10-30-1998

Congenital Stationary Night Blindness in the Dog: Common Mutation in the RPE65 Gene Indicates Founder Effect

Gustavo D. Aguirre
University of Pennsylvania, gda@vet.upenn.edu

Victoria Baldwin

Sue Pearce-Kelling

Kristina Narfström

Kunal Ray

*See next page for additional authors*

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Recommended Citation


PMID: 9808841

At the time of publication, author Gustavo Aguirre was affiliated with the James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University. Currently, he is a faculty member at the Vet Med school at the University of Pennsylvania.

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Abstract

**Purpose:** To clone and characterize the canine RPE65 cDNA from normal dog, examine for mutations, and establish if the mutation identified in Swedish briard dogs with retinal dystrophy is present in dogs of the same breed that originated from the United States and other countries, and are affected with congenital stationary night blindness.

**Methods:** Fifteen briard dogs were studied, of which 10 were affected with *csnb*, and five were clinically normal. In addition, we tested samples from four Swedish dogs, and samples from a briard affected with progressive retinal atrophy. RPE65 cDNA was cloned a from retinal cDNA library by PCR, and from canine retina by RT-PCR. ERG and morphology were used to characterize *csnb*.

**Results:** The normal RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence; the deduced amino acid sequence shares 98%, 97%, and 93% identity with homologous human, bovine, and rat sequences, respectively. A homozygous four nucleotide (AAGA) deletion, representing nucleotides 487-490 of wildtype RPE65 sequence, was found only in *csnb* and retinal dystrophy affected dogs; heterozygous animals had normal and mutant alleles. The mutation produces a frameshift, causing a deduced mistranslation with a premature stop codon. The mutation causes retinal dysfunction and RPE accumulation of lipid vacuoles.

**Conclusions:** Identification of the same mutation in *csnb* and retinal dystrophy confirms the molecular identity of the two disorders. A common mutation in dogs derived from different countries suggests a founder effect causing the propagation of a common mutant allele in the population at risk.

Disciplines
Comparative and Laboratory Animal Medicine | Genetic Phenomena | Medicine and Health Sciences | Veterinary Medicine

Comments
PMID: 9808841


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**Author(s)**
Gustavo D. Aguirre, Victoria Baldwin, Sue Pearce-Kelling, Kristina Narfström, Kunal Ray, and Gregory M. Acland

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Congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect

Gustavo D. Aguirre,1 Victoria Baldwin,1 Sue Pearce-Kelling,1 Kristina Narfström,2 Kunal Ray,1 Gregory M. Acland1

1James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA; 2Department of Medicine & Surgery, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden

Purpose: To clone and characterize the canine RPE65 cDNA from normal dog, examine for mutations, and establish if the mutation identified in Swedish briard dogs with retinal dystrophy is present in dogs of the same breed that originated from the United States and other countries, and are affected with congenital stationary night blindness.

Methods: Fifteen briard dogs were studied, of which 10 were affected with csnb, and five were clinically normal. In addition, we tested samples from four Swedish dogs, and samples from a briard affected with progressive retinal atrophy. RPE65 cDNA was cloned from retinal cDNA library by PCR, and from canine retina by RT-PCR. ERG and morphology were used to characterize csnb.

Results: The normal RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence; the deduced amino acid sequence shares 98%, 97%, and 93% identity with homologous human, bovine, and rat sequences, respectively. A homozygous four nucleotide (AAGA) deletion, representing nucleotides 487-490 of wildtype RPE65 sequence, was found only in csnb and retinal dystrophy affected dogs; heterozygous animals had normal and mutant alleles. The mutation produces a frameshift, causing a deduced mistranslation with a premature stop codon. The mutation causes retinal dysfunction and RPE accumulation of lipid vacuoles.

Conclusions: Identification of the same mutation in csnb and retinal dystrophy confirms the molecular identity of the two disorders. A common mutation in dogs derived from different countries suggests a founder effect causing the propagation of a common mutant allele in the population at risk.

