Naturally occurring rhodopsin mutation in the dog causes retinal dysfunction and degeneration mimicking human dominant retinitis pigmentosa

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Rhodopsin is the G protein-coupled receptor that is activated by light and initiates the transduction cascade leading to night (rod) vision. Naturally occurring pathogenic rhodopsin (RHO) mutations have been previously identified only in humans and are a common cause of dominantly inherited blindness from retinal degeneration. We identified English Mastiff dogs with a naturally occurring dominant retinal degeneration (see RetNet, http://www.sph.uth.tmc.edu/RetNet/; refs. 4 and 5). RHO caused by RHO mutations exhibits two different phenotypes (4, 5). One is an early-onset disorder with rapid loss of rods uniformly across the retina. The second has a protracted natural history of vision loss and puzzling features: rod vision can be normal early in life, and degeneration slowly spreads from a disease focus in one retinal region. Furthermore, and independent of disease stage, there is abnormally slow recovery of rod vision after bright light exposure. Variation in severity of this second phenotype may indicate that epigenetic factors play an important role in its progression (5–7) and suggests that it may be particularly amenable to preventive or ameliorative therapies.

Other than in humans, naturally occurring disease-causing RHO mutations have not been identified previously in mammals. Genetically engineered animals and mutagenized flies have been the mainstay for in vivo research and treatment attempts in the past decade (reviewed in ref. 8).

Naturally occurring hereditary retinal degenerations in dogs, termed progressive retinal atrophies (PRAs), are widespread and have provided several models of autosomal recessive and X-linked RP (9–13). We have now identified an autosomal dominant form of PRA, one that closely resembles the second human phenotype described above. This model should advance understanding of the pathophysiology of the disease and therapies for this major subset of dominant RP.

Materials and Methods

**Rhodopsin Gene Sequence Analyses.** Mutation analysis by comparative sequencing of the five canine RHO exons was performed by using published primers (14). Detection of the C to G transversion at nucleotide 11 followed PCR, using primers OPIAF (5'-GCA GCA CTC TTG GGA CTG AG) and OPIAR (5'-TGT AGT GGA GAG GTG TAC GC). Digestion of the 275-bp product with BamFI results in fragments of 202, 47, and 26 bp (wild type) or 249 and 26 bp (nt11C to G). For Northern analysis, wild-type cDNA was amplified with primers D849 and D855 (14) to generate a 701-bp probe. RNA isolation, electrophoresis, transfer, and hybridization were conducted with standard procedures, and the blot was hybridized with β-actin to confirm equal amounts of RNA loading.

**Clinical Electroretinograms (ERGs).** Clinical ERGs were recorded from halothane-anesthetized, dark-adapted dogs as described (15).

**ERG Photoresponses.** Dogs were dark-adapted overnight, premedicated, and anesthetized as described (16). Pupils were dilated with cyclopentolate (1%) and phenylephrine (10%). Pulse rate, oxygen saturation, and temperature were monitored. Full-field ERGs were recorded with Burian–Allen (Hansen Ophthalmics, Iowa City, IA) contact lens electrodes and a computer-based system. High-energy flashes (1-ms duration; maximum luminance of unattenuated white flash = 3.66 log scotopic (scot)-cd·s·m−2) were attenuated and spectrally shaped with Wratten (Eastman Kodak) filters. Dark-adapted photoresponses were elicited by a series of five stimuli presented with 2-min interstimulus intervals: one white, two blue (Wratten 47A), and two red (Wratten 26) flashes (3.66, 2.87, 2.27, 1.27, and 0.5 log scot-cd·s·m−2, respectively). White flash bleaches of 3.66, 5.0, and 5.8 log scot-cd·s·m−2 were used in bleaching adaptation experiments and white backgrounds in the range of −0.9 to 2.3 log scot-cd·m−2 were used in background adaptation experiments. The rod-isolated component of bleaching and background adaptation functions was estimated with the highest energy blue flash (2.87 log scot-cd·s·m−2).

**ERG Analyses.** Rod and cone components of dark-adapted photoresponse series were analyzed with a model of rod and cone

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Abbreviations: RP, retinitis pigmentosa; PRA, progressive retinal atrophy; ERG, electroretinogram; OCT, optical coherence tomography; LRP, longitudinal reflectivity profile; ONH, optic nerve head.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY092841).

