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Purpose. The purpose of this study was to evaluate a chromatic pupillometry protocol for specific functional assessment of rods, cones, and intrinsically photosensitive retinal ganglion cells (ipRGCs) in dogs.

Methods. Chromatic pupillometry was tested and compared in 37 dogs in different stages of primary loss of rod, cone, and combined rod/cone and optic nerve function, and in 5 wild-type (WT) dogs. Eyes were stimulated with 1-s flashes of dim (1 cd/m²) and bright (400 cd/m²) blue light (for scotopic conditions) or bright red (400 cd/m²) light with 25-cd/m² blue background (for photopic conditions). Canine retinal melanopsin/Opn4 was cloned, and its expression was evaluated using real-time quantitative reverse transcription-PCR and immunohistochemistry.

Results. Mean ± SD percentage of pupil constriction amplitudes induced by scotopic dim blue (scDB), scotopic bright blue (scBB), and photopic bright red (phBR) lights in WT dogs were 21.3% ± 10.6%, 50.0% ± 17.5%, and 19.4% ± 7.4%, respectively. Melanopsin-mediated responses to scBB persisted for several minutes (7.7 ± 4.6 min) after stimulus offset. In dogs with inherited retinal degeneration, loss of rod function resulted in absent scDB responses, followed by decreased phBR responses with disease progression and loss of cone function. Primary loss of cone function abolished phBR responses but preserved those responses to blue light (scDB and scBB). Although melanopsin/Opn4 expression was diminished with retinal degeneration, melanopsin-expressing ipRGCs were identified for the first time in both WT and degenerated canine retinas.

Conclusions. Pupil responses elicited by light stimuli of different colors and intensities allowed differential functional assessment of canine rods, cones, and ipRGCs. Chromatic pupillometry offers an effective tool for diagnosing retinal and optic nerve diseases.

Keywords: canine, chromatic pupillometry, intrinsically photosensitive retinal ganglion cells, melanopsin, retinal dystrophy

The pupillary light reflex (PLR) informs about retinal and visual pathway functions, and it is routinely evaluated during clinical ophthalmic examination. Pupillometry enables precise quantification of changes in pupil size for clinical and research purposes, improving upon traditional qualitative PLR testing based on gross observations of pupillary responses. The discovery of a subset of retinal ganglion cells (RGCs) known as intrinsically photosensitive RGCs (ipRGCs) provided new understanding of the PLR. The ipRGCs’ unique photopigment melanopsin, with a peak spectral sensitivity of ~480 nm, may provide a basis for using blue light PLR to differentiate diseases affecting inner retina, optic nerve, and central nervous system. Subsequently, chromatic pupillometry using red and blue light stimuli was developed in humans to permit detailed testing of retinal cell subpopulations; pupil constriction evaluated in the dark by dim and bright blue light stimuli (scotopic dim blue [scDB] and scotopic bright blue [scBB]) specifically measures rod- and ipRGC-mediated functions, respectively, whereas a bright red light stimulus with a blue-background (photopic bright red [phBR]) specifically measures cone function.

Chromatic pupillometry has also been introduced for assessment of the visual pathway in the dog, an important model species for inherited blinding disorders; however, the respective contributions of different canine cell populations to specific colored light responses are unknown. In this study, we assessed the validity of a human chromatopho quadruplet of well-characterized canine retinal and optic nerve disease models at Michigan State University and University of
Pennsylvania. Furthermore, in order to complement our functional results, we cloned the canine melanopsin/Opn4 gene and demonstrated for the first time melanopsin-expressing ipRGC in the normal and the diseased canine retina.

**METHODS**

**Animals**

We compared results of chromatic pupillometry testing in 37 dogs with different stages of primary loss of rod, cone, combined rod/cone, and optic nerve functions with those in 5 wild-type (WT) dogs (Table). Diseases included CNGB1-

