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Evaluation of Retinal Status Using Chromatic Pupil Light Reflex Activity in Healthy and Diseased Canine Eyes

Sinisa D. Grozdanic,¹ Milan Matic,¹ Donald S. Sakaguchi,² and Randy H. Kardon³

PURPOSE. To differentiate rod-cone-mediated pupil light reflexes (PLRs) from intrinsic melanopsin-mediated pupil light reflexes by comparing pupil responses with red and blue light stimuli of differing intensities in normal dog eyes and in those with sudden acquired retinal degeneration syndrome (SARDS) exhibiting a nonrecordable electroretinogram.

METHODS. The PLR was evaluated in 14 healthy dogs using a computerized pupillometry system and in five dogs with SARDS. Contraction amplitude, velocity, and implicit time of the PLR were studied as a function of peak wavelength (480 nm vs. 630 nm) and light intensity (−0.29 to 5.3 log units) to determine characteristics of the rod-cone versus predominantly melanopsin-mediated PLR activity.

RESULTS. The PLR in healthy, mildly sedated dogs could be elicited at low light intensities (−0.29 log units; 0.51 cd/m²). Canine SARDS patients displayed a complete absence of vision, electroretinographic amplitude, and PLR at low light intensity. However, in SARDS dogs, a pupil light reflex could be elicited with wavelengths corresponding to the melanopsin spectral sensitivity (blue light – peak at 480 nm) and at relatively high intensity (4.3 log units or higher), whereas red light (630 nm peak wavelength) was ineffective in eliciting any detectable PLR response even at light intensities of 6 log units (1,000,000 cd/m²).

CONCLUSIONS. The PLR in healthy canine eyes can be elicited at very low light intensities using red and blue wavelengths of light, but in dogs with blindness caused by SARDS, the pupil reacts only to high-intensity blue wavelength light, implying loss of the rod-cone-mediated PLR and most likely the presence of intrinsic, melanopsin-mediated, retinal ganglion cell-mediated PLR. (*Invest Ophthalmol Vis Sci.* 2007;48:5178–5183) DOI:10.1167/iovs.07-0249

The pupil light reflex (PLR) is an objective parameter of retinal and optic nerve function. Despite significant interest and data supporting the value of PLR analysis in the evalu-

ation of retinal and optic nerve diseases in humans, this method has not been widely used in experimental and clinically pathologic conditions in animals^{1–10} because of technical limitations in adapting computerized pupillometry to different species and because of interspecies differences in anesthetic effects on pupil size and reactivity to light, which can limit its use. Given that relatively few data are available concerning the physiological properties of the PLR among animal species, it is difficult to evaluate possible pathologic PLR changes in different animal models of eye disease.^{11–15} Although dogs are one of the most frequently used large animal models in which to test efficacy, safety, and toxicity in pharmacologic research, no data detail the physiological parameters of the PLR. The similarities between the dog lifestyle and that of humans, combined with centuries of inbreeding, have resulted in numerous spontaneously developing retinal and optic nerve diseases in dogs, which frequently share nearly identical genetic, morphologic, and physiological properties of human ocular diseases.^{16–20} Recent completion of the canine genome and decades of veterinary experience in recognizing, describing, diagnosing, and treating different ocular diseases in dogs provide an enormous source of information that can dramatically increase the use of dogs as large animal models for translational research in ophthalmology.^{21,22} The canine eye and retinal structure are more similar to the human eye than are those of the rodent. Moreover, the use of canine models of spontaneously occurring or experimentally induced ocular disease can lead to faster development of strategies for the successful treatment of blinding diseases in humans. To facilitate this translational process, it is important to refine methods that can be used to monitor retinal and optic nerve function. In addition to the use of evoked potentials, the PLR also has potential for monitoring afferent input. The discovery of the melanopsin-containing retinal ganglion cells and their mediation of the PLR have allowed better understanding of the neural input to the PLR and the conditions of light stimulus affecting it. Recent information about the physiology of the melanopsin-containing retinal ganglion cells and their activation by photoreceptors and by intrinsic phototransduction may provide a basis for using the PLR to differentiate diseases affecting the outer retina from those affecting the inner retina and optic nerve based on properties of the light stimulus, such as wavelength and intensity.

