this region was consistent with adjacent areas of the retina and with previous reports on rod photoreceptor morphology in *RPE65 -/-* dogs.[128] Future studies will use immunohistochemistry to evaluate for the presence of cone photoreceptor subtypes.

Taken together the data presented here provides a detailed and extensive summary description

of the phenotype and natural course of disease in the *RPE65 -/-* dog. Data presented here will be relevant for current and future studies evaluating the outcome of therapeutics for LCA type 2 utilizing this canine model of Leber Congenital Amaurosis Type II.

CHAPTER 5

Successful gene therapy in older RPE65-deficient dogs with subretinal injection of rAAV2/2.hRPE65p.hRPE65

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5.1 Abstract

Young RPE65-deficient dogs have been used as a model for human RPE65 Leber Congenital Amaurosis (RPE65-LCA) in proof-of-concept trials of adeno-associated virus (rAAV) gene therapy. However there are only reports of the outcome of rAAV gene therapy in five eyes of RPE65-deficient dogs older than two years of age. The purpose of this study was to investigate the success of this therapy in older RPE65-deficient dogs. Thirteen eyes were treated in dogs between aged between 2 and 6 years. rAAV2 expressing the human RPE65 cDNA driven by the human RPE65 promoter was given by subretinal injection. Twelve of the thirteen eyes had improved retinal function as assessed by electroretinography, and all showed improvement in vision at low lighting intensities. We conclude that functional rescue is still possible in middle-aged dogs and that the use of middle-aged and older RPE65-deficient dogs, rather than young RPE65-deficient dogs that have very little loss of photoreceptors more accurately models the situation when treating human RPE65-LCA patients.

5.2 Introduction

Mutations in *RPE65* cause between 6 and 15% of cases of Leber Congenital Amaurosis (LCA), a condition characterized by vision-loss in childhood.[31, 43] A deficiency of functional RPE65 results in failure of the retinoid cycle and lack of 11-*cis* retinal supply from the retinal pigment epithelium to the photoreceptors. Normal visual pigment formation cannot occur, resulting in severely reduced rod and cone photoreceptor sensitivity and associated visual impairment.[30] Briard dogs with a null mutation in *RPE65* develop a similar phenotype to LCA type II patients.[123-125, 128] Affected dogs have severely decreased photoreceptor sensitivity,

marked changes in the electroretinogram (ERG) and poor visual function, particularly in dim light.[123, 128, 131] Two engineered mouse models and a spontaneously occurring mouse model (the *Rpe65*$^{rd12/rd12}$ mouse) also exist and show similar alterations in photoreceptor function.[47, 67, 86]

Loss of function prior to marked degeneration of photoreceptor cells provides a "window of opportunity" for gene augmentation therapy. The initial proof-of-principle studies in young RPE65-deficient dogs showed that subretinally-delivered rAAV vectors expressing either human or canine RPE65, dramatically improved photoreceptor function as assessed by ERG, vision testing performance, and retinal-mediated visual cortex activity.[110, 114, 135, 137] Similar dramatic improvements in retinal function were noted in young *Rpe65* -/- and *Rpe65*$^{rd12/rd12}$ mice treated by rAAV subretinal injection.[126, 159, 161, 179] However, no LCA type II patient in the human clinical trials reported to date has shown an ERG improvement that anywhere near matches that achieved in the dog model.[163-165, 167] The likely reason for this difference is RPE65-deficient dogs appear to lose photoreceptors at a relatively slower rate than occurs in LCA type II patients.

Immunohistochemical studies recently reported from RPE65-deficient dogs up to 18 months of age showed good preservation of both cone and rod photoreceptors.[129] Our own studies with the RPE65-deficient dog demonstrate an earlier loss of rods and M/L cones and an even more rapid loss of S-cones.[180] Encouragingly we found that gene therapy was able to

preserve S-cones as had previously been reported in the mouse models.[106, 180] Degeneration of photoreceptor cells is a limiting factor for gene augmentation therapy. In addition to the loss of photoreceptors RPE65-deficient animals develop lipid inclusions within the retinal pigment epithelium. In the dog model the inclusions become quite large in size and it is conceivable that they may impact RPE health and could be another factor that might limit the success of gene augmentation therapy.[128]

Scientific reports of gene augmentation therapy in RPE65-deficient dogs include a total of 96 eyes from 85 dogs (Summarized in Supplementary Table 5.1). Of these, 89 eyes were treated when the dog was less than one year of age. These reports include only five eyes of four dogs that were older than 2 years of age at the time of treatment.[135, 156, 157] Of these, two eyes of two dogs aged between 2- 2.5 years showed rescue, while one eye of a dog 2.5 years of age had no evidence of rescue despite the expression of RPE65 in the injected region.[156, 157] An interpretation of failure of expressed RPE65 to rescue retinal function in this latter dog might be photoreceptor or retinal pigment epithelium pathology had progressed to such an extent that it was no longer possible to achieve rescue. This might represent the upper age at which gene augmentation therapy could be successful in the RPE65-deficient dog. One additional dog, four years of age at treatment, received bilateral injections and reportedly showed rescue although no details were given.[135] As the upper age limit at which gene augmentation therapy can be successful in the RPE65-deficient dog has not been clearly delineated we sought to investigate whether retinal function could be restored in older RPE65-deficient dogs. We treated 13 eyes of 9 dogs between 2 and 6 years of age and found rescue of retinal function on

ERG in 12 of 13 eyes and improved vision testing performance in all eyes. This is particularly relevant for the treatment of adult LCA type II patients where retinal pathology is frequently more advanced than in the young RPE65-deficient dogs used in the reported preclinical canine gene augmentation trials.

Table 5.1 Reported gene therapy in RPE65-deficient dogs and (eyes)

		Distribution of ages					
Study	Total number of RPE65-deficient dogs (eyes) given subretinal *AAV-RPE65*	Number of dogs (eyes) first reported in study	0 - 12 mths	1 - 2 yrs	2 - 3 yrs	3 - 4 yrs	4 - 5 yrs
Acland *et al* 2001	3 (3)	3 (3)	3	-	-	-	-
Narfström *et al* 2003a & b, Ford *et al* 2003, *Narfström et al* 2008	11 (11)	11 (11)	8	2	1	-	-
Acland *et al* 2005, Jacobs *et al* 2006	19 (29)	16 (26)	25	1	-	-	-
Jacobson *et al* 2006	16 (29)	16 (29)	29	-	-	-	-
Rolling *et al* 2006, LeMeur *et al* 2007	8 (8)	8 (8)	7	-	1#	-	-
Aguirre *et al* 2007	6 (12)	1 (2)	-	-	-	-	2
Bennicelli *et al* 2008	3 (5)	3 (5)	5	-	-	-	-
Jacobs *et al* 2009	5	0 (0)	-	-	-	-	-
Amado *et al* 2010	6 (12)	6 (12)	12	-	-	-	-
	TOTAL	**85 (96)**	**89**	**3**	**2**	**0**	**2**

Key

= older dog with failed rescue despite detectable RPE65 expression in treated region

5.3 Materials and methods

5.3.1 Animals

Nine crossbred RPE65-deficient dogs from a colony maintained at Michigan State University Comparative Ophthalmology Laboratory were used in this study. All animals were housed under 12:12 hour light–dark cycles and cared for in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research. All procedures performed were approved by Michigan State University's Institutional Animal Care and Use Committee.

5.3.2 Recombinant AAV2/2 construct and subretinal injection

A recombinant AAV2/2 vector was used, containing the human *RPE65* cDNA coding sequence driven by the human *RPE65* promoter (rAAV2/2.h*RPE65*p.h*RPE65*) as previously reported.[164] Injections were performed in the tapetal (central and dorsal) fundus consistent with the previously reported finding of greater ERG improvement in dogs treated in this location compared with those injected in the nontapetal (inferior) fundus.[110] The vector titers were determined by dot blot and the preparation diluted to provide two viral vector concentrations, $1x10^{11}$ and $1x10^{12}$ vg ml^{-1}. In 10 eyes subretinal injections were performed as previously described.[115] In 3 eyes a standard pars plana three-port 23-gauge vitrectomy was performed (Accurus, Alcon, Irvine, CA, USA). The prior vitrectomy made starting the subretinal bleb easier than if no vitrectomy was performed but was not anticipated to alter the outcome of the gene augmentation therapy. Two different volumes of viral construct were administered, 350 and 500 µl. Details of subretinal injections are provided in Table 5.1. All dogs were treated with

topical, subconjunctival and oral anti-inflammatory medications pre- and post-operatively as previously described.

5.3.3 Ophthalmic evaluation and fundus imaging

Ophthalmic examinations including: slit-lamp biomicroscopy with aqueous humor flare scoring, indirect ophthalmoscopy and wide-field fundic imaging (RetCam II, Clarity Medical Systems, Pleasanton, CA, USA) were performed pre-operatively, immediately post-operatively and at all subsequent study time points. Post-operative images were used to calculate subretinal injection bleb size Images that were separated into groups for statistical analysis based on whether the area centralis region of the retina was included or not included within the boundaries of the injection bleb. The position of the area centralis on post-operative images was determined based on the findings of Mowat *et al*.[116]

5.3.4 Electroretinography

To assess rod and cone photoreceptor function ERG tracings were recorded as previously described both pre-operatively and 4 months post-operatively.[178] Briefly the ERG assessment consisted of a dark-adapted intensity series from below response threshold up to a flash intensity of 2.38 log cdS/m^2. This was followed by light adaptation at 30 cd/m^2 for 10 minutes and recording of cone flicker responses to 33Hz of flashes at 0.39 log cdS/m^2 superimposed on a background light of 30 cd/m^2. For comparison of rod photoreceptor responses, the dark-adapted b-wave amplitude at 0.0 log cdS/m^2 was used; a flash intensity

which was below the response threshold for all eyes prior to treatment. The first intensity at which an ERG waveform could be recorded was noted and the amplitude and implicit time of subsequent a- and b-wave responses were measured, shape of waveforms assessed, and intensity: response curves plotted. To evaluate cone photoreceptor responses, the amplitudes of the light-adapted 33 Hz flicker responses were measured. Flicker responses were used because it is possible that the standard rod-suppressing background light might not completely desensitize rods in RPE65-deficient dogs.[181]

5.3.5 Vision testing

Vision testing was performed using a previously validated visual choice-based device consisting of a light-proof box with four possible exit tunnels, one of which is randomly selected to be open per run.[174] Evaluation of individual eyes was performed by placement of an eye mask over the contralateral eye, with trials completed at three different light intensities, bright through dim as previously described. The number of correct exit choices and mean time to exit the device were recorded and averaged over 7 runs at each light intensity. Vision testing was performed pre-operatively and 4 months post-operatively.

5.3.6 Statistical analysis

ERG and vision testing outcomes were analyzed using independent samples t-tests to evaluate for differences between average pre-operative and 4-month post-operative outcomes. Analysis was performed on b-wave amplitudes at 0.0 log cdS/m^2 and 33Hz cone flickers for ERG responses, mean number of correct exit choices and mean time to exit the device for vision

testing outcomes. To assess for correlation between age, bleb size, viral dose and ERG and vision testing outcomes, two-tailed Pearson analysis was performed. The ERG and vision testing results were further compared using a one-way ANOVA between three age groups at the time of treatment; 2-3 years (n=4), 3-5 years (n=5) and 5-6 years (n=4). Data were considered significant at $p < 0.05$.

5.4 Results

5.4.1 Subretinal injection of rAAV2/2.h*RPE65*.h*RPE65*

Details of the subretinal injections are shown in Table 5.2. At the time of injection, the dogs ranged in age from 2 to 6 years. Thirteen eyes of nine RPE65-deficient dogs were injected subretinally with rAAV2/2.h*RPE65*p.h*RPE65*. All injection blebs were created in the superior fundus and the mean proportion of the tapetal fundus occupied by the blebs was 44% (range 7–84%). Twelve of 13 eyes had blebs occupying between 32% and 84% of the tapetal fundus. Technical difficulties in establishment of an initial retinal detachment in the left eye of dog #05-067 resulted in a small bleb occupying only 7% of the tapetal fundus. Five of 13 eyes had bleb boundaries that included the area centralis and in 8 of 13 eyes the area centralis did not lie within the bleb boundaries; there was no significant difference in bleb size between these groups ($p = 0.061$). All injection blebs resolved by 1 week post-operativel

Table 5.2 Subretinal injection details and funduscopy findings

Dog #	Eye	Age at injection (yrs)	Titer (vg/ml-1)	Volume (uL)	Bleb size (proportion of tapetal fundus)	Injection location	Funduscopy (entire study duration)
05-070	OD	2.0	10^{11}	500	0.62	t	ns
06-077	OS	2.1	10^{12}	350	0.32	t	ns
05-069	OD	2.2	10^{11}	500	0.39	t	ns
05-067	OD	2.2	10^{11}	500	0.84	ac	ns
05-022	OD	3.0	10^{11}	500	0.49	ac	1,2
04-245	OD	3.1	10^{11}	500	0.34	t	1,2
03-084	OD	4.4	10^{11}	500	0.37	ac	ns
05-070	OS	4.8	10^{11}	350(v)	0.46	ac	ns
05-067	OS	4.8	10^{11}	500	0.07	t	ns
05-014	OD	5.1	10^{11}	350	0.35	t	1,2,3
04-245	OS	5.2	10^{11}	350	0.42	t	1
05-022	OS	5.3	10^{11}	350 (v)	0.65	ac	ns
03-040	OS	5.9	10^{11}	350 (v)	0.42	t	ns

Key

v = had vitrectomy prior to subretinal injection
t = tapetal fundus
ac = tapetal fundus including the area centralis
ns = no significant changes
1 = pigmentary spots
2 = regions of tapetal hyper-reflectivity (suggestive of retinal thinning)
3 = injection problems

5.4.2 Evaluation for ocular inflammation and fundus changes

Mild aqueous humor flare (graded as 1 on a scale of 1–4) was detected by slit-lamp biomicroscopy post-operatively in all treated eyes for up to one week. There were no vitreal changes that would indicate inflammation detected in any of the eyes. With indirect ophthalmoscopy a small pigmented scar at the subretinal injection site and a 'highwater' mark indicating the bleb boundary were appreciated in all treated eyes. Additionally, within the bleb boundary 4 of 13 eyes developed small pigment spots (Figure 5.1, B2 and D2) and 3 of 13 eyes developed patchy areas of tapetal hyperreflectivity suggestive of retinal thinning (Figure 5.1, B2). Summary findings for treated eyes are recorded in Table 5.2 with representative fundus images shown in Figure 5.1.

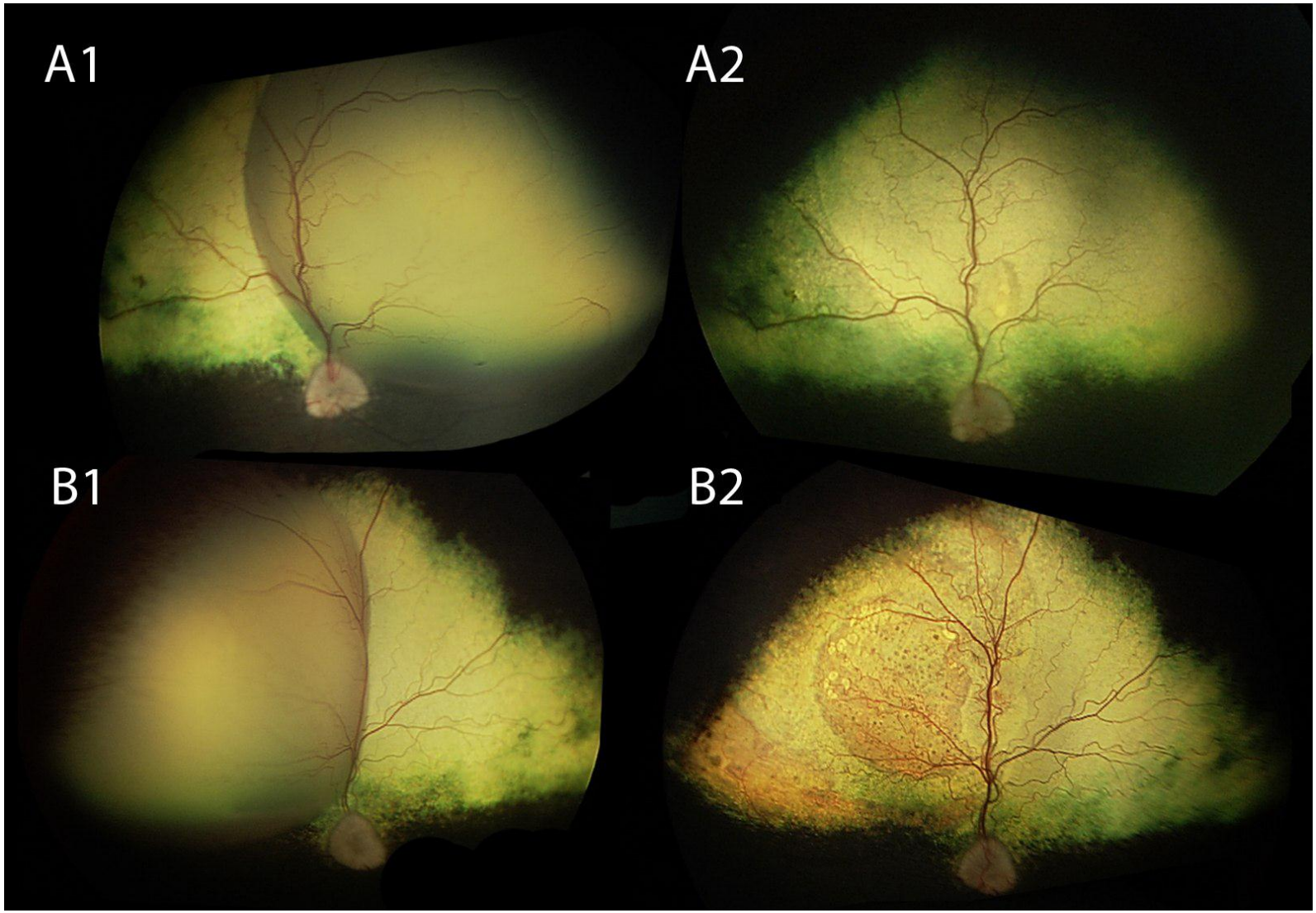


Figure 5.1 **Fundus images after subretinal injection**. Digital fundus images (Retcam II, Clarity Medical Systems) immediately post

injection showing the extent of the subretinal bleb (1), and the fundus appearance 4 months following treatment (2). A: Dog 05-022 OS, large subretinal bleb including the area centralis and no significant fundus changes. B: Dog 05-022 OD, large subretinal bleb that included the area centralis. Patchy tapetal hyper-reflectivity and pigment foci have developed post-injection (B2). C: Dog 05-067 OS, very small subretinal bleb after one previous failed injection attempt. There is obvious scarring at the injection sites 4 month post-injection (arrows in C2). D: Dog 04-245 OS, superior fundus injection that did not include the area centralis. A focus of pigment spots developed adjacent to the optic nerve head (arrow in D2).