**TABLE. Summary of Study Dogs**

| Disease                  | Mutated Gene     | Functional Change | Dog  | Sex | Age, y |
|--------------------------|------------------|-------------------|------|-----|-------|
| WT                       |                  |                   | N1   | F   | 1     |
| N2                       |                  |                   | F    |     | 1     |
| N3                       |                  |                   | F    | 0.6 |       |
| N4                       |                  |                   | M    | 1   |       |
| N5                       |                  | Rod function      | 1    |     | 0.3   |
| N6                       |                  | Rod function      | 2    |     | 5.2   |
| N7                       |                  | Rod function      | 3    |     | 0.8   |
| CNGB1-PRA                | CNGB1            | Rod function      | 1CNGB1 | M  | 0.3   |
| CNGB3-ACHM               | CNGB3            | Cone function     | 1CNGB3 | M  | 4.8   |
|                         |                  |                   | 2CNGB3 | F  | 4.6   |
|                         |                  |                   | 3CNGB3 | M  | 1.6   |
|                         |                  |                   | 4CNGB3 | F  | 1.3   |
|                         |                  |                   | 5CNGB3 | M  | 1.4   |
|                         |                  |                   | 6CNGB3 | M  | 0.6   |
| PDE6B-rcd1 (young)       | PDE6B            | Rod function      | 1PDE6B | M  | 0.7   |
|                         |                  |                   | 2PDE6B | F  | 0.7   |
|                         |                  |                   | 3PDE6B | F  | 0.4   |
| PDE6B-rcd1 (old)         | PDE6B            | Rod and cone function | 4PDE6B | F  | 6     |
|                         |                  |                   | 5PDE6B | F  | 5.5   |
| RD3-rcd2 (old)           | RD3              | Rod and cone function | 1RD3 | M  | 1.5   |
|                         |                  |                   | 3RD3 | F  | 3.7   |
|                         |                  |                   | 4RD3 | F  | 3.4   |
| PDE6A-rcd3 (young)       | PDE6A            | Rod function      | 1PDE6A | M  | 0.7   |
| PDE6A-rcd3 (old)         | PDE6A            | Rod and cone function | 2PDE6A | M  | 3.8   |
|                         |                  |                   | 1PDE6A† | F  | 4.4   |
|                         |                  |                   | 2PDE6A† | F  | 4.4   |
|                         |                  |                   | 3PDE6A† | F  | 2.1   |
|                         |                  |                   | 4PDE6A† | F  | 0.8   |
|                         |                  |                   | 5PDE6A† | F  | 0.8   |
|                         |                  |                   | 6PDE6A† | F  | 0.8   |
| PRCD-prcd                | PRCD             | Rod and cone function | 1PRCD‡ | F  | 8.5   |
|                         |                  |                   | 2PRCD | M  | 5.9   |
| IQCB1-crd2               | IQCB1            | Rod and cone function | 1crd2 | F  | 1.3   |
|                         |                  |                   | 2crd2 | F  | 1.5   |
|                         |                  |                   | 3crd2 | M  | 3.8   |
|                         |                  |                   | 4crd2 | M  | 3.8   |
| STK38L-erd               | STK38L           | Rod and cone function | 1erd‡ | M  | 8.4   |
|                         |                  |                   | 2erd‡ | M  | 3.5   |
|                         |                  |                   | 3erd M  | 3.5   |
| RPGR-XLPRRA2             | RPGR             | Rod and cone function | 1XLPRRA2 | F  | 7.2   |
|                         |                  |                   | 2XLPRRA2 | F  | 6.5   |
|                         |                  |                   | 3XLPRRA2 | F  | 5.8   |
| RD3-rcd2 (old) and NEHJ1-CEA | RD3 and NEHJ1 | Rod, cone, and RGC function | 2RD3§ | M  | 3.5   |

* Light intensity series was performed with multiple increases in stimulus intensity.
† ERG was completed during the same session as pupillometry.
‡ IHC was available.
§ Animal also had severe optic nerve head coloboma.
|| Chromatic pupillometry reproducibility evaluated by repeat testing within 4 to 5 months.

progressive retinal atrophy (PRA), CNGB3 achromatopsia (ACHM), PDE6B rod/cone dysplasia 1 (rcd1), RD3 rod/cone dysplasia 2 (rcd2), PDE6A rod/cone dysplasia 3 (rcd3), RPE65 Leber congenital amaurosis (LCA), PRCD progressive rod/cone degeneration (prcd), IQCB1 cone/rod dystrophy 2 (crd2), RPGR-X-linked progressive retinal atrophy 2 (XLPRRA2), STK38L early retinal degeneration (erd), and NEHJ1 Collie eye anomaly (CEA).16–39 Dogs were grouped and tested based on 4 previously reported retinal and optic nerve disease phenotypes, as follows: (1) Group 1 consisted of primary loss of rod function in dogs with CNGB1-PRA and young (<1 year of age) dogs with PDE6A-rcd3 and PDE6B-rcd1; (2) group 2 included primary loss of cone function in dogs with CNGB3-
Canine Chromatic Pupillary Response

Anesthesia

In an initial pilot study, 5 dogs underwent chromatic pupillometry recordings using injectable chemical restraint alone with intravenously administered dexmedetomidine (4 μg/kg; Dexdomitor; Zoetis, Florham Park, NJ, USA). This sedation alone was insufficient for reliable PLR recordings due to persistent fluctuations in pupil size and eye movements. Therefore, for all pupillometry recording sessions, dogs received isoflurane gas anesthesia and were positioned in sternal recumbency on a custom-made table equipped with a head rest. They were first premedicated using acepromazine, at an intravenous dose of 0.02 mg/kg (Acepject; Henry Schein, Dublin, OH, USA) and induced with propofol (Propoflo 28; Abbott, Abbott Park, IL, USA) given intravenously to effect (starting dose, 4 mg/kg). The dogs were then intubated and general anesthesia was maintained with isoflurane (2%–3% vaporizer setting; Isofact; Henry Schein) in oxygen. Heart rate, respiratory rate, and body temperature were monitored throughout the procedure. A portable multiparameter veterinary monitor (model PM-9000Vet; Shenzhen Mindray; Bio-Medical Electronics, Co., Ltd., Nanshan, Shenzhen, PR, China) was used to assess blood pressure, oxygen saturation, and end-tidal CO₂. Anesthesia level was evaluated by monitoring changes in respiration or heart rate.