To characterize the rod-cone and possible intrinsic melanopsin-mediated PLR activity in canine eyes, we performed PLR analysis in healthy dogs and in dogs with sudden acquired retinal degeneration syndrome (SARDS), under stimulus conditions in which light intensity and wavelength were varied. SARDS is characterized by the sudden onset of visual loss and the complete loss of photoreceptor activity documented by the absence of electroretinographic (ERG) amplitudes caused by acute damage to photoreceptor outer segments.^{23,24} Although SARDS has been recognized for almost 20 years, its etiology remains unknown,^{25,26} but the most striking feature observed in these patients is that they still exhibit pupil constriction after stimulation with a light stimulus of high-intensity months after blindness has developed. Characterization of a large animal model of blindness with complete absence of photoreceptor

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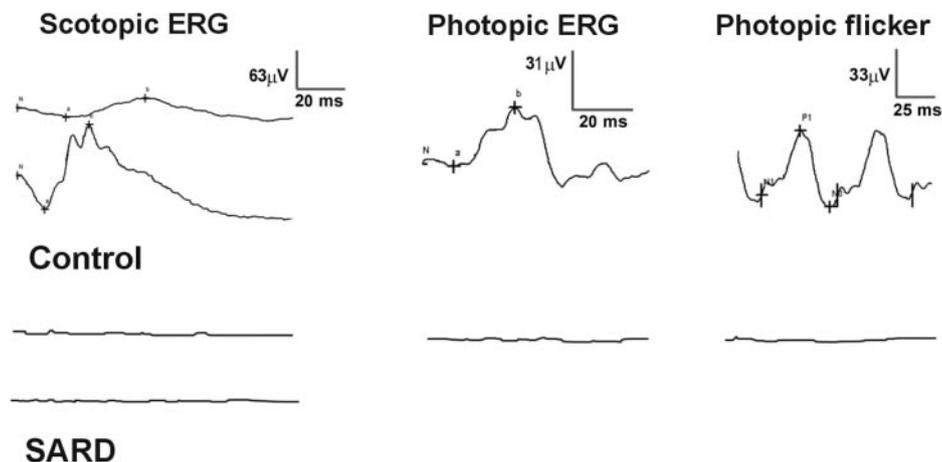


FIGURE 1. Electretinography tracings in a healthy dog (*top*) and a dog with SARDS (*bottom*). Electretinography analysis showed complete absence of the ERG amplitudes in the SARDS patient.

activity yet intact PLR activity offers a unique opportunity to study rod-cone and intrinsic melanopsin-mediated components of the PLR in the retina of a diurnal mammalian species (dog), which has a higher population of cones than the rodent retina.

MATERIALS AND METHODS

All animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Iowa State University Committee on Animal Care.

Fourteen healthy dogs (beagles) 6 to 8 months of age were used in this study. These animals underwent ocular examination (slit lamp biomicroscopy, intraocular pressure measurement, and indirect ophthalmoscopy) to rule out the possible presence of ocular disease before inclusion in the study. Five additional canine patients (different age, sex, and breeds) diagnosed with SARDS were also included in the study. Diagnosis of SARDS was established based on the following parameters: history of sudden onset of blindness, absence of any other neurologic deficits, relatively normal appearance of the optic nerve head and retina on ophthalmic examination, normal intraocular pressure, absence of PLR with weak light stimuli (less than 4.3 log units), near normal PLR with strong light stimuli (5.3 log units), and complete absence of ERG amplitudes (4.7 log units) in both eyes (Figs. 1 and 2).

Computerized Pupillometry

PLR was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA).²⁷ All dogs were mildly sedated with medetomidine (Domitor; Pfizer Animal Health, New York, NY) administered intramuscularly in a dose of 5 $\mu\text{g}/\text{kg}$ body weight. This dose was lower than the prescribed sedation dose for dogs because a higher sedation dose could cause pupil miosis and poor response to the light stimuli. The animals were confined to cages in a quiet room under mesopic conditions (dim light) for at least 15 minutes after sedation. Because of the low dose used for sedation, all computerized pupillom-