Figure 5.1 continued

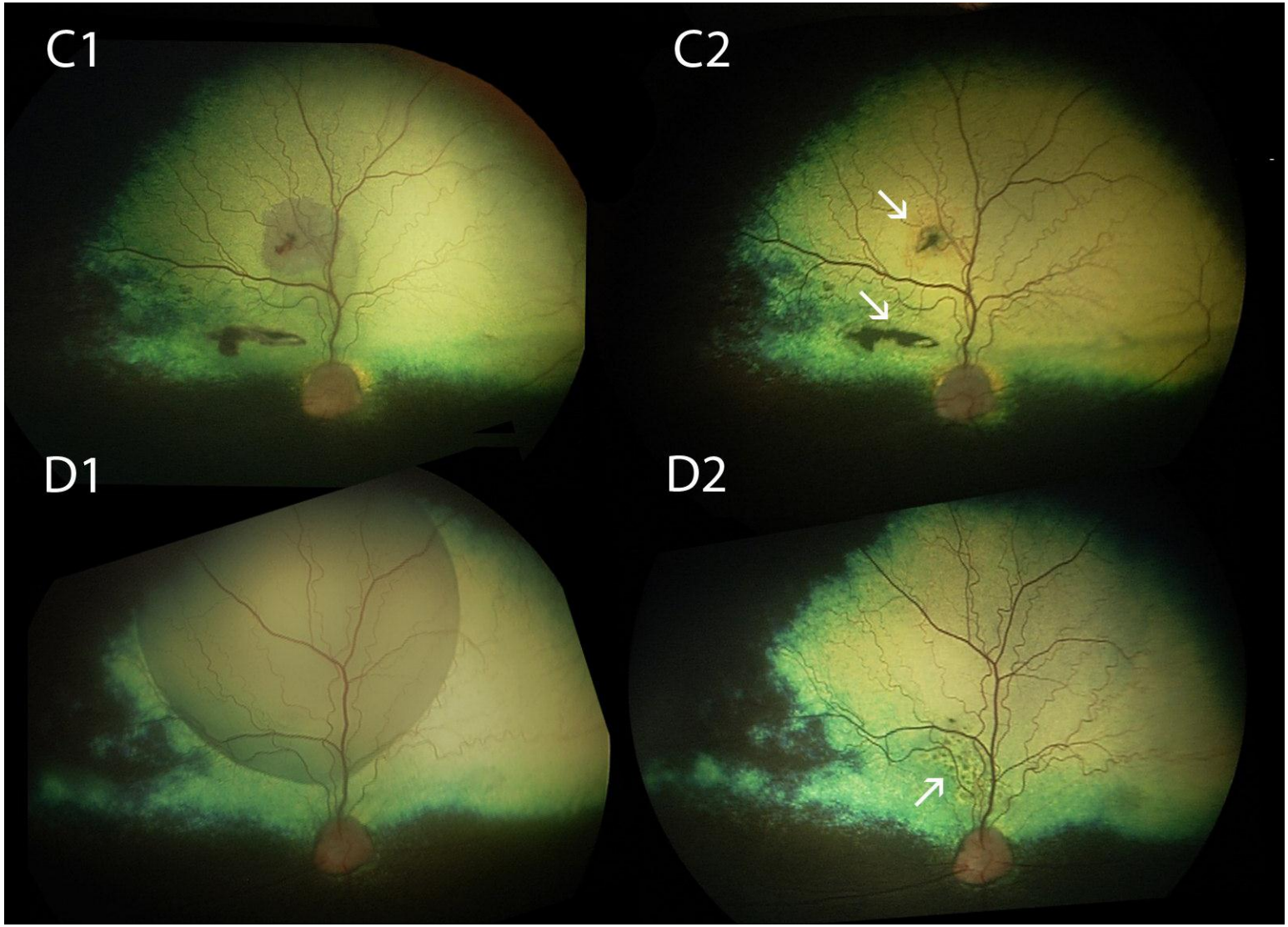


Figure 5.1 continued

5.4.3 Evaluation of retinal function

Retinal function as assessed by dark-adapted full-field flash and light-adapted cone flicker electroretinograms (ERG) showed rescue of rod and cone photoreceptor function in 12 of 13 eyes (Figures 5.2 and 5.3, and Table 5.3). There was significant, lowering of dark-adapted a- and b-wave response thresholds in the 12 eyes with ERG rescue ($p<0.001$) (Figures 5.2 and 5.3). The lowering of threshold ranged in magnitude from 1 to 3 log units (Figure 5.3). The shape and timing of the waveforms at the lower light intensities following treatment were typical for canine rod responses (Figure 5.2). Untreated REP65-deficient dogs never show ERG waveforms of this characteristic shape. The post-operative dark-adapted a- and b-wave amplitudes at 0.0 log cdS/m^2 were significantly increased ($p<0.001$) (Table 5.3). This flash intensity was chosen as an indicator of rod rescue as untreated dogs have no measureable response at this light intensity. Flicker responses were recordable in the 12 eyes with dark-adapted ERG rescue, a significant change, having been unrecordable in any of the eyes preoperatively ($p<0.001$). Light-adapted 33Hz flicker responses were used as a measure of cone function.

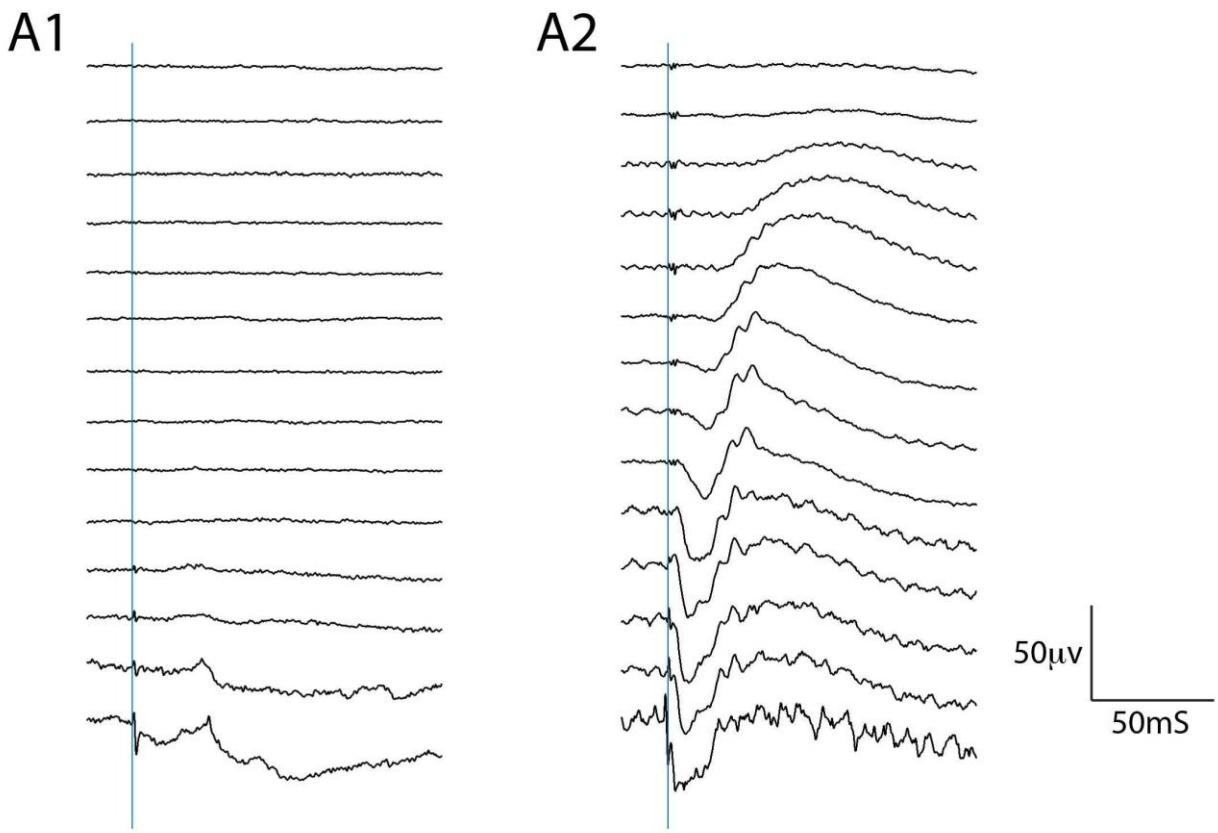


Figure 5.2 Rod and cone ERG responses. Dark-adapted rod ERG montages (A) and light adapted 33Hz flicker responses (B) from dog 05-022 OS (1). Pre-treatment ERG recordings (2). Four months post treatment recordings. Note: dark adapted flash intensities from top to bottom were -3.18, -2.79, -2.41, -2.0, -1.6, -1.19, -0.79, -0.39, 0.0, 0.39, 0.85, 1.36, 1.9, 2.38 log cdS/m^2. Blue lines indicate timing of light flashes

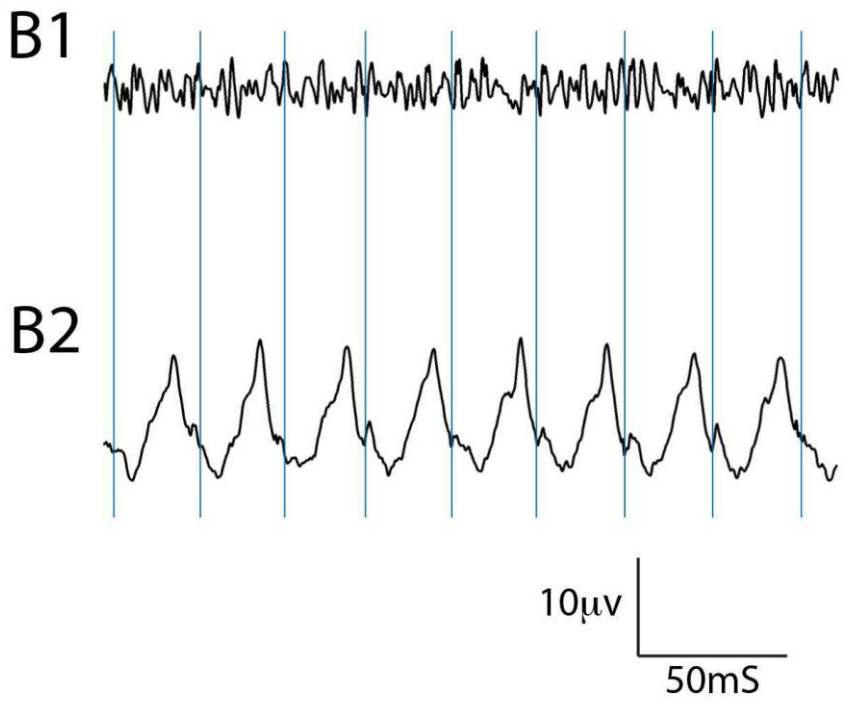


Figure 5.2 continued

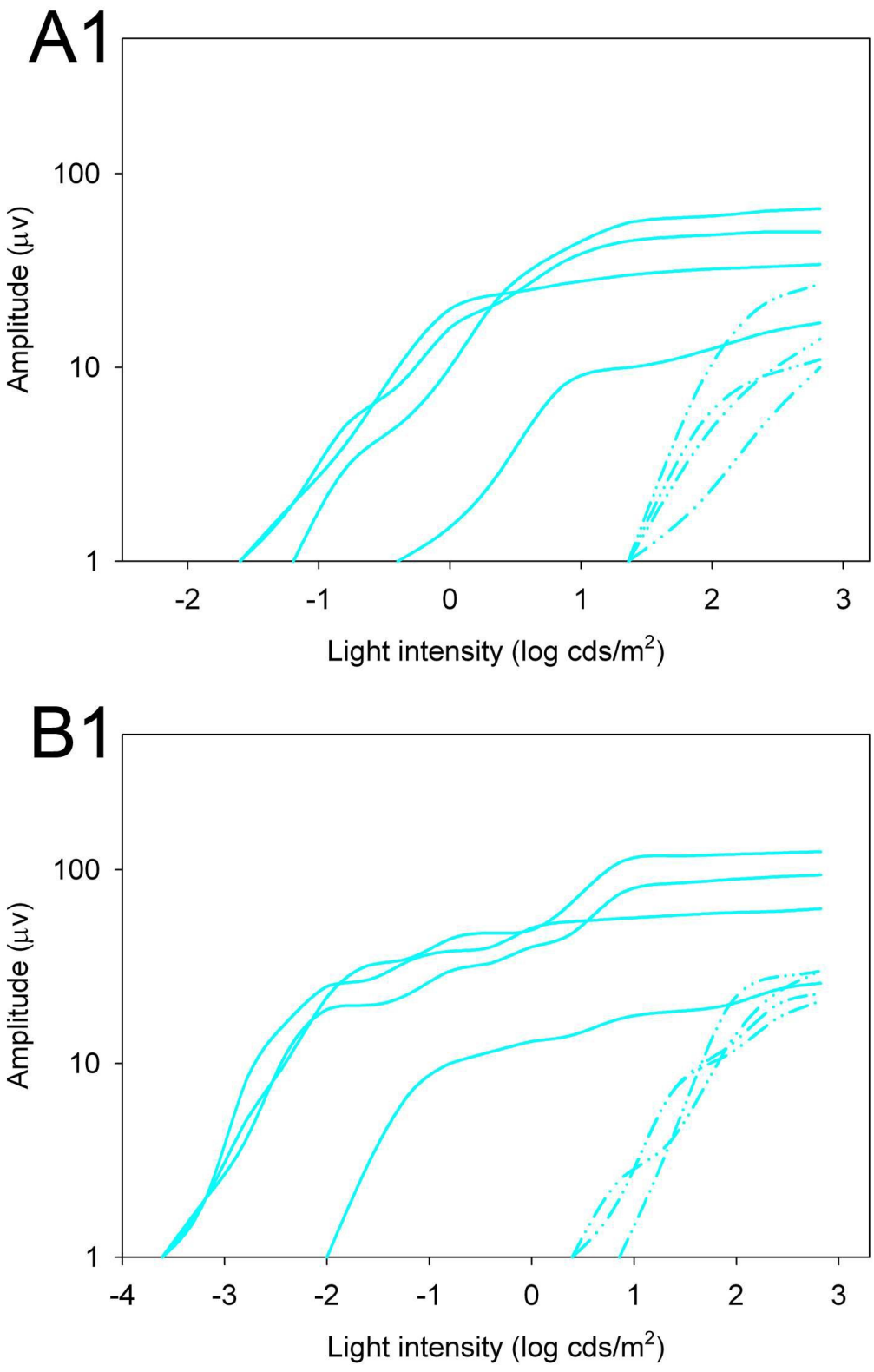


Figure 5.3 **Dark-adapted ERG intensity response series.** Dark-adapted intensity response

series of the a-waves (A) and b-waves (B) displayed on a log:log scale with each tracing representing a single dog pre- and post-treatment. Dashed lines = pre-treatment, solid lines = 4 months post-treatment. (1): Eyes treated at 2-3 years of age. (2): Eyes treated at 3-5 years of age. (3): Eyes treated at 5-6 years of age. Note the single eye in A3and B3 (04-245 OS) with the lack of improvement in a- and b-wave ERG threshold

Figure 5.3 continued

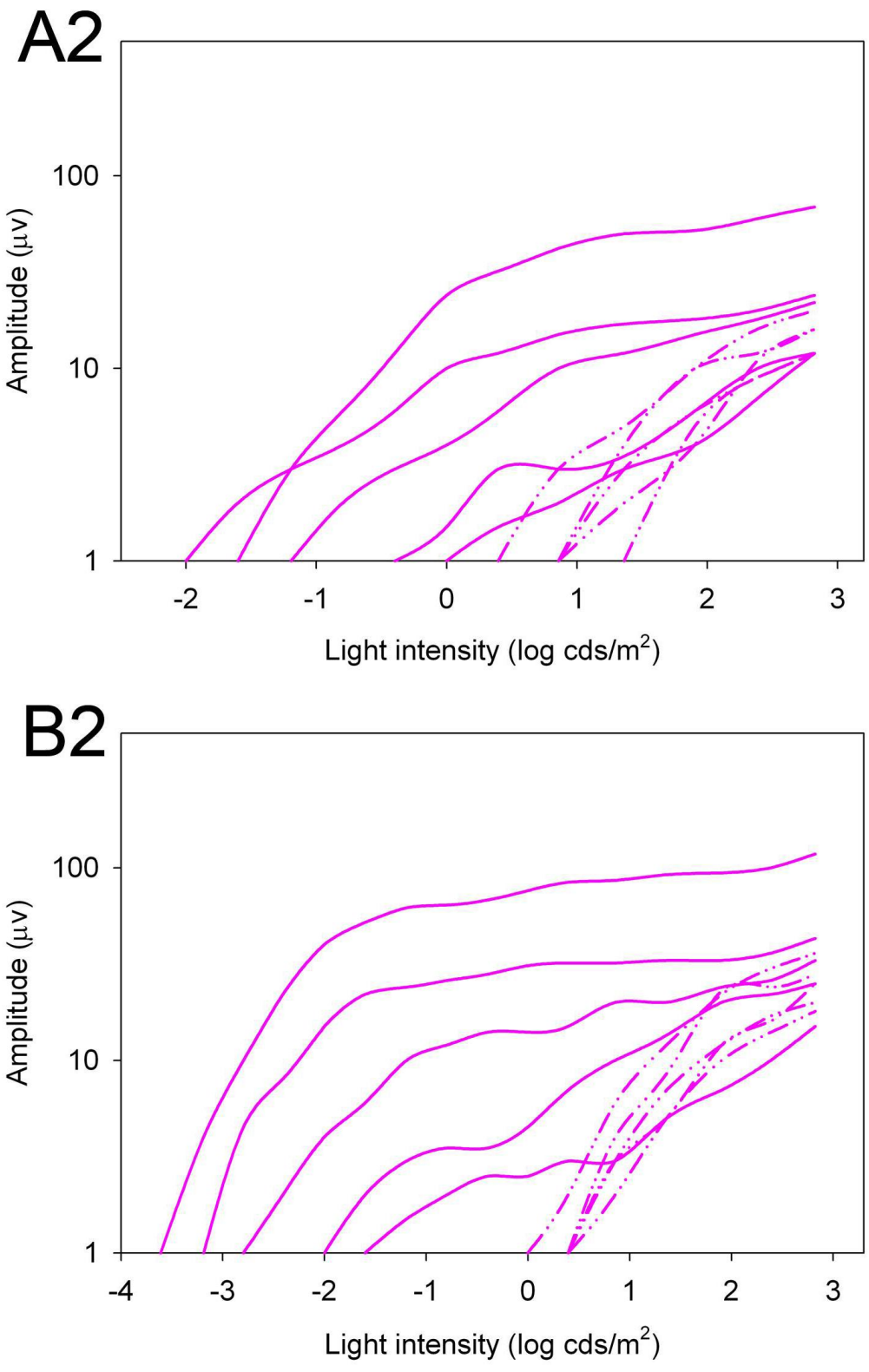
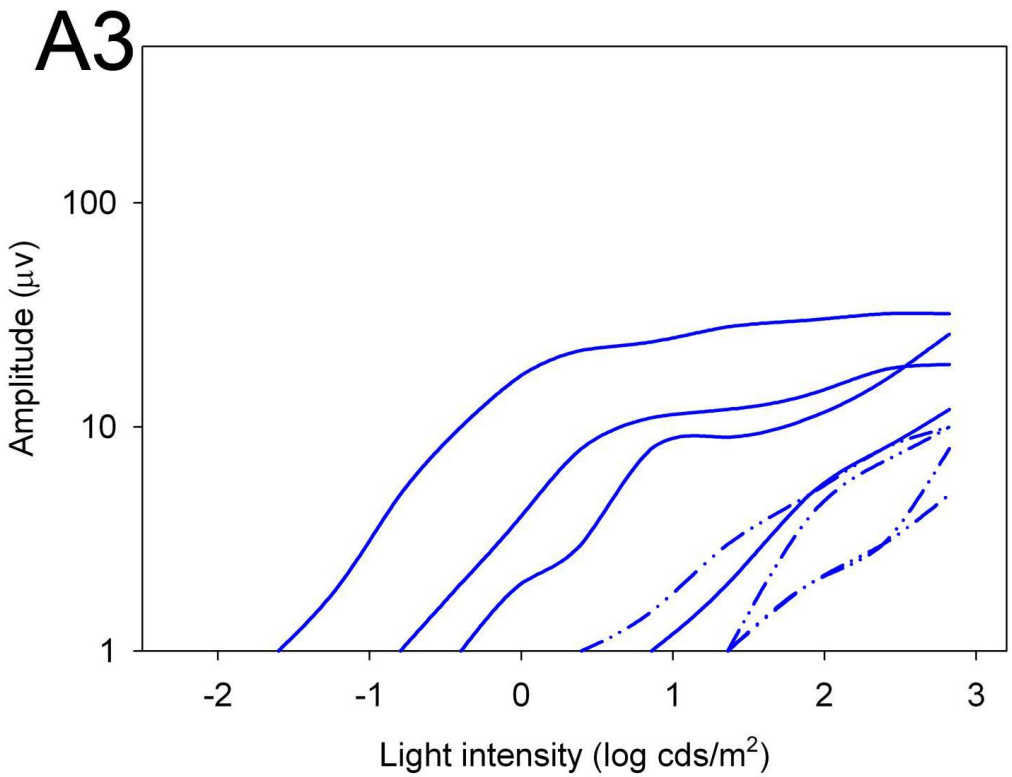