Chromatic Pupillometry

Both the left and right eyes were tested in each dog. The right eye was tested first. A Barraquer eyelid speculum was inserted to prevent interference by nictitating membrane and eyelids. Stay sutures (4-0 Perma-Hand Silk; Ethicon, Inc., Somerville, NJ, USA) were placed in the superior and inferonasal bulbar conjunctiva 2 mm posterior to the limbus. The stay sutures allowed globe manipulation and maintenance of the pupil in the central optical axis for pupillometry recording. The eye was lubricated (Optixcare Eye Lube; CLC Medica, Waterdown, Ontario, Canada) prior to the start of recording. The untested eye was covered with a black plastic ocular shield (Oculoplastik, Inc., Montréal, Canada) which contained a hypermellose 2.5% ophthalmic demulcent (Goniosoft; OCUsoFT, Inc., Rosenberg, TX, USA). After the right eye was tested, the left eye was tested using the same procedure.

Recordings were performed with the RETIport system using a Ganzfeld dome (Roland Consult, Havel, Germany). This system allowed timed delivery of a light-emitting diode (LED) light of a particular wavelength and intensity. An infrared-sensitive camera located within the Ganzfeld dome was used for real-time pupil recording. The system contained an automatic pupil detector that measured pupil size throughout recording, at a sample rate of 30 fps. The RETIport pupillometry system was developed for testing human subjects, but we found that actual canine pupil size correlated linearly with the system’s measurements, the machine consistently overestimating pupil size by ~3% (Supplementary Fig. S1). Actual pupil size was measured using a ruler placed temporal to the eye when the dog’s head was in the Ganzfeld dome. Because this pupillometry system was new, we continued to improve its adaptation for use in animals in collaboration with the manufacturer (Roland Consult).

Testing parameters were adapted from a previously published human protocol. After 10 min of dark adaptation (scotopic conditions), the eye was stimulated first with a 1-s flash of dim blue light (scDB: 1 cd/m², 470 nm) and then a 1-s flash of bright blue light (scBB: 400 cd/m², 470nm) following pupil recovery to baseline. After 5 min of light adaptation to a blue background (~800 nm, 25 cd/m²) the eye was stimulated with a 1-s flash of bright red light (phBR: 400 cd/m², 640 nm). The blue background light remained on throughout photopic-condition testing. Duplicate tests were performed for each light intensity tested in each eye, and the results were averaged. These intensities were photopically matched for the human eye, and we also found good agreement with canine spectral sensitivity (see Fig. 6).

During our evaluation of the system, we noted that, in dogs that retained cone function, a small light artifact (movement of the pupil detector) was observed for several milliseconds after onset of the bright red light stimulus but that the pupil response quickly overcame the artifact. In dogs with no cone function, the light artifact was seen throughout the time the bright red light stimulus was on. Due to the light artifact, we found that it was important to observe the real-time video recording of the bright red light testing with blue background to differentiate between light artifact and true pupil response.

Test-retest variability was assessed in 3 dogs (Table) by repeating the chromatic pupillometry testing protocol 4 to 5 months after the first recording (see Supplementary Fig. S5).

To determine if variations in constriction amplitude found between the WT dogs could be due to entrance pupil size, pupil responses were measured over a range of light intensity stimuli, under both scotopic and photopic conditions for the right eyes of 2 WT dogs and 2 CNGB3 mutant dogs. After 10 min of dark adaptation, alternating red and blue light stimuli were presented over an intensity range of 1 to 400 cd/m² in ~0.5-log steps (Supplementary Table S2). Light stimulus duration of 1 s was used. Duplicate tests were performed at each light intensity. We followed the protocol described above for the photopic condition, except that we added a blue background light to suppress rod function: The eyes were allowed to adapt for 5 min to the blue background light (25 cd/ m²) prior to testing, and the background light remained on throughout this testing.
Data Analysis

Data were exported to Excel software (Microsoft, Redmond, WA, USA) for analysis. Absolute PLRs were recorded and averaged for the duplicate tests at each light intensity. Baseline pupil size was taken as the median pupil size during the 1-s interval preceding onset of each respective stimulus. Normalized pupil size was then calculated by dividing the averaged absolute pupil size by the baseline pupil size. When both the right and the left eyes were tested, the normalized pupil sizes for both eyes were averaged. Maximum constriction amplitude was defined as the minimum pupil size attained after stimulus onset. Latency was defined as the time between stimulus onset and beginning of pupil constriction. To determine the onset of the PLR, we attempted to use a method based on filtering, followed by first and second derivatives of pupil movement but found it was unsuccessful due to the low sample rate of 30 fps. Therefore, we determined latency by measuring time from stimulus onset to the beginning of pupil constriction from each respective pupil response graph.

Anesthesia records of the tested dogs were evaluated to determine whether PLRs were affected by anesthesia level, by assessing heart rate, respiratory rate, isoflurane level, end tidal CO₂ level, and blood pressure.

Results are mean ± SD. Statistical significance of differences in latency and constriction amplitudes between WT dogs and affected dogs in the three different disease groups 1–3 for all testing protocols was determined by F test and unpaired Student’s t-test or Mann-Whitney U test. The relationship between baseline pupil size and constriction amplitude was evaluated by Pearson correlation. Statistical computations were performed in Microsoft Excel, GraphPad (www.graphpad.com/quickcalcs/contMenu, in the public domain), and VassarStats (www.vassarstats.net/index.html, in the public domain). Differences were considered statistically significant for P values of <0.05.