The briard dog is affected with a recessively inherited retinal disorder characterized by congenital night blindness with various degrees of visual impairment under photopic illumination. Vision in affected dogs ranges from normal day vision to profound day blindness [1]. The disease was initially described in Swedish dogs as a stationary disorder analogous to human congenital stationary night blindness (CSNB [2]). More recently, the disease has been described as having a progressive component, and has been termed hereditary retinal dystrophy [3,4]. Along with the visual impairment, affected dogs have an abnormal electroretinogram (ERG); in general, the recorded responses are normal in waveform, but show a marked diminution of response amplitudes, similar to a “Riggs type” ERG in man. The ERG recorded under DC conditions shows complete absence of the a-, b-, and c-waves, with the latter waveform being replaced by a very slow negative potential which develops when the stimulus intensity is greater than 3 log units above the normal b-wave threshold. The authors interpret the abnormalities in the a- and b-waves as representing a delay in rod phototransduction [5]. A similar disease is also recognized in other countries, including France, Canada, and the United States. In the US, the disease is termed congenital stationary night blindness, and csnb has been designated as the gene symbol for the disease locus. Apart from the above studies in Swedish briard dogs, no other systematic investigation of the disease has been reported, nor has there been definitive proof that csnb and retinal dystrophy represent the same disorder.

During a presentation on the mutation spectrum of the RPE65 gene in childhood onset retinal dystrophies at the 1998 ARVO meeting, Andreas Gal and associates stated that a 4-nucleotide (AAGA) deletion in the RPE65 gene was responsible for hereditary retinal dystrophy in the Swedish briard dog [6]. This presentation prompted us to clone and characterize the canine RPE65 cDNA from normal dog, examine for mutations in the coding sequence of this gene, and establish if the mutation described in Swedish briards is present in dogs of the same breed that originated from the United States and other countries. Our results indicate that the same four nucleotide deletion in the RPE65 gene is the mutation causing csnb in the briard dog.

METHODS

Animals: Briard dogs affected with csnb and related and unrelated phenotypically normal dogs have been examined to characterize the disease phenotype, and examine for mutations in the RPE65 gene. Overall, we have studied 15 briard dogs, of which 10 were affected with csnb, and five were clinically normal. These dogs came primarily from the US and Canada, of breeding stock that originated from the US and...
France. In addition, we tested samples from four Swedish dogs, both purebred briard or briard-beagle crosses, of which two were affected and two heterozygous for the reported four nucleotide deletion in the RPE65 gene. Lastly, we tested samples from four littermate briard dogs that we had examined previously. One individual from this last group of four dogs was clinically affected with a retinal degenerative disorder. This dog, at 6 years of age, showed evidence of night blindness, hesitant behavior in bright light, and ophthalmoscopically visible retinal thinning and vascular attenuation characteristic of mid-stage progressive retinal atrophy (PRA [7]). ERG testing confirmed the retinal disorder, and indicated that only cone mediated responses were recordable (Aguirre, unpublished data).

All dogs studied were subjected to a comprehensive clinical ophthalmic examination, including indirect ophthalmoscopy and slit lamp biomicroscopy. In addition, a selected number of dogs underwent ERG testing as previously described [8]. Briefly, the ERG was recorded from the hAlthone anesthetized dog using a stimulus protocol that, by differentially eliciting rod and cone components of the ERG, allowed their separate evaluation [7-10]. Signal averaging of very low amplitude responses also was conducted to examine the waveform of these responses.

The eyes from two dogs, 4.3 and 10.7 months of age, were removed following euthanasia by barbiturate overdose, and processed for microscopic examination using methods we have previously described for embedding either in plastic (4.3 months, both eyes) [10] or in the synthetic wax diethylene glycol distearate (DGD; 10.7 months, one eye) [11]; the tissues were sectioned at 1 µm and stained with azure II/methylene blue. The retina of the fellow eye of the 10.7-month-old dog was isolated under sterile conditions, and kept frozen at -70 ºC until used for these studies. All procedures involving animals were undertaken in strict compliance with the guidelines of the US Public Health Service’s policy on the Humane Care and Use of Laboratory Animals, and the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

**Genomic DNA and RNA samples:** Genomic DNA was isolated using standard techniques [12] from either blood samples collected in citrate anticoagulant tubes, or from splenic samples from deceased dogs. Retina from the enucleated fellow eye of the 10.7-month-old affected dog was utilized for RNA extraction; total RNA was isolated from retina using the guanidinium-phenol procedure previously described [13].