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photoisomerization rate was determined on dark-adapted retinas (5, 17). The ratio of this rate to the hyperbolic function fitted to the average LRP was used as an estimate of the background LRP at each location. A full retinal thickness map was generated by interpolation between the non-adjacent retinal thickness estimates.

**Clinical Retinal Examination and Definition of Disease Progression.** Animals were observed ophthalmoscopically for areas of retinal thinning as evidenced by hyperreflectivity of the fundus (first observed in RHO T4R/+ animals 6–12 mo old). Observation of retinal vascular attenuation (first observed after 12 mo) and pallor of the optic nerve head (ONH) (before 36 mo) define advancing stages of the disease.

**Optical Coherence Tomography (OCT).** Cross-sectional retinal reflectivity profiles were obtained with OCT from two normal and three RHO T4R/+ dogs. Principles of the technique (21) and our methods (22, 23) have been described. Multiple scans (4.5- or 8.0-mm-long, at various orientations) were performed to give maximal coverage of an area 9–12 mm in diameter, approximately centered on the ONH. A custom program was used to determine the precise location and orientation of each scan relative to the retinal features (blood vessels and ONH). The individual longitudinal reflectivity profiles (LRPs) that make up each scan were allotted to 0.3 mm × 0.3 mm bins based on their location. The LRPs in each bin were aligned with a dynamic cross-correlation algorithm (22, 23), were median-filtered to remove speckle noise, and were averaged. The difference between the most vitreal maxima and the most scleral minima of the slope in the average LRP was used as a measure of retinal thickness at each location. A full retinal thickness map was generated by interpolation between the non-uniformly spaced retinal thickness estimates.

**Histopathology and Immunocytochemistry.** Retinal sections for morphologic studies were prepared with either a triple fixation protocol (15) before embedding in plastic or 4% paraformaldehyde (24) and immunocytochemistry. For reconstructing the topographic distribution of disease, 1-μm retinal plastic sections, extending from the optic disk to the periphery (sora serrata), were evaluated from continuous overlapping 110-μm fields (25). For immunocytochemical studies, sections from DGD-embedded retinas were labeled with mAb K16-107C, directed at the C-terminal domain of opsin (26).

**Human Studies.** RHO mutation analyses in the patients have been reported (5, 27, 28). Bleaching and background adaptation functions were obtained as described (5, 28, 29). Thresholds as a function of background were fitted to the hyperbolic function

\[ T = \log \left( \frac{I_B}{I_0} \right) \]

where \( T \) is the log threshold, \( I_0 \) is the background luminance that raises the threshold by 0.3 log units, \( I_B \) is the background luminance, and \( n \) is the slope of the function. Note that the model used for the human psychophysical thresholds is the inverse of the function used for dog photoreceptor responses parameters. OCT methods have been published (30).

**Results**

**Identification of a Dominant Canine Progressive Photoreceptor Disease and the Causative Gene Mutation.** PRA in English Mastiff dogs was found to be inherited as an apparently autosomal dominant disease. Confirmation by test mating (Fig. 1a) prompted exon scanning of canine RHO in affected dogs. A single nonsynonymous C → G transversion at nucleotide 11...
changes Thr-4 to Arg (T4R) and cosegregates with disease in the test pedigree (Fig. 1 a and b). A mutation of Thr-4 to Lys (T4K) has been associated with human RP (31). Testing for T4R in PRA-affected (n = 26) and related -unaffected (n = 21) Mastiffs showed the association predicted for a mutation causing dominant disease (23/26 affecteds T4R/+; 3/26 affecteds T4R/T4R; 21/21 normals +/−). The absence of T4R from 136 clinically normal dogs from 17 other canine breeds further indicates T4R is not neutral but causes autosomal dominant PRA.

Clinical ERGs defined the disease as a progressive retinal degeneration. ERG rod- and cone-mediated responses were not significantly different between 2-mo-old normal and T4R/+ heterozygous affected dogs (Fig. 1d). By 12–18 mo of age, however, ERG b-wave amplitudes were severely abnormal in heterozygous affected dogs.