Electroretinography

Standard scotopic and photopic full-field electroretinograms (ERG) were recorded in selected dogs (Table) under isoflurane anesthesia during the same chromatic pupillometry recording session, using the Ganzfeld dome system (Roland Consult), jet contact lens electrodes (Fabrinal Eye Care, La Chaux-de-Fonds, Switzerland), and commercially available platinum subdermal needle electrodes (Grass Technologies, Warwick, RI, USA). In these dogs, chromatic pupillometry was only performed in one eye. The ERG was recorded first in the left eye followed by chromatic pupillometry in the right eye. The untested eye was covered with a black plastic ocular shield as described above. The left pupil was dilated with tropicamide 1% ophthalmic solution (Akorn Inc., Lake Forest, IL, USA) prior to electroretinography. Rod- and mixed cone/rod-mediated responses were recorded after 20 min of dark adaptation with scotopic single-white-flash stimuli at three different light luminances (0.01, 3, and 10 cd/m²). Multiple responses (25 for the 0.01 cd/m² single-white-flash and 10 for the 3, and 10 cd/m² single white flash) were averaged. Following 10 min of light adaptation to white background illumination of 30 cd/m², cone-mediated signals were recorded in response to a 1-Hz single white flash (5 and 10 cd/m²) and 30 Hz white flicker (3 cd/m²). Multiple responses (10 for the 1-Hz single flash and 90 0.05-s sweeps for the 30-Hz flicker) were averaged.

Melanopsin/Opn4 Sequencing and Cloning

Normal retinas were collected from a WT dog and flash-frozen immediately following euthanasia. Total RNA was isolated from the retina by using TRIzol reagent (Invitrogen, Carlsbad, CA,
USA) after homogenization. First-strand cDNA was synthesized from 2 μg of RNA, using High Capacity reverse transcription kit (Applied Biosystem, Waltham, MA, USA) according to the manufacturer’s instructions. The resultant cDNA was used in a 50-μL PCR reaction assay, using FailSafe PCR system (Epicentra, Madison, WI, USA) with the custom-designed primers Mel_cDNAF (forward 5'-ACCACCCCCAGGATGAAC-3') and Mel_cDNAAR (reverse 5'-CTGCAGGCTTGTCCCTGT-3'). Primers were designed using Primer3 (http://frodo.wi.mit.edu, in the public domain) and the predicted canine sequence (CanFam 2.0) assembly (http://genome.ucsc.edu/cgi-bin/hgGateway, in the public domain). Each 50-μL reaction mixture contained PCR-grade water, FailSafe PCR enzyme mixture, 1 μL (100ng/μL) of DNA template, 1 μL of both forward and reverse primers, and one of the 12 different FailSafe PCR 2× PreMixes (A–L). PCR assay was performed under the following conditions: initial denaturation at 94°C for 3 min, then 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min for 35 cycles, followed by final extension at 72°C for 5 min.

Electrophoresis of PCR products was then carried out on a 1.5% agarose gel containing ethidium bromide, and a single DNA band of the correct size was observed for FailSafe PCR 2× PreMix G. The DNA was purified using the QIAquick gel Extraction Kit (QIAGEN, Inc., Germantown, MD, USA) according to the manufacturer’s protocol. The purified 1,446-bp PCR product was cloned into the pCR4-TOPO TA vector using TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the provided protocol. The plasmid was prepared from an overnight culture, using the PureYield plasmid miniprep system (Promega, Madison, WI, USA), and 5

**FIGURE 2.** (A) Effects of mutations in PDE6B, CNGB1, and PDE6A on PLRs compared to average WT PLRs. Representative recordings are shown here. Recordings from all dogs are shown in Supplementary Figure S2. * indicates undetectable PLRs from PDE6A- and CNGB1-mutants overshadowed by a light artifact. Shaded area represents 1-s stimulus presentation. (B) Representative full-field ERGs. Compared to WT dogs, rod-mediated response were nonrecordable in young PDE6B- and PDE6A-mutants, whereas cone-mediated function was recordable but severely reduced in amplitude. (A) Calibration bars: 1 s (time, horizontal) and 20% (normalized pupil size, vertical).
to 10 individual clones were sequenced using Automated 3730 DNA analyzer (Applied Biosystems), aligned to the predicted sequence (CanFam 2.0 assembly; http://genome.ucsc.edu/cgi-bin/hgGateway, in the public domain) using Sequencher version 4.2.2 software (Gene Codes Corp., Ann Arbor, MI, USA), and submitted to GeneBank (accession number KU341721). The predicted amino acid sequence of the canine melanopsin was aligned with those of other species by using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/, in the public domain).

**qRT-PCR Analysis of Retinal Gene Expression**

Primers and TaqMan Probes were designed for canine melanopsin/Opn4 using Primer Express software version 2.0 (Applied Biosystems). Primer pair and probe were designed to neighboring exon sequences to ensure that only cDNA and not genomic DNA was amplified. Using cDNA synthesized as described above, qRT-PCR was performed with the custom-designed primer pairs MelDegE67F (forward, GCGGCTACAGAGAGAGTGGAA) and MelDegE67R (reverse, ACATGAACTCCCGTGCCAGC) and probe (CTTTCGCTGGGTATT) that spans the junction of exons 6 and 7. All qRT-PCR assays were performed in 96-well plates using 7500 model real-time PCR machine with 7500 v2.0.1 detection software (Applied Biosystems).