**Screening of a canine retinal cDNA library for RPE65 clone by polymerase chain reaction (PCR):** The canine retinal cDNA library was custom made (Stratagene Cloning Systems, La Jolla, CA) from poly-A+ RNA isolated from retinas of homozygous normal miniature poodles. RPE65 cDNA sequence was retrieved from the cDNA library by polymerase chain reaction (PCR) based screening of the library. A forward primer (RPE65-1: 5'-CAA TGC CCT TGT TAA TGT CTA CCC AG-3') and a reverse primer (RPE65-3: 5'-CCT GCT TAA TTG TCT CAA AGG TCT C-3') were designed from the consensus region of human (GenBank accession number U18991), bovine (GenBank accession numbers L11356 and X66277) and rat (GenBank accession number AF035673) RPE65 cDNA sequences. The gene specific forward primer, RPE65-1, was used in combination with a vector specific reverse primer (pBK-V: 5'-CCG CTC TAG AAG TAC TCT CGA GTT -3') to amplify the 3'-region of the canine homologue of RPE65 cDNA. Similarly, the gene specific reverse primer, RPE65-3, was used in combination with the vector specific forward primer (pBK-III: 5'-GGT CGA CAC TAG TGG A TC TAA CCC AG-3') to amplify the 5'-region of the canine RPE65 cDNA that would have an overlapping region with the amplified 3' cDNA fragment. PCR was done for 30 cycles (94 ºC for 1 min, 60 ºC for 1 min, 72 ºC for 2 min with a final extension at 72 ºC for 10 min) using 0.4 µM of each primer pair in a volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, and 0.2 mM each dATP, dCTP, dGTP and dTTP.

**Reverse transcription (RT)-PCR from canine retina:** A 540 nucleotide long 5'-region of canine RPE65 cDNA was successfully amplified by PCR from the retinal cDNA library, but the 3'-region of the cDNA did not yield PCR product containing the remainder of the coding sequence. To clone the 3' end, a new consensus primer (RPE65-8: 5'-TGC TTG CTC AAC TCA GTG CTT TCT G-3') was designed, as described above, from the 3'-untranslated region (UTR) of mammalian RPE65. A 1400 bp DNA fragment was amplified by RT-PCR using the RPE65-1 and RPE65-8 primer pair from total RNA isolated from retina using RNA PCR kit (Perkin Elmer, Foster City, CA). The identity of the amplified DNA fragment was confirmed to be RPE65 by direct sequencing of the PCR product, and comparison with previously published homologous sequences using the BLAST service. PCR conditions were as above.

**PCR using genomic DNA templates:** To identify the presence of a mutation in the canine RPE65 gene and examine for...
co-segregation of the mutation and the disease, PCR was undertaken for 30 cycles (94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min with a final extension at 72 °C for 5 min) using primers RPE65-1 and RPE65-3 selected from a single exon (putative exon 5) spanning the location of the mutation. The sizes of the DNA fragments amplified from homozygous normal and csnb-affected dogs were 109 bp and 105 bp, respectively. The amplified DNA fragments were electrophoresed in a 6% nondenaturing polyacrylamide gel using TBE buffer (0.089 M Tris-borate and 0.002 M EDTA, pH 8).

DNA sequencing: PCR amplified DNA fragments were used directly for sequencing after purification of the samples. Sequencing was accomplished by Taq cycle sequencing using BigDye™ fluorescent terminators (PE-Applied Biosystems, Foster City, CA) in an ABI 377 DNA sequencer (PE-Applied Biosystems) at the core sequencing facility of Cornell University. Sequence manipulation and comparison were undertaken using programs Seqed (ABI Applied Biosystems, Inc.) and Gene Jockey II (Biosoft, Cambridge, UK), and the Genbank BLAST service [14].

RESULTS

Clinical and morphologic characterization of csnb: We have examined 10 briard dogs affected with csnb that originated from stock in the US, Canada and France; in addition, we examined five clinically non-affected briards that were related to the affected dogs in this study. The obligate heterozygous animals had normal ophthalmologic examination; prior studies have shown that visual function and the ERG of heterozygous animals is normal (Aguirre and Acland, unpublished data). In contrast, the affected dogs had a severe impairment of visual function that primarily affected night vision, but, in some cases, day vision was affected to various degrees. When young, some of the animals had distinct nystagmus which disappeared with aging, but could be induced with excitement; no other abnormalities were recognized on clinical ophthalmic exam. The one older affected dog that was available for examination at 4 years of age also showed no abnormalities on ophthalmologic examination.

Electroretinography of affected dogs showed that the rod and cone mediated responses were severely depressed in amplitude in comparison to those recorded from normals (Figure 1). In general, the responses appeared diminutive or non-recordable under most recording conditions, especially when the retina was stimulated with weak illumination. Higher intensity flickering light stimuli that elicited cone-mediated responses often resulted in low amplitude signals (data not shown). Signal averaging showed the presence of small amplitude responses that often had a normal waveform, similar to a “Riggs type” ERG in man [2].