Photoreceptor-specific ERG function was evaluated with high-energy stimuli and a model of phototransduction activation (Fig. 1e). RHO T4R/+ dogs at 3–6 mo of age have rod and cone photoreponses within the normal range. Fully dark-adapted rod photoreceptors thus have normal circulating dark currents and a normal gain of phototransduction amplification despite the RHO mutation. Consistent with clinical ERG results in the heterozygous dogs, by 13 mo of age rod and cone photoresponses were abnormal. A third homozygous (T4R/T4R) dog at 3 mo had photoreponses similar to a 13-mo-old heterozygote (data not shown). The normal photoresponse sensitivity in the T4R/T4R dog suggests the mutant allele can produce a normal gain of phototransduction. The natural history of disease, however, seemed to be accelerated because of the increased mutant allele dosage. The reduced maximum amplitude in older T4R/+ and in young T4R/T4R dogs is consistent with loss of rods, shortening of their outer segments (the rhodopsin-containing cell component), or both.

Abnormal Photoreceptor Adaptation in RHO Mutant Dogs Imitates a Human Phenotype. Our studies of human RHO mutations (5, 27, 28) induced us to ask whether rod photoreceptor function in RHO T4R/+ dogs can recover normally in the dark after adapting light exposure. Rod recovery was probed with ERG photoreponses after different levels of adapting light flashes. Rod-isolated photoreponses of RHO T4R/+ dogs recovered similarly to those of wild-type dogs after the two dimmer adapting flashes (estimated to cause 75% and 78% bleaches, respectively). After the brightest adapting flash (99% bleach; Fig. 2a), however, photoreponses did not recover to baseline.

Analysis of recovery with the maximum amplitude parameter of rod photoreponses showed there was an initial period of near-normal recovery kinetics that was interrupted at approximately 15 min; the resulting abnormal plateau did not appear to change for the duration of the experiment (Fig. 2c). A T4R/T4R dog had adaptation kinetics similar to the T4R/+ dogs (not shown). Recovery of rod sensitivity was similar in normal and mutant animals. Dim adapting lights could be used both in wild-type and RHO T4R/+ dogs to simulate the photoreponse properties of mutant dogs during the abnormal plateau of bleach recovery (Fig. 2b). Analysis of photoreponse parameters as a function of increasing levels of background light showed hyperbolic saturation for maximum amplitude and sensitivity both for wild-type and RHO T4R mutant dogs (Fig. 2d). The decay of equivalent background obtained with a Crawford transformation (32) had dominant linear components on semilogarithmic plots supporting exponential decays of bleach byproducts; the rate of decay was −0.1 log min−1 (time constant = 4.3 min) for the maximum amplitude and −0.05 log min−1 (time constant = 8.7 min) for sensitivity (Fig. 2e).

Defective dark (or bleaching) adaptation in RHO T4R/+ dogs was indeed similar to that in patients with RP with certain RHO mutations (Class B1; ref. 5) (Fig. 2 f–h). In these patients, recovery of psychophysically determined rod thresholds is abnormal after a bright (>95% bleach) adapting exposure (Fig. 2f). Qualitatively, patients with T58R, G106R, and G190D RHO mutations showed an abrupt change of recovery kinetics occur-
show localized thinning with a narrow transition zone (~1.0 mm) separating normal and thin retina. Topographical maps of retinal thickness, based on OCT LRPs, provide further support for the localized distribution of the disease in its early stages (Fig. 3c). Intraretinal variation in severity also occurs in patients with adRP because of RHO mutations (5, 6, 27, 33). A visual field from such a patient shows a scotoma in the superior field (Fig. 3d). Average LRPs from a vertical scan (arrow on the visual field) indicate that the retina becomes progressively thinner as the scan passes into the dysfunctional region (Fig. 3e).

Morphologic examination of RHO T4R+/− retinas confirmed and extended the results of noninvasive studies (Fig. 4). Three observations confirm that RHO T4R+/− retinal photoreceptors develop normally, and the mutation does not impair RHO expression or trafficking. Firstly, rod photoreceptors in 8–9-week-old affected dogs (n = 8) were indistinguishable from wild-type +/− littermate controls (n = 9) (Fig. 4a). Secondly, rhodopsin localization with Abs directed at the N- (amino acids 3–8, data not shown) and C-terminal (amino acids 340–348; Fig. 4 h1 and h2) domains showed the normal pattern of label intensity and distribution restricted to rod outer segments. Finally, comparison of mRNA expression detected no difference in the intensity or size of the RHO transcript (Fig. 1c).