Relative melanopsin/Opn4 gene expression was compared with that of the GAPDH product and calculated as \[1/(2^{ΔCt\text{Gene} - ΔCt\text{GAPDH}})\]. The ratios of relative gene expression were calculated between the diseased eye and age-matched WT control, using the ΔΔCT method. Ratios different from 1 were considered relevant, indicating either increased (>1) or decreased (<1) gene expression.

**Immunohistochemistry**

Retinal IHC was performed as previously described. Immunohistochemical staining was performed using the antibodies shown in Supplementary Table S3. A custom-made rabbit anti-canine melanopsin antibody directed at a mixture of
FIGURE 4. (A) Representative PLRs of older dogs bearing mutations in PDE6B, RD3, STK38L, and PRCD. Recordings from all dogs with mutations in STK38L, IQCB1, RPGR, RPE65, and PRCD and older dogs carrying PDE6B, RD3, and PDE6A-mutations are shown in Supplementary Figure S4. Shaded area represents the 1-s light stimulus presentation. * indicates absent PLR to scBB in one older STK38L-mutant. Arrows indicate small residual rod function and near-normal preserved cone function in an older dog with mutated PRCD. (B) Representative full-field ERGs recorded from older dogs bearing mutations in PDE6B, STK38L, and PRCD. Responses of a WT dog are shown for comparison. For PDE6B- and STK38K-mutant dogs, rod- and cone-mediated function was non-recordable. For the PRCD-mutant dog, cone-mediated function was recordable, but the amplitude was reduced compared with that of the WT dog. (A) Calibration bars: 1 s (time, horizontal) and 20% (normalized pupil size, vertical).

FIGURE 5. All PLRs were absent in the dog affected by a mutation in RD3 and concurrent severe optic nerve head coloboma due to a mutation in NEHJ1. Shaded area indicates the 1-s light stimulus presentation. Calibration bars: 1 s (time, horizontal) and 20% (normalized pupil size, vertical).
RESULTS

Normal Chromatic Pupillometry Recorded From WT Dogs

We assessed pupillary responses in 5 WT dogs, using testing parameters developed based on a human protocol (Fig. 1). After a 10-min dark adaptation, mean pupil diameter was 10.8 ± 2.4 mm. Mean ± SD percentage of constriction amplitudes induced by scDB, scBB, and phBR stimuli in WT dogs were 21.3% ± 10.6%, 50.0% ± 17.5%, and 19.4% ± 7.4%, respectively. Mean latencies of PLRs elicited by scDB, scBB, and phBR stimuli in WT dogs were 0.26 ± 0.05 s, 0.28 ± 0.10 s, and 0.32 ± 0.03 s, respectively. The PLRs to the scBB stimuli were characterized by a sustained response (7.7 ± 4.6 min after the offset of the stimulus), which is thought to be melanopsin-driven. A small light artifact (movement of the pupil detector) was observed for several milliseconds after onset of the phBR light stimulus.

Individual dogs exhibited considerable variation in pupil constriction amplitudes (Fig. 1A), which was significantly correlated with baseline pupil size for scBB (r = 0.72, P < 0.05), but not for scDB (r = 0.46; P = 0.19) or phBR (r = −0.27; P = 0.45) stimuli (Fig. 1B). No other obvious cause for this variation was evident, including age, sex, or anesthesia level.

Primary Rod Disease Results in Absence of Pupillary Responses to scDB

Chromatic pupillometry results concurred with prior descriptions of functional phenotype of the respective diseases. In contrast with WT dogs, young dogs with PDE6A-rcd3 and PDE6B-rcd1, and dogs with CNGB1-PRA exhibited no measurable PLRs to scDB used to test rod function (Fig. 2, Supplementary Fig. S2). A sustained response was elicited by scBB, which represents the melanopsin-driven response, and persisted in all affected dogs.

As expected, based on known disease phenotypes, phBR evoked measurable PLRs in most of the affected dogs and were likely cone-mediated. In one of the younger (0.4-year-old) PDE6B-mutant dogs, ERG was recorded during the same session as chromatic pupillometry and showed an absence of rod-mediated response but preservation of reduced cone responses (Fig. 2B). Unexpectedly, no recordable responses to phBR were present in the PDE6A- and both CNGB1-mutant dogs as confirmed by video observation. It is possible that small cone responses were present, as shown in the ERG of the PDE6A mutant dog (Fig. 2B), but were overshadowed by the light artifact.

The mean constriction amplitude induced by scBB for all affected dogs (49.1% ± 13.4%) and phBR for mutant dogs excluding nonresponders (24.2% ± 23.6%) were unaltered with respect to those seen in WT dogs (P = 0.89 and P = 0.55, respectively). The mean latencies of responses to scBB and phBR in mutant dogs were likewise unaltered with respect to those seen in WT dogs (0.32 ± 9.56 s; P = 0.37; and 0.27 ± 9.32 s; P = 0.89, respectively).