Figure 5.3 continued



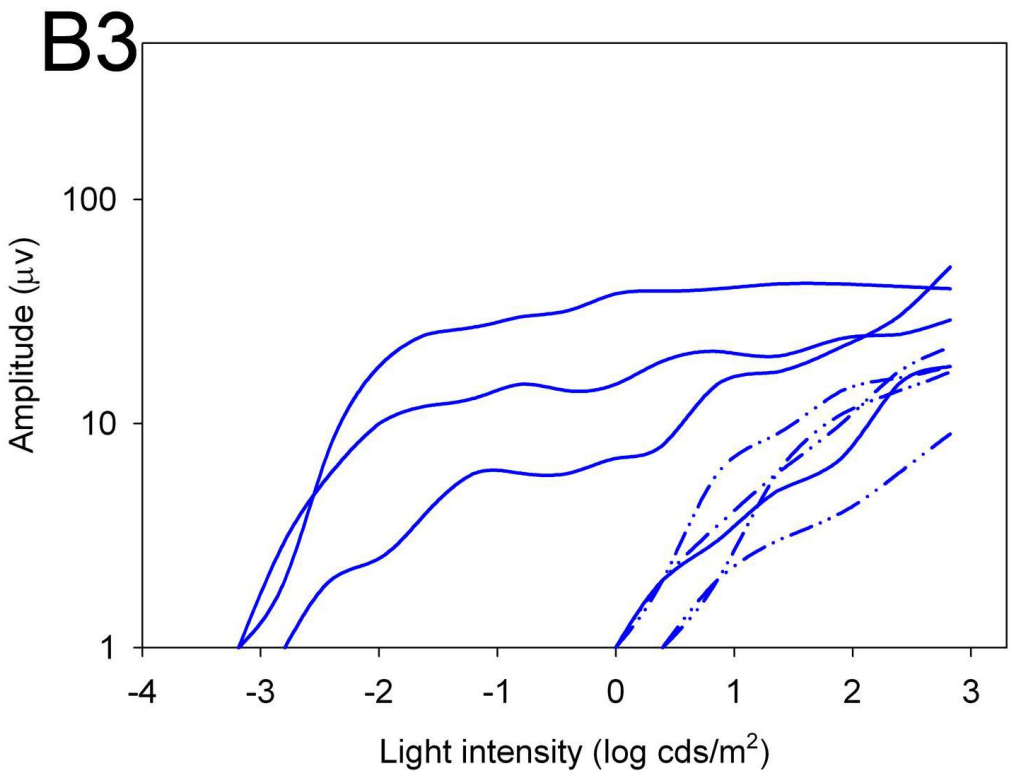


Figure 5.3 continued

Table 5.3 Overview of ERG outcomes

			Electroretinography			
			b-wave at 0.0 cdS/m^2		33 Hertz cone flicker	
Dog	Eye	Age at injection (yrs)	pre-injection	4mths post-injection	pre-injection	4mths post-injection
05-070	OD	2.0	nr	40	nr	12
06-077	OS	2.1	nr	13	nr	3
05-069	OD	2.2	nr	50	nr	12
05-067	OD	2.2	nr	49	nr	18
05-022	OD	3.0	nr	14	nr	7
04-245	OD	3.1	nr	4.5	nr	7
03-084	OD	4.4	nr	31	nr	8
05-070	OS	4.8	nr	76	nr	15
05-067	OS	4.8	nr	2.5	nr	4
05-014	OD	5.1	nr	7	nr	3
04-245	OS	5.2	nr	nr	nr	nr
05-022	OS	5.3	nr	38	nr	12
03-040	OS	5.9	nr	16	nr	4

Key
nr = not recordable

For further analysis treated eyes were divided into three groups: 2-3 years (n=4), 3-5 years (n=5) and 5-6 years (n=4), and ERG outcomes compared. There was no significant difference in rod or cone ERG measurements between age groups (p values for amplitude and threshold ranged from 0.18 to 0.47). We also investigated whether the ERG outcomes correlated with a number of variables including age at injection, size of the injection bleb, area centralis included within bleb boundary, viral genomes administered, volume of injection, and titer injected. Amplitude of the cone flicker responses significantly correlated with both area centralis included within bleb boundary ($p = 0.02$) and larger bleb size ($p = 0.008$) (Table 5.4). Although not reaching significance, a trend is suggested for correlation of b-wave amplitude at 0.0 log

cdS/m^2 with both area centralis included within the bleb boundary ($p = 0.053$) and larger bleb size ($p = 0.068$). No other significant correlations or trends were appreciated (Table 5.4).

One of 13 treated eyes (04-245 OS) failed to show evidence of ERG rescue. There was no obvious reason for failed rescue; there was a relatively large injection bleb and only small pigment foci within the bleb boundary noted on ophthalmoscopy (Figure 5.1, D2). This dog was 5.2 years of age at time of injection; the other eye had been previously treated (at 3.1 years of age) and had shown moderate improvement in ERG measures (Table 5.3). It is notable that despite the lack of ERG rescue, this eye had improved dim-light vision (see below).

Table 5.4 Variables evaluated for correlation with ERG outcomes

	Electroretinography					
	Dark adapted b-wave threshold		Dark adapted b-wave amplitude at 0 cdS/m^2		33 Hertz cone flicker amplitude	
	Pearson's correlation coefficient	p-value	Pearson's correlation coefficient	p-value	Pearson's correlation coefficient	p-value
Age at injection (years)	0.28	0.35	-0.22	0.47	-0.39	0.18
Vitrectomy (yes/no)	-0.18	0.56	0.42	0.15	0.24	0.43
Total vector dose (vg)	-0.09	0.75	-0.17	0.58	-0.26	0.39
Vector titer (vg/ml)	-0.07	0.82	-0.17	0.58	-0.28	0.34
Injection volume (uL)	-0.29	0.32	0.05	0.87	0.34	0.25
Bleb size (proportion of tapetum)	-0.019	0.95	0.52	0.068 *	0.69	0.008 **
Injection site included a/c (yes/no)	-0.25	0.40	0.55	0.053 *	0.60	0.02 **
Areas of tapetal h-r (yes/no)	-0.16	0.61	-0.44	0.13	-0.26	0.39

Key
a/c = area centralis
h-r = hyper-reflectivity
* = approaching a significant correlation
** = significant correlation

5.4.4 Evaluation of vision

Using an objective testing device vision we assessed two outcome parameters, exit choice and time to exit, under standard room light and at two dim-light intensities. Preoperatively under standard room light all dogs correctly and rapidly and exited the device, but at both dim lighting intensities all dogs were effectively choosing exits randomly and were very slow to exit the device. Four months post-operatively vision testing under room light remained unchanged, but at both dim-light intensities 13 of 13 dogs showed significant improvement in both correct exit choice (p values <0.001) and time to exit (p values <0.001) (Figure 5.4). Outcomes pre-treatment and 4-6 months post-treatment at the dimmest light intensity are also displayed for each treated eye in Table 5.5, showing the left eye of Dog # 04-245 with no recordable ERG improvement had a similar improvement in visual function to the other treated eyes.

There was no significant difference between the three age groups: 2-3 years (n=4), 3-5 years (n=5) and 5-6 years (n=4) for either correct exit choice (p = 0.83) or time to exit (p = 0.73) (Table 5.6). However, area centralis included within the bleb boundary correlated significantly with both correct exit choice (p = 0.01) and time to exit (p = p = 0.02) (Table 5.6).

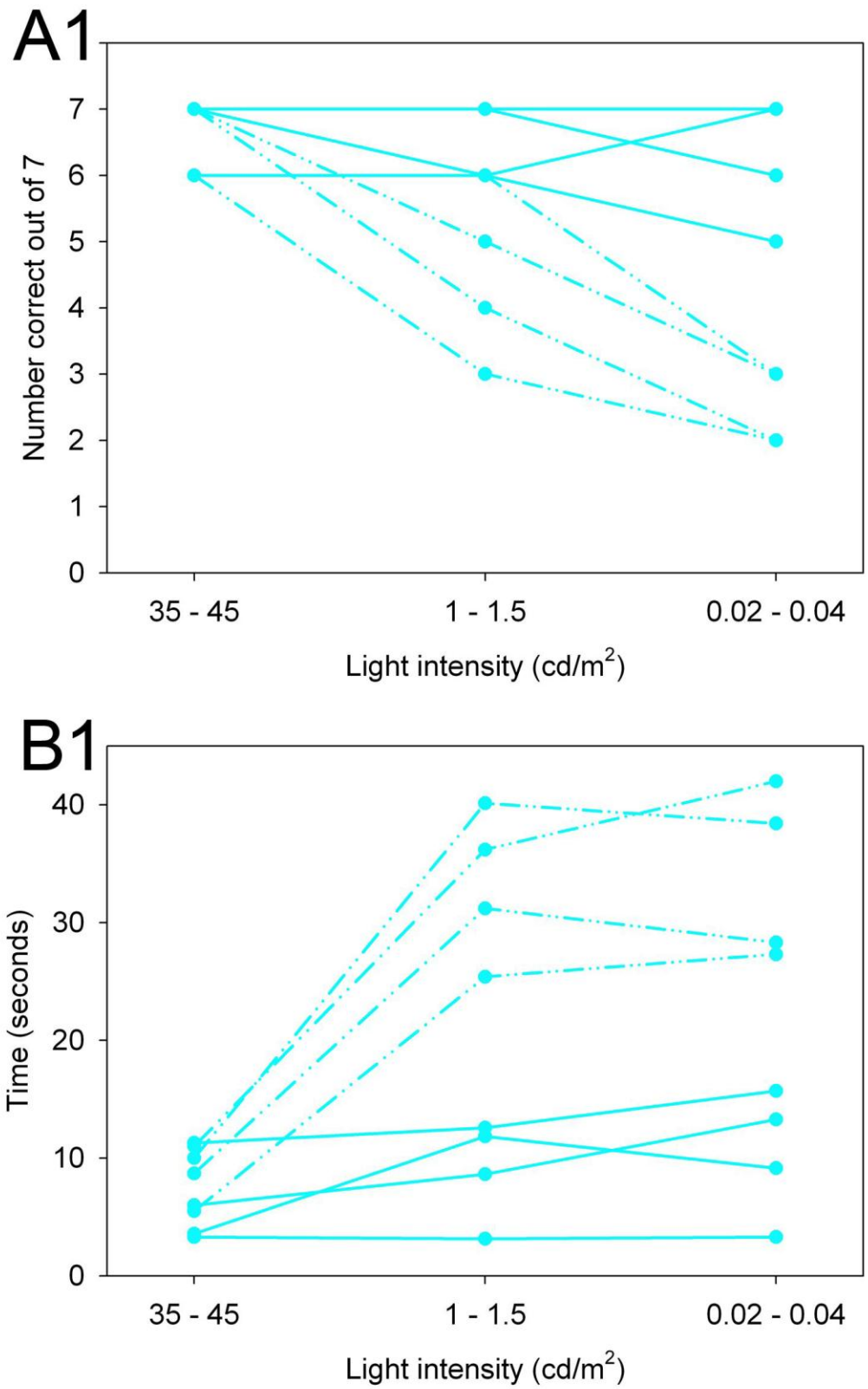
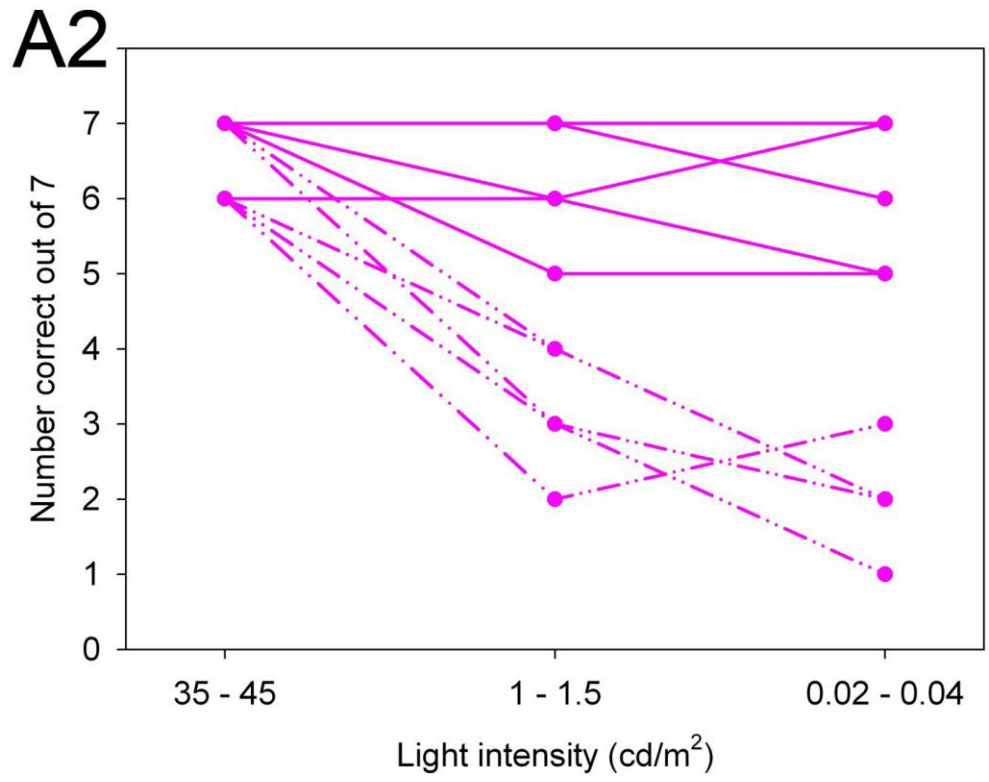


Figure 5.4 **Vision testing outcomes**. Vision testing results at three light intensities showing

the two outcome measures, number of correct exit choices of seven trials (A), and time to exit the device (B). Pre-treatment results are dashed lines and 4 months post-treatment are solid lines for each individual eye. (1): Eyes treated at 2-3 years of age. (2): Eyes treated at 3-5 years of age. (3): Eyes treated at 5-6 years of age. Note that prior to treatment there was a poor performance for both measures of outcome at the two lower light intensities. All dogs in all age groups showed an improvement in both measures of visual function after treatment.