The nonuniform degeneration of photoreceptors detected by both clinical examination and OCT was also observed morphologically (Fig. 4 a–g, j, and k). Photoreceptor disease and degeneration occurs in older animals, with advanced degeneration present at 4.5 and 11 years. In younger affected adult dogs, the disease is expressed with striking topographic variation. In different retinal locations, photoreceptors can be normal or show different gradations of disease (Fig. 4 a–g). The topographic distribution of disease was examined in an 11-mo-old RHO T4R+/− mutant dog by serial reconstruction of retinal sections from the major quadrants of the eye (Fig. 4j). In general, more severe disease (stages 3–6) was present in an area surrounding the ONH but centered in the temporal tapetal region of the fundus. Beyond this severely diseased region, there was an abrupt transition zone (Fig. 4j and k1–7) beyond which photoreceptors were structurally and quantitatively normal. Thus, at this age and stage of disease, despite the severe disease centrally, most of the retina was comprised of structurally intact photoreceptors.

**Discussion**

The phenotype of the RHO mutant dog is distinctly different from other canine retinal degenerations (15, 25), and these differences are precisely the similarities it shares with certain human RHO mutation phenotypes. Normal retinal structure, rhodopsin expression, receptor activation, and postreceptor signaling in young affected dogs suggest that the pathogenesis does not involve abnormal photoreceptor development. The defect in dark adaptation and the focal initiation of photoreceptor degeneration uniquely characterize both this canine disease and that in human patients with RP with class B1 RHO mutations (5). The compelling question then is how are these features linked together?

Rhodopsin, like other G protein-coupled receptors, has seven transmembrane α-helical segments, and N- (intradiscal or extracellular) and C-terminal (cytoplasmic or intracellular) domains (Fig. 5). The T4R mutation would be expected to affect the extracellular surface, a domain comprised of the N terminal and three interhelical loops (34). The mutation could alter one of two consensus glycosylation sequences in mammalian RHO (35) (sites 2–4 and 15–17). Although glycosylation has been implicated in RHO transportation, the consequences of its deficiency remain unclear. *In vitro* investigation of the T17M mutant indicated mislocalization (36) whereas analysis of a human postmortem donor retina from a patient with RHO T17M
failed to show evidence of missorting (7). The amount and distribution of mature RHO in nondegenerate areas of canine RHO T4R y1 retinas were indistinguishable from normal (Fig. 4). These findings taken together with the normal sensitivity of rod photoresponses in a T4R y1 T4R dog support the notion that the mutation at T4 is not critical for targeting of the mature RHO protein to the photoreceptor outer segment and the mutant molecule functions normally during phototransduction activation.

Of the seven human pathogenic RHO point mutations that we identified as having very prolonged recovery from light exposure (5, 27, 28), five are in the N-terminal and interhelical loops of the extracellular domain (Fig. 5). Two others are in transmembrane (TM) helices I and II. Evidence from in vitro studies (37) and the crystal structure of bovine RHO (3, 8) suggests that the extracellular surface and these TM domains closely interact with the bound chromophore. Misfolding of the mutant RHO molecules and abnormal chromophore binding have been among the mechanisms suggested to contribute to the pathophysiology in patients, but the exact molecular sequence leading to dysfunction and rod cell death remains unknown (8).

The extremely prolonged dark adaptation of photoreceptors harboring certain mutant alleles is likely to have a complex basis. After decades of debate about the sites in the visual system involved in normal recovery of sensitivity in the dark after exposure to light (38–42), there is recent consensus that latent transduction activity originating from bleach products within rods (32, 43) leads to bleaching adaptation in vivo. Several different bleach products probably contribute to varying extents at different times during adaptation (42, 44). Inactivation of these active bleach products and regeneration of RHO through the visual cycle pathway (45) define the observed kinetics of sensitivity recovery during bleaching adaptation. Among the active bleach products are photoisomerized rhodopsin, as well as phosphorylated and arrestin-bound forms. In addition, transduction activity originating from opsins and noncovalently bound forms of opsin and retinoid has been described. The relative activity and effective lifetimes of these moieties are currently not well established. Abnormally prolonged bleaching adaptation, such as in the RHO T4R y1 dog or in human RHO mutations, could be the result of slowed or deficient deactivation of one of these intermediate bleach products. Because the abnormality