Variations in pupil constriction amplitude were noted between individual PDE6B-mutant dogs (Supplementary Fig. S2), as we likewise found in WT dogs that were not attributable to differences in age, sex, or anesthesia levels. Constriction amplitudes evoked by scBB (P = 0.14) or phBR (P = 0.84) testing protocols were not significantly correlated with baseline pupil size.
Primary Cone Disease Reduces Pupil Responses to phBR

In 4 of 6 CNGB3-mutants, response to phBR was absent, except for the light artifact (Fig. 3A, Supplementary Fig. S3), supporting the notion that this stimulus specifically elicits cone-mediated PLRs. Responses to both scDB and scBB were well preserved. The results of chromatic pupillometry corresponded with the ERGs, as rod function was normal but cone function was lost in dogs with CNGB3-ACHM (Fig. 3B). Although most chromatic pupillometry results showed loss of cone function as expected based on prior phenotypic descriptions, the remaining 2 of 6 CNGB3-mutant dogs (3CNGB3 and 5CNGB3 dogs) did exhibit persistent pupillary responses to phBR (Fig. 3A, arrow), consistent with incomplete ACHM (Koehl K, et al. IOVS 2015;56:ARVO E-Abstract 4651). Their mean constriction amplitudes were markedly reduced compared with that in WT dogs (9.1% ± 2.4% vs. 19.4% ± 7.4%, respectively). Mean latency of response to phBR was similar to that seen in WT dogs (0.26 ± 0.05 s). Sample size (n = 2) in this CNGB3-subset did not permit statistical analysis.

The mean pupil constriction amplitudes evoked by the scDB were significantly greater (51.2% ± 6.2%; P = 0.02) than that seen in WT dogs (21.3% ± 10.6%), but those to scBB were similar (65.4% ± 8.7%; P = 0.16). Mean latency of responses to scDB (0.29 ± 0.06 s) and scBB (0.27 ± 0.07 s; P = 0.78) were unaltered compared with those in WT dogs (P = 0.26 and P = 0.78, respectively). Comparable to WT dogs, individual CNGB3 mutants exhibited variations in pupil constriction amplitude that were not attributable to age, sex, or anesthesia level (Supplementary Fig. S3). We found a significant positive correlation between baseline pupil size and constriction amplitude for scDB (r = 0.72; P < 0.05) but not for scDB (r = -0.34; P = 0.34).

Response to scBB Persists in Advanced Outer Retinal Diseases

With advanced retinal disease due to various vision-impairing mutations, pupillary responses to scDB and phBR were reduced or absent, consistent with progressive loss of rod and cone function (Fig. 4A, Supplementary Fig. S4). With one exception (see below), the response to scBB was maintained in all dogs, even when rod- and cone-mediated retinal functions were not detectable, providing strong evidence that this stimulus is specific for inner retina. Mean PLR latency was approximately doubled in these dogs (0.61 ± 0.26 s vs. 0.28 ± 0.10 s, respectively) by 0.33 s compared to that in WT dogs (P = 0.0005), consistent with a melanopsin-mediated pupillary response without rod or cone influence.44 The scBB-evoked mean constriction amplitude was significantly decreased by 11.7% compared to that in WT dogs (38.3% ± 13.9% vs. 50.0% ± 17.5%, respectively; P = 0.03), indicating loss of contribution of rods and cones to the initial constriction amplitude.44

One of the older prcd-affected dogs had both rod and cone function preserved, evident in responses seen both by chromatic pupillometry and ERG (Fig. 4). In an older STK38L-mutant, PLR to all light stimuli, including scBB, was absent. Such loss of inner retinal response is due to advanced retinal degeneration also involving the inner retina (see Presence of ipRGC and Melanopsin Expression in Diseased Retinas below).36,45 As the function of STK38L is not known to impact inner retinal responses.

Variations in pupil constriction amplitude were also noted between individual mutant dogs (Supplementary Fig. S4b), as in WT dogs, which was not attributable to age, sex, or anesthesia level but potentially could result from individual variations in disease severity. Baseline pupil size was not significantly correlated with constriction amplitudes seen with scBB (P = 0.14) or phBR (P = 0.43).

Loss of ipRGC Function

The dog with severe optic nerve head coloboma associated with NEH1 mutation was also affected with RD3 rcd2. As expected, all pupillary responses were absent, including response to scBB (Fig. 5).

Variations in Constriction Amplitude: Reproducibility and Light Intensity Series

Three dogs (1 WT and 2 CNGB3-mutants with incomplete ACHM) were retested twice within 4 to 5 months. We found that recorded PLRs were highly reproducible, supporting the diagnostic value of chromatic pupillometry in dogs (Supplementary Fig. S5).

We hypothesized that the individual variations in constriction amplitudes seen between WT dogs could have been due to differences in entrance pupil size, and hence, amount of light entering the eyes. To test this idea and assess the role of light stimulus intensity on pupil responses under scotopic and photopic conditions, we tested responses to a light intensity series in 2 WT dogs and found that stimulus intensity and constriction amplitude are related (Fig. 6).

Under scotopic conditions (Fig. 6, left column), saturation of peak amplitudes occurred at high light intensities (32–400 cd/m²) and recovered rapidly with the red light stimuli, whereas PLRs to the photopically matched blue stimuli were characterized by a sustained postillumination response for intensities ≥32 cd/m², which we attribute to melanopsin-driven response.5 Contrasting with PLRs under scotopic conditions, under photopic conditions with a blue background light of 25 cd/m² (Fig. 6, middle column), PLRs to red stimuli were fairly similar to PLRs seen in photopically matched blue stimuli, presumably both were cone-mediated. No sustained response was seen to blue stimuli under photopic conditions, suggesting that the blue background suppressed ipRGC activity. A light artifact was noted for approximately 0.1 s after red and blue stimuli onset at high light intensities (100 and 400 cd/m², respectively). In addition to the WT dogs, 2 CNGB3 mutants were tested under photopic conditions to determine the stimulus intensity at which light artifacts occur. PLRs were absent in all blue and red light stimuli. A small light artifact was only noted at a high red light intensity of 400 cd/m² (Fig. 6, right column).