Figure 5.4 continued



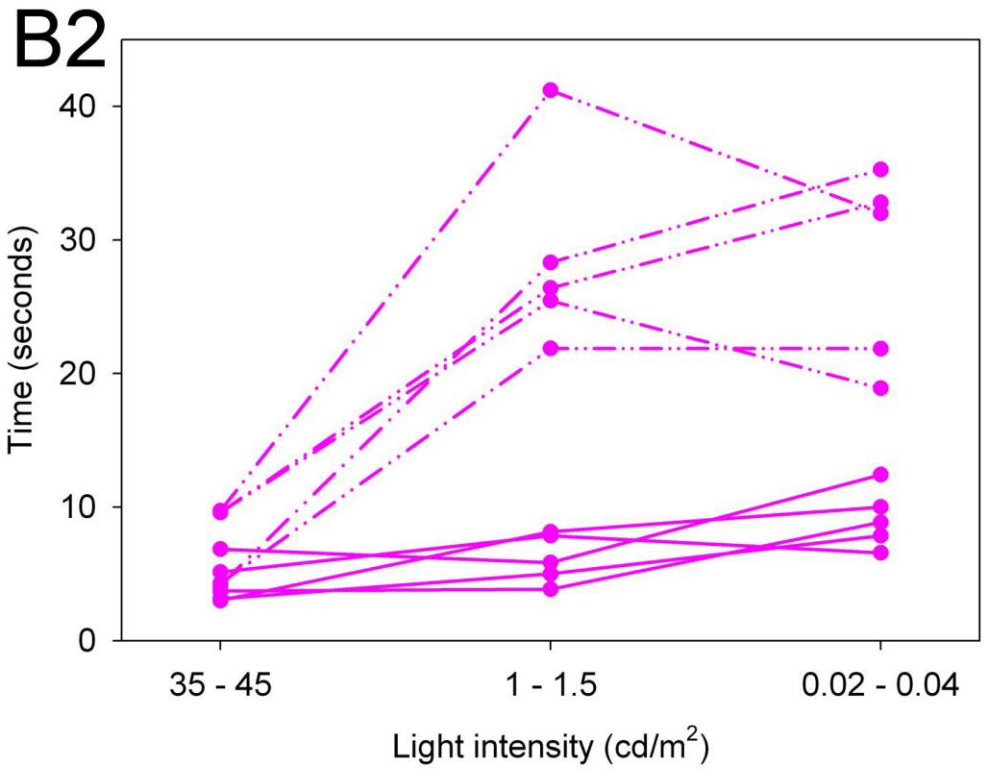


Figure 5.4 continued

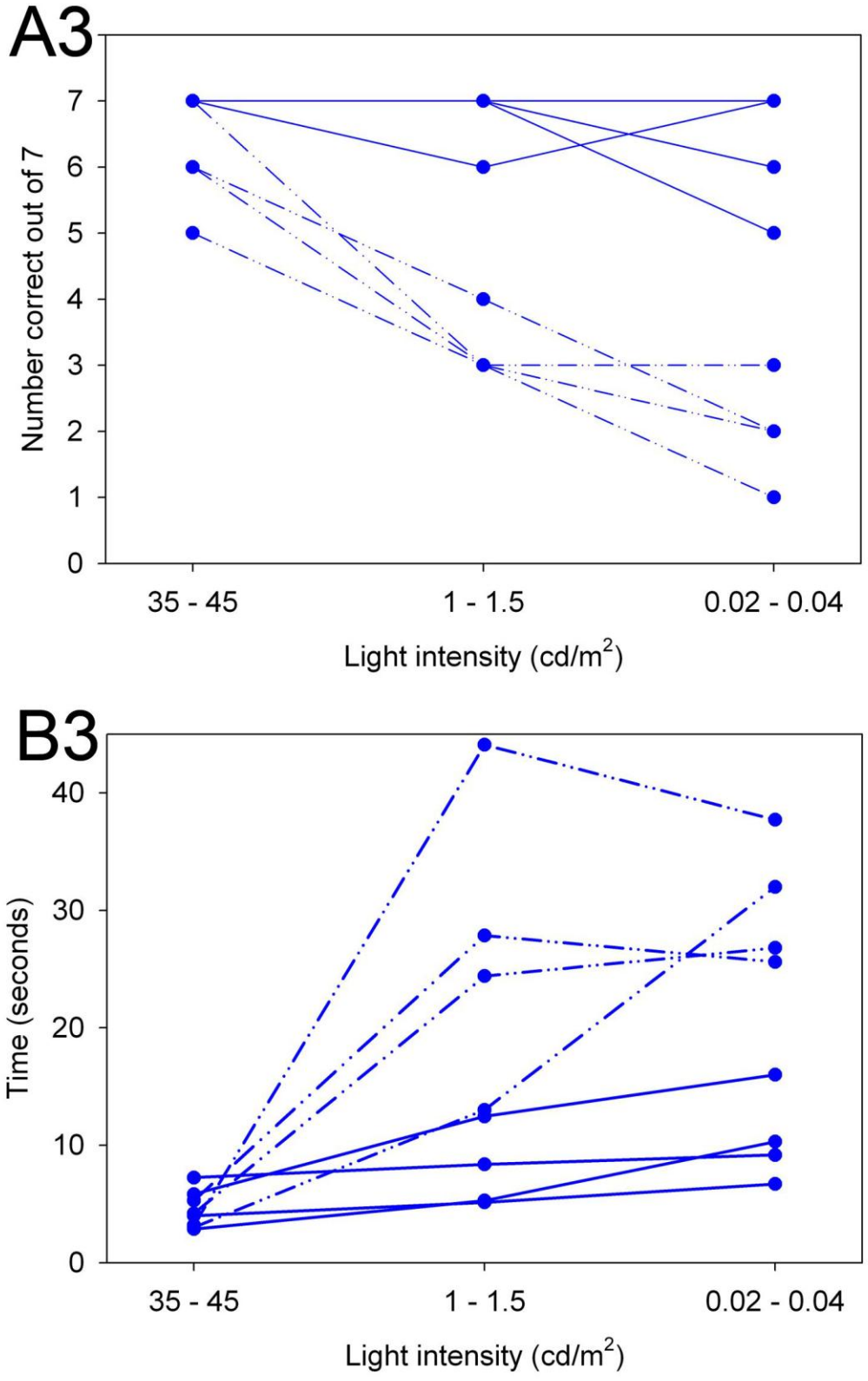


Figure 5.4 continued

Table 5.5 Overview of vision testing outcomes

			Vision testing (at 0.02-0.04 cd/m^2)			
			Correct exit (of 7 trials)		Time to exit (seconds)	
Dog	Eye	Age at injection (yrs)	pre-injection	4-6mths post-injection	pre-injection	4-6mths post-injection
05-070	OD	2.0	3	6	39	15.7
06-077	OS	2.1	3	7	27.3	9.1
05-069	OD	2.2	3	5	28.3	13.2
05-067	OD	2.2	3	7	42	5.4
05-022	OD	3.0	2	7	18.9	6.6
04-245	OD	3.1	3	5	21.8	10
03-084	OD	4.4	1	7	35.2	12.4
05-070	OS	4.8	3	7	29.0	8.3
05-067	OS	4.8	3	6	17.8	8.9
05-014	OD	5.1	1	5	37.7	11.0
04-245	OS	5.2	2	5	30.5	10.3
05-022	OS	5.3	2	7	25.6	5.1
03-040	OS	5.9	3	7	26.3	11.6

Key

nr = not recordable

Table 5.6 Variables evaluated for correlation with vision testing outcomes

	Vision Testing			
	Correct exit choice (of 7 trials)		Time to exit (seconds)	
	Pearsons correlation coefficient	p-value	Pearsons correlation coefficient	p-value
Age at injection (years)	0.03	0.91	-0.16	0.61
Vitrectomy (yes/no)	0.47	0.1	-2.9	0.32
Total vector dose (vg)	0.25	0.42	-0.05	0.87
Vector titer (vg/ml)	0.24	0.41	-0.06	0.82
Injection volume (uL)	-0.11	0.72	0.19	0.52
Bleb size (proportion of tapetum)	0.16	0.59	-0.34	0.26
Injection site included a/c (yes/no)	0.68	0.01 **	-0.62	0.02 **
Areas of tapetal h-r (yes/no)	-0.35	0.24	-0.08	0.78

Key
a/c = area centralis
h-r = hyper-reflectivity
** = significant correlation

5.5 Discussion

In this study we found that gene augmentation therapy using a rAAV2 vector expressing human *RPE65* under control of the human *RPE65* promoter improved visual function in 13 of 13 eyes of RPE65-deficient dogs that were treated at between 2 and 6 years of age. Functional rescue was still possible in these dogs despite the progressive loss of photoreceptors and accumulation of lipid droplets in the retinal pigment epithelium that is known to develop with age in this model.[127, 128]

Prior to this study, gene augmentation therapy results in only four RPE65-deficient dogs older than 2 were reported. Results were mixed, with rescue achieved in 4 of 5 eyes. The one eye which failed to show either ERG or vision testing rescue from a dog injected at 2.5 years of age was subsequently shown by immunohistochemistry to have appropriate *RPE65* transgene expression in the RPE suggesting failure was not due to technical injection issues or failure of RPE transduction by the viral vector.[156, 157] With only one dog treated older then 2.5 years, this treatment failure could indicate that gene therapy outcomes may be less successful in older RPE65-deficient dogs. However the results of our current study suggest that this is not the case; with all eyes treated showing improvement in vision at dim-light levels using an objective vision testing measure. Furthermore, 12 of the 13 eyes treated had an improvement in both rod and cone function as assessed by ERG; with lowering of dark-adapted response thresholds, the appearance at the lower flash intensities of waveforms with a shape typical of rod-mediated

responses, increases in waveform amplitudes and recordable cone flicker responses (prior to therapy cone flicker responses were not recordable from these eyes).

In one of the older dogs in the current study (aged 5.2 years at the time of injection) there was no detectable rescue of ERG function, although dim-light vision was improved. This eye had one of the larger subretinal injection blebs and it did not develop any funduscopic lesions in the injection area that would indicate a degenerative process as a result of the injection. It may be that in this dog there were not enough photoreceptors with restored function to generate a recordable electrical response. Clearly it requires fewer photoreceptors to make a detectable difference in visual function compared to the number required to make a difference in recordable ERG. This can be demonstrated in dogs with retinal degenerative conditions that have useful vision and yet an ERG response is not recordable (Petersen-Jones personal observations). There is a similar finding in the RPE65-LCA patients treated by gene augmentation therapy, where even in those patients with the greatest improvement in visual function the full-field flash ERG was not improved.[163-165] The findings from this single dog in our study may indicate this age is approaching the upper limit at which ERG rescue is possible, for our colony at least, and the use of RPE65-deficient dogs older than 6 years of age for testing gene augmentation therapy may more closely mimic the challenges that are faced in treating human patients. Although important for proof-of-concept and other preclinical studies, the use of young RPE65 deficient dogs does not accurately mimic the situation faced in

the clinics where patients with low numbers of remaining potentially responsive photoreceptors are being treated.

It is important to note that we did not find a correlation of ERG outcomes (b-wave amplitude at 0.0 log cdS/m^2 and cone flicker amplitudes) with age, the only significant correlations with ERG outcomes being whether the area centralis was included in the injected area and the proportion of the retina injected. The correlation of positive ERG outcomes with inclusion of the area centralis in the treated area may be because the central retina in the normal canine has a greater number of photoreceptors per unit area than the peripheral retina. Also previous studies have shown that in RPE65-deficient dogs the photoreceptors of the peripheral retina are completely degenerate by between 5 and 7 years of age.[116, 128] It is also possible that although the ERG stimulus used a Ganzfeld bowl and the pupils were fully dilated, the degree of light stimulation of the central retina might still be greater than that of the peripheral retina. The lack of correlation of outcome with age was unexpected as the older dogs will have lower numbers of surviving photoreceptors, but we should consider here the relatively low numbers of dogs used and a possible masking effect of other variables such as dose, volume injected, size of the subretinal injection and precise region of the retina treated (it is difficult to control the spread of the subretinal injection bleb and therefore difficult to standardize between animals).

The treatment itself is not without potential deleterious effects and indeed ophthalmoscopically detectable fundus changes developed in the region of the subretinal injection bleb in some animals. These included patchy tapetal hyperreflectivity, which is a clinical indication of retinal thinning, and development of pigment spots that possibly indicated changes in the retinal pigment epithelium. Patchy retinal thinning was recorded in a previous gene augmentation therapy trial in RPE65-deficient dogs.[157] The current generation of viral vectors used in retinal gene therapy require delivery by subretinal injection to transduce the retinal pigment epithelium. Creating a retinal detachment has deleterious effects on the retina with studies indicating that there is significant induction of cytokines associated with apoptotic pathways within 72 hours of retinal detachment.[182, 183] It is not clear whether the retinal changes that developed in the injected area are the result of the physical detachment of the retina or a result of the viral vector and expression of the transgene or a combination of both. Patchy retinal degeneration has also been recorded in a reporter gene study in normal dogs, and developed only in the eyes receiving the highest titers; the timing of the degeneration suggested that this was an immune reaction to either the high levels of the expressed transgene (green fluorescent protein) or to the high concentration of AAV particles.[184]

This study adds information on the outcome of gene augmentation therapy in older RPE65-deficient dogs, adding 13 eyes of dogs over 2 years of age to the 5 recorded in the literature. More importantly it shows that the use of older RPE65-deficient dogs in gene augmentation therapy will be valuable in investigating the factors that limit the success of therapy, such as the numbers of remaining photoreceptors, and the effect of lipid droplet accumulation in the RPE.

Another important feature that makes the canine RPE65-deficient model particularly relevant is the presence in the dog of an *area centralis* and visual streak. These retinal regions have greater photoreceptor densities compared to the peripheral retina.[116] This more closely reflects the photoreceptor distribution in humans than murine models that do not have regions of higher photoreceptor density.

CHAPTER 6

Gene therapy in the second eye of RPE65-deficient dogs improves retinal function

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Gene Therapy (2011) 18, 53-61

6.1 Abstract

The purpose of this study was to evaluate whether immune responses interfered with gene therapy rescue using subretinally delivered recombinant adeno-associated viral vector serotype 2 carrying the *RPE65* cDNA gene driven by the human RPE65 promoter (r*AAV2.hRPE65p.hRPE65*) in the second eye of *RPE65* -/- dogs that had previously been treated in a similar manner in the other eye. Bilateral subretinal injection was performed in nine dogs with the second eye treated 85-180 days after the first. Electroretinography (ERG) and vision testing showed rescue in 16 of 18 treated eyes, with no significant difference between first and second treated eyes. A serum neutralizing antibody (NAb) response to rAAV2 was detected in all treated animals, but this did not prevent or reduce the effectiveness of rescue in the second treated eye. We conclude that successful rescue using subretinal r*AAV2.hRPE65p.hRPE65* gene therapy in the second eye is not precluded by prior gene therapy in the contralateral eye of the *RPE65* -/- dog. This finding has important implications for the treatment of human LCA type II patients.

6.2 Introduction

Leber congenital amaurosis is a severe early-onset inherited form of retinal degeneration that shows genetic heterogeneity. The estimated prevalence of LCA in the North American Population is 1: 81,000.[30] The condition is characterized by severe visual impairment in dim light, typically progressing to complete blindness in the second decade of life. LCA type II results from mutations in the *RPE65* gene, and accounts for 10–15% of LCA cases.[43, 185] *RPE65*

encodes a protein that forms an essential component of the visual cycle and is expressed within the retinal pigment epithelium.[43] The visual cycle is responsible for the supply of the chromophore, 11-*cis* retinal, to the photoreceptor cells for combination with the rod and cone opsins to form the visual pigments. RPE65 is an isomerohydrolase that converts esters of vitamin A to 11-*cis* retinol for subsequent oxidation to 11-*cis* retinal prior to transport to the photoreceptors. A spontaneous 4 basepair deletion in *RPE65* in the Briard breed of dog results in a premature stop codon and an absence of *RPE65* gene product, resulting in a very similar phenotype to LCA type II.[124] Affected dogs have markedly reduced vision and an abnormal electroretinogram with greatly elevated threshold of responses.[124, 125] The similarities between the human and canine disease resulting from *RPE65* mutations, make the *RPE65* -/- Briard a valuable large animal model for LCA type II.

Dramatic restoration of vision with gene therapy was first reported in the canine *RPE65* -/- model of LCA.[114] A number of studies have shown rod and cone photoreceptor rescue using rAAV vectors to deliver a normal copy of the *RPE65* gene via a subretinal injection in the RPE65-deficient Briard.[110, 114, 126, 133, 153, 186-188] Based on the great success of the canine trials, phase I/II clinical trials of rAAV-*RPE65* gene replacement therapy in human LCA patients have started with the first reported results showing great promise.[163, 189, 190] Thus far in all human patients only one eye has been treated. A critical aspect of the management of LCA type II individuals will be the ability to achieve rescue in the second eye. There are concerns

that immune responses to the viral capsid and transgene may limit rescue achieved by repeated administration.

Immune responses following rAAV-mediated gene delivery have been analyzed in several detailed studies in animal models, but have generated some contradictory reports and remain inconclusive, with immune responses appearing to depend on the route of administration, vector dose and species differences.[191] There are conflicting reports on the success of repeated gene therapy in non-ocular tissues. In some studies re-administration of rAAV at later time points was less successful than the initial administration because of neutralizing antibodies (NAb) to the viral capsid proteins.[192-195] Serotype switching and transient immunosuppression have been used to try and overcome this obstacle.[193, 194] Other studies have reported additional transduction events and successful transgene expression after readministration of rAAV, despite the presence of serum NAb to the vector.[196, 197] In ocular tissues, the site of vector administration is reported to impact on the degree of immune interference with subsequent rAAV administration.[198] A study evaluating bilateral intravitreal rAAV2 injections 1 month apart in C57BL/6J mice found that the second eye had very poor reporter gene expression compared with the first treated eye.[173] In contrast, the immune privilege of the subretinal space appears to allow for successful readministration. Subretinal injection of rAAV to the contralateral eyes of two previously treated monkeys achieved reporter gene expression at an equivalent level to that seen in the first treated eye.[199] Similarly in the *Rpe65*$^{rd12/rd12}$ mouse, administration of rAAV-*RPE65* to the

subretinal space of the second eye also achieved rescue similar to that obtained in the first eye.[172] In addition, prior intravitreal injection of the first eye did not interfere with the degree of reporter gene expression achieved by subretinal injection in the second eye of C57BL/6J mice.[200]

Short duration of rAAV-mediated transgene expression in a number of clinical trials has been linked to the very high level of pre-exposure to wild type AAV in the human population.[201] However, a much more limited systemic response to vector has been observed when rAAV was administered to the brain, which is an immune privileged site, with no anti-vector antibodies detected in the CNS itself and only a minority of patients developing circulating Nab.[202] The eye is also a site of immune privilege, and thus is a good target for gene delivery. Three clinical trials for LCA type II have been initiated, in which one eye of each patient has been treated with rAAV2. All three trials have shown the vector to be well tolerated, with no evidence of antibody responses against the *RPE65* gene product in any patient, and only limited transient NAb responses against the rAAV2 capsid in a minority of patients.[163, 189, 190]

Establishing if repeat subretinal injection of rAAV-*RPE65* can achieve rescue in the second eye of *RPE65* -/- dogs is required prior to including treatment of the second eye in human clinical trial protocols. Previous studies have reported bilateral subretinal injections in *RPE65* -/- dogs but these have mostly been performed concurrently. Acland *et al* reported bilateral subretinal rAAV2-*RPE65* injections in nine dogs, of these only one dog (BR29 in Table 1) had the second

eye injected at a later date and outcome for this eye was not shown.[110] Bennicelli *et al* reported bilateral subretinal injections of rAAV-*RPE65* in two dogs, but both eyes were treated at the same time.[126] In this study, we sought to investigate the rescue achieved in the second eye by gene replacement therapy in *RPE65* -/- dogs that had previously had the same vector construct administered by subretinal injection in the contralateral eye. We report that successful rescue in the second treated eye of *RPE65* -/- dogs occurs at a level comparable with that achieved in the first eye. This finding has important implications for the treatment of human LCA type II patients.

6.3 Materials & Methods

6.3.1 Subjects

Nine *RPE65* -/- dogs were used (Table 6.1), from a colony maintained at Michigan State University Comparative Ophthalmology Laboratory. All animals were housed under 12:12 hour light:dark cycles and cared for in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Procedures performed were approved by Michigan State University's Institutional Animal Care and Use Committee.