etry experiments were conducted in a sound isolated room to prevent excessive excitation caused by noise stimuli from surrounding rooms. We noticed that even minor noise could increase the level of excitation in animals during recording sessions, which would result in transient pupil dilatation and poor response to low-intensity light stimuli. The computerized pupillometer was attached to two infrared sensitive CCTV video cameras for simultaneous visual monitoring of both pupils, which were mounted on the goggles so that pupils could be monitored even if the animal was moving its head (Fig. 1A). However, a single-channel computerized pupillometer was used to record the movement of only one pupil while the stimulus light was alternated between the right eye and the left eye. For this study, the pupil recording was randomly selected (right or left) for each animal. Fifteen different intensities of light stimulus were delivered in the range of -0.3 to 1.75 log units (cd/m^2). Stimulus duration was 0.2 seconds, with intervals of 5 seconds between stimuli. Stimuli were delivered through the goggles using four green (light stimulus-eliciting PLRs) and three infrared (iris-illuminating) diodes per eye. Custom-made software routines (Winnana Software; R.H. Kardon, University of Iowa) were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to determine objectively the timing and amplitude of the pupil reflex responses (Figs. 3 and 4).

Analysis of the melanopsin-mediated responses was performed using a light source (Melan-100 unit; BioMed Vision Technologies, Ames, IA). Because PLRs could not be elicited in SARDS dogs using the green light-emitting diode stimulus from the goggles, we used the light unit (Melan-100; BioMed Vision Technologies), which has a powerful diode-based light source with a narrow wavelength—blue light (480 nm) and red light (630 nm)—and a stimulus of 5 seconds' duration because stimuli in the range of 0.2 to 1 seconds' duration could not elicit PLR response in SARDS dogs. Given that PLRs in SARDS dogs could not be elicited with a light intensity of 3.47 log units and because some PLR activity was detected with light intensity of 4.3 log units ($n = 3$ dogs), we used a blue light stimulus with a light intensity of 5.3 log units to

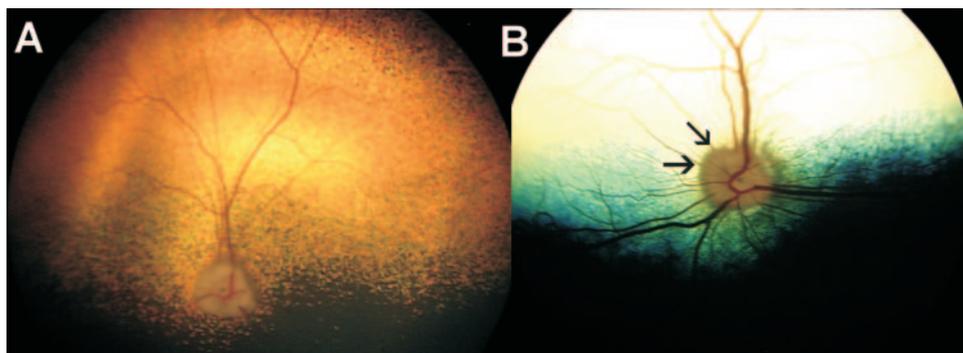


FIGURE 2. Fundus appearance of a SARDS dog (A) and a healthy control dog (B). SARDS patients have relatively mild fundus changes (primarily vascular attenuation). *Arrows:* arterioles at the level of the optic nerve head in a healthy dog, which are almost completely absent in the SARDS patient. Image is overexposed to allow better visualization of the optic nerve head vasculature.

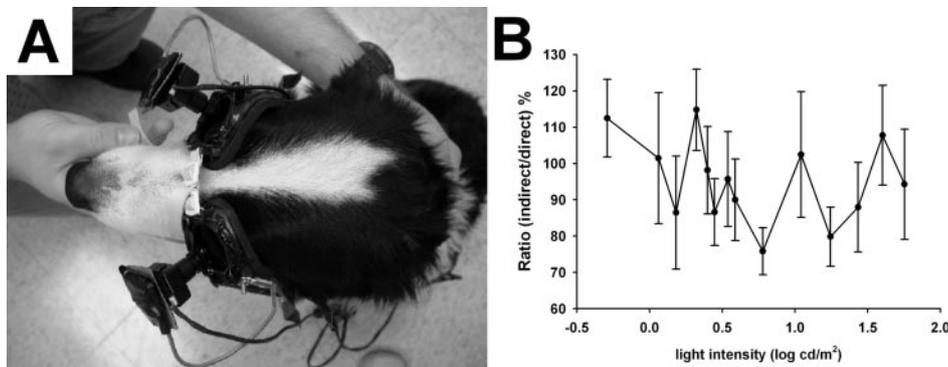


FIGURE 3. Computerized pupillometry in the canine model. (A) PLR parameters were evaluated with a computerized pupillometer connected to goggles with light-emitting diodes and two infrared CCTV cameras for the monitoring of PLR parameters. (B) Indirect PLR was slightly smaller and significantly different compared with the direct response. Graph represents pupil constriction data as a ratio between indirect and direct response. Average value of the indirect response was $95.2\% \pm 3\%$.

elicit a PLR in SARDS dogs. Red light (630 nm), did not elicit PLR responses at all tested light intensities (3.47, 4.3, 5.3, 5.7, and 6 log units) in SARDS dogs.