Presence of ipRGC and Melanopsin Expression in Diseased Retinas

To permit melanopsin quantification and analyses in dogs by enabling design of antibodies and qRT-PCR primers, we cloned the canine melanopsin/Opn4 gene. The full-length cDNA contains a 1,446 bp open reading frame encoding a 482-amino acid protein (Supplementary Fig. S6). Alignment of the isolated canine amino acid sequence with those of mouse, chicken, lizard, and human melanopsin revealed substantial homologies. The highest sequence similarity (89.6%) was with the cat homolog, whereas weaker similarity (46.6%) was evident with the Italian wall lizard homolog. Dog melanopsin showed approximately 75.4% amino acid sequence similarity to that of mouse and rat homologs and approximately 78.9% to that of human homolog.

Using qRT-PCR, we observed a significant decrease in melanopsin/Opn4 expression in advanced stages of prcd compared to that in age-matched WT dogs (P < 0.05) (Fig. 7A). Melanopsin/Opn4 expression levels in early onset retinal
degeneration in \textit{RPGR}- and \textit{PDE6B}-mutant dogs was also decreased, but this finding was inconclusive due to small sample sizes. In contrast, melanopsin/\textit{Opn4} expression was variable but not significantly altered in \textit{CNGB3}-ACHM, a nondegenerative retinal disease ($P = 0.193$). Melanopsin immunostaining of representative retinal sections from WT and \textit{PDE6B}-mutant dogs confirms the presence of ipRGCs in WT and young and old \textit{PDE6B}-mutant dogs, consistent with positive PLRs to scBB (Fig. 7B).

Immunolabeling of representative retinal sections confirmed the previously reported histologic phenotype of the studied diseases, including the preservation of RGCs in most affected dogs (Supplementary Fig. S7). In contrast, an \textit{STK38L}-mutant dog (1erd) showed loss of normal retinal architecture by IHC,\textsuperscript{36,41,45} with complete loss of RGCs due to severe retinal degeneration, thus accounting for the observed absence of any scBB response.

**DISCUSSION**

Our findings validate the use of an available human chromatic pupillometry clinical testing protocol for dogs.\textsuperscript{10} The evaluation of a previously established human protocol was justified by the fact that the absorption spectra of canine photoreceptors are similar to those in humans (Supplementary Table S4). Standard pupillometry has been used in dogs before, and chromatic pupillometry has been suggested, but we have now confirmed the validity of this method in this species.\textsuperscript{11–13,46–48}

By taking advantage of purpose-bred dogs with well-defined retinal disease phenotypes, we verified that pupil constriction evaluated in the dark by dim and bright blue light is specific for rod and ipRGC functions, respectively, and that a bright red light stimulus on a blue-lit background is specific for cone function. For example, we found that young dogs affected by \textit{PDE6B-rcd1}, which are expected to have a complete lack of rod function with initially intact cone function, exhibited no PLR response to scDB.

As reported previously in humans, in dogs a blue background used in the cone protocol also suppresses rod and ipRGC functions, respectively, and that a bright red light stimulus on a blue-lit background is specific for cone function. For example, we found that young dogs affected by \textit{PDE6B-rcd1}, which are expected to have a complete lack of rod function with initially intact cone function, exhibited no PLR response to scDB.

In contrast, an \textit{STK38L}-mutant dog (1erd) showed loss of normal retinal architecture by IHC,\textsuperscript{36,41,45} with complete loss of RGCs due to severe retinal degeneration, thus accounting for the observed absence of any scBB response.
complete ACHM is paradoxical pupillary constriction in darkness,\textsuperscript{50–52} seen when a patient is moved to darkness after exposure to bright light for several minutes. However, we did not observe paradoxical pupil constriction in our CGNB3-ACHM dogs. Whether this was due to species difference or other factors remains to be determined.

We found that a bright blue light stimulus successfully isolated ipRGC function. This response was well preserved unless inner retina and optic nerve were impaired, as seen in dogs affected with severe optic nerve head coloboma or advanced retinal degeneration. The complete loss of RGCs seen by IHC in one STK38L-mutant dog corresponded with the absence of its response to scBB. This finding suggests that, in the future, ipRGC response testing may be exploited to quantify optic nerve damage by determining the extent to which the scBB response correlates with the number of viable optic nerve axons.