Table 6.1 Overview of all dogs treated and details of subretinal injections

Dog	Sex	Eye injected	Age at injection (days)	Titer (vg/ml)	Volume (µl)	Total Dose (vg)	Proportion of tapetal fundus injected	Post injection ocular changes
1	F	First (a)	179	10^{11}	250	2.5×10^{10}	0.11	none
		Second (b)	271	10^{11}	500	5×10^{10}	0.43	none
2	F	First (a)	172	10^{11}	250	2.5×10^{10}	0.44	none
		Second (b)	263	10^{11}	500	5×10^{10}	0.37	tapetal hyper-reflectivity
3	M	First (a)	179	10^{11}	250	2.5×10^{10}	0.45	none
		Second (b)	271	10^{11}	500	5×10^{10}	0.48	mild tapetal hyper-reflectivity
4	F	First (a)	172	10^{11}	250	2.5×10^{10}	0.06	majority choroidal injection
		Second (b)	263	10^{11}	500	5×10^{10}	0.43	none
5	M	First (a)	137	10^{11}	250	2.5×10^{10}	0.53	mild tapetal hyper-reflectivity
		Second (b)	221	10^{11}	500	5×10^{10}	0.49	none
6	M	First (a)	137	10^{11}	250	2.5×10^{10}	0.55	none
		Second (b)	221	10^{11}	500	5×10^{10}	0.62	none
7	M	First (a)	574	10^{12}	500	5×10^{11}	0.52	mild tapetal hyper-reflectivity
		Second (b)	755	10^{12}	250	2.5×10^{11}	0.33	none
8	M	First (a)	176	10^{12}	500	5×10^{11}	0.17	none
		Second (b)	359	10^{12}	250	2.5×10^{11}	0.24	cataract

9	M	First (a)	176	10^{12}	500	5×10^{11}	0.46	none
		Second (b)	357	10^{12}	250	2.5×10^{11}	0.44	none

Table 6.1 continued

6.3.2 rAAV2/2 construct and subretinal injection

A recombinant AAV2/2 vector was used, containing the human *RPE65* cDNA coding sequence driven by the human *RPE65* promoter (rAAV.h*RPE65*p.h*RPE65*), flanked by AAV2 ITRs encapsidated in an AAV2 shell. Viral vector was produced by Targeted Genetics (Seattle, WA, USA), with the use of a B50 packaging cell line.[203] The rAAV titers were determined by dot blot, generating vector concentrations of $1x10^{11}$ and $1x10^{12}$ vg/ml. Subretinal injections were performed as previously described.[115] Two different volumes of injection were administered 250 µl and 500 µl (Table 6.1). Dogs were treated with oral prednisone 0.5 mg/kg daily 1 week before vector administration. Immediately after subretinal injection, all dogs received a subconjunctival injection of 2mg dexamethasone solution and 8mg gentamicin. Oral prednisone was administered post-operatively at 1 mg/kg daily for week 1, 0.5 mg/kg for week 2, 0.25 mg/kg for week 3, and 0.125 mg/kg for week 4.

6.3.3 Ophthalmic evaluation and fundic imaging

To monitor for any resultant ocular inflammation complete ophthalmic examination was performed, including slit lamp biomicroscopy (model SL14; Kowa, Tokyo, Japan), indirect ophthalmoscopy (Welch Allyn, Skaneateles Falls, NY, USA) and fundus photography (RetCam II, Clarity Medical Systems, Pleasanton, CA, USA). Examination was performed pre-injection and then after injection every other day for the first week, twice weekly for the first 2 months, then monthly. Wide angle digital fundus images captured immediately post-subretinal injection were used to calculate the proportion of the tapetal fundus injected, with measurements performed

using Photoshop CS4 (Adobe, San Jose, CA, USA). The extent of the tapetum was similar between all dogs; therefore this method of measuring the subretinal bleb was comparable between eyes.

6.3.4 Electroretinography

ERG recordings were performed pre-injection, and 2, 4 and then between 9-12 months after injection for all treated eyes. A final ERG was recorded at 20-24 months post treatment for 15 of the 18 eyes. ERGs were recorded under inhalant isoflurane anesthesia as previously described, except ERG-Jet corneal contact lens electrodes were used.[204] Briefly, globes were positioned in primary gaze using stay sutures of 4-0 silk (Ethicon, Inc Piscataway, NJ, USA) and the pupils were dilated with 1% tropicamide (Mydriacyl, Alcon Laboratories, Honolulu, HI) and 10% phenylephrine hydrochloride (AK-Dilate, Akorn Inc, Buffalo Grove, IL). Full-field flash ERGs were recorded using ERG-Jet lenses (Microponent, Le Cret-du-Locie, Switzerland) and the UTAS-E 3000 electrophysiology unit with a Ganzfeld (LKC Technologies Inc; Gaithersburg, MD). Band pass filter cut off was set at 0.5 to 500 Hz. Dark-adapted ERG responses were recorded following 1 hour of dark adaptation, from a series of 16 white flash stimuli (-3.6 -3.18, -2.79, -2.41, -2.0, -1.6, -1.19, -0.79, -0.39, 0.00, 0.39, 0.85, 1.36, 1.9, 2.38 and 2.82 log cdS/m^2). Interstimulus intervals were increased from 1 second at low intensities to 360 seconds at the highest intensity to avoid light-adapting the rods[204]. Following exposure to a background light of 30 cd/m^2 for 10 minutes, cone mediated flicker responses were recorded at 33 Hz (0.39 log cdS/m^2).

For the assessment of rod responses the dark adapted b-wave amplitude at 0.0 log cdS/m^2 was used. This flash intensity was below the response threshold for all pre-treatment RPE65-/- eyes. Dark adapted ERGs were assessed for threshold of response and shape of waveform. Dark adapted b-wave intensity:response curves were plotted. The a- and b-wave amplitudes were measured for each averaged response. The a-wave amplitude was measured from the onset of light stimulus to the trough of the first negative wave; b-wave amplitude from the trough of the first negative wave to the peak of the first positive wave. To evaluate cone responses, the amplitudes of light-adapted 33 Hz flicker responses were analyzed. Flicker responses were chosen rather than single flash light-adapted responses because of the concern that rods not supplied with 11-*cis* retinal have markedly reduced sensitivities and may still retain some recordable function even in the presence of a background light that saturates normal canine rods. The origin of the sometimes relatively large amplitude response to bright flash stimuli in untreated *RPE65* -/- dogs is not established. For the 33Hz flicker responses, amplitude (trough to peak) was measured.

6.3.5 Vision testing

Vision testing was performed using a vision testing device as previously described.[174] Evaluation was performed twice, before treatment and 70-255 days post-injection for all dogs. Each treated eye was individually assessed by placement of an eye mask over the contralateral eye. Vision was tested by seven repeated trials at three different light intensities (0.02-0.04,

1.0-1.5 and 35-45 cd/m^2). Average time to exit the device and the number of first correct choices of exit tunnel were recorded.

6.3.6 Detection of neutralizing antibodies to rAAV

Serum was collected pre-injection, every 2 weeks until 28 weeks post injection, then at 9 and 12 months after treatment of each eye. Aqueous humor was collected from all eyes immediately after the first injection. Aqueous and serum were stored at -80°C. To determine NAb titers, serial dilutions of serum were prepared in triplicate and 1 x 10^8 vg rAAV.CMV.GFP was added to each sample. Plates were incubated at 37°C for 1 hour, then the contents of the wells were added to 96-well plates of 293T cells containing 2.5 x 10^4 cells per well. Plates were incubated for 48 h and then the number of GFP positive cells per well was counted using an inverted fluorescence microscope. The titer of NAb was defined as the highest dilution that produced 50% fluorescence compared with the rAAV2-positive media only control.

6.3.7 Detection of total IgG and IgM to recombinant RPE65

Aqueous and serum was collected and stored as for detection of NAb to rAAV. In all, 96-well Maxisorp microtitre plates (NUNC, Roskilde, Denmark) were coated in recombinant human RPE65 (gift from Professor Martin Warren, University of Kent, UK) (1 in 5000 dilution in 100 µl of phosphate buffered saline (PBS)) overnight at room temperature, then washed with PBS +

0.05% Tween-20 (PBS-T). Plates were blocked with 1% bovine serum albumin (BSA) in PBS for ≥ 1 hour at room temperature, then washed and samples (1:100 or 1:200 dilution) applied. Plates were incubated at room temperature for 1.5 hours and washed 3 times with PBS-T. Bound canine IgG or IgM was detected with sheep-anti-canine IgG-HRP or goat-anti-canine IgM-HRP (AdB Serotec, Kidlington, UK) for 1.5 hours at room temperature, then washed 3 times with PBS-T and color was developed with TMB substrate (Pharmingen, Oxford, UK) and quenched with 1 N HCL. Absorbance at 450 nm was quantified using a plate reader (E Max, Molecular Devices, Wokingham, UK).

6.3.8 Statistical Analysis

The ERG results and vision testing outcomes data were analyzed using PASW Statistics 17.0 (SPSS Inc., Chicago, IL). Independent samples *t*-tests were used to test for differences between average pre- and post-injection outcomes as well to assess for any difference between first and second eye injected at all time points. Analysis was performed on 33Hz flicker responses and b-wave amplitudes at 0 log cdS/m^2. Power analysis calculations were performed using the program G*power, version 3.1.2 (Dusseldorf, Germany). For correlation between age, bleb size, viral dose and ERG outcomes, 2 tailed-Pearson analysis was performed to assess the relationship between the variables. For vision testing outcomes, mean time to exit, and mean number of correct exits, independent samples *t*-tests were again used to test for differences between pre- and post-injection outcomes as well to assess for any difference between first

and second eye injected. Independent samples *t*-tests were chosen above more complex tests due to the relatively low sample size of this study. Data were considered significant at $P < 0.05$.

6.4 Results

6.4.1 Subretinal injection of rAAV2/2.h*RPE65* in *RPE65* -/- crossbred dogs

Both eyes of nine *RPE65* -/- crossbred dogs were injected subretinally with r*AAV2.hRPE65p.hRPE65* with the second eye treated 85-180 days after the first eye (Table 6.1). The age of the dogs at the time of treatment ranged from 137 to 755 days. The injections were made in the superior fundus of both eyes and the mean proportion of the subretinal bleb in relation to tapetal area was 40% (range: 6 – 62%). The retinal detachments created by the subretinal injections typically resolved over the subsequent few days. All eyes had complete retinal reattachment on indirect ophthalmoscopic examination by one week after injection with the exception of eye 6a which still had a small subretinal bleb remaining at that time point (Figure 6.1 (c)). The small retinotomy created by the cannula sealed in all cases with complete reattachment of the retina at the injection site. The injection site was often visible as a small-pigmented scar (Figures 6.1 (c and g). A 'highwater' mark indicating the edge of the detachment could be detected on careful funduscopic examination.

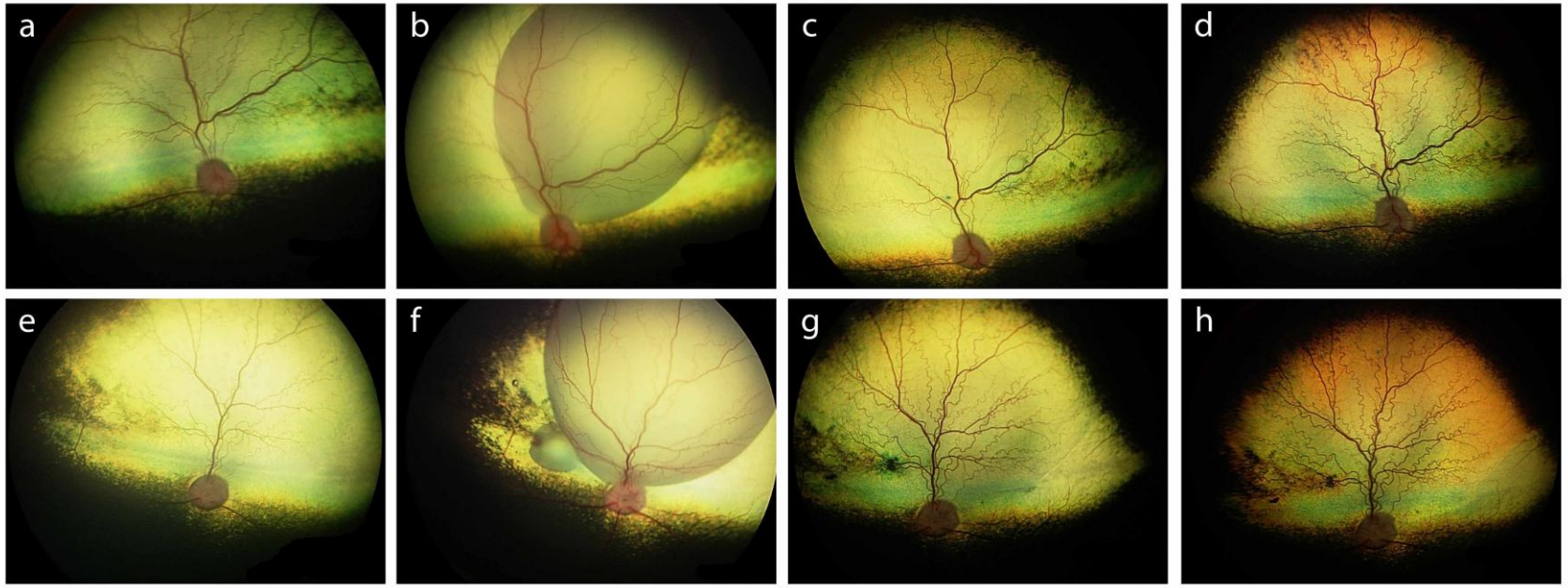

Figure 6.1 Fundus images pre- and post-treatment. Wide-angle fundus images (RetCam II, Clarity Medical Systems) of first and second subretinally injected eyes of a *RPE65* -/- dog (dog 6). The right eye (OD) was injected first and the left eye (OS) was injected 90 days later. (a) OD pre-injection, (b) OD immediately after subretinal injection, (c) OD 1 week post-injection, (d) OD 1 year post-injection, (e) OS pre-injection, (f) OS immediately after subretinal injection, (g) OS 1 week post-injection, (h) OS 1 year post-injection.

6.4.2 Evaluation for ocular inflammation

Eyes were examined for ocular inflammatory responses throughout the study. As anticipated following the subretinal injection, mild inflammation, as indicated by lowered intraocular pressure (compared with pre-injection levels) and very mild aqueous flare (ranging from trace flare to 1 on a scale of 1-4), was appreciated for up to 7 days. The tract of the cannula through the vitreous could be observed. However, no vitreal changes that would indicate an inflammatory reaction were detected in any of the eyes. There was no difference in the post-surgical inflammatory response between first and second injected eyes and in all cases this was a very mild response, which in our experience is typical for that seen in dogs following this sort of surgical intervention. Ophthalmoscopically detectable fundic changes over the period of the study were minimal in 14 of 18 treated eyes, with a small scar often seen at the site of cannula penetration of the retina; representative fundus images from both eyes of a treated dog are displayed in Figure 6.1. Four eyes showed changes in tapetal reflectivity. In three eyes (3b, 5a, 7a) small foci of tapetal hyper-reflectivity were scattered across the area of the created subretinal bleb. In one eye (2b) a larger area of tapetal hyper-reflectivity developed, covering the majority of the treated fundus, consistent with retinal thinning as described in a previous report.[187]

6.4.3 Evaluation of retinal function

Retinal function was assessed with full-field flash electroretinography (ERG). Rod and cone photoreceptor rescue was observed for all treated eyes with the exception of eyes 4a (very

small retinal bleb with majority of vector administered choroidally due to poor injection) and 2b (as anticipated due to aforementioned changes). Representative dark-adapted ERG tracings and light adapted cone (33Hz) flicker responses measured before and after treatment are shown in Figures 6.2 and 6.3.

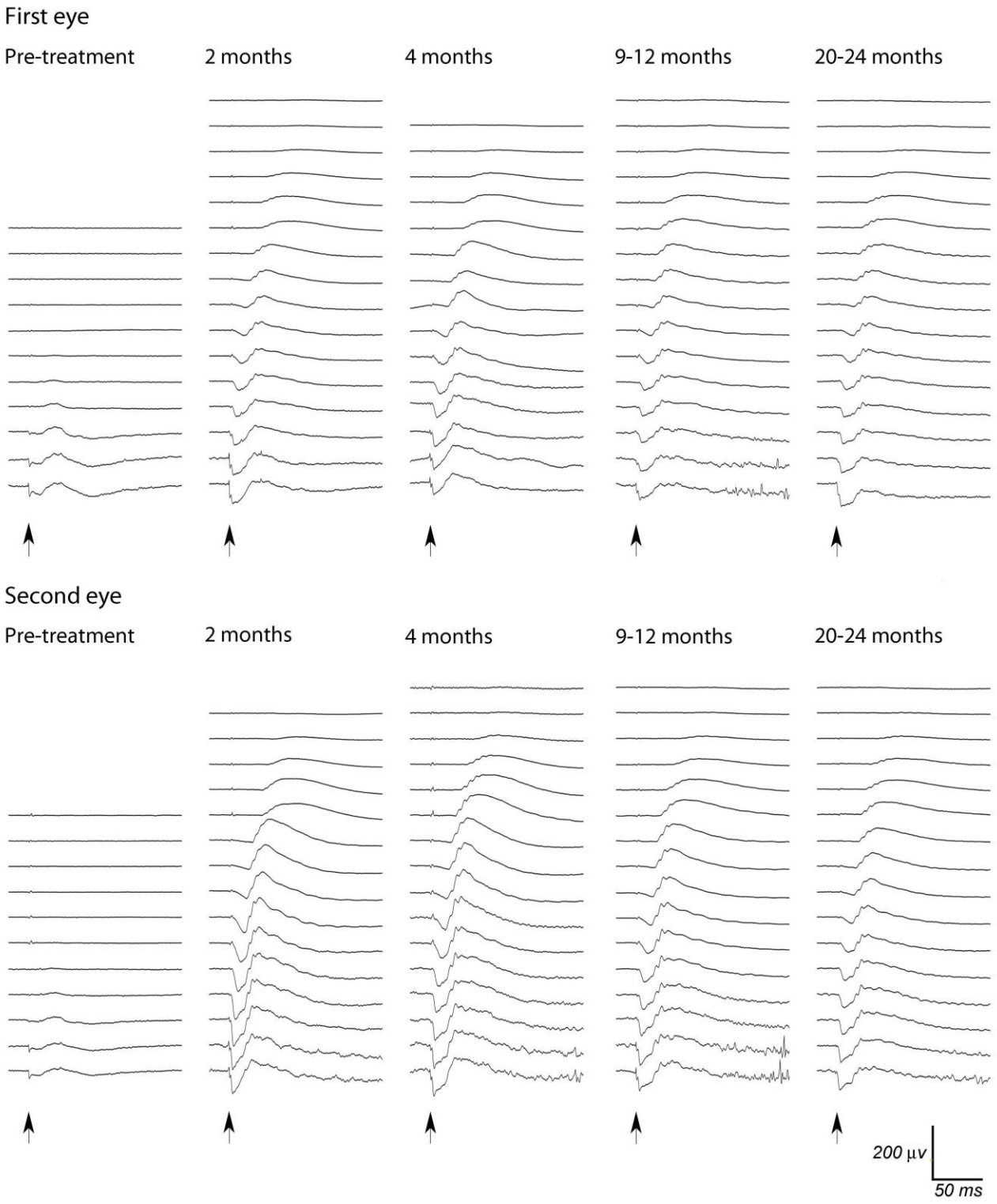

Figure 6.2 Representative dark-adapted rod ERGs. Dark-adapted ERGs from first and second subretinally injected eyes of dog 6 are displayed. The second eye was injected 90 days

after the first eye. Flash intensities from top to bottom were, 2.82, 2.38, 1.9, 1.36, 0.85, 0.39, 0.0, -0.39, -0.79, -1.19, -1.6, -2.0, -2.41, -2.79, -3.18, -3.6 log cdS/m^2. Arrows indicate timing of flashes.