Electroretinography

Electroretinography was used to establish diagnoses of SARDS in five dogs. Pupils were dilated with 1% tropicamide, and animals were dark adapted for 20 minutes. Contact lens electrodes (ERG Jet electrode; LKC Technologies, Gaithersburg, MD) were used to record ERGs from both eyes. The reference electrode was positioned in the forehead region between both eyes, and the ground electrode was placed on the back of the head (occipital region). Both electrodes were placed subcutaneously. An ERG system (Roland Consult, Brandenburg, Germany) was used to deliver light stimuli and to collect signals from the lens electrode. Scotopic electroretinography (-0.658 log units [rod response]; 4.7 log units [combined rod-cone response]), photopic electroretinography (1.32 log units rod saturating illumination with 1.892 log units for flash stimulus), and photopic flicker (1.892 log units) routines were used to evaluate retinal function in healthy and

SARDS dogs. All SARDS patients displayed a complete absence of retinal electrical activity during ERG recording.

Statistical Analysis

Statistical analysis was performed by with the use of a paired *t*-test and graphics software GraphPad (GraphPad, San Diego, CA). $P < 0.05$ was considered significant.

RESULTS

Evaluation of the PLR in Healthy Dogs

The baseline pupil diameter for healthy dogs was 8.3 ± 0.6 mm (mean \pm SEM), whereas the constriction diameter at the highest light intensity (1.75 log units, 56.6 cd/m^2) for the direct pupillary response (light stimulus to the monitored eye) was 5.7 ± 0.6 mm. For the indirect response (light stimulus opposite the monitored eye), it was 6 ± 0.6 mm. The indirect, or consensual, pupil response displayed a significantly smaller