Light microscopic examination of the retinas showed pathologic changes limited to the retinal pigment epithelium (RPE) and photoreceptor layers. These abnormalities were most distinct in plastic embedded sections of the retina of the younger dog (4.3 months), but were also evident in the eye from the older affected dog that was embedded in DGD. The photoreceptor outer segments appeared normal, particularly in the periphery, but showed slight disorganization in the posterior pole and equator (Figure 2A). Additionally, there was an uneven shortening of the rod inner segments that caused the rod outer segments to have a variable length, even when structurally normal (Figure 2B). The shortening of the rod inner segments resulted in increased prominence of the cones in the photoreceptor layer. The most remarkable abnormalities, however, were present in the RPE, and consisted of the accumulation of cytoplasmic inclusions of variable size. These inclusions were single to multiple, and were vacuolated or appeared homogeneous (Figure 2C-E). The RPE inclusions appeared to coalesce and were much larger in the 10.7-month-old dog. The RPE appeared somewhat reactive in that the cells were slightly hypertrophied, and their apical surfaces were irregular. At the two ages examined, there were no other pathologic changes in the retina, and no evidence of photoreceptor degeneration or cell death as indicated by the presence of an outer nuclear layer of normal thickness.

Characterization of the canine RPE65 cDNA: Overlapping fragments of normal canine RPE65 cDNA were amplified from the retinal cDNA library by PCR, and from retinal RNA by RT-PCR. The characterized region of normal canine RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence predicted to encode a protein of 533 amino acids (61 kDa), 27 nucleotides of 5'-UTR and 94 nucleotides of 3'-UTR (Figure 3). Over the coding region, the canine RPE65 gene shares 88-89% nucleotide sequence identity with homologous human and bovine sequences, and 83% identity with rat sequence. The deduced amino acid sequence shares 98%, 97%,
and 93% identity with homologous human, bovine, and rat sequences, respectively. csnb results from the same mutation in the RPE65 gene causing retinal dystrophy in Swedish briard dogs: Once we characterized the normal canine RPE65 cDNA, the cDNA was amplified from csnb-affected retina and compared with the normal. We observed that the four nucleotide (AAGA) deletion reported to cause retinal dystrophy in Swedish briards [6] is present in csnb-affected briards in USA and Canada. The deleted nucleotides (AAGA) represent nucleotides 487-490 of wildtype canine RPE65 sequence, and correspond to nucleotides 340-343 of human exon 5 (Genbank accession number U20479). The mutation produces a frameshift, causing a deduced mistranslation of (now) nucleotides 487 through 645, with a stop at (now) codon 205 (nucleotides 643-645 of mutant sequence). No other disease causing mutations were identified in the sequence obtained from the affected dogs.

To identify the mutation from genomic DNA in susceptible dogs, a region of putative exon 5 encompassing the site of the mutation was amplified. As shown in Figure 4, PCR using genomic DNA from normal and csnb-affected dogs resulted in amplification of DNA fragments 105 bp and 109 bp long, respectively. As expected, PCR product from an obligate heterozygote showed a double band indicating that only one allele contained the mutation. Also, the presence of two different hybridized bands with slower mobility indicates that the target site of the mutation is a polymorphism or a pseudogene. We observed that the four nucleotide (AAGA) deletion reported to cause retinal dystrophy in Swedish briards is present in csnb-affected briards in USA and Canada. The deleted nucleotides (AAGA) represent nucleotides 487-490 of wildtype canine RPE65 sequence, and correspond to nucleotides 340-343 of human exon 5 (Genbank accession number U20479). The mutation produces a frameshift, causing a deduced mistranslation of (now) nucleotides 487 through 645, with a stop at (now) codon 205 (nucleotides 643-645 of mutant sequence). No other disease causing mutations were identified in the sequence obtained from the affected dogs.

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Figure 4. Sequencing of the amplified DNA fragments from the normal and csnb-affected pedigree revealed deletion of four nucleotides (AAGA).