Figure 6.2 continued

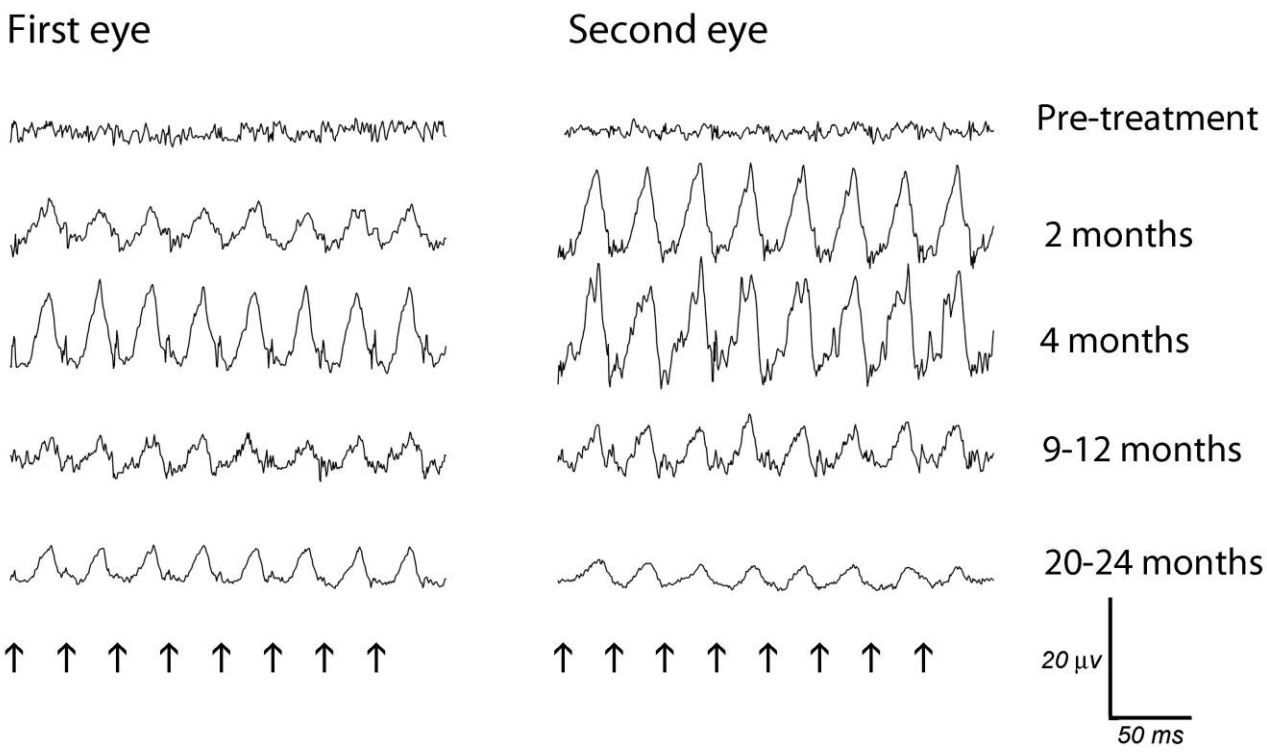

Figure 6.3 Representative cone flicker ERGs. Cone flicker responses from first and second injected eyes of dog 6. Flicker frequency was 33 Hz, at a light intensity of 0.39 log cdS/m^2. The second eye was injected 90 days after the first eye. Note that there was a marked reduction in flicker amplitudes from the peak response by the 20-24 month timepoint. Arrows indicate timing of flashes.

Dark adapted a- and b-wave intensity-response curves were generated. These showed that there was an increased a- and b-wave amplitude and lower response threshold for post-injection ERGs relative to pre-injection recordings in 16 of the 18 eyes. These improvements in ERG waveforms were comparable between first and second injected eyes (Figure 6.4).

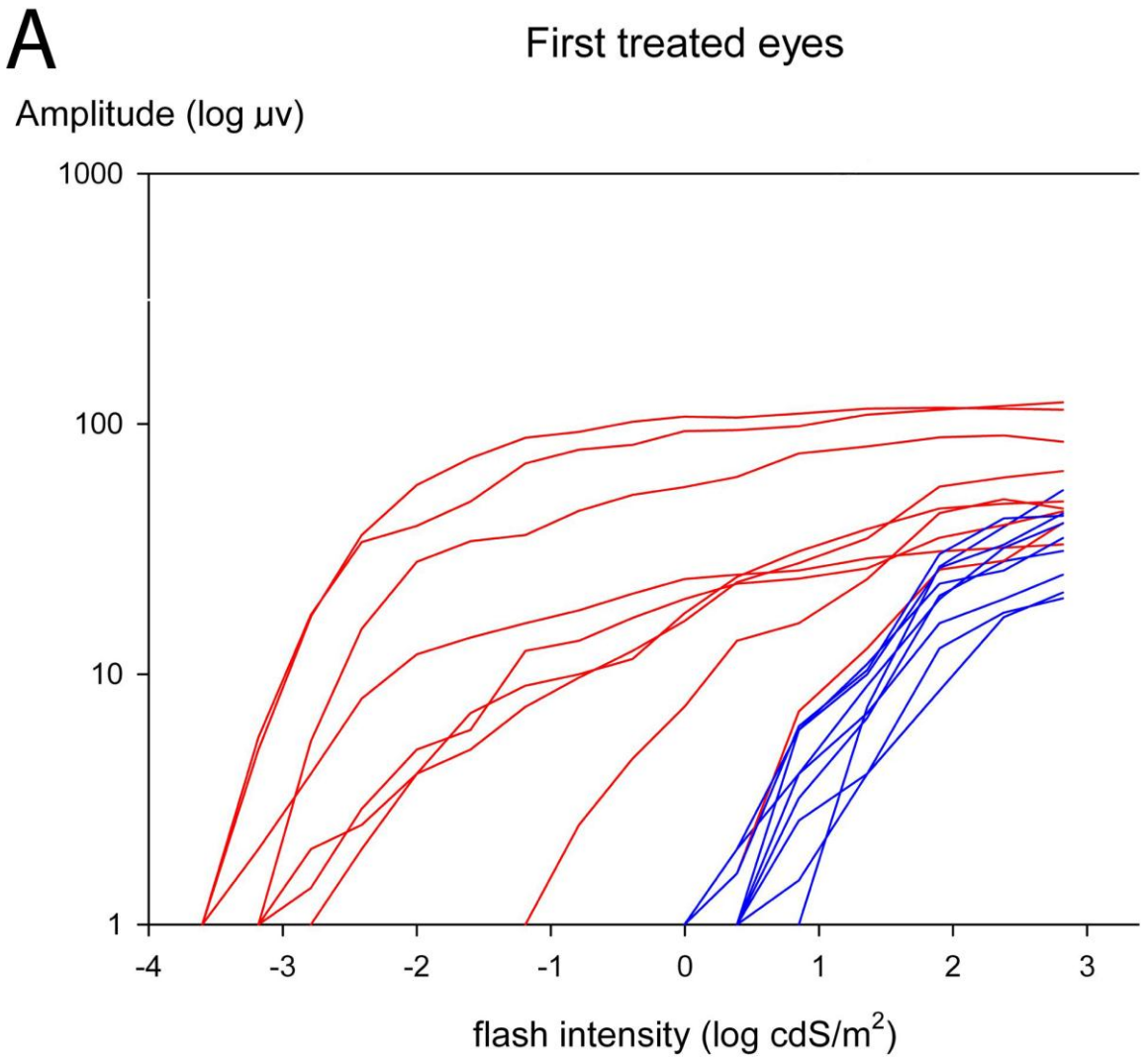

Figure 6.4 Rod ERG intensity response curves for all treated eyes. Montage of dark-adapted b wave log intensity:response curves for all first treated eyes (A) and second treated eyes (B). Pre-injection intensity:response curves are shown in blue, 4 months post-injection are shown in red.

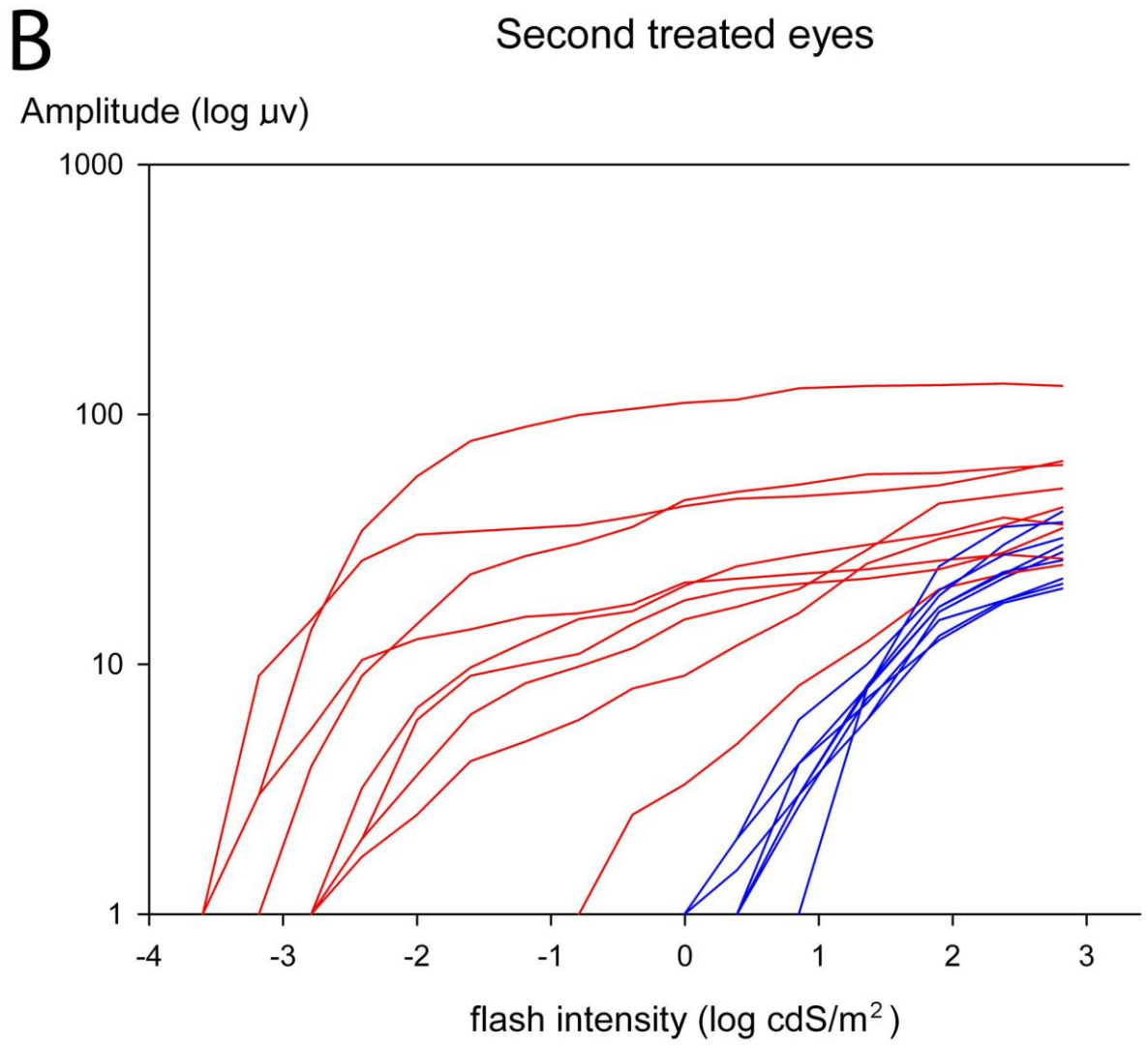

Figure 6.4 continued

As a measure of predominately rod rescue, the amplitude of the dark-adapted b-wave at a flash intensity of 0.0 log cdS/m^2 was selected for further analysis. This flash intensity was below the response threshold for all untreated *RPE65* -/- dogs. Cone function was assessed by the amplitude of the 33Hz flicker response. This response was either unrecordable or of a very low amplitude response for eyes before injection. Using these parameters, recovery of rod and cone photoreceptor function was observed at all time points post-injection for all eyes except 2b and 4a. There was no significant difference in the degree of rod or cone rescue between first and second treated eyes at any time point (Table 6.2 and 6.3). A power analysis was performed to indicate the magnitude of difference in the mean b-wave amplitude at 0.0 log cdS/m^2 and the mean cone flicker amplitudes that given the sample size and variability would have reached significance (at $P<0.05$). These values were 13.3 µV and 2.5 µV respectively.

Table 6.2 Rod and cone ERG responses of all eyes. ERG results for individual dogs comparing pre-treatment and 4 months post treatment for first and second injected eyes. (Rod responses recorded at 0 log cdS/m^2, cone responses recorded from 33Hz flicker)

Dog	Eye	Rod a-wave pre-injection (µv)	Rod a-wave 4 months post-injection (µv)	Rod b-wave pre-injection (µv)	Rod b-wave 4 months post-injection (µv)	Cone flicker pre-injection (µv)	Cone flicker 4 months post-injection (µv)
1	First (a)	0	2	0	7	0	4
	Second (b)	0	4	0	21	0	4
2	First (a)	0	28	0	94	0	9
	Second (b)	0	1	0	3	0	0
3	First (a)	0	4	0	20	0	5
	Second (b)	0	3	0	15	0	2
4	First (a)	0	0	0	0	0	0
	Second (b)	0	4	0	9	0	3
5	First (a)	0	5	0	16	2	8
	Second (b)	0	19	0	45	0	7
6	First (a)	0	22	0	56	0	4
	Second (b)	0	44	0	111	0	14
7	First (a)	0	10	0	24	0	3
	Second (b)	0	11	0	21	0	3
8	First (a)	0	8	0	18	0	4
	Second (b)	0	5	0	18	0	2
9	First (a)	0	50	0	107	0	15
	Second (b)	0	24	0	43	0	5
Normals*		46 (+/-18)		124 (+/-38)		21(+/-9)	

*Shows the ERG results from 18 *REP65*+/+ eyes of dogs of similar breeding. (Mean +/-SD)

Table 6.3 ERG difference between eyes and over time. (I and II) mean dark-adapted b wave amplitude (µV) at 0 log cdS/m^2, and (III and IV) mean light adapted 33 Hz flicker amplitude, for all dogs pre and post injection, with P-values comparing amplitude of response for first and second treated eyes.

Eye	pre-treatment	2 months	4 months	9-12 months	20-24 months	P value (pre vs 4 mth)	P value (2mth vs 4mth)	P value (9-12mth vs 20-24mth)	P value (4mth vs 20-24mth)
I) First (a)	0	25	38	36	27	<0.001*	0.122	0.633	0.235
II) Second (b)	0	30	32	30	25	<0.001*	0.573	0.364	0.348
p value (1^{st} vs 2^{nd})		0.748	0.722	0.814	0.887				
III) First (a)	0	4.6	5.8	5.9	3.0	<0.001*	0.214	0.519	0.10
IV) Second (b)	0	4.5	4.4	4.0	1.9	<0.001*	0.322	0.157	0.041*
p value (1^{st} vs 2^{nd})		0.954	0.474	0.303	0.350				

* Significant difference

The mean post-injection amplitudes of rod and cone responses did not change significantly over the period of the study (Table 6.3). However there was a trend towards an increase in b wave amplitude between the 2- and 4-month ERGs, and a decline between the 9-12 and 20-24 month ERGs. This observation was consistent between the two eyes. When the effect of age at injection, total viral dose, injection volume, vector concentration, subretinal bleb size and NAb response on rod and cone rescue was assessed, only the subretinal bleb size correlated significantly with the amplitude of rod and cone ERG responses (Table 6.4).

Table 6.4 Factors affecting rod and cone ERG outcomes. Impact of variables affecting the mean rod and cone ERG rescue at 4 months post-injection (P values displayed)

Variable (P value)	Rod b (0 log cdS/m^2)	Cone (33Hz) flicker
Age at injection	0.438	0.229
Total dose of vector for both first and second injected eyes	0.564	0.601
Total dose of vector – first injected eyes	0.42	0.56
Total dose of vector – second injected eyes	0.69	0.64
Volume of injection	0.653	0.843
Concentration (vp/ml)	0.511	0.565
Subretinal bleb size	0.024*	0.0034*
Peak NAb response	0.161	0.123
Change in NAb levels (before and after injection)	0.146	0.102
NAb level at time of second injection with ERG outcome in second eye.	0.718	0.909

* significant correlation

6.4.4 Evaluation of vision

A vision testing apparatus that assessed the dog's ability to see an open exit tunnel was used to quantitatively measure visual function under varying light levels as previously described.[174] Visual function was evaluated at normal room light and two lower light intensities, by recording the time to exit the device and the first exit tunnel entered. A significant improvement in visual function relative to pre-treatment values was seen at the two lower light intensities for both parameters evaluated (Figure 6.5). Importantly, there was no significant difference in visual function between first and second treated eyes (Table 6.5).