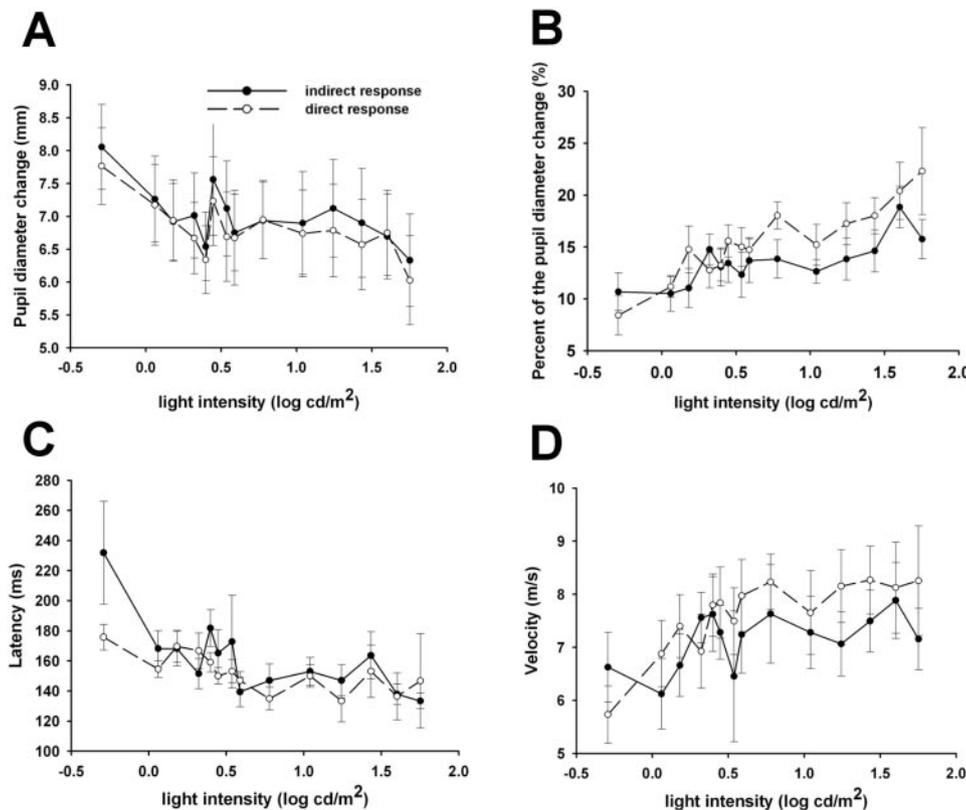


FIGURE 4. Pupillographic analysis of healthy dog retinas showed significantly lesser values for the indirect PLR compared with the direct PLR for the pupil diameter change (A; $P = 0.002$), percentage of the pupil diameter change (B; $P = 0.015$), and velocity (D; $P = 0.018$) but not latency (C; $P = 0.06$). Error bars represent SEM.

constriction diameter than the direct response ($P = 0.002$, paired t -test; Fig. 4A). Maximal percentage changes in diameter were observed at the highest light intensity and were $22.3\% \pm 4.2\%$ for direct PLR and $18.8\% \pm 2\%$ for indirect PLR ($P = 0.015$, paired t -test; Fig. 4B). The average ratio between the indirect PLR and the direct PLR was $95.2\% \pm 3\%$ (Fig. 3B).

The time delay between the onset of light and the beginning of pupillary contraction is the latency time. The latency time for the direct PLR was 133.3 ± 5.3 ms, and for the indirect PLR it was 146.6 ± 31.3 ms. The difference between direct and indirect response was not statistically significant ($P = 0.06$, paired t -test; Fig. 4C). At the highest tested light intensity, the velocity was 8.2 ± 1 mm/s for the direct PLR and 7.1 ± 0.5 mm/s for the consensual PLR ($P = 0.018$, paired t -test; Fig. 4D).

Evaluation of the PLR Activity in SARDS Dogs

Baseline pupil diameters were 9.6 ± 0.7 mm for SARDS eyes and 8.3 ± 0.6 mm for normal eyes. Retinal illumination using 2 and 3 log units light intensity (blue and white light) did not elicit PLR in SARDS patients. With further increases in light intensity, a very mild PLR was elicited at 4.3 log units (3 of 5 dogs). To elicit consistent pupil responses, we used 5.3 log units light intensity of blue (480 nm) and red (630 nm) light. Stimulation of the SARDS patients with red light of different intensities (3.47, 4.3, 5.3, 5.7, and 6 log units) did not elicit any PLR (Figs. 5, 6, 7), whereas stimulation with blue light (480 nm, 5.3 log units) consistently elicited PLR (pupil constriction diameter was 3.8 ± 0.4 mm). Statistical analysis showed a significant difference between the pupil diameter after red and blue illumination ($P = 0.0004$, paired t -test; Fig. 7).

We were able to detect PLR responses in healthy dog eyes at the very low light intensity (-0.29 log units), which is the light intensity used for recording rod responses in canine retinas (Fig. 1, first row, upper tracing from healthy canine eye). We observed significantly more pronounced PLR deficits at low light stimulus conditions in dogs with prominent rod damage and normal cone function (Grozdanic S, unpublished observation, 2006), further supporting our conclusion that canine PLRs are mediated by the rod-cone system and intrinsically sensitive melanopsin-containing RGCs.

DISCUSSION

The PLR is an objective indicator of retina and optic nerve function after light stimulation. A very important clinical application of the PLR observation is assessing a disease-induced afferent deficit, which can affect retina, optic nerve, and anterior, pregeniculate visual pathways (chiasm, optic tract, and

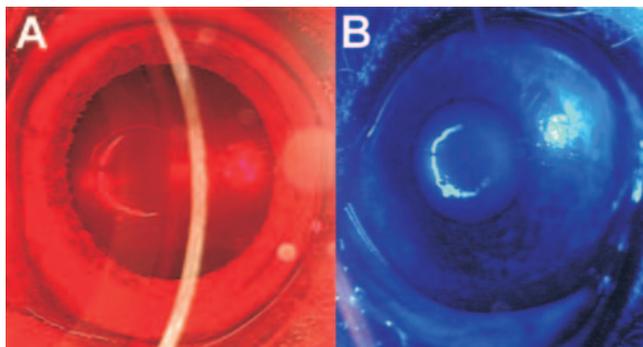


FIGURE 5. Pupil photography of a SARDS patient. Good pupil constriction is observed with the *blue* light stimulus (480 nm, 5.3 log units), whereas no pupil constriction was observed with the *red* light stimulus of the same light intensity (630 nm, 5.3 log units).