Fig. 4. Retinal disease morphology and RHO immunocytochemistry. (a–g) Sections from an 11-mo-old RHO T4R y1 retina. (a) Normal section of T4R y1 retina. (b) Early rod loss is associated with drop out of diseased rods and shortened outer segments in remaining rods. (c and d) The degenerative phase is characterized by rod loss with preservation of cones. (e–g) End-stage atrophy results in the progressive and sequential loss of all photoreceptors and retinal pigmented epithelium. (h1 and h2) Immunocytochemistry in a 2-mo-old RHO T4R y1 retina shows a normal pattern of intense opsin labeling limited to the outer segments. (i1 and i2) Examination of an 11-mo-old T4R y1 retina shows RHO label is present in the short, disorganized, outer segments of the few remaining rods adjacent to regions devoid of opsin staining where only cones remain. Calibration marker = 10 μm. (j) Location of 7 1-μm retinal plastic sections, extending from the optic disk to the periphery, taken to reconstruct the topographic distribution of disease. (k) Sections were evaluated in continuous overlapping fields and assigned stage 0–6 to correspond with disease severity as defined in a–g, respectively. All regions are drawn to scale. More severe disease (stages 3–6) was observed surrounding the ONH and centered in the temporal tapetal region of the fundus. Dotted line indicates approximate area of retinal thinning apparent on gross examination of the fixed eyecup; colored line represents inferred margin of degenerate area from evaluation of fixed sections.

Fig. 5. A secondary structure model of rhodopsin indicating the transmembrane helices (I–VII), interhelical loops of the extracellular domain (E1–E3), the intracellular and extracellular domains, and the locations of residues (circles) altered in canine (black) and human (gray) RHO mutations leading to a similar phenotype.
occurs relatively late during development and after intense adapt-
ing lights, a late intermediate with relatively low activity is likely responsible. Alternatively, long-lived products resulting from photoreversal of mutant rhodopisin could cause the abnormal adaptation (46). Whether photoreceptor degeneration in the RHO mutant dog leads from the incomplete recovery after light exposure (7, 47–49) awaits further study.

Why should there be regions of severe structural damage sur-
rrounded by areas of structurally normal retina when the mutant rhodopisin is presumably distributed uniformly? The normal region of observed structural damage may arise from topographic expression of genes (50–52), nonuniform distribution of light absorption across the retina, or both. The initial focus of disease in the canine T4R RHO mutant retina is mainly in the temporal tapetal retina. This region is the area of central vision in the dog and the area of highest photoreceptor population density. It may well be that this area receives the greatest light exposure naturally. Light damage has been suggested to cause regressive disease in RHO mutations in humans (6, 7). Thus, the history of exposure to light may be a critical factor controlling the observed regional and temporal variation in the onset and progression of retinal degen-
eration in Mastiff autosomal dominant PRA.

Identification of this nonhuman RHO mutant large animal offers opportunities for evaluation of mechanism and therapy in humans affected with RP as a result of RHO mutations. The specific class of G-protein mutations includes the P23H RHO allele that accounts for a major percentage of adRP in the U.S. (4). The effect of the mutant (missense) allele dosage on disease severity, a controversial topic in the human literature (e.g., refs. 53–55), may be resolvable in the canine model. Adverse effects of environmental light and benefits of supplemental nutrients found in transgenic rodent models of human RHO mutations (e.g., refs. 47 and 56) can now be extended to a large animal. The temporal, functional, and structural progression of disease in the canine model offers an ideal time window of opportunity for gene therapy (16, 57). Affected dogs are clinically normal for at least several months before retinal degeneration develops, but at this stage retinal dysfunction can be clearly monitored by dark adapta-
tometry. The therapeutic approach in the heterozygote, assuming a deleterious effect of the mutant allele, would require a vector designed to deliver a “knockdown” construct capable of destroying the mutant allele rather than simply replacing it with the wild-type allele. The RHO mutant dog thus provides an invaluable tool to evaluate such therapies before commencement of human clinical trials.

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