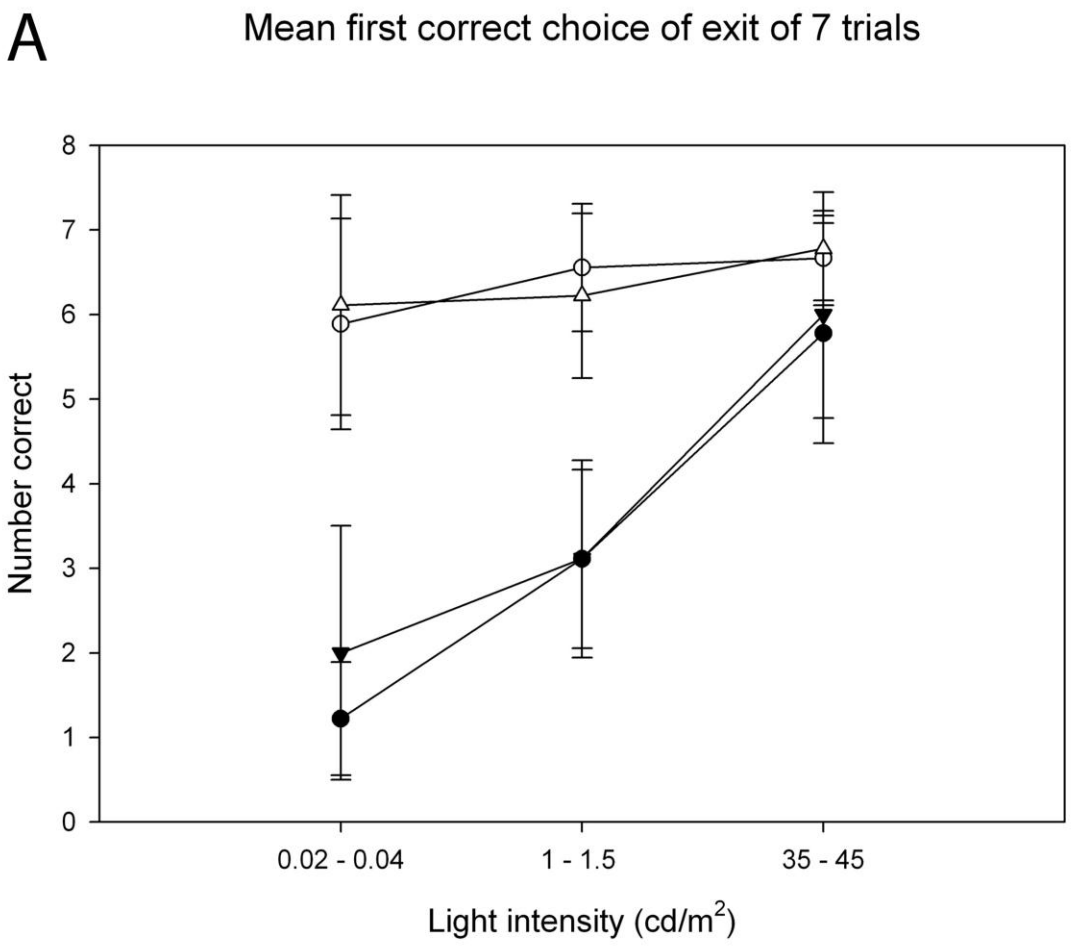

Figure 6.5 Vision testing outcomes pre- and post-treatment. Pre-injection values are compared with post-injection for first and second subretinally injected eyes. Testing was performed at three light intensities; 0.02-0.04, 1.0-1.5, and 35-45 cd/m^2 for all trials. (A) mean time to exiting the vision testing apparatus is shown, averaged from seven trials per eye, (B) mean outcome for the first correct choice of four exit tunnels is displayed as number correct of seven trials. Error bars = standard deviation.

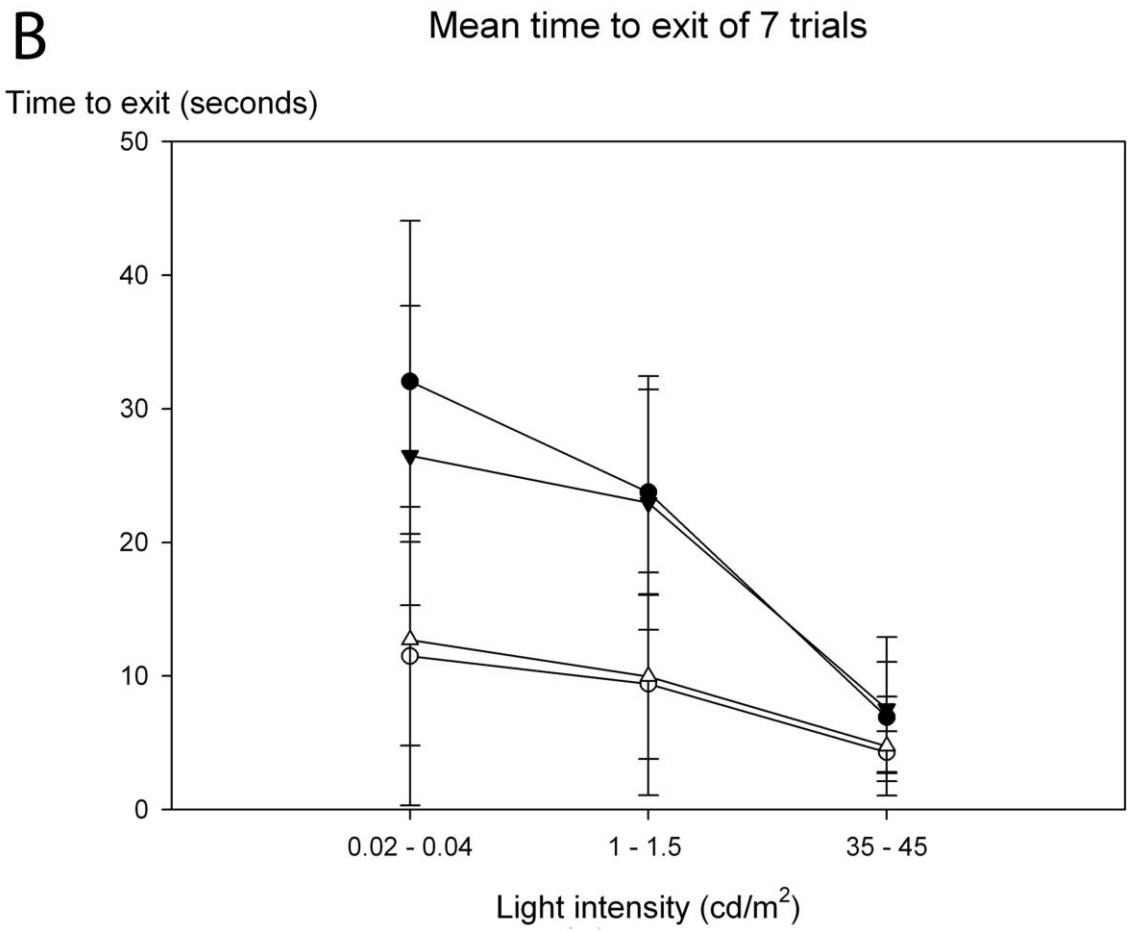

Figure 6.5 continued

Table 6.5 P values for the vision testing assessments

P values	Time to exit of 7 trials			First correct choice of exit (7 trials)		
Light intensity (cd/m^2)	35-45	1.0-1.5	0.02-0.04	35-45	1.0-1.5	0.02-0.04
P - pre vs post (1^{st} eye)	0.063	<0.001*	0.002*	0.052	<0.001*	<0.001*
P - pre vs post (2^{nd} eye)	0.025*	0.001*	<0.001*	0.043*	<0.001*	<0.001*
P - 1^{st} vs 2^{nd} eye (pre)	0.797	0.848	0.327	0.714	1.0	0.174
P - 1^{st} vs 2^{nd} eye (post)	0.739	0.982	0.877	0.694	0.525	0.716

* Significant difference

6.4.5 Immunology

Evaluation for serum NAb to rAAV2 capsid showed that prior to gene delivery, 1 out of 9 dogs (dog 5) had detectable circulating NAb against rAAV2. Four weeks after vector delivery, all dogs had substantially increased titers of NAb that declined by 12 months, but remained above pre-treatment levels. The NAb titer increased again in 7 out of 9 dogs after the second delivery of vector and peaked at a higher level after the second injection compared with the first injection. A large variation was observed in the NAb titers between dogs; four weeks after the first injection the NAb titer ranged from 1/100 to 1/32,000 and four weeks after the second injection titers ranged from 1/125 to 1/35,000 (Table 6.6). Aqueous humor collected from the second injected eye at the time of injection did not have detectable levels of NAbs (data not shown). Notably, higher serum Nab titers to rAAV2 capsid were observed in dog 4 after the first injection and again after the second injection relative to the other dogs. The response after the first injection correlated with choroidal administration of vector construct in this eye. Enzyme-linked immunosorbent assay performed on serum to evaluate for potential humoral immune responses directed to RPE65 showed no detectable change in antibody response specific to RPE65 in any dog after first or second subretinal injection of rAAV2 (data not shown). An analysis was performed on the ERG results of the second injected eyes to see if there was a correlation in ERG outcome and the NAb level at the time of the second injection. There was no correlation between the second eye b-wave at 0.0 log cdS/m^2 at 4 months and the NAb at the time of injection ($P=0.718$), nor between cone flicker outcome at 4 months and NAb at the time of injection ($P=0.909$). The analysis was repeated following adjustment of the ERG amplitudes for the size of the bleb and again there was no significant correlation.

Table 6.6 Serum neutralizing antibody response. rAAV2 serum Nab titers pre- and post-injection for first and second injected eyes

Dog	Eye injected	Titer pre- injection	Titer 4 weeks post injection
1	First (a)	1 in 2	1 in 300
	Second (b)	1 in 150	1 in 125
2	First (a)	1 in 2	1 in 100
	Second (b)	1 in 150	1 in 8,500
3	First (a)	1 in 2	1 in 2,000
	Second (b)	1 in 800	1 in 800
4	First (a)	1 in 2	1 in 32,000
	Second (b)	1 in 8,500	1 in 35,000
5	First (a)	1 in 150	1 in 5,000
	Second (b)	1 in 2,000	1 in 8,000
6	First (a)	1 in 3	1 in 3,000
	Second (b)	1 in 1,000	1 in 5,000
7	First (a)	1 in 2	1 in 200
	Second (b)	1 in 25	1 in 25,000
8	First (a)	1 in 2	1 in 3,500
	Second (b)	1 in 200	1 in 3,500
9	First (a)	1 in 2	1 in 750
	Second (b)	1 in 1,000	1 in 7,500

6.5 Discussion

In this study we sought to establish whether successful rescue could be achieved in the second eye of *RPE65* -/- dogs following previous gene therapy treatment of the fellow eye. We found that rescue achieved in the second eye was comparable to that in the first treated eye, with no evidence of interference of RPE transduction by immune reaction to the first treatment. These findings have significant implications for treatment of human Leber Congenital Amaurosis type II patients.

Consistent with other studies evaluating immune responses to rAAV, we saw elevated serum NAb titers after all injections. After injection of the first eye, the maximum titer observed was 1/32,000 in dog 4. In eye 4a the majority of vector was injected choroidally resulting in a very small subretinal bleb (6% of tapetal fundus). The NAb titers in this animal were much higher than all other animals and since the choroid is not protected by the blood retinal barrier this finding is not surprising. The highest NAb titer after exclusively subretinal administration was 1/5000. The titers declined by 12 months post-injection but remained higher than baseline pre-injection NAb levels. The serum NAb response was higher after the second injection than after the first injection, similar to a prime-boost effect, but this did not appear to affect the rescue of the second injected eye compared with the first. NAb were not detected in the second eye at the time of injection, indicating that the serum NAb did not cross the blood-aqueous barrier in the untreated eye. The NAb response showed no significant correlation with the degree of mean rod or cone rescue achieved. It is of interest that eye 4a, which received vector

choroidally, had a lack of detectable ERG and vision rescue. The lack of resuce in this eye may be accounted for by the very small subretinal bleb that was achieved, but we cannot rule out an effect of the presence of a high NAb titer. While both rod and cone photoreceptor rescue was seen in the second injected eye of this dog (4b), it was observed that the amplitude and threshold of ERG responses for eye 4b were lower than anticipated by the size of the subretinal bleb achieved in this eye. It is possible that the lower than anticipated rescue seen in eye 4b is a function of the higher systemic NAb titer seen after the first injection. However with only one dog with this complication any correlation between the high systemic antibody level at the time of injection of the second eye and outcome cannot be proven.

In addition to the importance of route of administration, trials of repeated AAV gene therapy in other organ systems have shown that the vector dose is important in determining the degree of immune response and success of transgene expression. Consistent with this, we have previously observed transgene expression in both eyes of *Rpe65*^{Rd12} mice when a lower concentration ($1x10^{11}$vg/ml) of vector construct was used, whereas a higher concentration ($5x10^{11}$vg/ml) resulted in higher NAb titers and variable transgene expression in the second eye.[172] In the larger canine eye we have achieved successful photoreceptor rescue at both high and low concentrations ($1x10^{11}$ and $1x10^{12}$ vg/ml) in both first and second treated eyes without evidence of any significant variation in NAb response. Similarly administration of a larger total dose of vector did not have a major effect on immune responses or degree of

rescue in the first or second treated eyes. This variation may reflect the higher relative dose of viral particles in the smaller murine eye or species specific immune responses.

Importantly, we observed no ocular or systemic adverse effects after successful subretinal gene delivery. The mild post-injection inflammatory response seen in all injected eyes was typical for that seen after this sort of intervention in dogs. Aqueocentesis itself in the dog induces a mild inflammatory response and has been used in canine studies to test efficacy of anti-inflammatory medications.[205] The mild inflammation resolved completely, and there was no indication of any ongoing inflammatory reaction in any eye. Despite choroidal administration of vector and subsequently elevated serum NAb titers, eye 4a did not show evidence of an inflammatory response greater or more persistent that that in other eyes. In this eye an anticipated degree of post-operative aqueous flare was noted, comparable with that observed in other treated eyes. Small foci of tapetal hyper-reflectivity were observed in 3 of 18 injected eyes, consistent with focal retinal thinning as described in a previous report.[187] The presence of the tapetum in the dog allows for detection of retinal thinning by ophthalmoscopic examination more readily than in species with no tapetum. Previous studies have documented the histologic appearance of lesions resulting from subretinal injections in dogs and include damage at the injection site, retinal thinning and focal defects in reattachment resulting in retinal ‘ripples’.[126, 157, 158] The small areas of tapetal hyper-reflectivity that we detected in 3 of the 18 eyes did not preclude successful recovery of rod and cone function in these eyes. Reasons for the more diffuse tapetal hyper-reflectivity and presumed retinal thinning in the

injected area of eye 2b are not clear but may reflect the potential detrimental effects of the creation of a retinal detachment. Certainly this finding correlated with failed recovery of rod and cone ERG responses in this eye.

Improved rod and cone function was seen in 16 of 18 injected eyes, this success rate is comparable with that reported in previous studies evaluating unilateral injections.[110, 114, 126, 133, 153, 186-188] However the amplitudes of rescued cone responses were somewhat variable between eyes. Consistent with this finding Acland *et al* describe rod rescue in 23 of 26 eyes, but saw cone rescue in only 8 of these 23 eyes using more stringent criteria for rescue.[110] In considering these outcomes, previous biochemical and immunochemistry data show that only the area of the subretinal bleb regains functional *RPE65* expression and subsequent 11-*cis* retinal production.[110] Similarly, PCR results have shown that viral DNA only persists in the neural retina, RPE and choroid of the injected area.[114] Concordant with these reports we saw a significant effect of subretinal bleb size on the amplitude of dark adapted rod ERG responses, and cone (33Hz) flicker responses. In fact subretinal bleb size explained the majority of variability observed in ERG responses between treated eyes in this study. While we sought to create a consistently sized subretinal bleb located over the area centralis, the formed blebs varied somewhat in size and location, consistent with a previous description.[187] The area centralis has the highest cone density and corresponds to the human macula, although it has a lower cone density and does not have a rod-free region.[206] The location and size of the

subretinal bleb likely plays a significant role in determining measurable cone photoreceptor rescue.

Improved rod and cone ERG responses were observed for the duration of the study. However although the mean rod responses at 4 months and those at 20-24 months were not statistically different, there was a statistically significant decline in cone flicker responses between the two time-points. These findings were consistent between first and second injected eyes. These results are similar to those reported by Narfström *et al*, who describe a peak rod ERG response at 3–6 months post-injection and a peak cone ERG response at 18 months post-injection, both rod and cone responses declining thereafter.[153] In contrast Acland *et al* followed 2 eyes out to 3 years and did not report a significant decline in rod or cone function.[110] Bennicelli *et al* report a decline in response from initial ERG at 5 weeks to second ERG at 3 months, but also describe one treated eye followed out to 3 years whose rod ERG varied by less than 10%.[126] The cause for this apparent variability in the maintenance of ERG responses in the longer term is not clear and its potential significance to human clinical trials of gene replacement therapy for LCA type II remains to be determined.

We have demonstrated functional rescue of the ERG and improved visual outcomes in the *RPE65* -/- dog injected subretinally with rAAV2.h*RPE65p*.h*RPE65* into both eyes, 85-180 days apart. This demonstrates that vector of the same serotype and expressing the same transgene can be effectively re-administered to treat the contralateral eye of previously treated *RPE65* -/-

dogs without evidence of an untoward effect of the therapy. These results support the inclusion of treatment of the second eye in current clinical trials of gene therapy in LCA type II patients.

CHAPTER 7 Discussion and future directions

7.1 Evaluating outcomes of gene therapy in the RPE65-deficient dog

After extensive preliminary work in canine and murine models of RPE65-LCA, phase I/II gene therapy trials in human patients were initiated, demonstrating both safety and efficacy. To date there have been no detectable systemic safety concerns, though some ocular adverse events have been reported, these have been attributed to the surgical procedure.[163-171] With regard to efficacy there has been variably improved visual function, determined by different measures out to 3 years after the treatment.[163-171] This has led to questions of how subretinally delivered AAV mediated gene therapy for RPE65-LCA can be improved. The crucial role of the RPE65-deficient dog in pre-clinical trials along with the previously described advantages of studying the canine eye have made it the model of choice for evaluating such questions. With a large colony of RPE65-deficient dogs at MSU that had been used for studies including subretinal injection of different AAV2/2 vector titers in support of the regulatory application for the first human phase I/II gene therapy trial (NCT00643747), the canine model and required techniques were well established at MSU's comparative ophthalmology laboratory.[164] We were therefore well positioned to evaluate two questions that arose from the phase I/II clinical trials; how much will the degree of photoreceptor preservation effect treatment outcomes, and is treatment of the second eye safe and effective. To evaluate the effect of treatment in older dogs with photoreceptor loss it was important to more fully describe the RPE65-deficient dog phenotype with increasing age. As vision testing is a key part of outcome assessment it was also important to evaluate whether there were non-visual

factors that affected the validity of repeated use of this technique on the same dogs.

After demonstrating evidence for the validity of repeated vision testing using Gearhart *et al*'s previously described device and detailing the progression of the phenotype of the RPE65-deficient dog over a wide age range we proceeded to address those two questions. First we showed that rAAV mediated gene therapy treatment of RPE65-deficient dogs 2-6 years of age with more advanced retinal degeneration is successful, with age not having a significant impact on outcomes. Second we demonstrated that gene therapy treatment of the second eye of RPE65-deficient dogs is equally as successful as treatment of the first eye, without an effect on outcomes from immune responses due to prior treatment of the first eye.