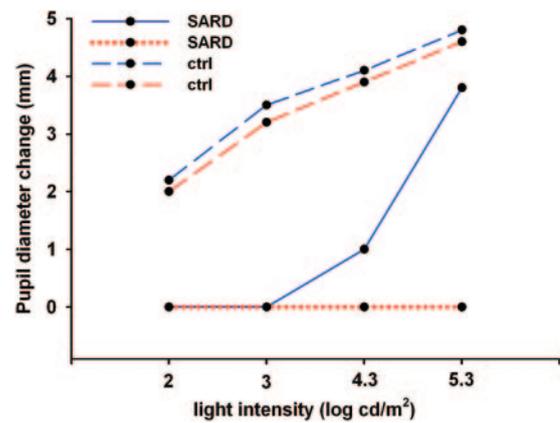


FIGURE 6. PLR analysis showed that blue light could elicit PLR responses in SARDS patients at 4.3 and 5.3 log unit light intensity, whereas the red light did not elicit detectable response at all tested light intensities (*bottom*). In healthy dogs, the blue light always elicited slightly stronger PLR responses than the red light of same intensity (*top*).

midbrain pathways). The past 5 years have seen a revolutionary advancement in the understanding of the physiological basis of photoreceptor and nonphotoreceptor-mediated PLR activity.²⁸⁻³¹ Discovery of the vitamin A-based photosensitive pigment (melanopsin) in retinal ganglion cells projecting to mid-brain structures mediating PLR activity, circadian rhythm regulation, and visual processing provides a principal explanation for the existence of intact PLR activity in eyes with virtually complete damage to the photoreceptor layer.^{29,31,32} Traditionally, clinical testing of PLR activity is performed with nonchromatic white light stimuli of different light intensities. Our study in healthy dogs and dogs with SARDS has demonstrated that rod-cone-mediated PLR activity can be elicited with light stimuli below the 3 log units light intensity, whereas possible exclusive melanopsin-mediated PLR could be elicited with much a higher light stimulus (4.3 log units or higher). Furthermore, we demonstrated that the separation of melanopsin and nonmelanopsin (rod-cone-mediated) PLR components can possibly be achieved by using light stimuli of different wavelengths (blue vs. red response). Because melanopsin sensitivity peaks close to 480 nm of wavelength (blue light), blue colored light stimuli of sufficient light intensity can be conveniently

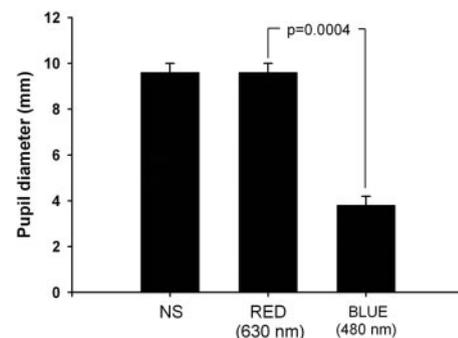


FIGURE 7. Pupil constriction analysis in SARDS dogs ($n = 5$). Red light stimulus (5.3 log units) did not elicit pupil constriction, and the pupil remained large, similar to the no light stimulus condition (NS, no light stimulus; pupil diameter after dark adaptation). The blue light stimulus of the same light intensity caused strong constriction, resulting in a small pupil. Pupil constriction in SARDS dogs could not be elicited with light stimuli below 4.3 log units of light intensity. Observed spectral response properties of the PLR in SARDS dogs coincided with the melanopsin peak spectral sensitivity in the blue bandwidth range.