**Cosegregation of the RPE65 mutation in csnb-affected biparietal pedigree:** Once the four nucleotide deletion in the RPE65 gene was identified in csnb-affected dogs, we examined a biparietal pedigree informative for csnb to determine if the mutation cosegregated with the disease. In the animals available for molecular testing, we found complete cosegregation of the mutation with the disease; affected dogs were homozygous for the four nucleotide deletion while obligate carriers were heterozygous for the normal and mutant allele.
Progressive retinal atrophy (PRA) in the briard dogs is not associated with the RPE65 mutation causing csnb and retinal dystrophy: To determine if this mutation in the RPE65 gene is associated with PRA in the briard, we tested samples from four littermate dogs of this breed in which a diagnosis of PRA had been made in one of them on the basis of the characteristic visual, ophthalmoscopic and ERG abnormalities. We found no abnormality in the region of the RPE65 gene that harbors the mutation responsible for csnb, either in the PRA-affected dog or in its normal littermates (Figure 4, right panel).

DISCUSSION

The clinical, electrophysiologic and pathologic features of retinal dystrophy in the briard dog have been reported in a series of very detailed studies from Sweden [1-5]. The disease has a characteristic clinical phenotype, consisting of profound visual impairment present soon after the dog is sufficiently mature to test visual function (5-6 weeks of age), and a normal appearing fundus, at least for the first 3-4 years of age. Older dogs may show subtle retinal abnormalities indicative of a slowly progressive retinal degenerative process. The ERG responses, both rod and cone mediated, are also abnormal, and the DC ERG suggests a defect in the phototransduction process [5]. Surprisingly, the photoreceptor cells do not show extensive pathologic abnormalities, at least in the early stages of the disease, that would be expected for animals with such functional deficits. The RPE has shown a dramatic accumulation of lipoidal inclusions [3,15] that, until now, appeared to be an unexplained byproduct of the disease process (see below).

Because of the clinical similarities in phenotype between retinal dystrophy and csnb, a disease identified in briard dogs from the United States and Canada, we examined a selected population of briards using methods which would evaluate the clinical, functional and morphologic characteristics of csnb in a manner that was analogous to the studies done on the Swedish dogs. With the limitation imposed by using slightly different methods, our results are totally compatible to those published by Narfström and associates in their studies [1,2,4]. At least on a clinical and morphologic basis, we can conclude that csnb and retinal dystrophy appear to represent the same disorder. Based on the four nucleotide deletion in the RPE65 gene that was reported to be causally associated with retinal dystrophy in briards [6], we cloned and characterized the canine RPE65 cDNA to determine if a mutation in this gene is present in csnb, and if it is the same as that causing retinal dystrophy in the Swedish dogs.

Previous reports have indicated that the RPE65 gene is exclusively expressed in the RPE [16,17]. However, we decided to characterize the cDNA from a retinal cDNA library on the premise that even a low level of expression of the gene in the tissue would be sufficient for amplification of the coding sequence, and characterization of the UTR. The characterized region of normal canine RPE65 cDNA spans 1724 nucleotides (GenBank accession number AF084537), and includes 1602 nucleotides of coding sequence predicted to encode a protein of 533 amino acids (61 kDa), 27 nucleotides of 5'-UTR and 94 nucleotides of 3'-UTR. Comparison of the sequence between the normal and csnb-affected dog indicated that in the affected there was a four nucleotide deletion (AAGA) in the putative exon 5 of the RPE65 gene that was the same as described by Gal and associates for dogs with retinal dystrophy. The deleted nucleotides (AAGA), represent nucleotides 487-490 of wildtype canine RPE65 sequence, and correspond to nucleotides 340-343 of human exon 5. The mutation produces a frameshift, causing a deduced mistranslation with a stop at (now) codon 205 (nucleotides 643-645 of the mutant sequence), and a presumably non-functional RPE65 gene product.

To establish if the observed mutation was causally associated with the disease, we amplified from genomic DNA a region of the putative exon 5 encompassing the site of the mutation. In a three generation pedigree informative for csnb, we could establish the cosegregation of the mutant allele with 100% concordance. These dogs were part of a larger sample of 15 briard dogs whose disease status was known, and derived from breeding stock that originated from the US and France. In all cases, affected dogs showed the homozygous four nucleotide deletion of the RPE65 gene, while obligate heterozygous dogs had the mutation in only one allele. Lastly, we tested four Swedish briard or briard-beagle crosses, two affected and two heterozygous for retinal dystrophy [1], and found the same mutation. Identification of the same mutation in briards with csnb retinal dystrophy confirms the molecular identity of the two disorders. Furthermore, because some of the dogs tested in this study were apparently unre-
lated, the finding of a common mutation in dogs derived from different countries suggests a founder effect causing the propagation of a common mutant allele in the population at risk.