Only blue light of intensities ≥32 cd/m\(^2\) evoked sustained postillumination pupil responses in dogs; these exceed those reported in humans (10 cd/m\(^2\)).\textsuperscript{10} Such sustained responses are thought to be melanopsin-driven.\textsuperscript{5,6,8} In macaques, for example, after pharmacologic blockade of the classic rod and cone photoreceptors, the melanopsin-driven pupillary responses persisted after light stimulus offset.\textsuperscript{5} This sustained response has been defined as postillumination pupil response (PIPR).\textsuperscript{55} Recent studies have used PIPR to assess the function of the melanopsin signaling pathway and to measure disease-related progressive changes in ipRGC function.\textsuperscript{53–57} In patients with advanced glaucoma, the PIPR was reduced compared to that in normal age-matched patients.\textsuperscript{54,55} Also, redilation time was found to be slower for patients lacking rods or cones.\textsuperscript{44} We observed a sustained PIPR in all dogs that had intact inner retinas. Because the pupillometry system recorded only to 14 s after light offset, we were unable to calculate the full extent of PIPR.\textsuperscript{53,54}

Our findings in ipRGC testing revealed interesting effects upon PLR latency in dogs with losses of rod and cone function. Latency of the PLR response for scBB in dogs with selective loss of either rod or cone function was unaltered with respect to that of WT dogs, but detectable PLR latencies were significantly prolonged in dogs with combined loss of both rod and cone functions. The rapid pupillary constriction evoked by light stimulus onset is mediated by rods and cones.\textsuperscript{44} Accordingly, as found in human studies,\textsuperscript{44} onset of pupil responses in affected dogs lacking both rod and cones was abnormally slow, as expected if mediated purely by melanopsin, which has long latency.

Our findings also provide the first demonstration of melanopsin/Opn4-expressing ipRGCs in the normal and diseased canine retina. Melanopsin/Opn4 mRNA expression was decreased in a canine model of late-onset retinal degeneration but was unchanged in non-degenerative retinal disease such as ACHM.

Although our findings strongly support the prospects for using chromatic pupillometry as an additional diagnostic tool for testing of canine outer and inner retinal function, the current testing protocol still needs to be optimized, and there are limitations to this study. Most importantly, improvements are needed in the Roland system to eliminate the artifact noted with phBR light stimulation for the cone-testing protocol to eliminate the red light artifact, which impedes the ability to detect very small responses. This may account for the absence of detectable cone response in a young PDE6A-mutant dog. It is possible that the stopband filter (\(\lambda_{c}\)) of 700 nm for the infrared camera in the Ganzfeld dome was not high enough to prevent light admission from the 640-nm red LED, causing a light artifact. Due to the light artifact, we found that it was important to observe the real-time video recording of the phBR light testing with blue background, to determine if there was a true pupil response.

Although we found good test-retest reproducibility for individual dogs, there was considerable variation in constriction amplitudes between dogs, which may prevent interpretations about the numbers of photosensitive cells. Although pupillometry is an objective means for precise quantitative measurement of changes in pupil size; certain factors can affect pupil size and contraction, including retinal illumination, accommodative state of the eye, age, and emotional conditions.\textsuperscript{58–61} Important sources of such variability, confirmed by our results, are differences in baseline pupil size and amount of retinal illumination. Kardon et al.\textsuperscript{52} determined in eyes with darkly pigmented irides that baseline pupil size strongly influence retinal illumination. Such variability could also be due to mechanical effects of small pupil size on iris movement.\textsuperscript{63} We used a closed-loop paradigm, such that PLR was measured in the same eye that received the light stimulation; hence, retinal illumination was affected by baseline pupil size. To minimize the influence of pupil size, an open-loop paradigm could be used in which one pupil is dilated pharmacologically and stimulated with light but recordings are taken from the undilated contralateral eye.

Another potential cause of such variability may be anesthesia level and anesthetic drug used, but by monitoring relevant variables measured on our dogs during anesthesia, including heart rate and blood pressure, we found that these variables were very consistent, showing little variability. Significant differences in pupillometry parameters have been reported in humans treated with different anesthetics.\textsuperscript{64} The medications used for anesthesia in that particular study, ketamine and xylazine, reportedly elicit mydriatic effects in dogs. The anesthetics we used were acepromazine, propofol, and isoflurane. Administration of acepromazine in dogs has been shown to cause miosis, and halogenated agents (isoflurane) cause mydriasis, whereas propofol does not alter pupil reactivity.\textsuperscript{65,66} Despite these reported effects of anesthetic agents on the pupil, we found that the dogs’ eyes dilated well to baseline pupil size after dark adaptation.

Further research work is needed to define conditions that minimize individual variability in pupil constriction amplitude. As handheld pupillometry devices become more readily available, it will be possible in the future to eliminate use of anesthetic agents for PLR testing. With a handheld device, anesthesia and head positioning in the Ganzfeld dome would be less critical, allowing easy performance of chromatic pupillometry, and perhaps decreasing the individual variability of pupil constriction. A recent study found that the handheld pupillometer was easy to use on dogs, and its use was equally facile in conscious and anesthetized dogs.\textsuperscript{64}

**Conclusions**

In summary, we validated the use of a chromatic pupillometry protocol in dogs to assess rod, cone, and ipRGC function. We found that chromatic pupillometry results in dogs with specific retinal diseases correlated well with the known disease phenotype, including ERG, IHC, and qRT-PCR results. These findings are translationally relevant, as dogs have become increasingly important for the research of inherited retinal diseases and development of novel therapies.\textsuperscript{15,67} For example, our pupillometry approach may assist evaluation of the outcome of retinal gene therapy by complementing ERG recordings and visual behavioral testing to enable complete functional assessment of retinal and central visual pathways.\textsuperscript{49,68–74}
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