used to evaluate function of melanopsin-containing retinal ganglion cells²⁸ lacking in rod-cone input (which is most likely the case in dogs with SARDS) and, hence, optic nerve function. Use of red light (630 nm), with a wavelength that does not overlap with melanopsin sensitivity, can be conveniently used to test for the cone photoreceptor-mediated component of the PLR.^{28,30} Previous studies of photopigments in dogs with visual behavior and electroretinography testing showed that canine retina has one rod pigment with peak sensitivity at 508 nm and two cone pigments with peak sensitivities at 430 to 435 nm (short-wavelength sensitivity) and 555 nm (long-wavelength sensitivity).^{33,34} Given that we used a red light of high wavelength (630 nm), it is likely that we managed to activate primarily the cones with long-wave sensitivity pigment because previous studies demonstrated end spectral sensitivity in the range of 650 nm. However, we cannot absolutely exclude the possibility that red light of 630 nm and high intensity did not also activate the rod system because of a very wide range of sensitivity of rod pigments in canine retina (peak sensitivity at 508 nm but end-of-sensitivity curve well in the range of 630 nm).³⁴ Because SARDS dogs do not have any PLR responses with red light but have good responses with blue light (480 nm) and do not have detectable rod- or cone-mediated ERG activity, we hypothesized that observed responses are indeed a result of melanopsin-mediated activity rather than residual photoreceptor activity (with a short-wavelength sensitivity³⁴), which could not be detected by standard electroretinography. Several factors support our hypothesis. The wavelength of blue light used in our study (480 nm) corresponds to the previously published peak spectral sensitivity of melanopsin in different species and falls between peak spectral sensitivity for cone and rod pigments in canine retina; we had to use the blue light of very high intensity to reliably induce PLR responses in SARDS dogs (5.3 log units = 200,000 cd/m²). Although we showed that latency data in healthy canine eyes is in the range of 150 to 220 ms, we never managed to induce PLR responses in SARDS dogs with light stimuli of 0.2 to 1 seconds, which again is indirect evidence that observed pupil light responses in SARDS dogs correspond by increased latency to previously described melanopsin-mediated responses.

How can these findings be used to improve diagnostic capabilities for different retinal and optic nerve diseases? In the past few years we have extensively studied PLR properties in different canine models of retina and optic nerve disease and have determined that chromatic evaluation of the PLR is an essential test for two reasons. One is to differentiate between retina and optic nerve disease. The other is that PLR combined with electroretinography can precisely localize the pathologic process. Canine patients with different forms of retinal detachment are usually characterized by weak or completely absent PLR with the red stimulus but near normal PLR with the blue stimulus because of primary RPE-photoreceptor abnormalities and intact inner retinal function.³⁵ We also had a chance to examine canine patients with different forms of photoreceptor degenerative diseases and determined that even in the early stages of retinal degeneration, the PLR is characterized by normal or decreased constriction amplitude but significant pupillary escape when red light stimulus was used. However, in advanced cases of retinal degeneration when complete blindness developed, red light responses were minimal or completely absent, but blue responses were characterized by decreased amplitude of constriction and always detectable prominent pupillary escape.³⁵ It has been recently described that melanopsin-containing RGCs physiologically react to rod and cone electrical activity.^{30,36} Because degenerating photoreceptors have decreased physiological capacity to convert light stimuli to an electrical stimulus, deficient photoreceptor function can result in the lower capacity of PLR, driving RGCs

to sustain pupil constriction, which ultimately results in the prominent pupillary escape when photoreceptor pathway activation (red light) is dominant. Dogs with advanced retinal degeneration are frequently characterized by minimal (or completely absent) responses to red light and by pupillary escape when the blue light stimulus is used. Hence, in the case of early retinal degeneration, the PLR response with a red light stimulus may act as a precise sensor of photoreceptor-deficient activity. Numerous studies have demonstrated that advanced retinal degeneration results in a complex reorganization of the inner retina synaptic architecture, which can be a serious limiting factor for the possible use of subretinal prosthesis or stem cell-based therapeutic strategies.³⁷⁻⁴⁰ It is likely that the presence of the decreased pupil response (or the presence of the pupillary escape) to blue light can be the result of melanopsin-containing RGC degenerative changes,⁴¹ which may serve as a prognostic parameter when determining whether possible regenerative or prosthesis-based strategies yield positive results for patients with advanced retinal degenerative diseases.

The presence of unique canine diseases (such as SARDS) can offer excellent tools for better understanding of melanopsin function in diurnal mammalian species. Use of SARDS canine patients, combined with functional MRI, can provide an exceptional opportunity to better understand the regulation of non-visual light-induced activity of the CNS.⁴²

Although this work provides a better understanding of the physiological properties regulating PLR activity in a large animal model with diurnal behavior (dog), the presence of different retinal pathologic entities in different breeds of dogs can be used to develop effective diagnostic strategies for similar diseases in human patients based on chromatic evaluation of the PLR activity.

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