Progressive retinal atrophy also is present sporadically in the briard breed, and the clinical and functional abnormalities identified in the intermediate stages of the disease could be compatible with those present in older dogs affected with csnb. To exclude the four nucleotide deletion in the RPE65 gene from causal association with PRA, we tested samples from four littermate briard dogs, one PRA-affected and three non-affected. For the region of the RPE65 gene examined by PCR, we did not find the four nucleotide deletion that results in csnb. Thus, this mutation could be excluded as a cause of PRA in this dog breed.

In their 1997 paper, Gu and associates described five different mutations in the RPE65 gene responsible for autosomal recessive childhood-onset severe retinal dystrophy [18]. Most patients had severe visual deficits present at birth or within the first decade of life. Ophthalmoscopic abnormalities varied from vascular attenuation and optic disc atrophy without bone spicules, to lesions typical of advanced retinitis pigmentosa in adults. In these patients, the disease progresses to severe visual impairment and blindness, and concomitant ophthalmoscopic abnormalities indicative of advanced retinal degeneration [18]. Similar abnormalities have been described in a second study of the disease [19]. More recently, mutations in this gene have been causally associated with autosomal recessive RP or Leber congenital amaurosis [20]. Although the profound visual deficit early in life is similar in the human and dog, the lack of ophthalmoscopically visible advanced retinal degeneration in adult dogs is not, and may indicate a difference in the temporal course of the photoreceptor disease. After all, most dogs affected with the different forms of PRA show evidence of advanced fundus pathology by 5 years [7], an age that would be comparable to a 35-year-old human.

Mice with an RPE65 gene knockout were recently created [21]. Homozygous mutant mice show irregularities of the rod outer segments by 15 weeks of age, and these changes are associated with a 4.5 log unit increase in the dark adapted threshold, and a small amplitude ERG that is almost identical to that recorded under light adapted conditions. Even though the rod ERG is abolished, the results indicate that the cone ERG is normal (Redmond TM, Personal Communication, May, 1998). A recent commentary has suggested that the RPE65 gene product functions in retinoid metabolism in the RPE and retina [22]. Based on this putative function, the normal cone ERG function in the absence of rod mediated activity could be interpreted as supporting the hypothesis that rod and cones have different and independent pathways for visual pigment regeneration [23]. This difference, however, does not appear to exist in the dog since cone ERG function was compromised in all dogs with the mutation, and profound impairment of day vision was present in some of the affected animals. This issue merits further investigation as it may play a significant role in the evaluation of mice or dogs following RPE cell transplantation or vector-mediated gene therapy for the experimental treatment of the disease.

The salient pathologic abnormality in the retina of dogs with the four nucleotide deletion in the RPE65 gene, documented in this study or reported previously [3,15], is the accumulation of lipoidal inclusions of variable size within the pigment epithelium. Mice with the RPE65 gene knockout have no rhodopsin in the dark adapted retinal rods, but accumulate all-trans retinyl esters in the pigment epithelium (Redmond TM, Personal Communication, May, 1998). The accumulation of all-trans retinyl esters in the RPE suggests that the RPE65 protein functions in one of the metabolic steps involved in the conversion of all-trans retinyl esters to 11-cis retinol [22]. Although the precise function of the RPE65 protein in RPE retinoid metabolism is still to be determined, deficiency of the protein, either in naturally occurring cases or in transgenic knockout mice, results in the accumulation of all-trans retinyl esters in the RPE. Based on prior studies of vitamin A metabolism in the frog eye, these retinyl esters probably accumulate in oil droplets within the RPE which is the major storage depot for esterified vitamin A in the RPE [24]. These lipoidal inclusions are present in the RPE of affected dogs, and their number and size increases with age as reported here and in other studies [3,15].

**ACKNOWLEDGEMENTS**

Supported in part by the Briard Club of America, American Kennel Club-Canine Health Foundation, The Morris Animal Foundation/The Seeing Eye, Inc., The Foundation Fighting Blindness, NEI/NIH Grant EY 06855, and Swedish Medi-
REFERENCES


The typographical corrections below were made to the article on the date noted. These changes have been incorporated in the article and the details are documented here.

17 November 1998: In the caption of Figure 3A, the word “gray” was changed to “purple” and the color of the gray text was changed to purple.