7.2. Evidence for validity of repeated vision testing

The vision testing device described by Gearhart *et al* had been shown to be accurate for discriminating affected from unaffected dogs with inherited retinal dystrophies, and also for distinguishing between different retinal dystrophies.[174] However we sought to perform repeated vision testing on the same dogs, including comparison of outcomes before and after gene replacement therapy. So it was important to provide evidence that the vision testing results obtained from the device did not improve with repeated testing, consistent with measurement of visually guided behavior without influence of non-visual cues. Of note, of the other described techniques used for assessment of canine vision in studies of canine models of inherited retinal dystrophies none have yet been evaluated in this way despite common use for repeated testing.[114, 133, 134] We found no evidence for improvement of vision testing

outcomes with repeated testing using this device. These findings supported the suitability of using the device for repeated vision testing to assess response to therapy in the further studies in this thesis, giving confidence that any improvement in vision would be due to the therapy administered alone.

Our described evidence for no improvement in vision testing outcomes with repeated testing using Gearhart *et al's* device also has relevance for future studies evaluating other vision-restoring therapies using canine models. With canine models of human retinal dystrophies becoming increasingly important for evaluating such therapies.[110-113] As evidenced by our finding of increased outcome variability where device transit time was measured, other techniques that include only a measure of time are likely to provided less consistent results.[134] The inclusion of a choice based measure of outcome, unique to Gearhart *et al's* device, along with our findings of repeatability argue for the merit of using this device in future studies evaluating canine vision.

7.2.1 Limitations and further questions

Our finding of a slowing in exit time with repeated testing was postulated to be due to familiarity with the device and decreased impetus to exit. However, an effect of other behavioral variables is also possible, and this decline in outcomes warrants further evaluation. This could be by comparison of outcomes with and without a greater impetus to exit the device, such as food rewards. Additionally, a limitation of the device pointed out by Gearhart *et al*, is that it does not assess visual acuity.[174] This is a common outcome assessed in human clinical

trials for inherited retinal diseases, and such a means of assessing visual acuity would be desirable for canine studies; however, tests to assess canine visual acuity are likely to require a significant investment in time to train the studied dogs.

7.3 Relevance of the description of the RPE65-deficient dog phenotype

While younger RPE65-deficient dogs had been widely studied as part of pre-clinical gene therapy trials, published details on the phenotype of older affected dogs are limited. For our study evaluating treatment outcomes in older dogs it was important to document the changes in retinal structure, ERG and vision. We found a decline in light- and dark-adapted ERG amplitude but stability of other measured ERG parameters, and stable vision testing outcomes despite the progressive retinal thinning seen in these dogs. A similar finding has been reported in RPE65 knockout mice where the minimal light responsiveness did not decline significantly with age despite the loss of photoreceptors.[207] We also know from studies of RPE65 knockout mice that opsin levels decrease significantly with age, correlated with a decrease in the number of photoreceptors and lowered ERG amplitudes.[207] Of note, in this same study 11-*cis* retinal injections resulted in the regeneration of similar amounts of rhodopsin and improved rod function to similar levels irrespective of age at time of treatment.[207] This led Rohrer *et al* to conclude that opsin levels decrease due to a loss of photoreceptors but the remaining photoreceptors and components of the phototransduction cascade as well as the retinal circuitry remain functional in the aged RPE65 knockout mouse.[207] These features are consistent with a genetic mutation that has a severe effect on function but only results in a slow loss of cells, as is the case for RPE65 deficiency, and contrasts somewhat with other

models of photoreceptor dystrophies loss of function results primarily from photoreceptor loss.[208-211]

Our documentation of the phenotypic changes of these colony dogs over a wide age range also provides information that may prove important for future studies utilizing these dogs. Notable is the demonstrated consistency of assessed parameters between the affected dogs in our colony. This finding of a relatively uniform phenotype is consistent with descriptions of the phenotype of murine models of RPE65-deficiency.[212] While there appear to be some differences between dogs from different colonies it is important to consider the different methodologies and techniques used in studying the different colonies. The variable presence of nystagmus and reported differences in photoreceptor preservation may argue for a true difference; however, this remains speculative.[114, 126-129, 136, 137] If a true phenotype difference is found studies to identify possible modifier loci may be valuable.

7.3.1 An effect of age and/or disease on ERG amplitude

There was an apparent correlation between the decline in photoreceptor number and the progressive decline in the low amplitude of light- and dark-adapted flash ERG responses. How this amplitude decline compares to that seen with normal aging in dogs is speculative as this has not been described. We can, however, anticipate a decline of some magnitude from results of studies in mice where the amplitudes of rod- and cone-mediated ERG's declined significantly with age.[213] This was reported to occur in the absence of changes to other ERG parameters or an age-related decline in either rod or cone photoreceptor density.[213] Similar findings

have been reported in humans, with rod and cone ERG amplitudes by age 70 declined to almost half those of 20 year old individuals.[214, 215] In people a mild retinal thinning has also been documented with age but this is much less dramatic, showing a decrease of approximately 0.53 µm per year (mean thickness 249 µm), and wide variability between individuals was also observed [216]. Future studies might seek to confirm and detail the anticipated decline in ERG amplitude and other changes in ERG parameters with age in the normal dog.

7.3.2 Further questions raised by the descriptive study

We and others have noted that the light adapted single flash ERG responses recorded from RPE65-deficient dogs are of similar amplitude to those of unaffected normal dogs while the cone flicker responses are typically unrecordable or very reduced in amplitude.[114] This feature that has also been observed in *Rpe65*^{R91W} knockin mice.[47] In these cases it has been suggested that the background light used is not adequate to suppress the responses from the desensitized rods, resulting in a light-adapted flash ERG response comprised of cone responses and incompletely light-adapted rod responses.[47, 114] In the normal situation the contribution of rod photoreceptors to the light-adapted flash ERG is minimal. The hypothesis of incomplete light adaptation of the rods in models of RPE65 deficiency is supported by several points. First unbound rod opsin induces persistent low-grade rod signaling.[95] Second, a report describing low amplitude photopic ERG recordings in double knockout *Rpe65*^{R91W} mice with absent rod function due to blocked rod transducin signaling, showing the light-adapted ERG was no longer 'falsely elevated'.[109]

The progressive accumulation of lipid droplets containing retinyl esters in the RPE has been observed in both canine and murine models.[67, 86, 104, 105, 127, 128] At present it is not known if these RPE lipid accumulations are a pathologic feature of significance, or whether *RPE65* gene replacement therapy slows their development and progression.

A previously undescribed feature of the canine RPE65-deficeint phenotype that we have described is the development of a region of ONL thinning and photoreceptor loss in the *area centralis* of RPE65-deficient dogs. The *area centralis* is the region of highest photoreceptor density in the canine retina. Further studies are required to determine the photoreceptor subtype(s) lost in this area. It is conceivable that the rapid death of the photoreceptors in the region is because of their high stacking density here results in greater competition for available residual chromophore. In the canine *area centralis* the rod photoreceptor packing density is reported to be approximately 500,000 cells/mm^2, almost 5 times greater then in the region of maximal density in humans (130,000 cells/mm^2 located 4-6mm from the foveola), perhaps explaining why this focal region of retinal thinning has not been observed in the fundus of RPE65-LCA patients.[116, 217-219] This may also explain why this feature has not been described in murine models of RPE65-deficiency, as mice lack a region of higher photoreceptor density. The reason this feature has not been described in other RPE65-deficient dog colonies may be its small size and difficulty appreciating it under bright fundus illumination levels. However, as it has not been reported in other colonies a role of background genetics or other modifying loci cannot be excluded and again would argue for a comparative genomic study.

Future studies will look at whether the progression of this feature is halted or impeded by *RPE65* gene replacement therapy at an early age. Other studies might include an evaluation of chromophores in this region and examination of other RPE65-deficient dogs from other colonies using similar examination techniques.

A final question is how RPE65-deficient individuals and animals with a mutation that results in a non-functional protein have any remaining vision, since RPE65 is the isomerohydrolase required for regeneration of 11-*cis* retinal. It has been proposed that an alternative as yet undiscovered chromophore mediates the remnant vision.[54] This alternate chromophore would similarly have an alternate regenerative biochemical pathway, also as yet undiscovered. Studies of RPE65 knockout mice have demonstrated 9-*cis* retinal plays a role in the rod photoreceptor mediated vision, present in the retina and RPE of RPE65 knockout mouse, it forms light sensitive isorhodopsin independent of RPE65.[220] However, the exact source of 9-*cis* retinal is currently unknown. A role of alternate pathways and chromophores are not new ideas, the relatively slow rate of the canonical visual cycle and competition with rods for available 11-*cis* retinal, have previously led to the suggestions that cones use an alternate mechanism for recycling of chromophore to allow the preferential rapid supply of recycled 11-*cis* retinal to the cones.[221] Further Wang *et al* argue for the existence of a second, cone-specific retinoid cycle.[221] Evidence for this includes the closer physical proximity of rods to the apical processes of RPE cells that would favor traffic of chromophore to rods over cones, and the tendency of cone pigments to dissociate spontaneously into opsin and 11-*cis* retinal, possibly allowing the retinoid to be lost to adjacent rod photoreceptors.[221]

7.4. Successful treatment of older RPE65-deficient dogs

Prior to this study, despite reports of gene therapy treatment of over 100 eyes of RPE65-deficient dogs, only 5 eyes of dogs aged over 2 years have been treated with mixed success.[135, 156, 157] Of these, one eye of a dog 2.5 years of age had no evidence of rescue despite the expression of RPE65 protein in the injected area, though a 4 year-old dog is reported to have received bilateral injections and to show rescue with no details given.[135, 157] We report successful treatment of 13 eyes of RPE65-deficient dogs aged 2-6 years with no appreciable decline in ERG or vision testing outcomes. This was despite the documented progressive photoreceptor loss reported in our study describing the phenotype of these dogs and by other authors.[127, 128] This was an unanticipated finding and indicates that the threshold for achieving successful rescue in the canine model is 6 years or above. Our results here are in accordance with those of a study in RPE65 knockout mice, describing the regeneration of similar amounts of rhodopsin and improved rod photoreceptor function to similar levels after 11-*cis* retinal injections, irrespective of age and the degree of photoreceptor degeneration.[207] Certainly treatment of older mice with more advanced retinal degeneration has resulted in poorer outcomes eyes.[40] However there was marked retinal degeneration in these mice, and perhaps similar results could be anticipated if RPE65-deficient dogs older then 6 years were treated in future studies. While we do not have results that support selecting for RPE65-LCA patients with a lesser degree of retinal architecture changes in efforts to improve outcomes (whether by age or patient screening by OCT imaging), we do not have evidence to argue against this. We hypothesize that treatment of RPE65-deficient dogs 6 years of age and older may more closely emulate the human treatment challenges. Future studies are also

proposed to benefit from *in-vivo* evaluation of retinal thickness by OCT pre and post-treatment. Since completion of this study outcomes of treatment of additional RPE65-LCA patients have been reported, and the treatment of younger patients has resulted in variable outcomes to date.[170, 171]

7.4.1 Effect of treatment area and location on ERG and vision testing outcomes

In evaluating for an effect of different variables on treatment outcomes we appreciated a correlation between size of the treated area and cone ERG amplitude. A similar effect was observed for both rod and cone amplitude in our study of the safety and efficacy of treating the second eye of RPE65-deficient dogs. Noting here that studies using immunohistochemistry have shown that *RPE65* is only expressed in the treated area.[114] The conclusion that treatment of as much of the retina as possible may improve outcomes is in accordance with the theory and practice of one of the groups performing RPE65-LCA clinical trials.[164] Various techniques can help facilitate this, such as core vitrectomy, injection bleb massaging, and multiple injections. However the size of the treated area may be less important than injection location, with our study of treatment of older dogs showing an effect of injection location on cone ERG rescue and both vision testing outcome measures. This effect of improved outcomes was seen if the region of highest rod and cone photoreceptor density in the canine retina, the *area centralis,* was included in the injected area.. Other authors have described similarly improved outcomes when the superior retina of RPE65 deficient dogs was treated, the region where the *area centralis* is located, relative to treatments in the inferior retina.[114] At this time, the human clinical trials lack consensus for an effect of treatment area on visual acuity, in terms of both treatment size

and location.[163-171] Some of the difficulty here is that while each group of investigators used similar approaches, delivering rAAV2/2 to the RPE by subretinal injection, there were important differences between the trials. These include the use of different promoters in the vector construct, and treatment of RPE65-LCA patients of different ages with different degrees of residual visual function pre-treatment.

7.5 Treatment of the second eye of RE65-deficient dogs is safe and effective

Treatment of both eyes was explored as a method to improve overall vision, particularly since in the Phase I/II clinical trials it was the worst affected eye that was treated.[163-165] However an important question was whether this would result in untoward immune responses that would preclude or limit the success of treatment in the second eye, or even result in potentially damaging ocular inflammation. Our study of treatment of the second eye showed improved ERG and vision testing outcomes in RPE65-deficient dogs with no diminution of measured outcomes in the second treated eye and no evidence of untoward immune responses. These results provided evidence to support inclusion of treatment of the second eye in the RPE65-LCA clinical trials. Since publication of our findings and those of a very similar but smaller study by Amado *et al*, the safe and effective gene therapy treatment of the second eye of 3 previously treated RPE65-LCA patients has been reported.[222, 223] Bennett *et al*, using fMRI, also report that treatment of the second eye improved cortical responses received from the first treated eye as well as the second treated eye of RPE65-LCA patients.[223] A finding suggested to be a consequence of improved binocularity.[223] In concert these studies provide evidence to proceed with gene therapy treatment in both eyes of RPE65-LCA patients. However a potential

downside to performing bilateral treatments is that any adverse event may preclude future therapy should there be additional advances in the treatment of inherited retinal diseases such as RPE65-LCA. At this time due to the potential for improved outcomes in future the more conservative approach of treating only one eye is likely to be recommended.

7.6 Future directions

7.6.1 Improving viral vector transduction efficiency

Efforts to improve the efficiency of gene transfer to the RPE cell nucleus offer a means of improving therapeutic outcomes. The evaluation of methods for improving transduction efficiency is likely to be performed in the RPE65-deficient dog prior to inclusion in future RPE65-LCA clinical trials. Self-complementary (sc) rAAV vectors containing two dimeric inverted repeat copies of the gene to bypass the rate-limiting step of synthesis of double stranded DNA in the RPE cells have been evaluated in this way.[115] With stronger and more rapid GFP expression in the outer retina demonstrated by subretinal injection of scAAV2/5 when compared to single stranded AAV2/5.[115] However this technique is currently limited by the 4.7kb size constraint of the AAV vector.[147, 150] While the human *RPE65* cDNA is only 2.1kb (www.genecards.org), it is typically placed under 1.6 kb fragment of its natural promoter meaning an scDNA insert would be two strands of 2.1kb plus 2 strands of 1.6kb, totaling 7.4kb.[157, 224] In future this approach may prove useful in light of a recently published report describing packaging of AAV5 capsids with up to 8.9 kb of transgene.[225] Though further studies will be required as a recent report suggested that the AAV packaging limit of 4.7kb is not exceeded, rather the transgene is

split into fragments across independent vector particles, which limits gene transfer efficacy.[226]

7.6.2 Evaluation of non-viral methods of gene transfer

The use of non-viral gene transfer is attractive as it would avoid immunologic reactions against viral vectors, and overcome the limited packaging capability, particularly of rAAV vectors. Non-viral methods might allow delivery of entire genomic DNA fragments, including gene regulatory elements and intronic sequences that could conceivably improve transgene expression efficiency.[141] Additional advantages of non-viral gene therapy may include lower production costs and a likely classification as a drug rather then a biologic which would further reduce costs and administrative efforts.[141] At present, though approaches such as electroporation and the use of nanoparticles have been studied in murine models and appear to hold promise, these approaches have not been shown to match the transfection rates and transgene persistence achieved with viral vector-mediated gene transfer.[227-229] As further work is carried out in these promising areas the confirmation of safety and efficacy in the RPE65-deficeint dog will likely be required prior to initiation of human clinical trials.

7.6.3 Gene therapy treatment of other ocular diseases

The relevance of these studies is not limited to treating RPE65-LCA. Efforts to optimize outcomes will be relevant for the treatment of other retinal dystrophies. Recently Beltran *et al* reported the successful gene therapy treatment of two canine models of X-linked retinitis pigmentosa.[113] In addition to treatment of inherited diseases, gene therapy is being explored

for treatment of common pathways of non-inherited disorders. Most prominently, augmentation of common anti-angiogenic factors to combat vascular diseases such as age related macular degeneration and diabetic retinopathy has now entered phase I clinical trials, excellently described in a recent review by Campochiarro *et al*.[230] Moving beyond the retina other immune privileged ocular tissue such as the cornea has been successfully treated using AAV vectors to deliver a gene to down-regulate the vascular endothelial growth factor (VEGF) in situations of corneal neovascularization.[231] These and future studies are indebted to the pioneering work performed in clinical and pre-clinical trials of gene therapy for RPE65-deficiency.

7.6.4 Beyond gene therapy for inherited retinal dystrophies

Despite the promise of gene replacement therapy for inherited ocular diseases, given the diversity of mutations that cause these disorders, such an individualized approach to therapy may be less practical then methods of delaying or preventing common pathologic outcomes such as photoreceptor cell death. In the case of RPE65 deficiency where there is a paucity of 11-*cis* retinal, oral and intravitreal administration of retinoid have been studied and had mixed success.[111, 232, 233] To target the photoreceptor degenerative process administration of neuroprotective agents has been investigated. One of these agents, ciliary neurotropic factor (CNTF) has been commonly studied, and has been shown to promote both rod and cone photoreceptor survival.[234-237] Finally, of the different approaches being evaluated, stem cell therapy is a rapidly developing field that offers the promise of replacing non-functioning photoreceptors or other retinal cells. A sub-population of retinal Müller glial cells with stem cell

characteristics has been recently identified in the adult human retina, these have been proposed to be easier to differentiate into retinal cells than embryonic stem cells, offering the prospect of efficient retinal transplantation.[238, 239] Hurdles remain, including degree of donor cell integration, and ability to evade immune rejection, though again the immune-privileged nature of the subretinal space offers advantages. Certainly with such prospects on the horizon the future looks bright.

7.7 Conclusion

A large part of this thesis was aimed at providing information that could help answer some of the questions that have arisen from the RPE65-LCA gene therapy clinical trials. Using the RPE65-deficient dog model we have provided evidence that gene therapy treatment of the second eye is safe and effective. Treatment of the second eye of RPE65-LCA patients has since been successfully performed. We also showed that treatment of RPE65-deficient dogs 2-6 years of age improves retinal function and vision testing outcomes with no diminution of outcomes despite progressive photoreceptor loss. We have proposed a future study to evaluate for a threshold in dogs aged 6 years or older where retinal degeneration may impede successful treatment. Further, given the increasing use of canine models of inherited retinal disease our evidence for a no improvement in outcomes with repeated testing using an objective vision testing apparatus is of value for future studies. Our detailed description of the phenotype of the RPE65-deficient dog provides valuable important for current and future studies using this important model of RPE65-LCA and highlights additional areas of future research interest. This

includes further evaluation of a newly described region of photoreceptor loss at the canine *area centralis.*

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